

# Functional analyses of differentially expressed isoforms of the Arabidopsis inositol phosphorylceramide synthase

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**Abbreviations:** AbA – aureobasidin A; BSA – bovine serum albumin; Cer – ceramide; IPC – inositol phosphorylceramide; NDB C<sub>6</sub>-ceramide – 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl)sphingosine; PI – phosphatidylinositol; SD – synthetic minimal media with glucose; SGR – synthetic minimal media with galactose; SM – sphingomyelin

## **Abstract**

Sphingolipids are key components of eukaryotic plasma membranes that are involved in many functions, including the formation signal transduction complexes. In addition, these lipid species and their catabolites function as secondary signalling molecules in, amongst other processes, apoptosis. The biosynthetic pathway for the formation of sphingolipid is largely conserved. However, unlike mammalian cells, fungi, protozoa and plants synthesize inositol phosphorylceramide (IPC) as their primary phosphosphingolipid. This key step involves the transfer of the phosphorylinositol group from phosphatidylinositol (PI) to phytoceramide, a process catalysed by IPC synthase in plants and fungi. This enzyme activity is at least partly encoded by the *AUR1* gene in the fungi, and recently the distantly related functional orthologue of this gene has been identified in the model plant Arabidopsis. Here we functionally analysed all three predicted Arabidopsis IPC synthases, confirming them as aureobasidin A resistant AUR1p orthologues. Expression profiling revealed that the genes encoding these orthologues are differentially expressed in various tissue types isolated from Arabidopsis.

**Key words:** Arabidopsis, AUR1, expression, inositol phosphorylceramide, IPC synthase, sphingolipids

## Introduction

The diverse, amphipathic sphingolipids consist of a long chain base backbone with long-chain fatty acid and polar alcohol attachments. These lipid species are ubiquitous membrane components of eukaryotic cells, as well as being found in some prokaryotic organisms and viruses (Smith and Merrill 2002). Studies in mammals and yeast have shown that they are important structural components of membranes and also serve as bioactive molecules involved in cell signalling and regulation (Dickson, et al. 2006; Fernandis and Wenk 2007; Hanada, et al. 1992). The unmodified sphingolipid, ceramide, is an intermediate in complex sphingolipid biosynthesis in the Golgi apparatus. These complex species ultimately concentrate in the outer leaflet of the plasma membrane where, with sterols, they form lipid raft microdomains (Futerman and Hannun 2004). Rafts have been proposed to be central to a multitude of cellular processes, from the polarized trafficking of lipid-modified proteins (Brown and London 1998) to the formation of signal transduction complexes (Magee, et al. 2002; Pierce 2002). Furthermore, sphingolipid metabolites such as ceramide and phosphorylated sphingosine (sphingosine-1-phosphate), are central to intracellular signal transduction processes that regulate cell growth, differentiation and apoptosis (programmed cell death – PCD; Futerman and Hannun 2004).

The biosynthesis of sphingolipids shows commonality between mammals, fungi and plants up to the formation of ceramide (phytoceramide in plants and fungi), but the predominant complex phosphosphingolipid species subsequently synthesized differs. Mammals produce sphingomyelin (SM; a ceramide unit with a phosphorylcholine moiety), whereas fungi and plants synthesize inositol phosphorylceramide (IPC) by the transfer of the phosphorylinositol group from phosphatidylinositol (PI) to phytoceramide, a reaction catalysed by IPC synthase. This enzyme was shown to be at least partly encoded by the *AUR1* gene in yeast and other fungi (Nagiec, et al. 1997), and recently an accessory protein involved in IPC synthase activity (KEI1p) in these organisms has been identified (Sato, et al. 2009). However, the plant enzyme remained unidentified although the activity had been

measured in bean microsomes (Bromley, et al. 2003). Then, during a screen for factors involved in PCD defence mechanisms, an *AUR1* functional orthologue was identified in *Arabidopsis*. However, the encoded protein demonstrated little homology to the yeast AUR1p, having most similarity to the more recently identified animal sphingomyelin synthases (Wang, et al. 2008). This lack of similarity to the long known fungal enzyme (Nagiec, et al. 1997) lay behind the previously fruitless search for the plant orthologue (Dunn, et al. 2004).

Here we demonstrate that all three orthologues identifiable in the *Arabidopsis* genome database represent aureobasidin A resistant functional orthologues and that, through phylogenetic analyses, these represent a new group of sphingolipid synthases within the wider enzyme family. Furthermore, we show the expression profile of all three isoforms in the tissues of *Arabidopsis*. This demonstrated that there is differential expression of IPC synthase, an enzyme central to the synthesis of plasma membrane sphingolipids (Dunn et al. 2004) and a regulator of PCD (Wang, et al. 2008) in this plant species.

## Materials and Methods

### Complementation of auxotrophic AUR1 mutant yeast with AtIPCS1-3

Arabidopsis IPC synthase 1, 2 and 3 (AtIPCS1 - At3g54020.1, AtIPCS2 - At2g37940.1 and AtIPCS3 - At2g29525.1) were amplified from Arabidopsis cDNA using primers

5' AtIPCS1 EcoRI gaattcATGACGCTTTATATTCGCCGCG and 3' AtIPCS1 Sall gtcgacGAGCAGAGATCTCATGTGCC;

5' AtIPCS2 EcoRI gaattcATGACACTTTATATTCGTCTGTG and 3' AtIPCS2 Sall gtcgacTCACGCGCCATTCATTGTG;

5' AtIPCS3 EcoRI gaattcATGCCGGTTTACGTTGATCGCG and 3' AtIPCS3 Sall gtcgacTCAATGATCATCTGCTACATTG.

The products were subsequently cloned into the yeast vector pRS426MET25, creating pRS426 AtIPCS1-3. In the YPH499–HIS–GAL–AUR1 *S. cerevisiae* strain expression of the essential *AUR1* gene (Nagiec, et al. 1997) is under the control of the *GAL1* promoter and is repressed in the presence of glucose (Denny, et al. 2006). YPH499–HIS–GAL–AUR1 was transformed with pRS426 AtIPCS1-3 and pRS426 AUR1 and functionally complemented transformants selected on non-permissive SD medium containing necessary nutritional supplements (Denny, et al. 2006).

### Diffusion assay of complemented auxotrophic AUR1 mutant yeast

YPH499–HIS–GAL–AUR1 pRS246 AtIPCS1-3 and YPH499–HIS–GAL–AUR1 pRS246 AUR1 were assayed for susceptibility to aureobasidin A (Takara) and myriocin (Sigma) as previously described (Nagiec, et al. 2003). Briefly,  $2.4 \times 10^7$  logarithmically dividing cells were embedded in 15 ml of YPD-agarose (1% yeast extract, 2% peptone, 2% dextrose, 0.8% agarose) on 100 mm<sup>2</sup> square Petri dishes (Sarstedt). Inhibitors were applied in Me<sub>2</sub>SO at the concentrations described below and the dishes incubated at 30°C.

### **Microsomal assay of AtIPCS**

For *in vitro* assay microsomal membranes were prepared from YPH499–HIS–GAL–AUR1 pRS246 AtIPCS1-3 and YPH499–HIS–GAL–AUR1 pRS246 AUR1 and assayed as described (Mina, et al. 2009). In brief, assays were performed in 50µl of 100mM Tris HCl pH 7.4, 10mM EDTA and 6mg/ml defatted BSA, with or without 1mM PI (soybean, Avanti Polar Lipids; predominant species C16:0-C18:2) and 5µM aureobidin A, and with 2 µl of microsomes (10 mg/ml protein) and 2µl of 5mM NBD C<sub>6</sub>-Ceramide (Molecular Probes) in DMSO. After incubation at 30°C for 60 min the reaction was stopped by the addition of 150µl of chloroform/methanol/water (10:10:3 v/v/v) and the lipid fraction isolated by phase separation. This process facilitated reproducible quantitative analyses of IPC formation. Following equilibration (Synergy HT, Bio-tek) samples were analysed by high performance thin-layer chromatography (Denny, et al. 2001; Ralton and McConville 1998), imaged using a FLA3000 scanner (Fuji) and quantified using the Aida V3.11 software package.

### **Plant growth and harvesting of material**

*Arabidopsis thaliana* (Col-0) plants were grown under long-day conditions (16 h day/8 h night) and the following tissues were harvested at specific development stages (Boyes, et al. 2001). Rosette leaves were collected at stage 3.90, cauline leaves and stem were collected at stage 5.10, roots and flowers were collected at stage 6.50, siliques were collected at stage 8.00.

### **RNA Isolation and RT**

Plant tissue was harvested and immediately frozen in liquid nitrogen. Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen), including DNase I digestion, according to the manufacturers instructions.

RNA integrity was tested by electrophoresis on 1% agarose gels and was quantified by measuring the absorbance at 260 nm. The  $A_{260\text{ nm}}/A_{280\text{ nm}}$  ratios of purified RNA samples were in the range of 2.0 to 2.1 and were determined to be free of genomic DNA by the absence of larger, intron containing, PCR

products.

RT reactions were carried out on 1.0µg of total plant RNA using Superscript III reverse transcriptase (Invitrogen) and oligo dT primer according to the manufacturers instructions.

### **Real-Time qRT-PCR conditions and analysis**

Steady-state levels of RNA for each AtIPCS gene in plant material were analysed using SYBR green to monitor DNA synthesis with a Rotor-Gene RG-3000 (Corbett Research) instrument. Amplification was achieved with the following gene-specific primers; AtIPCS1-forward 5'-AGCCTCTTGATTATTGCGTC-3' and AtIPCS1-reverse 5'-AACAACGGCATTGCTCCCT-3' to give a 145 bp product; AtIPCS2-forward 5'-AGCCTCTTGATCATTGCCTC-3' and AtIPCS2-reverse 5'-GACTGCTGTGTTGCTCCCA-3' to produce a 145 bp product; AtIPCS3-forward 5'-TGGCTTATGGCAGTAATACAG-3' and AtIPCS3-reverse 5'-GCCAGAAATGGCAGAACGTTCT-3' to produce a 141 bp product. To determine the highest specificity and sensitivity qPCR profile for each AtIPCS transcript, titration experiments were performed over a range of MgCl<sub>2</sub> concentrations (1.5 to 5 mM) and primer concentrations from (0.1 to 0.5 µM). Concentrations were considered optimal at the lowest C<sub>T</sub> value that reproducibly gave an amplicon of the correct size in the absence of primer dimers. Based on results of these optimization experiments, qPCR assays for the AtIPCS1 transcript contained 3 mM MgCl<sub>2</sub> and 0.5 µM of each primer; the AtIPCS2 transcript 3 mM MgCl<sub>2</sub> and 0.4 µM of each primer; and the AtIPCS3 transcript 4 mM MgCl<sub>2</sub>, 0.4 µM of the forward and 0.5 µM of the reverse primer. The reactions (total volume 20 µl) also contained 2 µl of 10X PCR buffer, 10 pg of cDNA, 200 µM dNTPs, SYBR Green I dye and 2 Units of Taq polymerase. Assays were carried out using the following conditions: 1 cycle of 10 min of 95°C, followed by 30 cycles of 10 sec of 95 °C, 15 at 52 °C and 20 sec of 72 °C. Amplicon dissociation curves were recorded at the end of the PCR cycles. External standard curves were constructed using the three AtIPCS cDNAs and relevant gene-specific primer pairs and used to determine the number of transcripts for each gene.

## Results

### **AtIPCS1-3 complement an *AUR1* auxotrophic mutant yeast and confer aureobasidin A resistance**

Interrogation of the Arabidopsis genome database with the predicted protein sequence of the protozoan IPC synthase from *Leishmania major* (*LmIPCS*) (Denny, et al. 2006) identified opening reading frames encoding three highly related sequence orthologues – Arabidopsis IPC synthase 1 (*AtIPCS1* - At3g54020.1), *AtIPCS2* (At2g37940.1) and *AtIPCS3* (At2g29525.1) – demonstrating 26%, 29% and 31% identity to the protozoan protein. Subsequently, *AtIPCS2* has been isolated, characterised and designated ERH1 (Wang, et al. 2008). The open reading frames of *AtIPCS1-3*, when cloned into an URA3 selectable expression vector to create pRS426 *AtIPCS1-3*, restored the growth of the *AUR1* auxotrophic mutant YPH499-HIS-GAL-*AUR1*, as did the ectopic expression of *S. cerevisiae* *AUR1p* (*AUR1*) (figure 1A). These data indicated that *AtIPCS1, 2* and *3* are functional orthologues of fungal *AUR1p*, a protein that forms at least part of an IPC synthase (Nagiec, et al. 1997).

By diffusion assay (Nagiec, et al. 1997; Nagiec, et al. 2003) the efficacy of two different classes of inhibitor were assayed against YPH499-HIS-GAL-*AUR1* pRS426 *AtIPCS1, 2* and *3*. The transgenic yeast were resistant to aureobasidin A (AbA, a fungal IPC synthase inhibitor) at 100 $\mu$ M (figure 1B), a concentration shown to inhibit the growth of *LmIPCS* complemented yeast (Denny, et al. 2006), but remained sensitive to myriocin (which inhibits the first step in sphingolipid biosynthesis - serine palmitoyltransferase, SPT). Indeed, yeast complemented with the plant enzymes appeared to be more sensitive to the SPT inhibitor than the *AUR1* control, a similar observation had previously been made with regard to the *LmIPCS* complemented yeast (Denny, et al. 2006). The *in vitro* assay for *AtIPCS1-3* described below (figure 2A) confirmed that the Arabidopsis enzymes are AbA insensitive. In contrast, the IPCS activity from Golden butterwax bean extracts has been shown to be acutely

sensitive to this inhibitor (Bromley, et al. 2003). The reasons for this difference in AbA sensitivity are unclear and no data are currently available, for the bean sequence, to help elucidate this discrepancy.

### **AtIPCS1-3 are functional IPC synthases**

*In vitro* assay of microsomes prepared from YPH499-HIS-GAL-AUR1 pRS426 AtIPCS1-3 demonstrated that the three plant enzymes are active IPC synthases (figure 2A). However, their turnover appeared low when compared to an *S. cerevisiae* AUR1 control. This was also noted by Wang et al (2008) and was unaffected by the use of NBD C<sub>6</sub>-phytoceramide rather than C<sub>6</sub>-ceramide as acceptor substrate. In support of the data shown above (figure 1B), AtIPCS1-3 activity was insensitive to the addition of AbA at a concentration (5  $\mu$ M) that completely inhibited the yeast enzyme (figure 2A). Thereafter this inhibitor was applied to the reaction mix for assay of AtIPCS1-3 at 5  $\mu$ M.

In the experiment (figure 2A) above the plant (and yeast) enzymes are assayed in the presence of exogenous soybean PI. To ascertain the effect of adding this exogenous donor substrate samples were assayed with and without PI in the reaction mix (figure 2B). All samples showed a similar level of activity in the absence of donor substrate but, surprisingly, only AtIPCS2 demonstrated any significant increase in turnover on the addition of PI. This indicated that the AtIPCS1 and 3 are unable to utilize substrate from this source effectively. Similar results were obtained utilizing bovine PI which has previously been demonstrated to be effectively utilized by the protozoan orthologues from both *L. major* and *Trypanosoma brucei* but not by *S. cerevisiae* AUR1p (Mina, et al. 2009). Despite this, these data confirm AtIPCS1-3 as functional orthologues of AUR1p, forming at least part of an IPC synthase.

### **The plant IPC synthases form a new class of sphingolipid synthase**

The key motifs, D3 and D4, of the sphingolipid synthase family are conserved in the plant IPC synthases (Wang, et al. 2008). However, unlike their protozoan and mammalian counterparts they lack a D1 domain (Huitema, et al. 2004). In this they resemble the fungal IPC synthases and it may be hypothesized that D1 is involved in the binding of ceramide, a substrate for the protozoan and mammalian enzymes, whereas another unidentified region is involved in binding the phytoceramide substrate to the plant and fungal IPC synthases. Despite the conservation of some of these motifs the plant sequences are divergent with respect to primary sequence and so, until recently, remained cryptic within the genome databases (Wang, et al. 2008). This divergence was illustrated by phylogenetic analysis of predicted SM and IPC synthase sequences which demonstrated that the plant enzymes, the three orthologues identified in the *Oryza sativa* (rice) database are included, form a new clade of sphingolipid synthases (figure 3).

### **Expression profiling of AtIPCS1-3**

Estimates of the relative abundance of transcripts can be made from their representation within the EST databases. For the three AtIPCS genes described a similar number of AtIPCS1 and AtIPCS3 transcripts are present, whilst the largest number is for AtIPCS2 (Table 1; data derived from TAIR BLAST version 2.2.8). However, these data do not give any information regarding the tissue location of the transcripts and are therefore of limited use when investigating the expression of gene families. Tissue-specific transcript abundance data can be obtained from multiple microarray experiments using Genevestigator (Grennan 2006). Only AtIPCS2 is represented on the Affymetrix arrays that are used to derive these data and this shows that the gene is expressed in cauline leaves, roots and rosette leaves at similar levels and these are approximately twice the expression levels seen in stems, flowers and siliques (Table 2).

Given the limitations of these data sets, in order to establish a complete expression profile for each AtIPCS isoform, real time quantitative RT-PCR was performed on RNA isolated from various Arabidopsis tissues (figure 4).

AtIPCS2 was the most highly expressed of the three isoforms in all tissues. In agreement with the Genevestigator data, the highest levels of AtIPCS2 transcript were seen in root, rosette leaves and cauline leaves and these were five to ten fold greater than the levels of transcripts in siliques, stems and flowers. The patterns of expression determined by these two methods are in general agreement but the relative levels differ, this may reflect differences in the plant material used in the each case. Genevestigator uses a large number of microarray experiments to generate data and is therefore robust, however the data represents plant material grown under different conditions and which may have been harvested at different times within a developmental stage. The quantitative RT-PCR data presented here is determined from plants grown and harvested under identical conditions and therefore represents transcript levels at defined developmental stages in these tissues.

AtIPCS1 transcripts are expressed at low levels in all tissues, ranging from 0.05% of AtIPCS2 levels in rosette leaves to 2.8% of AtIPCS2 levels in stems. While AtIPCS3 transcripts are also present in low levels in cauline leaves, rosette leaves, roots and stems (ranging from 0.02% to 0.67% of AtIPCS2 transcripts) they are present at similar levels to AtIPCS2 transcripts in stem (84 % of AtIPCS2 levels) and flower (74% of AtIPCS2 levels).

The data presented here adds to existing studies by providing tissue specific expression data for all the isoforms of AtIPCS which may indicate a specific role for AtIPCS3 in stems and flowers.

## Discussion

The data presented describe the analyses of all three orthologues of the recently identified Arabidopsis IPC synthase (Wang, et al. 2008). They all represented functional orthologues of *S. cerevisiae* IPC synthase, AUR1p, and demonstrated activity in an *in vitro* assay. However, unlike the yeast activity the Arabidopsis enzyme is resistant to aureobasidin A, a non-competitive inhibitor of the fungal IPC synthase with an unknown mechanism of action (Zhong, et al. 1999). Conversely, it has previously been shown that the IPCS activity in Golden butterwax bean extracts was acutely sensitive to this inhibitor (Bromley, et al. 2003). The reasons for this discrepancy are unclear, however it is notable that similarly profound differences in aureobasidin A sensitivity have been observed within the kinetoplastid parasite IPCS orthologues. Whilst the *L. major* enzyme is relatively refractory to the drug, the *T. brucei* sphingolipid synthase (TbSLS) is highly sensitive (Mina, et al. 2009). No reason for this can be deduced from the primary sequence data and it may be envisaged that this diversity is due to subtle differences in 3-dimensional enzyme structure.

In the *in vitro*, microsome-based assay all three Arabidopsis isoforms demonstrated IPC synthase turnover. However, although the presence of PI (from soybean or bovine sources) led to a significant increase in turnover with AtIPCS2, AtIPCS1 and 3 were refractory to the addition of this donor substrate. This indicated that PI from these sources was utilized inefficiently by these Arabidopsis isoforms. Similarly, it has been noted that mammalian PI is not efficiently utilized by the *S. cerevisiae* IPC synthase activity (Mina, et al. 2009). Notably, unlike the yeast phospholipids (Guan and Wenk 2006), plant and mammalian PIs exhibit varying degrees of polyunsaturation (Thompson and MacDonald 1975; Thompson and MacDonald 1976). It may be hypothesized this polyunsaturation may confer structural constraints and make it less acceptable to the *S. cerevisiae* enzyme. However, the lack of reactivity of AtIPCS1 and with plant derived PI (soybean, C16:0-C18:2) is less easy to explain. It is possible that the endogenous yeast PI has a higher affinity for these isoforms and outcompetes the exogenous substrate.

Attempts to minimize the available quantity of endogenous PI to test this were made by detergent washing as previously (Mina, et al. 2009). However, this led to apparent inactivation of the enzymes. Alternatively, significant differences may exist in the substrate requirements of these Arabidopsis isoforms. Notably, AtIPCS1 transcript level is low in all plant tissue and AtIPCS3 in many, including siliques, perhaps reflecting the level of acceptable donor PI in seeds (such as soybeans, the source of PI in these experiments).

Despite these variances, all the plant IPC synthase orthologues identified share the motifs predicted to form the catalytic triad that promotes nucleophilic attack on lipid phosphate ester bonds which, in the case of IPC synthase, is thought to lead to the transfer of an inositol phosphate group from PI to the 1-hydroxyl group of (phyto)ceramide releasing diacylglycerol as a by-product (Huitema, et al. 2004; Neuwald 1997). Therefore it is likely that the eukaryotic sphingolipid synthases possess a common mechanism of action, indicating a single evolutionary origin. In contrast, the prokaryotic SM synthase lacks the catalytic triad (Luberto, et al. 2003) and is likely to have evolved independently. Phylogenetic analyses show the plant proteins forming a distinct clade, thereby defining a new class of eukaryotic sphingolipid synthases.

It has been demonstrated, by RNAi of the first enzyme in the biosynthetic pathway - serine palmitoyltransferase, that sphingolipid biosynthesis *per se* is essential for the viability of Arabidopsis (Chen, et al. 2006). Furthermore, recent work has suggested that the plant IPC synthase is pivotal in pathogen-resistance associated PCD. By analyses of leaf tissue from an ERH1 (AtIPCS2) insertion mutant Wang et al (2008) detected a significant increase in ceramide levels, an observation consistent with the fact that ceramide is a substrate for IPCS. Importantly, ceramide is a key regulator of PCD (Futerman and Hannun 2004) and the accumulation of this factor was coincident with PCD at the site of powdery mildew infection in Arabidopsis (Wang, et al. 2008). This work is further supported by similar observations in accelerated-cell-death (*acd11* and *acd5*) mutants of Arabidopsis in which a sphingosine transfer protein (ACD11) or a ceramide kinase (ACD5) are affected (Brodersen, et al. 2002; Liang, et al. 2003). Given the prominence of

AtIPCS2 as a putative regulator of PCD the differential expression levels of each of the three functional Arabidopsis orthologues may shed light on this key process in the whole plant.

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**Table 1**

**EST numbers for AtIPCS genes derived from TAIR BLAST version 2.2.8.**

	EST
AtIPCS1	32
AtIPCS2	57
AtIPCS3	25

**Table 2**

**Microarray Data for AtIPCS2 in the Genevestigator database**

Root	Rosette Leaf	Cauline Leaf	Stem	Flower	Siliques
2383	2135	2509	1167	998	705

## Figure 1

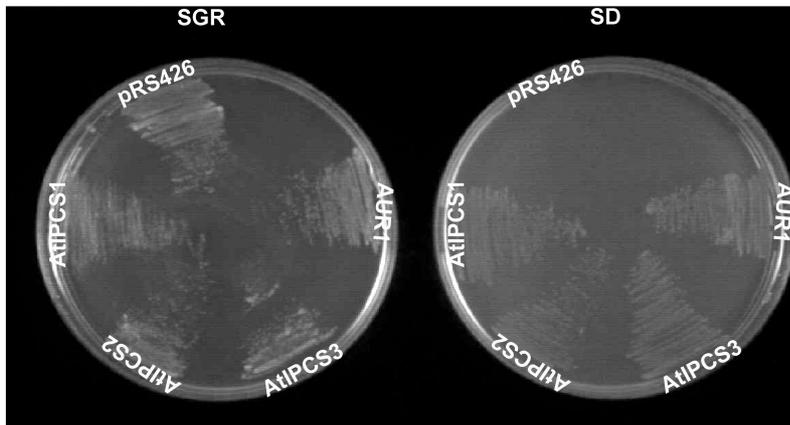
### **AtIPCS1-3 complements a yeast *AUR1* auxotrophic mutant and confers aureobasidin A resistance**

- A. YPH499-HIS-GAL-AUR1 auxotrophic yeast transformed with pRS246MET25 either empty or bearing AUR1 or AtIPCS1-3 and grown under permissive (SGR) or non-permissive conditions (SD).
- B. 100 $\mu$ M aureobasidin A (AbA) in DMSO, 1mM myriocin (Myr) in DMSO and DMSO alone spotted in 2 and 3  $\mu$ l quantities onto YPH499-HIS-GAL-AUR1 complemented with AUR1 or AtIPCS1-3 on non-permissive SD plates.

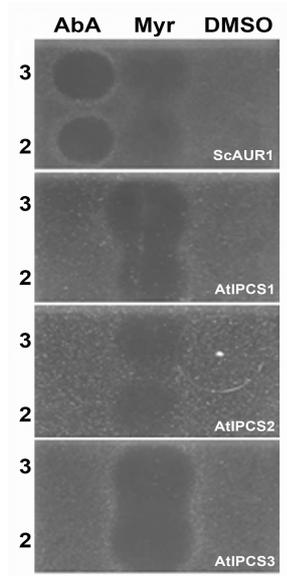
pRS426 – YPH499-HIS-GAL-AUR1 pRS426MET25; AUR - YPH499-HIS-GAL-AUR1 pRS426 ScAUR1; AtIPCS1-3 - YPH499-HIS-GAL-AUR1 pRS426 AtIPCS1-3. Grown on either permissive (non-glucose containing) SGR -HIS -URA or non-permissive (glucose containing) SD -HIS -URA.

Figure 1

A



B



## Figure 2

### AtIPCS1-3 encode functional IPC synthases

*In vitro* assay of microsomal extracts from YPH499-HIS-GAL-AUR1 pRS246 AtIPCS1-3 and YPH499-HIS-GAL-AUR1 pRS246 AUR1.

- A. Assay in the presence of phosphatidylinositol (PI) with (+) or without (-) the specific fungal inhibitor aureobasidin A (AbA). In contrast to the *S. cerevisiae* IPC synthase (AUR1), the plant activity encoded by AtIPCS1-3 is insensitive to AbA at 5  $\mu$ M.

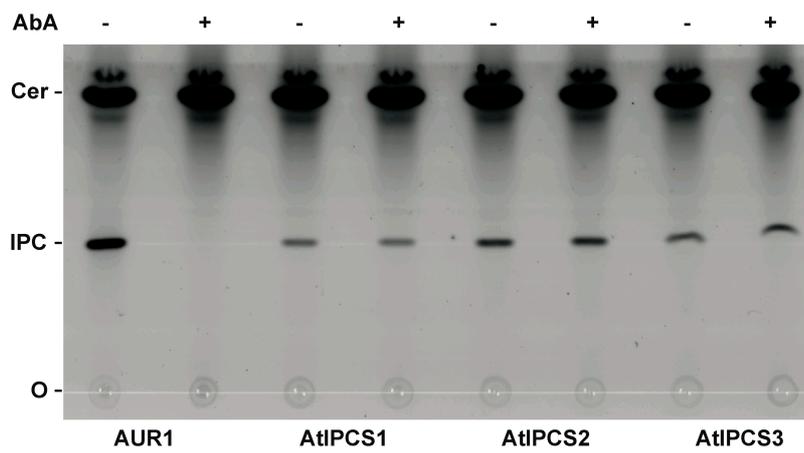
Markers of NDB C<sub>6</sub>-ceramide and NBD C<sub>6</sub>-IPC, and the origin (O) indicated.

- B. Assay with (+) or without (-) donor substrate PI in the presence of 5  $\mu$ M AbA. Enzyme turnover is only significantly enhanced by the addition PI in the case of AtIPCS2.

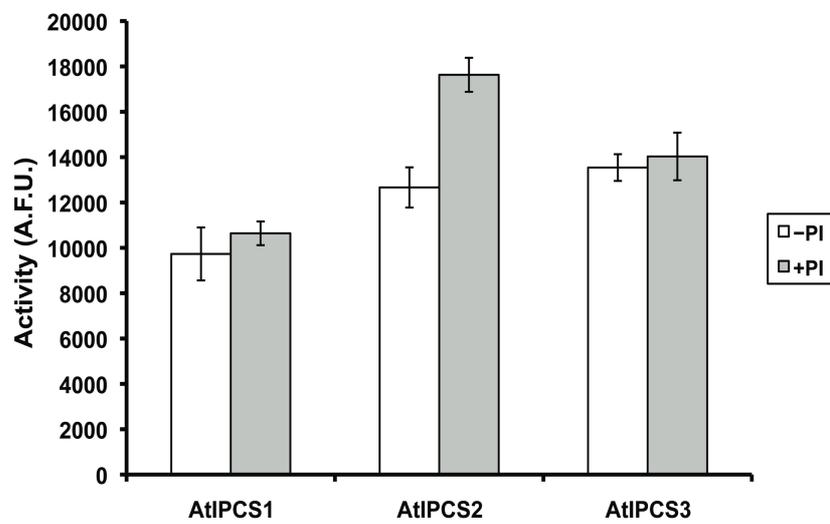
Mean of 3 separate experiments, standard error shown. A.F.U. – Arbitrary Fluorescence Units.

Figure 2

A



B



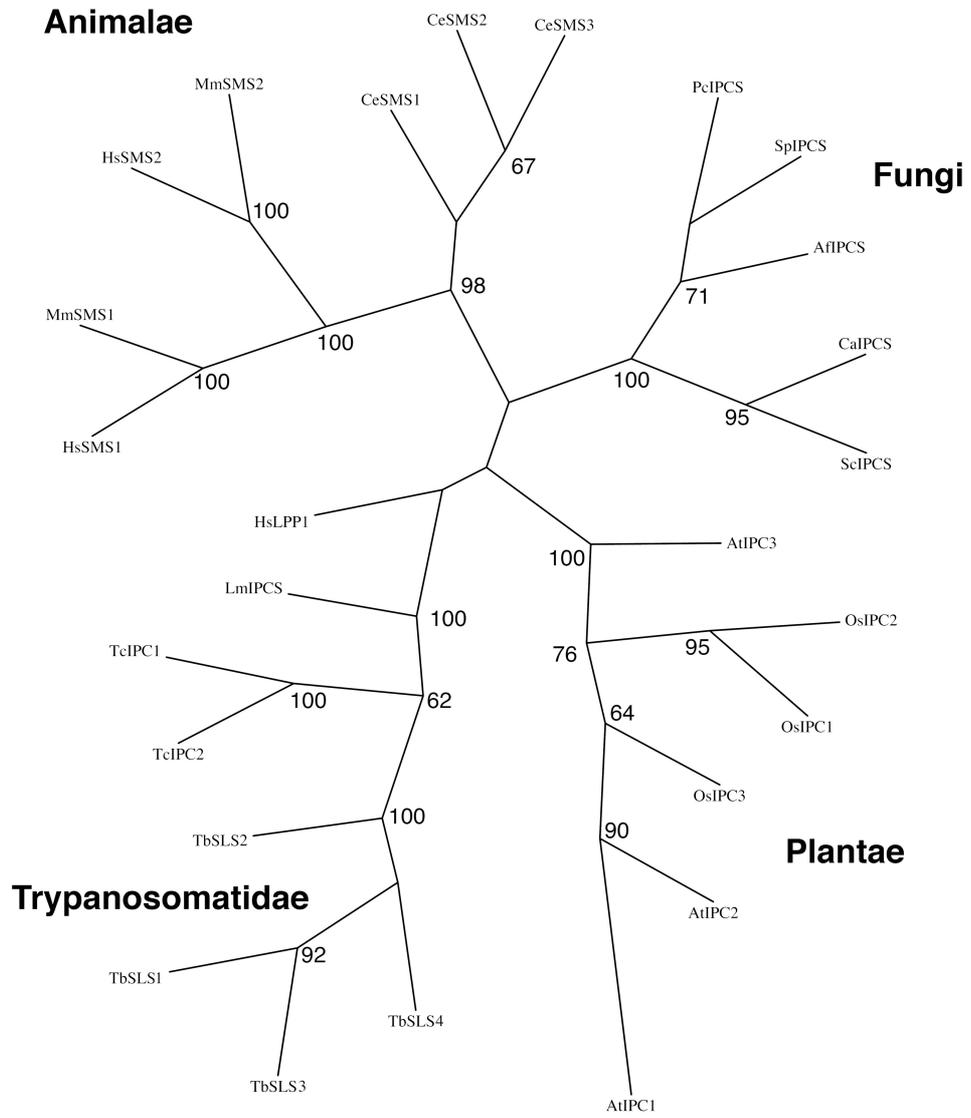
### Figure 3

#### The plant IPC synthase defines a new class of sphingolipid synthases

Maximum parsimony analysis of Animalae, Fungi, Trypanosomatidae and Plantae sphingolipid synthase sequences. Bootstrap scores >60 indicated.

*Homo sapiens* LPP1 (outgroup) accession number: O14494; *Arabidopsis thaliana* IPCS1-3 accession numbers: At3g54020.1, At2g37940.1, At2g29525.1; *Oryza sativa* IPCS1-3: NP\_001044812, NP\_001055712, NP\_001055096; *T. brucei* SLS1-4: Tb09.211.1030, Tb09.211.1020, Tb09.211.1010, Tb09.211.1000; *T. cruzi* IPCS1&2: Tc00.1047053506885.124, Tc00.1047053510729.290; *L. major* IPCS: LmjF35.4990; *Aspergillus fumigatus* AUR1p: AAD22750; *Candida albicans* AUR1p: AAB67233; *Pneumocystis carinii* AUR1p: CAH17867; *Saccharomyces cerevisiae* AUR1p: NP\_012922; *Schizosaccharomyces pombe* AUR1p: Q10142; *Caenorhabditis elegans* SMS1-3: Q9U3D4, AAA82341, AAK84597; *Homo sapiens* SMS1&2: AB154421, Q8NHU3; *Mus musculus* SMS1&2: Q8VCQ6, Q9D4B1.

Figure 3



## **Figure 4**

### **Steady-state levels of AtIPCS isoform mRNAs in plant tissues**

The numbers of AtIPCS1-3 transcripts were determined in total RNA from various tissues, by quantitative RT-PCR using a standard curve for each transcript. AtIPCS2 (At2g37940.1) is the most abundant form in roots, leaves and siliques, whereas stems and flowers have similar numbers of AtIPCS2 and AtIPCS3 (At2g29525.1) transcripts. AtIPCS1 (At3g54020.1) transcript is uniformly low in all tissues.

Figure 4

