Final Technical report: Functional analysis and genetic manipulation of plant ABCB organic ion transporters

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1.Demonstration of effective substrate uptake assays in *S. pombe* system

Several heterologous systems have been developed to study auxin transport activity of ATP Binding Cassette (ABCB/ PGP) transporters and other auxin transport families (PINs and AUX1). A common heterologous system good for all ABCB and auxin transporters was not developed due to differences in glycosylation and membrane composition between plant and previously used budding yeast *Saccharomyces cereviseae* or mammalian systems. The lack of cell wall also places potential limitations on mammalian systems. Kinetic analysis is also difficult for auxin transporters in mammalian systems. The fission yeast *Schizosaccharomyces pombe* shows plant like polar sterol-rich membrane domains and relatively small glycans in N-glycosylation. *S. pombe* contains only 11 ABC transporter genes, and in many other transporter families, single gene is found. Because of this, we have made an extensive effort to develop the *S. pombe* system for studies of plant transport proteins. This has included development of knockout lines for all ABC transporters and a number of other membrane transporters. We expressed both AtABCB1 and AtABCB19 in *S. pombe* ABC transporter double mutant *mam1Δpdr1Δ* under the inducible nmt41 promoter. *S. pombe* cells expressing AtABCB1 and AtABCB19 accumulated less ^{3}H-IAA compared to vector control, which is consistent with the efflux activity of AtABCB1 and AtABCB19. Second, we generated mutant lacking auxin effluxer like 1 (*AEL1*) using disruption plasmid and expressed and analyzed PIN proteins in the mutant *ael1Δ.* Third, we expressed AUX1 in *vat3Δ S. pombe* mutants and observed auxin uptake activity of AUX1. The auxin import activity kinetics can be analyzed for AUX1 and ABCB4 transporters as in Fig. 1.

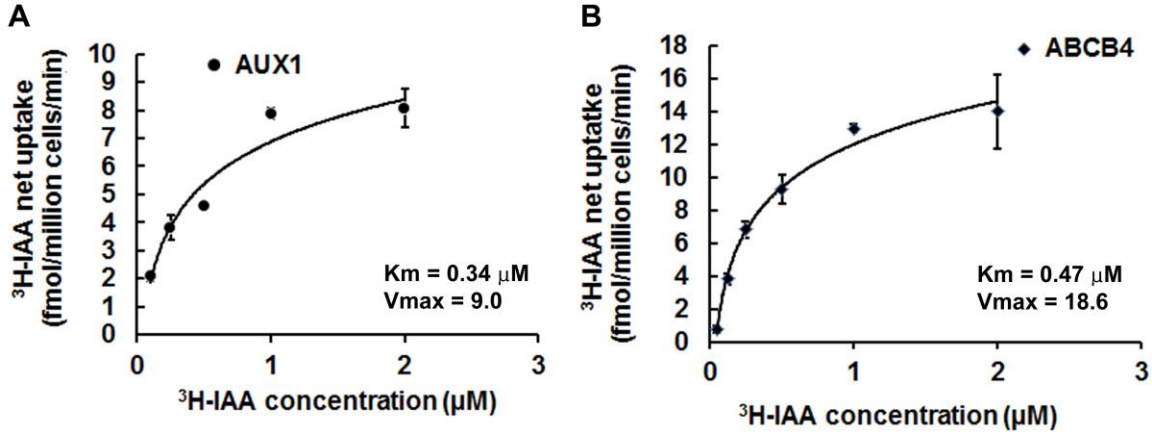


Figure 1. Auxin transport kinetics for AUX1 and ABCB4 **A.** ^{3}H-IAA uptake by AUX1 exhibits an apparent Km of 340 nM (8 min. assay). **B.** ^{3}H-IAA uptake by ABCB4 exhibits an apparent Km of 470 nM (4 min. assay).

2. phototropin1 Kinase Inhibits ABCB19 Transporter Activity

To understand the regulation of ABCB19 activity, we identified the interaction between ABCB19 and photo receptor phot1 in phototropism. Our attempts to express functional phot1 and B19 simultaneously in either insect cells or *Schizosaccharomyces pombe* were unsuccessful, precluding the possibility of investigating substrate phosphorylation by co-expression analysis in these systems. As an alternative, functional B19 generated in membranes of *S. pombe* was mixed with active phot1 enriched from insect cells for in vitro phosphorylation analysis. Although the addition of *S. pombe* membranes reduced the level of phot1 autophosphorylation from insect cells, increased phosphorylation of B19 in the presence of phot1 was apparent using this approach (Figure 2A). Phosphorylation of B19 in the presence of phot1 was verified by immunoprecipitation (Figure 2B), suggesting that B19 is a substrate target for phot1 kinase activity. B19 is a stable plasma membrane protein that does not exhibit the dynamic relocalization characteristics of PIN transporters. We therefore rationalized that B19 phosphorylation by phot1 could impact its transporter activity as opposed to its subcellular trafficking. To address this, we examined Arabidopsis ABCB transporter function in HeLa cells. Expression of B19 and B1 resulted in auxin efflux in this system, whereas expression of phot1 was without effect (Figure 2C). In each case, light treatment of HeLa cells resulted in a modest increase in auxin efflux activity. However, co-expression with phot1 reduced B19 transporter activity, particularly following irradiation. Introduction of a single point mutation (D806N) shown previously to inhibit phot1 kinase activity abrogated this effect, indicating that phot1 activation leads to a loss of B19 transporter function.

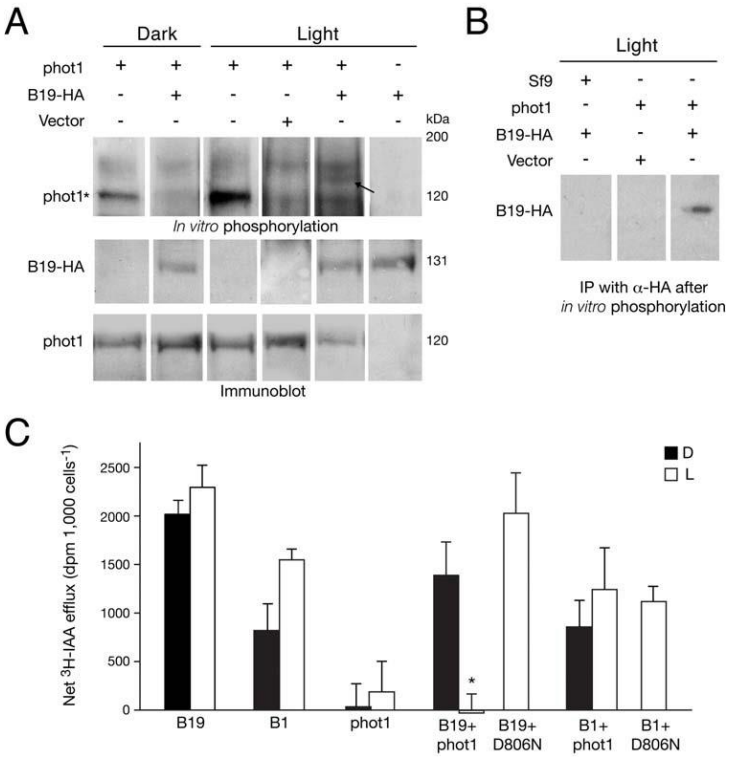


Figure 2. phot1 phosphorylation and regulation of B19 auxin transporter activity. (A) In vitro phosphorylation of B19 in the presence of phot1(upper panel). Protein levels were monitored using anti-HA and anti-phot1 antibodies, respectively (lower panels). (B) Phosphorylation of B19-HA by phot1 in (A) was confirmed by immunoprecipitation (IP) of B19-HA using anti-HA antibodies (right lane). All samples were treated with light. (C) phot1 regulation of B19-mediated auxin efflux in HeLa cells. Cells were prepared under a green safe light then incubated in darkness.

3. ABCB structural models: mutational analysis of predicted binding sites AtABCB19.

To gain a clearer understanding of how eukaryotic ABCB proteins interact with their transport substrates, we employed the same approaches used to develop HsABCB1 models to compute structures of plant ABCB transporters that exhibit a high degree of substrate specificity. Sequence and structural comparisons showed that characterised plant and mammalian ABCB transporters share a common architecture. Our analysis also identified candidate substrate binding sites in the transmembrane domains of the proteins near the inner leaflet of the plasma membrane. A general transport mechanism is proposed for ABCB proteins based on the predicted substrate binding pockets in the transmembrane domains (TMDs), the reported movement of nucleotide-binding domains (NBDs), and the deduced movement of TMDs driven by ATP hydrolysis. Re-examination of recent mutational analyses indicates that these new structural models are consistent with the mechanism proposed for the Sav1866-like architecture. Furthermore, the conserved “gate” sequences in the animal ABCB1 transporters explain their substrate promiscuity, while a divergent “gate” region in plant ABCBs accounts for their substrate specificity. Finally, these comparative models suggest potential domains that regulate the directionality of the plant ABCB transporters. However, they also suggest that eukaryotic uptake and conditional uptake/export transporters are structurally and evolutionarily distinct from prokaryotic uptake transporters. Our data provide a significant framework for the experiments in investigation of binding sites and directionality for ABCBs. Site-directed mutagenesis was designed based on the predicted substrate binding sites and “gate” sites. Site-directed mutagenesis constructs were generated…. (from mark)

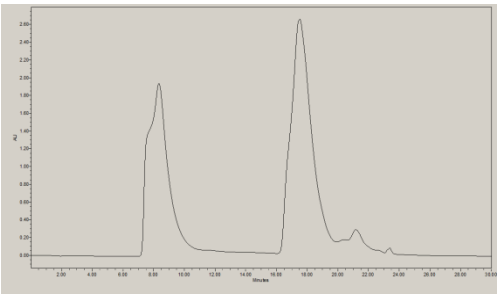
4. **Arabidopsis ABCB4 is a root-localised auxin efflux transporter with reported auxin uptake activity in low auxin concentrations**. Results reported here demonstrate that ABCB4 is a substrate activated regulator of cellular auxin levels. The contribution of ABCB4 to shootward auxin movement at the root apex increases with auxin concentration, but in root hair elongation assays ABCB4-mediated uptake is evident at low concentrations as well. Uptake kinetics of ABCB4 heterologously expressed in *Schizosaccharomyces pombe* differed from the saturation kinetics of AUX1 as uptake converted to efflux at threshold indole-3-acetic acid (IAA) concentrations. The concentration dependence of ABCB4

appears to be a direct effect on transporter activity, as ABCB4 expression and ABCB4 plasma membrane (PM) localisation at the root apex are relatively insensitive to changes in auxin concentration. However, PM localization of ABCB4 decreases with 1-naphthylphthalamic acid (NPA) treatment. Unlike other plant ABCBs studied to date, and consistent with decreased detergent solubility, ABCB4pro:ABCB4-GFP is partially internalised in all cell types by 0.05% DMSO, but not 0.1% ethanol. In trichoblasts, ABCB4pro:ABCB4-GFP PM signals are reduced by >200 nM IAA and 2,4-dichlorophenoxyacetic acid (2,4-D). In heterologous systems and *in planta*, ABCB4 transports benzoic acid with weak affinity, but not the oxidative catabolism products 2-oxindole-3-acetic-acid and 2-oxindole-3-acetyl-b-Dglucose. ABCB4 mediates uptake, but not efflux, of the synthetic auxin 2,4-D in cells lacking AUX1 activity. Results presented here suggest that 2,4-D is a non-competitive inhibitor of IAA transport by ABCB4 and indicate that ABCB4 is a target of 2,4-D herbicidal activity

New Technologies added to the project

Enzymatic generation of ^{14}C-labelled ferulic acid

A major limitation for the progress of the proposed project has been unavailability of radiolabelled aromatic organic acid substrates for comparative analyses of transporter activity. To date, the lab has used LC-MS of native and ^{13}C-labelled compounds for this purpose. The resulting studies have been useful, but required the use of higher substrate concentrations than are desirable. The lab has now enzymatically synthesized ^{14}C ferulic acid as described below and is using this labeled substrate for analyses of the rice ABCB10 and other transporters.LC-MS analyses confirm that the ferulic acid is not metabolized in *S. pombe*. These initial studies confirm that the labeled ferulic acid transport mediated by OsABCB10. Based on the success of this approach, the lab has enzymatically synthesized ^{14}C ring-labeled *trans* cinnamic acid (starting with ^{14}C ring-labelled phenylalanine) that will be used to enzymatically generate substrates for the project.



Reverse-phase HPLC separation of caffeic acid precursor (peak 1) and enzymatically synthesized ferulic acid (peak 2). ^{14}C ferulate was enzymatically synthesized by caffeyl O-methyl transferase after addition of ^{14}C *S*-adenosyl methionine and caffeic acid. Conversion rates of 60% of SAM were observed. Samples were purified by Oasis MAX-SPE before HPLC separation.

Dynamic imaging of substrate transport

In the proposed project, the validation of substrate specificity modifications analysed in yeast occurs in Arabidopsis and involves microdeposition of ^{13}C, ^{14}C, or ^{3}H – labelled compounds at discrete sites on growing plants followed by measurement of substrate movement in plant tissues. These measurements are determined by destructive sampling, extraction, and liquid scintillation counting or LC-MS. These methods are well established and have been refined continuously

in the lab for more than a decade. However, the goal of real time, non-destructive monitoring remains an important objective. The lab is able to simultaneously monitor indole-3-acetic acid (IAA) uptake and efflux along the root surface using a self-referencing ion-specific electrode (McLamore et al., 2010), but has been unable to employ this technology for measurements in shoots or internal tissues.

Use of conjugated auxins to selectively inhibit ABCB transport
In collaboration with Dr. Ken-ichiro Hayashi, University of Okayama, benzoxy conjugates of ABCB transport substrates were designed. In the Murphy lab, these conjugates were successfully tested for their ability to inhibit transport of aromatic organic acids in yeast and in plant systems without being trasnsported themselves.

Publications derived from the project:
Kubeš M, Yang H, Richter GL, Cheng Y, Młodzińska E, Wang X, Blakeslee JJ, Carraro N, Petrášek J, Zažímalová E, Hoyerová K, Peer WA and Murphy AS (2011) The Arabidopsis concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis 69:640-654

Bailly A, Yang H, Martinoia E, Geisler M, Murphy AS (2011) Plant lessons: exploring ABCB functionality through structural modeling. Frontiers in Plant Science, **2:**108, doi:10.3389/fpls.2011.001

Yang H, Richter GL, Wang X, Młodzińska E, Carraro N, Ma G, Jenness M, Chao D, Peer WA, Murphy AS (2012) Sphingolipids and sterols are required for post ER trafficking and plasma membrane stability of the Arabidopsis ABCB19 auxin transporter.

Kubeš M, Yang H, Richter GL, Cheng Y, Młodzińska E, Wang X, Blakeslee JJ, Carraro N, Petrášek J, Zažímalová E, Hoyerová K, Peer WA and Murphy AS (2011) The Arabidopsis concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis 69:640-654

Bailly A, Yang H, Martinoia E, Geisler M, Murphy AS (2011) Plant lessons: exploring ABCB functionality through structural modeling. Frontiers in Plant Science, **2:**108, doi:10.3389/fpls.2011.001

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New Technologies
Fluorescent organic acid probes
Enzymatic generation of ^{14}C-labelled ferulic acid

Generation of benzoxy conjugates of aromatic organic acids for use as non-trasnported competitive inhibitors of ABCB activity