# Auxin Analysis of Two 35S:ARK1 Mutant Lines of *Populus*

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## Abstract

The **purpose** of this study was to quantify the differences in endogenous levels of auxin in wild-type *Populus tremula* x *alba* (cultivar 717-1B4) and two independent transgenic lines of poplar, both exhibiting over-expression of the *ARBORKNOX1* (*ARK1*) gene. We **hypothesized** that the transgenic poplars would show altered auxin levels as compared to the wild-type. **Additionally**, we suspected that different cell types within a given individual plant would show altered hormone ratios when compared to one another. Therefore, we estimated endogenous levels of total auxin present per gram of fresh tissue in the leaves, stems, and roots of these three poplar lines by measuring the amounts of indole-3-acetic acid (IAA) present in each sample. We developed our final protocol based on a set of experiments completed with extra wild-type material; these experiments helped us determine appropriate initial tissue extraction, solid phase extraction, and derivatization procedures, as well as GC-MS parameters that would allow us to **detect and quantify IAA** in each sample. We used isotopically labeled IAA internal standard in order to estimate endogenous auxin that is present in the transgenic and wild-type samples. Analysis of the transgenic samples is currently in progress.

## Introduction

*ARK1* is a gene normally expressed in shoot apical meristems and vascular cambia of plants, and it tends to be down-regulated in terminally differentiated cells of leaves and secondary vascular tissues (Groover *et al.*, 2006). The two mutant lines of poplar used in this study (both of which were transformed in the Groover lab, with *ARK1* under the control of the strong 35S promoter) show unusual inhibited differentiation of leaves, internode elongation, and the appearance of secondary vascular cell types (*i.e.*, root cell types) in stems and at the tips of leaves (Figure 1). This phenotype could be due to an altered ratio of cytokinin to auxin as compared to wild-type plants, as it is this ratio that determines cell fate (*i.e.*, shoots or roots). **Specifically, we suspect that levels of auxin are higher in the two transgenic poplar lines than in wild-type 717-1B4 poplar, and that auxin levels will occur in a decreasing gradient from shoot to root tissues**, because auxin tends to stimulate root formation over shoot formation, and these transgenic plants show root formation, instead of shoots, at the shoot apical meristem. Endogenous amounts of auxin can be estimated by measuring IAA, one of the most common types of auxin found in plant tissue (Bialek & Cohen, 1992).

The **objectives** of this study are two-fold, to: (1) Develop & troubleshoot a protocol for IAA analysis in poplar, which has not previously been done, and using this new protocol, (2) Estimate endogenous IAA levels in the samples submitted to us from the Groover lab.

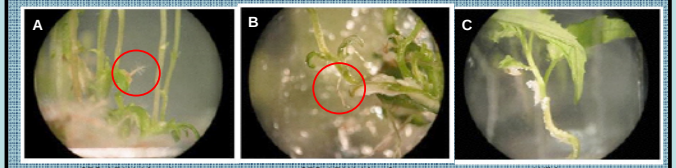


**Figure 1.** Two independently-transformed lines of poplar, *Populus tremula x alba* cv. 717-1B4. *ARK1* is over-expressed by the strong 35S promoter, resulting in unusual root formation as compared to wild-type plants (C). Red circles show roots forming on shoots (A) and stems (B) while wild-type (C) shows no unusual root formation. Images by A. Groover.

## Methods & Materials

We based our protocol for analysis of IAA in *Populus* tissue on one developed by J. Cohen (2006) for *Arabidopsis* tissue. Our revised version of this protocol consists of four major parts:

1. **Extraction** of IAA (plus other compounds) from poplar tissue. This involves **grinding** the frozen tissue, and **adding extraction agent** (65% IPA, 35% 0.2M imidazole) and a known amount of $^{13}$C-IAA internal standard. The length of time for extraction we modified based on a recent poplar study (Salyaev *et al.*, 2006).
2. **Solid-phase extraction** (SPE) to partially purify the tissue extract; this step removes remnants of tissue and compounds, based on their charge & hydrophobicity.
3. **Derivatization (methylation)** of IAA to stabilize the compound to make it more volatile (and, therefore, easier to detect in Step 4).
4. **Analysis** of extract via **GC-MS** to detect IAA, followed by **quantification** of endogenous IAA, relative to the amount of $^{13}$C-IAA internal standard present.

## Results

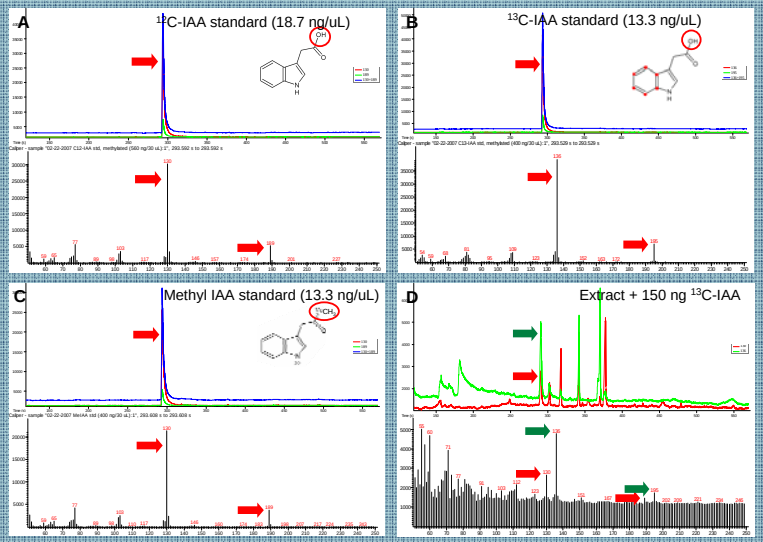


**Figure 2.** Four examples of gas chromatograms and mass spectra. Chromatograms (top graph in each panel) show time in seconds on the x axes and relative intensity on the y axes. Spectra (bottom graph in each panel) show mass-to-charge ratio (m/z) on x axes and relative peak intensity on y axes. **(A)** Characteristic chromatogram and spectrum for methylated $^{12}$C-IAA standard, a control, injected onto GC-MS at 18.7 ng/uL. Strong peaks for 130 and 189 m/z fragments at ~300 seconds are typical for this analyte, diagrammed above (red circle shows site of methylation). Note also the typical ratio between 130 and 189 m/z peaks in spectrum. **(B)** Characteristic chromatogram and spectrum for methylated $^{13}$C-IAA standard, a second control, injected at 13.3 ng/uL. Strong peaks for 136 and 195 m/z fragments at ~300 seconds are typical for this analyte, diagrammed above (red circle shows site of methylation; red dots show which carbons are heavy isotopes). **(C)** Characteristic chromatogram and spectrum for methyl IAA standard, a third control, injected at 13.3 ng/uL. This is a pre-methylated compound; note -$CH_3$ group (red circle) on diagram. Strong peaks for 130 and 189 m/z fragments at ~300 seconds are typical for this analyte. **(D)** Characteristic chromatogram and spectrum for plant extract (wild-type 717-1B4) spiked with 150 ng $^{13}$C-IAA as internal standard. Note that chromatogram is many more peaks, indicating the presence of compounds other than our analyte of interest. Green arrows correspond to internal standard; red arrows correspond to endogenous IAA. Amount of endogenous IAA can be calculated based on relative ratio between endogenous IAA and the internal standard.

To date, we have successfully modified the original *Arabidopsis* protocol (Cohen, 2006) for *Populus* tissue. We have optimized our SPE, methylation, and analysis steps (data not shown), and we are currently testing the effect of initial extraction time on the amounts of endogenous and internal standard IAA recovered. Figure 2 shows characteristic chromatograms and spectra for three controls (panels A-C), which are run every time we run a sample set, and an example of a more complicated set of graphs for an actual plant extract (in this case, from wild-type 717-1B4 tissue). This figure illustrates the characteristic peaks that indicate the presence or absence of IAA in our samples, whether that sample is a standard or a plant extract. It should be noted that evolution time and peak intensity depends, in part, on the parameters under which the GC-MS is run, which is why all three controls *must* be run every time! (For instance, changing the GC's ramping temperature will change the evolution time of our analyte of interest.) The evolution time and peak intensities of the controls help us determine where in the chromatogram to look for peaks indicating IAA's presence in our samples. Once we identify the peaks in the chromatograms of our samples that correspond to our analyte (in this case, IAA), the ratio of the parent ion to the molecular ion fragment confirm that identification.

Because $^{13}$C-IAA is six mass units heavier (due to the 6 heavy carbons in the phenol ring of IAA) than endogenous IAA, the mass spectrum of the internal standard is offset by 6 units. This results in the parent ion and molecular ion fragments appearing at 136 and 195 m/z (Figure 2B), rather than 130 and 189 m/z (Figure 2A). As a result, the endogenous IAA is easily distinguished from the internal standard; in the extracts of the plant tissue that have been spiked with internal standard, the area under the peaks corresponding to endogenous IAA can be compared to that under the peaks corresponding to the internal standard (heavy isotope) of IAA (Figure 2D). This is the key to being able to (back)calculate the amount of endogenous IAA in the sample. Keeping the amount of $^{13}$C-IAA standard constant but altering the amount of $^{12}$C-IAA standard in a given sample allowed us to develop a standard curve of expected ratios, against which to compare the extracts of our sample tissues (Figure 3).

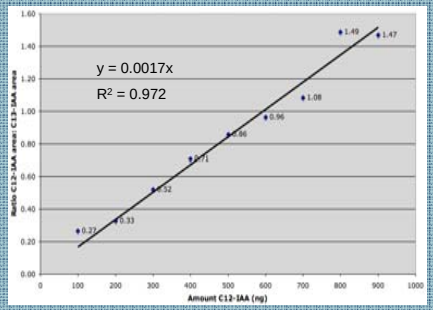


**Figure 3.** Standard curve of ratios of $^{12}$C-IAA peak area (130/189 m/z fragments) to $^{13}$C-IAA peak area (136/195 m/z fragments), calculated automatically from the corresponding chromatograms, when each are present in known quantities. Amount of $^{13}$C-IAA held constant at 400 ng; amount of $^{12}$C-IAA was altered as indicated on the x-axis. Resulting ratio is shown on the y-axis. The formula shown gives slope of the line of best fit (high $R^2$ value indicates highly accurate prediction), and will be used to calculate amount of endogenous IAA present in our samples.

## Discussion

**Development** of the protocol was easy; **optimizing** (and troubleshooting) the protocol has been challenging.

Use of the **$^{13}$C-IAA internal standard** allows us to determine how much of each sample was lost during the extraction, SPE, and methylation steps; in addition, it allows us to calculate the amount of endogenous IAA present per gram of fresh tissue.

Currently we are testing the **length of extraction time** and its effect on total amount of endogenous IAA extracted.

**Analysis** of the transgenic samples will soon be underway.

We predict that samples from the two **transgenic lines will contain higher auxin levels** than seen in the wild-type.

We also predict that **shoot tissues in all three lines will show the highest auxin levels**, and that root tissues will show the least auxin accumulation of all three tissue types.

## References

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