2β-Deoxy-Kdo Is an Inhibitor of the *Arabidopsis thaliana* CMP-2-Keto-3-Deoxymanno-Octulosonic Acid Synthetase, the Enzyme Required for Activation of Kdo Prior to Incorporation into Rhamnogalacturonan II

Dear Editor,

Gram-negative bacteria utilize the acid sugar 2-keto-3-deoxymanno-octulosonic acid (Kdo) as an essential component of the lipopolysaccharide (LPS). The enzyme CMP–Kdo synthetase (KdsB) is required to activate Kdo prior to incorporation into the LPS, utilizing Kdo and CTP to form CMP–Kdo and pyrophosphate. The potential of KdsB as a target for antibacterial agents has long been recognized and a number of inhibitors have been developed (Hammond et al., 1987). In eukaryotes, Kdo has been found in the embryophyte cell wall as a component of the pectic polysaccharide rhamnogalacturonan II (RG-II) and interestingly as a major component of the cell walls of thecae and scales of prasinophyte algae (Becker et al., 1995). RG-II is a complex, structurally conserved polysaccharide with a critical but as-yet only partially characterized function (O'Neill et al., 2004; Bar-Peled et al., 2012). RG-II has four side chains (A–D) and Kdo occurs only in side chain C, which consists of the disaccharide α-L-Rhap-(1→5)-α-D-Kdo-(2→3). The Kdo biosynthesis pathway is conserved in plants but traditional gene knockout approaches to study the effect of disrupting Kdo biosynthesis have been limited by the lethality of these mutants (Delmas et al., 2008).

This study characterizes the activity of the *Arabidopsis* KdsB enzyme, demonstrating that the enzyme catalyzes activation of Kdo to CMP–Kdo. It is also shown that 2β-deoxy-Kdo (Supplemental Figure 1), an *in vitro* inhibitor of bacterial KdsB, inhibits the *Arabidopsis* KdsB enzyme and that exogenous application to seedlings causes defects in root growth.

A multiple sequence alignment using KdsB homologs from a selection of plant species together with the *Escherichia coli* sequence reveals distinct conserved domains are retained, concentrated around key residues of the active site (Supplemental Figure 2). To further assess the structural conservation between the enzymes, the crystallographically determined *E. coli* structure (PDB ID: 3K8D) (Heyes et al., 2009) was used to generate a predicted model of the *Arabidopsis* enzyme. When superimposed, it is clear that the *E. coli* and predicted *Arabidopsis* active sites assume near identical conformations (Supplemental Figure 3).

The *AtKdsB* coding region from nucleotide +127 was cloned into pDEST17 Gateway vector (Invitrogen) to produce a truncated ΔAtKdsB protein including an N-terminal 6 His tag. The recombinant protein was produced by expression in *E. coli* BL21(DE3)pLysS cells, purified under native conditions (Supplemental Figure 4) and the *in vitro* activity measured by following the formation of pyrophosphate. The dependence of the rate on Kdo concentration displays typical Michaelis–Menten kinetics (Figure 1A). Under the conditions used, the V_{max} was determined to be 14.62 ± 0.58 μm min^{-1}, with a k_{cat} value of 8.77 ± 0.95 s^{-1} and K_m for Kdo of 88.59 ± 11.2 μm, comparable to those calculated for the bacterial KdsB enzyme. Previous studies have shown some activity with the alternative nucleotide UTP (Ray et al., 1981; Royo et al., 2000a; Kobayashi et al., 2011). However, other reports suggest strict nucleotide specificity (Misaki et al., 2009). Alternative nucleotide donors UTP and ATP were tested and showed virtually no activity under the experimental conditions used (Figure 1B).

The substrate analog 2β-deoxy-Kdo has been shown to act as a potent competitive inhibitor of *E. coli* KdsB (Heyes et al., 2009). The ability of 2β-deoxy-Kdo to inhibit the *Arabidopsis* KdsB enzyme was tested by measuring the rates of pyrophosphate formation by ΔAtKdsB over a range of Kdo and 2β-deoxy-Kdo concentrations (Figure 1A). The data could be fitted to the Michaelis–Menten model for full competitive inhibition with an R^2 value of 0.94 and K_i of 1.26 ± 0.15 μm confirming that 2β-deoxy-Kdo acts as a competitive inhibitor of AtKdsB. In addition, Isothermal Titration Calorimetry was used to study the interaction between the 2β-deoxy-Kdo inhibitor and ΔAtKdsB yielding a complex dissociation constant of 1.4 μm (Supplemental Figure 5).

In order to establish whether 2β-deoxy-Kdo affected *Arabidopsis* growth and development, seeds were germinated

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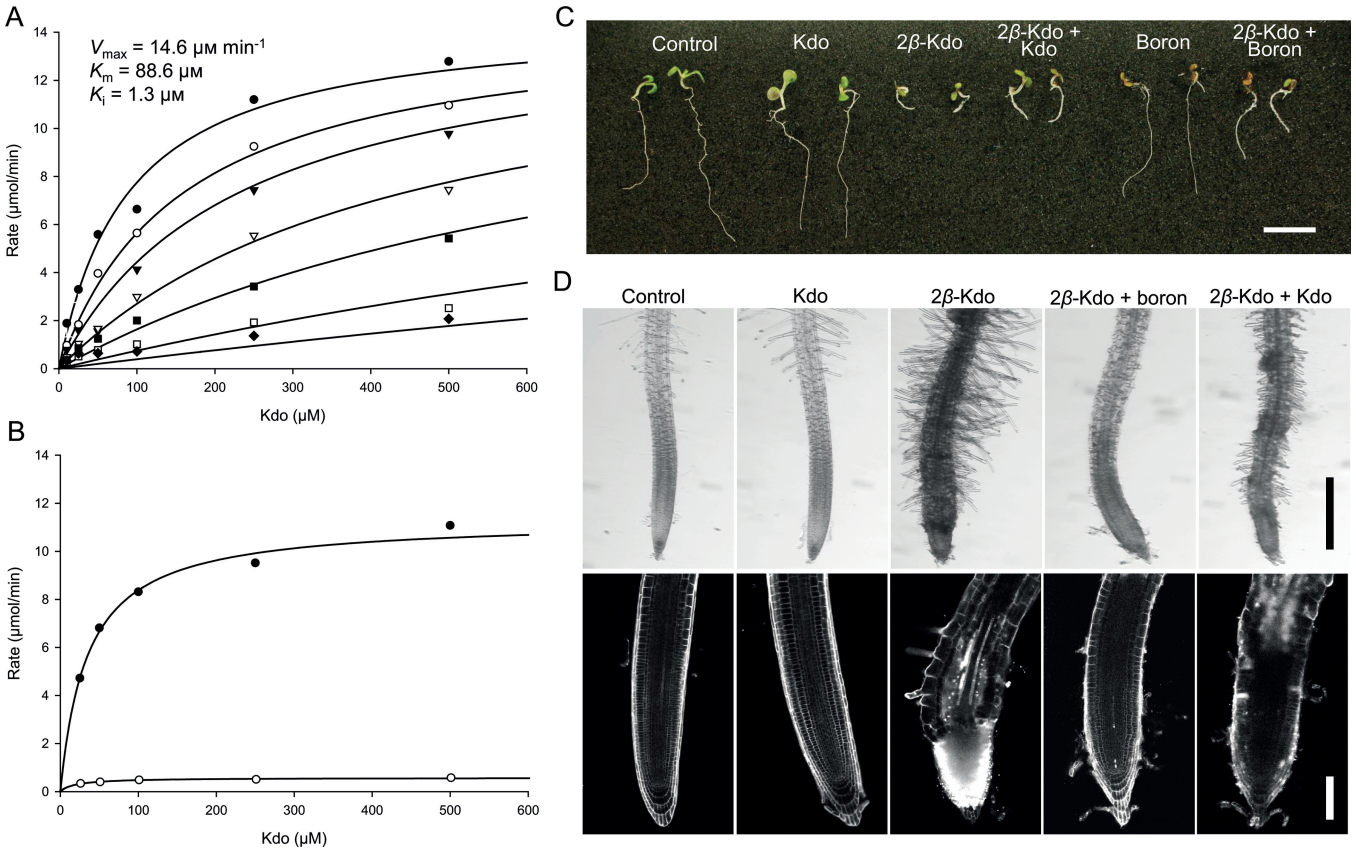

Figure 1. Kinetic Characterization of ΔAtKdsB and Inhibition Studies with 2β-deoxy-Kdo.

(A) The activity of 10 nm ΔAtKdsB was assayed over a range of Kdo concentrations at fixed 2β-deoxy-Kdo concentrations as follows: 0 μM (●), 1 μM (○), 2 μM (▾), 5 μM (▿), 10 μM (■), 25 μM (□), and 50 μM (♦). Data points are averages of three replicates and the curves are non-linear regression lines fitted to the data using the Michaelis–Menten equation for competitive inhibition.

(B) The effect of alternative substrates on the rate of ΔAtKdsB-catalyzed pyrophosphate formation using either 100 μM CTP (●) or UTP (○) in combination with Kdo. Activity similar to that of UTP was found for ATP, as well as for sialic acid with CTP (data not shown).

(C) Exogenous application of 2β-deoxy-Kdo inhibits root elongation that can be partially rescued with Kdo or boron. Seven-day-old *Arabidopsis* seedlings grown on liquid medium with no additives (Control) or supplemented with either Kdo (150 μM) or 2β-deoxy-Kdo (150 μM), boric acid (1.5 mM), or combinations of 2β-deoxy-Kdo (150 μM) with boric acid (1.5 μM) or 2β-deoxy-Kdo (150 μM) with Kdo (500 μM). Scale bar = 10 mm.

(D) Effect of 2β-deoxy-Kdo on root development. Stereo and confocal microscopy of seven day-old seedlings grown with no addition or supplemented with either 150 μM Kdo, 150 μM 2β-deoxy-Kdo, 2β-deoxy-Kdo plus 1.5 mM boric acid, or 2β-deoxy-Kdo plus 500 μM Kdo. Scale bars = 500 μM and 100 μM (upper and lower panels, respectively).

on liquid MS medium containing 150 μm 2β-deoxy-Kdo. After 7 d, seedlings treated with 2β-deoxy-Kdo exhibited reduced primary root and aerial growth compared to the controls (Figure 1C). Kdo added at the same concentration as 2β-deoxy-Kdo was used as an additional control and did not affect growth. Testing a range of 2β-deoxy-Kdo concentrations (10 μM, 20 μM, 50 μM, and 150 μM) showed that, at concentrations of 50 μM and above, root elongation was severely inhibited (Supplemental Figure 6).

It is likely that the growth and development defects associated with the application of 2β-deoxy-Kdo to *Arabidopsis* seedlings are due to specific inhibition of the AtKdsB enzyme resulting in an altered RG-II structure or reduced RG-II abundance. Application of Kdo at concentrations in excess of the inhibitor (150:500 μM, 2β-deoxy-Kdo:Kdo) was shown to partially rescue the root growth defects resulting from 2β-deoxy-Kdo treatment, suggesting the action of the inhibitor is indeed specific to AtKdsB (Figure 1C).

It is possible to rescue the phenotypes of several mutants with altered RG-II structure by application of exogenous boron (O'Neill et al., 2001). Inhibitor-treated seedlings were supplemented with excess boron, which was able to partially rescue 2β-deoxy-Kdo-treated seedlings (Figure 1C), suggesting that *in vivo* inhibition of AtKdsB leads to alterations in RG-II structure that reduces boron-mediated cross-linking.

Closer inspection of the root tip and elongation zones of 2β-deoxy-Kdo treated seedlings reveal that inhibitor application causes reduced cell elongation at the root tip (Figure 1D). This results in small, tightly packed root cells and the presence of differentiated cells close to the root tip as well as the appearance of densely packed root hairs. A longitudinal section obtained by confocal microscopy shows a root tip

region in which the propidium iodide stain, which is normally restricted to the cell wall, has entered the cells, indicative of cell death or defects in the cell wall integrity (Figure 1D and Supplemental Figure 7). Basal to the root tip zone, cells were radially expanded compared to non-treated seedlings and mature vascular tissue was observed close to the root tip. Application of excess boron partially rescues the primary root elongation and spatial organization of root tip cells of 2β-deoxy-Kdo-treated seedlings, supporting the conclusion that the inhibitor indirectly causes a reduction in boron-mediated RG-II cross-linking.

It is apparent that, despite their very different cell wall structures, the pathways for Kdo synthesis and activation are highly conserved between plants and Gram-negative bacteria (Royo et al., 2000b). Using recombinantly expressed *Arabidopsis* KdsB, it has been shown that the *in vitro* CMP–Kdo synthetase activity is similar to that previously reported for the bacterial enzyme. It was also found that the *in vitro* activity of *Arabidopsis* KdsB is inhibited by the substrate analog 2β-deoxy-Kdo and exogenous 2β-deoxy-Kdo application inhibits root cell elongation. These results open up the possibility to utilize 2β-deoxy-Kdo as a tool to study the structure–function relationship of RG-II in plants.

A reduced ability to form RG-II dimers has been shown to affect cell wall thickness and porosity and several dwarfed mutants with defects in RG-II structure can be rescued by exogenous boron application. The findings presented here show that 2β-deoxy-Kdo also appears to reduce RG-II boron cross-linking efficiency, raising the question of why inhibiting the synthesis of a residue found on side chain C which has not been directly implicated in boron cross-linking would have this effect. One explanation is that the complex structure of RG-II determines its secondary structure and that this is critical for dimer formation, thus driving RG-II structure conservation. Alternatively, it is possible that addition of the RG-II sidechains follows a sequential order such that blocking addition of sidechain C could hamper or abolish the synthesis of some or all of the other side chains.

The identification of 2β-deoxy-Kdo as an *in vivo* inhibitor of AtKdsB also creates an opportunity to apply this inhibitor in a forward genetic screen for the identification of novel genes involved in RG-II biosynthesis. Indeed, with no *a priori* knowledge of any Kdo interacting genes (other than those involved in Kdo and CMP–Kdo synthesis), such an unbiased approach represents the best possibility for identification of novel RG-II/Kdo associated genes.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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Kevin M. Smyth, Halina Mikolajek, Jörn M. Werner and Alan Marchant

Centre for Biological Sciences, University of Southampton, Southampton, SO17 1BJ, UK

[1] To whom correspondence should be addressed. E-mail A.Marchant@soton.ac.uk, tel. +44(0)23 80594363.

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