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Targeting a promising new herbicide mode of action: chemical and genetic approaches to elucidate the role of IPC synthase in plants

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy from the University of Durham by

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September 2017

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Targeting a promising new herbicide mode of action: chemical and genetic approaches to elucidate the role of IPC synthase in plants

ELIZABETH C. E. PINNEH

Abstract

Worldwide there are currently 479 recorded unique cases of herbicide resistant weeds, with 251 species (146 dicots and 105 monocots). According to current records, weeds have developed resistance to 22 of the 25 known herbicide sites of action and to 157 different herbicides. Research into sphingolipid synthesis has revealed a potential novel site of action involving the non-mammalian enzyme inositol phosphorylceramide synthase (IPCS). Two approaches to understanding the function of the enzyme in plants have been employed in this study. The genetic approach, which involves the overexpression and knockdown of the three IPCS orthologues in *Arabidopsis thaliana*, has given insights into the global function of the enzyme in plants at a phenotypic and transcriptomic level. Analyses of the RNASeq data show that *At*IPCS is a global negative regulator of plant defense, downregulating genes involved in plant defense against herbivory attack, nematodes, fungal and bacterial pathogens. RNASeq data in conjunction with phenotypic data, indicate that IPCS may play a role in floral transition from the vegetative phase to the reproductive phase, with the transgenic lines displaying an early flowering phenotype. *At*IPCS transcript levels is also shown to affect plant post-embryonic development. In parallel, high throughput screening facilitated the identification of chemical inhibitors of plant IPCS. Using a system of *Arabidopsis thaliana* as a model dicot and *Oryza sativa* as model monocot, two classes of inhibitors were identified and demonstrated differential activity: the phenylamidines and triazinones. In addition, *in vivo* screening of the phenylamidines demonstrated herbicidal activity, indicating that the necessary selectivity for herbicide development is achievable.

Table of contents

| | |
|---|----|
| Abstract | 3 |
| Abbreviations | 7 |
| Declaration | 8 |
| Statement of copyright | 8 |
| Acknowledgements | 9 |
| Dedication | 10 |
| CHAPTER 1 | 11 |
| Introduction..... | 11 |
| 1.1 Thesis synopsis | 12 |
| 1.2 Crop production and herbicide resistance | 13 |
| 1.2.1 Health and environmental concern of herbicide usage | 18 |
| 1.3 Sphingolipids | 19 |
| 1.3.1 LCB Variation..... | 21 |
| 1.3.2 Sphingolipid diversity in plants | 23 |
| 1.3.2.1 Glycosyl inositol phosphoceramides | 24 |
| 1.3.2.2 Glycosylceramides | 27 |
| 1.3.2.3 Ceramide | 28 |
| 1.3.2.4 LCB..... | 29 |
| 1.4 Sphingolipid biosynthetic pathway | 29 |
| 1.4.1 The <i>de novo</i> pathway | 30 |
| 1.4.2 Salvage pathway | 32 |
| 1.5 Programmed cell death in plants | 33 |
| 1.6 IPC Synthase..... | 35 |
| 1.6.1 IPCS topology..... | 36 |
| 1.6.2 Catalytic mechanism..... | 37 |
| 1.7 Targeting IPCS | 39 |
| 1.7.1 Anti-protozoal and anti-fungal applications..... | 40 |
| 1.7.2 Herbicide application | 42 |
| 1.7.3 Inhibitors of IPCS | 43 |
| 1.8 Project aims | 44 |
| 1.9 References..... | 46 |
| CHAPTER 2 | 54 |
| Construction of plant IPCS complemented yeast strain and assay development | 54 |
| 2.1 Chapter synopsis | 55 |
| 2.2 Bioinformatics analysis of plant IPCS | 56 |
| 2.2.1 Phylogenetic analysis..... | 57 |
| 2.2.2 Prediction of transmembrane topology | 59 |
| 2.2.3 Identification of cis-elements and transcription factors upstream of <i>At</i> IPCS promoter..... | 63 |

| | |
|---|-----|
| 2.3 Cloning and expression of plant IPCS to complement a mutant yeast strain..... | 66 |
| 2.3.1 Complementation Assay | 69 |
| 2.3.2 Diffusion Assay | 70 |
| 2.3.3 Verification of <i>At</i> IPC3 and <i>Os</i> IPC3 activity | 72 |
| 2.5 Conclusion | 81 |
| 2.6 References..... | 82 |
| CHAPTER 3..... | 85 |
| Screening for inhibitors of plant IPCS..... | 85 |
| 3.1 Chapter synopsis | 86 |
| 3.2 Approaches to screening for inhibitors | 87 |
| 3.3 Hypothetical effects of an IPCS inhibitor | 88 |
| 3.4 Primary screening | 92 |
| 3.5 Secondary screening | 101 |
| 3.6 <i>In vivo</i> screening | 112 |
| 3.7 Conclusion | 114 |
| 3.8 References..... | 115 |
| CHAPTER 4 | 118 |
| Validation of IPCS transgenic lines for functional genomics analyses..... | 118 |
| 4.1 Chapter synopsis | 119 |
| 4.2 RNA interference, over-expression and localisation of <i>At</i> IPC3-1 using gateway cloning..... | 120 |
| 4.2.1 <i>At</i> IPC3-1 localisation..... | 121 |
| 4.2.2 <i>At</i> IPC3-1 RNAi transgenic lines..... | 124 |
| 4.2.3 <i>At</i> IPC3-1 overexpression transgenic lines | 130 |
| 4.3 Phenotypic analysis of transgenic lines | 132 |
| 4.4 Transcript quantification of <i>At</i> IPC3 homozygous lines from European seed bank | 136 |
| 4.5 Conclusion | 137 |
| 4.6 References..... | 138 |
| CHAPTER 5..... | 139 |
| RNASeq analyses..... | 140 |
| 5.1 Chapter synopsis | 140 |
| 5.2 Analyses of the transcriptome..... | 140 |
| 5.3 Genes downregulated in response to the overexpression of <i>At</i> IPC3, 2 and 3 | 144 |
| 5.5 Genes upregulated in response to the overexpression of <i>At</i> IPC3, 2 and 3..... | 162 |
| 5.6 Genes downregulated in response to <i>At</i> IPC3 homozygous knock down..... | 176 |
| 5.7 Genes upregulated in response to a decrease in <i>At</i> IPC3 expression | 185 |
| 5.8 Conclusion | 189 |
| 5.9 References..... | 191 |
| CHAPTER 6..... | 198 |
| 6.1 Conclusion | 199 |
| 6.2 Future work..... | 201 |

| | |
|---|-----|
| 6.3 References..... | 204 |
| CHAPTER 7..... | 205 |
| 7.1 Experimental section..... | 206 |
| 7.1.1 Materials | 206 |
| 7.1.2 Instruments and Equipment | 208 |
| 7.2 Table 7-1: Buffer composition..... | 209 |
| 7.3 Table 7-2 Media composition..... | 209 |
| 7.4.1 DNA Extraction from Bacteria | 212 |
| 7.4.2 RNA Extraction from plant..... | 212 |
| 7.4.3 cDNA synthesis | 212 |
| 7.4.4 Quantitative reverse-transcription real-time PCR | 213 |
| 7.4.5 Polymerase chain reaction protocol..... | 214 |
| 7.4.6 T4 ligase cloning..... | 214 |
| 7.4.7 Gateway cloning | 215 |
| 7.4.8 In-Fusion Cloning | 216 |
| 7.5.1 Frozen Stocks..... | 217 |
| 7.5.2 Yeast Transformation..... | 217 |
| 7.5.3 Plasmid Shuffle..... | 218 |
| 7.5.4 Yeast culture scale-up | 218 |
| 7.5.5 Preparation of crude microsomal membranes..... | 218 |
| 7.5.6 Diffusion Assay | 219 |
| 7.5.7 High performance thin layer chromatography (HPTLC) assay | 220 |
| 7.6.1 Frozen stocks for screening | 220 |
| 7.6.2 Primary screen, cellular assay..... | 220 |
| 7.6.3 Secondary screen, enzyme assay | 221 |
| 7.7.1 Agrobacterium transformation | 222 |
| 7.7.2 Agrobacterium mediated plant transformation | 222 |
| 7.7.3 Plant growth conditions | 223 |
| 7.7.5 Tobacco infiltration..... | 223 |
| 7.7.6 <i>In-vivo</i> screening..... | 224 |
| 7.9.1 RNASeq Experiment | 225 |
| 7.9.2 RNASeq analysis | 225 |
| APPENDICES | 228 |
| APPENDIX A – Transcription factor binding site motif sequence | 229 |
| APPENDIX B – pESC-LEU vector map..... | 230 |
| APPENDIX C – Vector maps of expression plasmids used for plant genetic work..... | 231 |
| APPENDIX D - Structure of compounds tested against <i>AtIPCS1</i> complemented yeast strains..... | 233 |
| APPENDIX E – Primer sequences | 234 |
| APPENDIX F – RNA quality control for RNASeq experiment..... | 236 |

Abbreviations

| | |
|-------------------|---|
| IPCS | Inositol phosphorylceramide |
| IPCS | Inositol phosphorylceramide synthase |
| ALS | Actolactate synthase |
| ACCase | Acetyl CoA carboxylase |
| PS | Photosystem |
| PI | Phosphoinositol |
| EPSP | 5-enolpyruvylshikimate-3-phosphate synthase |
| HPPD | p-Hydroxyphenylpyruvate dioxygenase |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| LCB | Long-chain base |
| GSL | Glycosphingolipids |
| VLCFAs | Very long chain fatty acids |
| GluCer | Glycosylceramide |
| GIPC | Glycosyl inositol phosphoceramide |
| AUR1p | AUR1 protein (fungal IPCS) |
| AtIPCS | <i>Arabidopsis thaliana</i> inositol phosphorylceramide synthase |
| OsIPCS | <i>Oryza sativa</i> inositol phosphorylceramide synthase |
| LmjIPCS | <i>Leishmania major</i> inositol phosphorylceramide synthase |
| FDGlu | Fluorescein di-(β -D-glucopyranoside) |
| AbA | Auerobasidin A |
| DMSO | Dimethyl sulfoxide |
| PDR | Pleotropic drug resistance |
| BLAST | Basic Local Alignment Search Tool |
| IC ₅₀ | Half-maximal Inhibitory Concentration |
| PCR | Polymerase chain reaction |
| HTS | High Throughput Screening |
| Monocot | Monocotyledons |
| Dicot | Dicotyledons |
| SOC1 | Suppression of overexpression of <i>CONSTANS 1</i> |
| SM | Sphingomyelin |
| SMS | Sphingomyelin Synthase |
| 5FOA | 5-fluoroorotic acid |
| NBD ceramide | N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ceramide |
| NBD-IPC | N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-inositol phosphorylceramide |
| NBD-phytoceramide | N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phytoceramide |

Declaration

The work presented herein was carried out by the author in the Department of Chemistry and in the Department of Biosciences at the University of Durham from October 2013-September 2017. Some parts of the work were carried out by the author at Bayer Crop Science, Frankfurt, Germany, from February 2016-June2016 and in March 2017. No part of this work has been submitted for any other degree at this or any other University.

Statement of copyright

The copyright of this thesis rests with the author. No quotation from it should be published without the author's prior written consent and information derived from it should be acknowledged.

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Dedication

I dedicate this work to my grandparents:

Lucy and Dumbiri Ogbu

Florence Omolabake and Anthony Abari Pinneh

CHAPTER 1

INTRODUCTION

1.1 Thesis synopsis

The aim of this work was to characterise the role of inositol phosphorylceramide synthase (IPCS) in plants, and to identify putative inhibitors of the enzyme, that can be further developed for use as herbicides. In Chapter 1, an introduction to the need for the identification of new herbicide mode of action is outlined, as is the reasoning for pursuing IPCS as an herbicidal target. In this chapter, sphingolipid structural variation and biosynthesis, in plants and mammals is reviewed and known inhibitors of IPCS orthologues in fungi and protozoa are examined. In addition, current understanding of the role of sphingolipids in relation to programmed cell death in plants is presented. In chapter 2, bioinformatics analysis is carried out on the plant IPCSs from different plant species to glean information on how similar or different they are from each other, and to find out biological pathways they could affect by analysis of transcription factor binding sites in the promoter region. In that same chapter, assay parameters for the screening campaign were developed and validated. In chapter 3 and 4, results from the primary and secondary screening, as is the work done to validate *AtIPCS1-3* over-expresser and RNAi transgenic lines in *A. thaliana* is shown. In chapter 5, RNASeq analysis of *AtIPCS1* t-DNA mutant and transgenic lines over-expressing *AtIPCS1*, 2 and 3 are presented which reveal the role of plant *AtIPCS*. Conclusions and future work are discussed in chapter 6 and in chapter 7 the methods and materials are covered.

1.2 Crop production and herbicide resistance

World food production is heavily reliant on the use of herbicides and pesticides to reduce the detrimental effects of pests and weeds on crop yield. With global population projected to increase by 2 billion by the year 2050¹, there comes the need for increased agricultural production of staple crops which the global community rely on to survive. It is currently estimated that 12.5% of the global population are undernourished¹, this could increase in the coming years due in part to the unfolding effects of climate change on agricultural output. It is also projected that by the 2080s agricultural yield in developing countries will decline by 9% due to high carbon emissions even when the benefit of carbon fertilization is taken into consideration, and on a global scale this decrease in yield will be 3%, with yield decrease being greatest in South East Asia and sub-Saharan Africa² (Figure 1-1).

A study conducted by the World Bank study forecasted 50 million more people could be at risk of undernourishment because of climate change by the year 2050³; without mitigating measures to combat climate change the number of malnourished people globally is set to grow to 321 million within the 2050 decade and by 391 million in the decade of 2080⁴, with the most affected areas being developing countries.

Cereal crops are the most common food source for human consumption. According to the Food and Agricultural Organization of the United Nations (FAO) 2013 report approximately 2.3 billion tonnes of cereals are currently produced globally¹; roughly 1 billion tonnes is destined for food use, 750 million tonnes will be employed as animal feed, and the remaining 500 million tonnes will be processed for industrial use as seed or wasted¹. The growth rate of world cereal production fell to 1 percent per annum in the 1990s, down from 1.6 percent in the 1980s and almost 3 percent in the 1970s; between 2000 and 2003, growth was almost zero due to low prices and a surplus in stock¹.

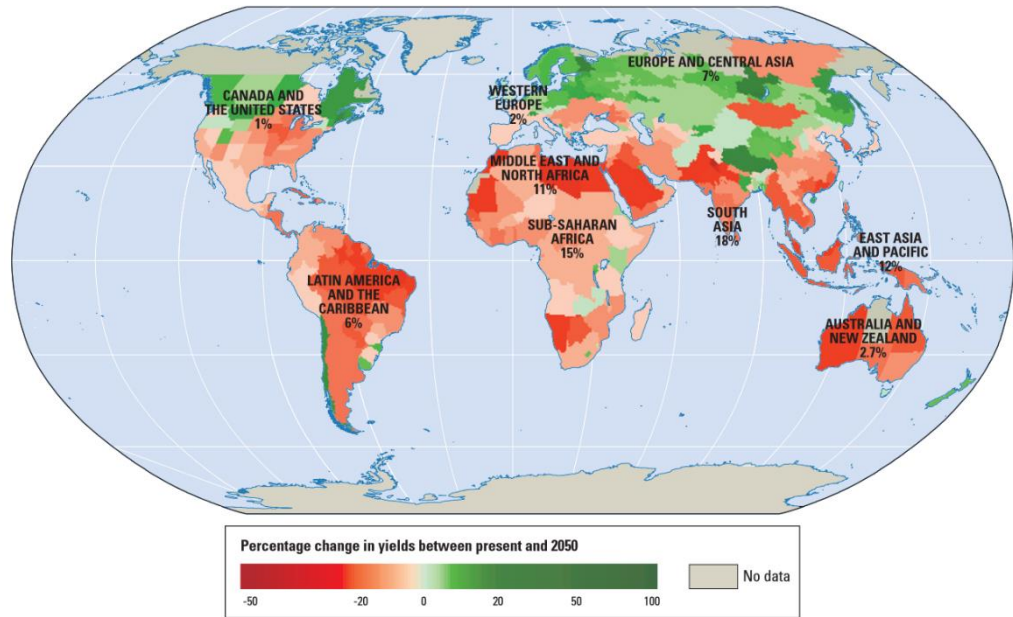


Figure 1-1: Global map of agricultural production projected for the years 2046-2055².

The challenge of increasing crop yield globally is further compounded by the decrease in available arable land; currently 40% of arable land is used to produce staple food which is expected to decrease to 30% by 2025 in order to provide land for renewable resources, and to feed companion and farm animals⁵. Another challenge is combating pesticide resistance which encompasses all plant protection agents, including but not restricted to fungicides, insecticides and herbicides. Global usage of pesticides is approximately 2 million tonnes, with 45% of this usage accounting for Europe, 25% in the US and the other 30% in the rest of the world⁶. The reliance on plant protection agents, in particular herbicides, which account for 47.5% of worldwide pesticide consumption⁶, mirrors the changes in farming practices from mechanical to chemical solutions for combating weeds. This has resulted in widespread weed resistance to herbicides with deleterious effects on crop yield. The control of weeds up until the end of the 20th century was primarily achieved by physical and mechanical removal of weeds combined with crop rotation⁷. The research conducted in World War II for biological warfare and research into

chemicals that could control insects carrying malaria, paved way for the synthetic chemical industry and modern herbicides⁸.

Herbicides are classified by activity, chemical family and site of action. The classification of herbicides by their site of action refers to the target enzyme and the subsequent effects on biochemical pathways as a result of inhibiting that enzyme. There are currently 21 known sites of action (Table 1) with some sites of action being unknown for certain herbicides such as Difenzoquat.

The first herbicide discovered was in 1940 by W. G. Templeman. 2,4-Dichlorophenoxyacetic acid (2,4-D)⁹ a plant hormone, was found to selectively kill broad-leaved weeds in the midst of cereal crops¹⁰. This was followed by the discovery and development of atrazine (Figure 1-2) from the triazine class of herbicides used on broadleaf weeds amongst maize and sugarcane⁹. The introduction of the herbicide glyphosate (N-(phosphonomethyl)glycine) by Monsanto in 1974 under the trade name 'Roundup' revolutionized the use of herbicides for farming in America; this herbicide was found to be non-specific, killing a wide range of weeds including grasses, broadleaf weeds and woody plants⁹. The subsequent introduction of glyphosate resistant crops resulted in its widespread use in both crop and non-crop lands¹¹ for over three decades. Recently, it has been found that there is widespread glyphosate resistance across America, a consequence of the evolution of glyphosate resistant weed biotypes found in rigid and Italian ryegrass, mareetail, goosegrass, common ragweed, waterhemp and velvet leaf¹¹. This glyphosate resistance has been found in the majority of soybean, cotton and corn farms and has been reported at 69% of 144 waterhemp population sites sampled in Missouri and 64% of 500 in Iowa in the year 2011-2012¹².

Glyphosate resistance has affected crop yield and profits and has resulted in the use of alternative herbicides to kill resistant biotypes. The cost of using herbicides in the south

Table 1: Classification of herbicides based on site of action, adapted from Heap 2017²

| Herbicide group | Site of action | Example of Herbicide |
|--|---|-----------------------------------|
| ALS inhibitors | Inhibits acetolactate synthase the key enzyme in the biosynthesis of branched amino acids isoleucine, leucine and valine | Chlorsulfuron |
| Photosystem II inhibitors | Inhibits photosynthesis by binding to the QB binding site on D1 protein of photosystem II complex in the thylakoid membrane | Atrazine |
| ACCase inhibitors | Inhibits acetyl CoA carboxylase which is involved in the de novo synthesis of fatty acids | Sethoxydim |
| PSI Electron Diverter | Herbicides accept electrons from photosystem II and are reduced to form herbicide radicals | Paraquat |
| Synthetic Auxins | Acts similar to endogenous auxins but the mechanism is not well understood; they acidify the cell wall by stimulating the activity of membrane bound ATPase pumps | 2,4-D |
| EPSP synthase inhibitors | Inhibits 5-enolpyruvylshikimate-3-phosphate synthase resulting in depletion of aromatic amino acids needed in biosynthetic pathways involved in growth | Glyphosate |
| PSII inhibitor (Ureas and amides) | Urea and amides inhibits photosynthesis by binding to the QB binding site on D1 protein of photosystem II complex in the thylakoid membrane | Chlorotoluron |
| Microtubule inhibitors | Binds to tubulin thereby inhibiting microtubulin polymerization at the assembly end | Trifluralin |
| Lipid Inhibitors (thiocarbamates) | Inhibitors of several plant processes including biosynthesis of fatty acids, lipids, proteins, isoprenoids, flavanoids and gibberellin | Triallate |
| PPO inhibitors | Inhibits Protoporphyrinogen oxidase involved in chlorophyll and heme biosynthesis | Oxyfluorfen |
| Carotenoid biosynthesis (unknown target) | Targets enzymes involved in carotenoid biosynthesis | Amitrole |
| PSII inhibitors (Nitriles) | Nitriles inhibit photosynthesis by binding to the QB binding site on D1 protein of photosystem II complex in the thylakoid membrane | Bromoxynil |
| Long chain fatty acid inhibitors | Inhibit the synthesis of very long chain fatty acids (VLCFA) | Butachlor |
| Carotenoid biosynthesis inhibitors | Inhibition of phytoene desaturase, an enzyme involved in carotenoid biosynthesis | Diflufenican |
| Antimicrotubule mitotic disrupter | Unknown mode of action | Flamprop-methyl |
| HPPD inhibitors | Inhibits p-Hydroxyphenylpyruvate dioxygenase | Isoxaflutole |
| Glutamine synthase inhibitors | Inhibits glutamine synthase which converts ammonia and glutamate to glutamine. | Glufosinate-ammonium |
| Cellulose inhibitors | Inhibits enzymes involved in cell wall biosynthesis | Dichlobenil |
| Mitosis inhibitors | inhibits cell division, microtubule organisation and polymerization | Propham |
| Cell elongation inhibitors | Unknown mode of action | Difenzoquat |
| Nucleic acid inhibitors | Unknown mode of action | Monosodium methanearsonate (MSMA) |

of America has increased from \$50-\$75 per hectare to about \$370 per hectare as a result of glyphosate resistance¹².

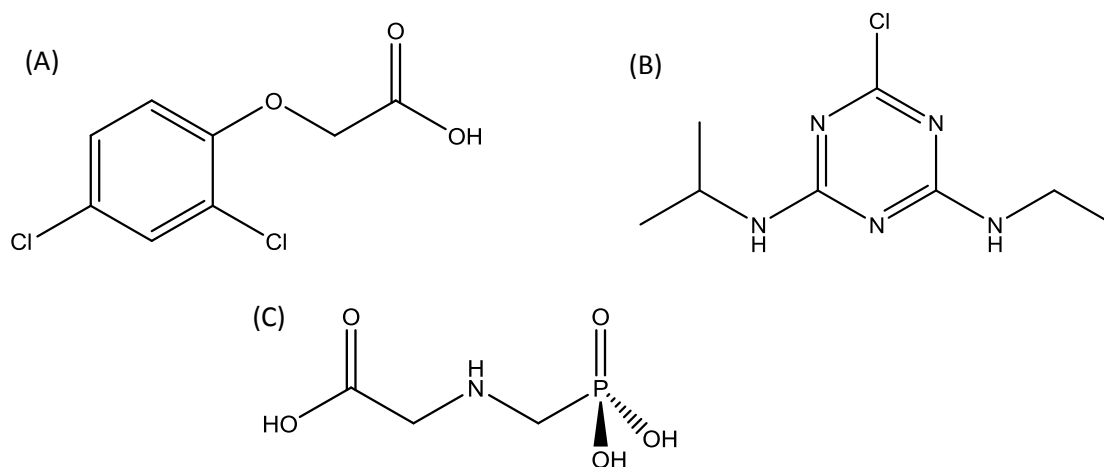


Figure 1-2: Chemical structures of first generation herbicides (A) 2,4-Dichlorophenoxyacetic acid (2,4-D) (B) Atrazine (C) Glyphosate.

Areas farming cotton have been heavily affected resulting in a shift away from growing cotton with a 60% and 70% decrease in area growing cotton in Tennessee and Arkansas respectively¹². According to the International Survey of Herbicide-Resistant Weeds (<http://www.weedscience.org>), there are currently 430 unique cases of herbicide resistant weeds globally, with resistance to two or more herbicides found in 235 species (138 dicots and 97 monocots)¹³. It has also been recorded that weeds have evolved resistance to 22 of the 25 known herbicide sites of action and to 154 different herbicides. Herbicide resistant weeds have been reported in 82 crops in 65 countries¹³. The Poaceae family have 74 recorded cases of weed resistance with 21 cases reported in the Brassicaceae family to date. The yield of staple crops are affected by herbicide resistant weed species; wheat is affected the most with 65 herbicide resistance species, followed by corn, rice and soybeans which have 58, 50 and 46 herbicide resistant species respectively¹³. Globally there has been a rapid increase in the number of unique resistance cases between the years 1975-2014 and the highest recorded number of herbicide-resistant weeds are recorded in the US (145), followed by Australia (69), Canada (60), China (37) and France (35)¹³.

The shift in farming methods in developing countries from mechanical removal of weeds to the use of herbicides means that herbicide resistance is a global problem. Countries such as China and Brazil now rely heavily on herbicides for weed control and are reporting a rapid increase in herbicide resistance cases with China currently ranked fourth in the world with 37 cases and Brazil ranked joint seventh with Germany on 31 cases. The full extent of herbicide resistance in most parts of Africa and Asia is not yet known, with no recorded cases in most countries on both continents.

1.2.1 Health and environmental concern of herbicide usage

In addition to herbicide resistance another major cause for concern in the use of herbicides are the health and environmental effects. The triazine herbicides have been linked to an increased risk of breast cancer¹⁴, and phenoxy herbicides contaminated with dioxins have also been linked to mortality of workers exposed to the herbicide from diseases including neoplasms, soft-tissue sarcoma, and non-Hodgkin's lymphoma¹⁵. Some herbicides have been found to contaminate groundwater; a study carried out by Thurman and colleagues in the United States found that several herbicides including atrazine, alachlor and simazine exceeded the Environmental Protection Agency promulgated maximum contaminant levels for drinking water¹⁶.

The health and environmental concerns of herbicide use combined with the growing cases of herbicide resistance on a global scale has led to a search for new chemically formulated herbicides with a specific site of action and minimal impact on the environment, animals and humans. The advent of research into sphingolipid synthesis has brought about a possible new site of action involving the non-mammalian enzyme, inositol phosphorylceramide synthases (IPCS). This enzyme catalyzes the transfer of phosphoinositol from phosphatidylinositol to the C-1 hydroxyl group of phytoceramide thereby generating the precursor inositol phosphorylceramide (Figure 1-3), for the

synthesis of the major sphingolipids in plants, glycosyl inositol phosphoceramides (GIPC). Exploitation of the divergence in sphingolipid biosynthesis in plants and animals can be used to create herbicides with minimal risks to mammals; fine-tuning of an IPCS inhibitor could result in the selective inhibition of monocots over dicots or vice versa, creating the pretext for the formation of herbicides that specifically kill certain types of weeds.

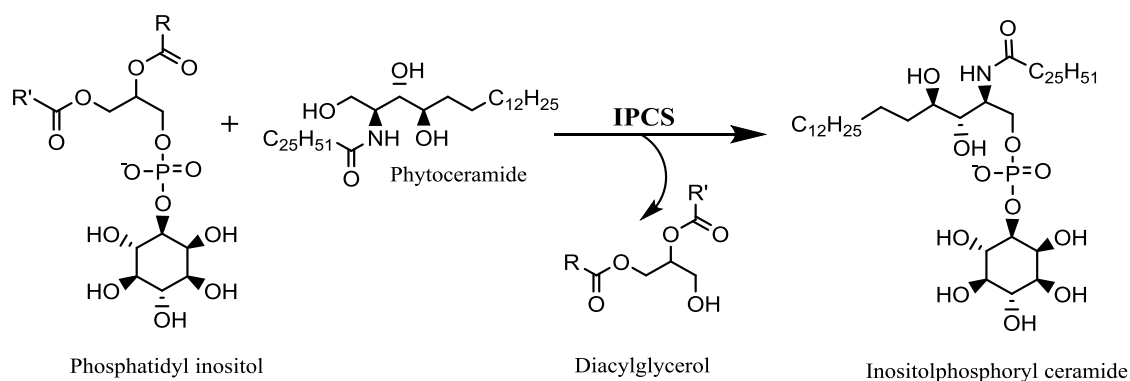


Figure 1-3: IPCS catalyses the transfer of phosphoinositol to phytoceramide thereby generating inositol phosphoryl ceramide and the by-product diacylglycerol.

1.3 Sphingolipids

Sphingolipids are a class of lipids that are essential components of all eukaryotic cell membranes and have been found to be crucial for survival across all kingdoms. In mammals they have been found to be involved in embryogenesis¹⁷, differentiation¹⁸, cell adhesion¹⁹, and signal transduction^{20, 21}. The model organism *Saccharomyces cerevisiae* has an abundance of sphingolipids comprising about 30% of the phospholipids found in the plasma membrane and these have been shown to be essential in growth regulation and cell integrity²²⁻²⁴. This class of lipids were first identified by Johann Tudichum during research into the effects of cholera on the brain²⁵. The findings of his research published under the title ‘a treatise on the chemical constitution of the brain’ in 1884 identified sphingomyelin, cerebroside and sulfatide, which were isolated from fractional crystallization of ethanolic brain extracts²⁶.

This class of macromolecules consists of a sugar residue, a fatty acid and an organic base which Tudichum named 'sphingosine' after the Greek sphinx, referring to the enigmatic structure of sphingolipids. Sphingosine was structurally characterized as 2*S*,3*R*,4*E*-2-amino-octadec-4-ene-1,3-diol in 1947 by Carter who also proposed the designation of lipids derived from sphingosines as sphingolipids. The basic unit of a sphingolipid is a sphingoid long-chain base (LCB), usually sphingosine, sphinganine (dihydro-sphingosine) or 4-hydroxysphinganine²⁷ and a fatty acid component linked to carbon-2 of the LCB via an amide bond yielding ceramide, with different groups attached at the 1-hydroxyl position resulting in different types of complex sphingolipids (Figure 1-4). The presence of a phosphate group at the 1-hydroxy position of ceramide yields ceramide-1-phosphate, and an O-glycosidically linked glucose or galactose substituent results in the formation of the compounds: glucosylceramide and galactosylceramide (cerebroside)²⁸. The higher glycosylated ceramide species, such as sulfatides²⁹ are derived from galactosylceramide by addition of a sulphate group to the 3-position of the sugar residue, whilst gangliosides contain a sialic acid (mostly N-acetyl neuraminic acid) moiety in the carbohydrate head group³⁰.

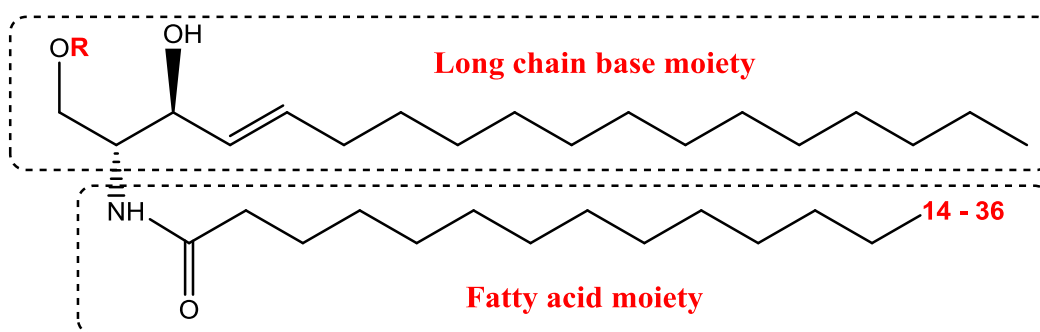


Figure 1-4: General structure of a sphingolipid which consists of an LCB moiety, a fatty acid moiety and different groups attached to the LCB component, where R can be a variety of head groups such as glucose, phosphate, phosphocholine, phosphoinositol or the simplest head group H to generate ceramide.

Sphingolipid variation results from differences in the LCB moiety and the groups attached to the LCB component; this is seen in the variation found in hydroxylation patterns,

number and stereochemistry of the double bonds, chain length and the branched side chains.

1.3.1 LCB Variation

In mammals, the LCB moiety is mostly (*E*)-sphing-4-enine (sphingosine, d18:1⁴), whereas in yeast the predominant LCB is 4-hydroxysphinganine (phytosphingosine, t18:0) formed by desaturation or hydroxylation of sphinganine (d18:0) at C-4³⁰. The predominance of 18 carbon sphingoid bases (d18:0, d18:1, and t18:0) in most mammalian sphingolipids is consistent with the preference of mammalian serine palmitoyltransferase (SPT) for saturated fatty acyl-CoAs composed of 16 carbons atoms in length³¹, combined with the abundance of palmitoyl-CoA³². Sphingoid bases with chain lengths of 12 to 26 carbons have been reported in human skin³¹ which belongs to a special class of ceramide containing ω -hydroxy fatty acid³⁰.

Plant sphingoid bases are composed of eight different C₁₈-sphingoid bases derived from sphinganine³³ (Figure 1.5). *Cis*- or *trans*-desaturation at C-8 results in the unsaturated plant LCBs: (*E/Z*)-sphing-8-enine (d18:1⁸), (*4E,8E/Z*)-sphinga-4,8-dienine (d18:2^{4,8}) and (*8E/Z*)-4-4-hydroxy-8-sphingenine (t18:1⁸); d18:1⁴ is absent whilst the major LCBs in *Saccharomyces cerevisiae*, d18:0 and t18:0 are present in minor proportions^{34, 35}. Other LCBs are present in plants as minor components³⁶.

Plants are uniquely different from animals and yeast in that the major LCBs t18:1(*8E/Z*) and d18:2 (*4E/(8E/Z)*) are not found in these organisms. The proportion of sphingosine found in different species appears to segregate along taxonomic lines, with the *Solanaceae* (tomato and tobacco) having large proportions and the *Fabaceae* (pea and soybean) an intermediate amount, whilst *Brassicaceae* (*Arabidopsis*) have very low to non-existent levels of sphingosine³⁷. The major difference between sphingoids found in

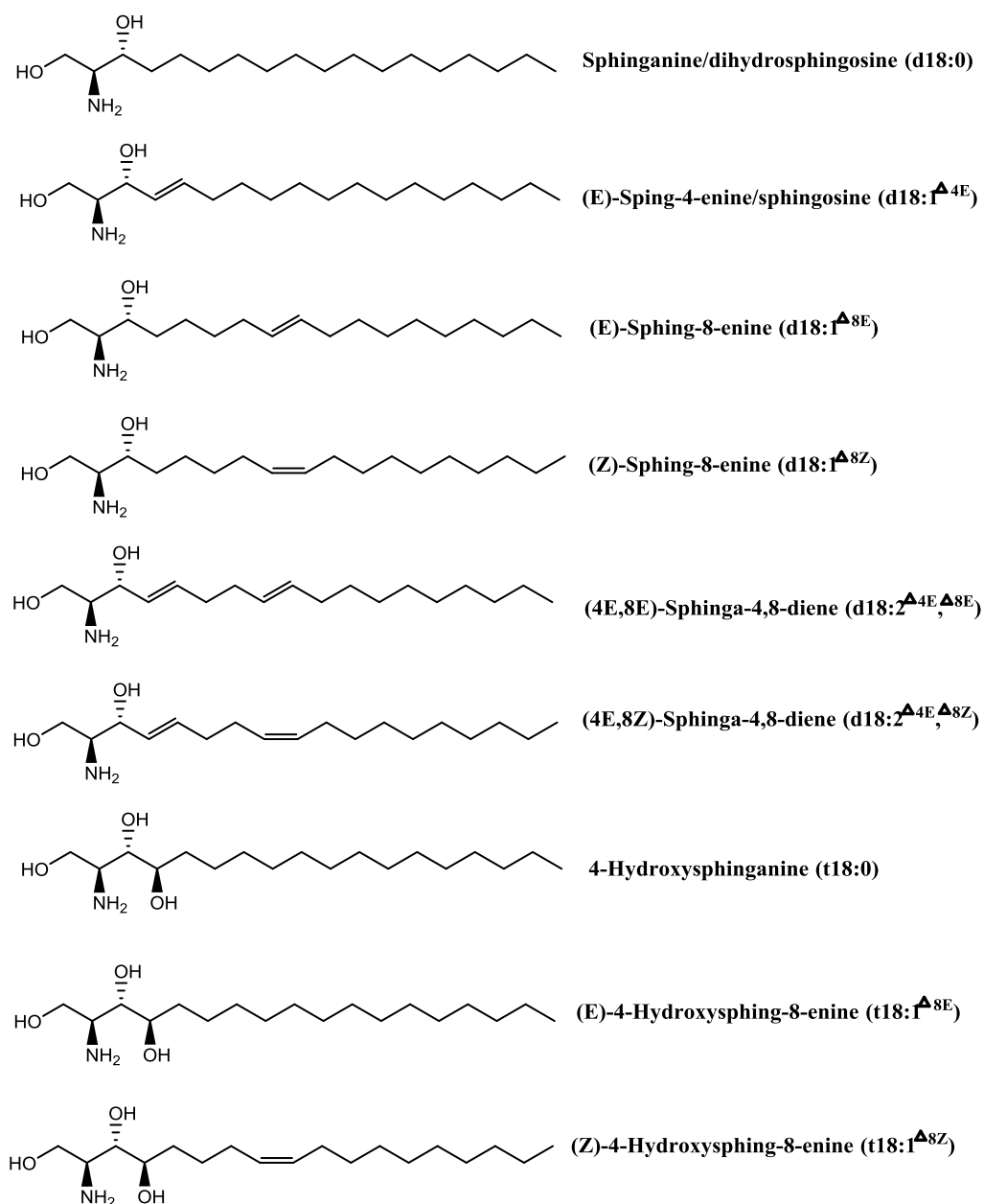


Figure 1-5: The structures and shorthand designation of the different sphingolipids found in plants, fungi and mammals whereby the LCB can be dihydroxy or trihydroxy; the number before the colon is the number of carbon atoms present in the compound and the number after the semi colon is the degree of unsaturation. Adapted from Pata *et. al*³³.

plants and fungi as compared to mammals is the lack of the C4-C5 double bond which shows a higher pattern of hydroxylation at C4, usually with a longer acyl chain³⁸. Sphingolipid variation in other species is even more complex, with nematodes having both iso- branched and anteiso-branched (4E,6E-d13:2)³⁹ sphingoid bases (Figure 1-6A and 1-6B) . In *Drosophila* a sphingoid possessing a conjugated diene has been found⁴⁰

(Figure 1-6C), whilst in aquatic organisms, unique sphingoids bearing a cyclopropane ring have been isolated from marine sponges (Figure 1-6D)^{41, 42}.

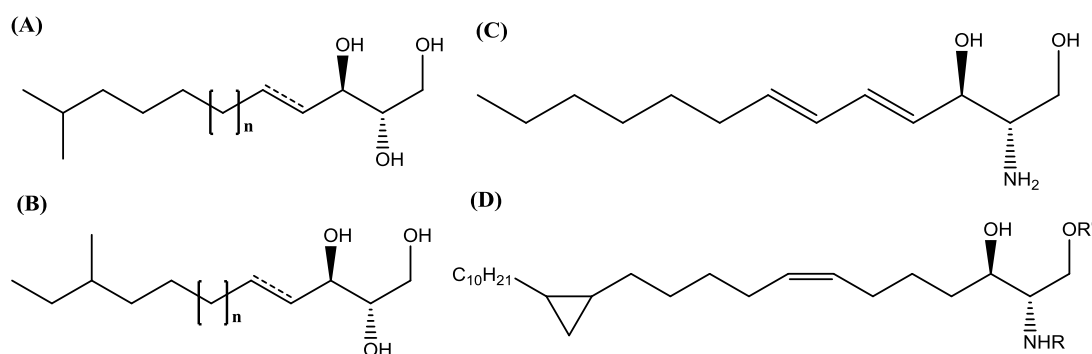


Figure 1-6: Structural variation in sphingoid bases in different species: (A, B) Nematode (C) *Drosophila* (D) *Plakortis simplex*. Figure adapted from Pruett *et. al*⁴².

1.3.2 Sphingolipid diversity in plants

There are at least 500 (and perhaps thousands of) different molecular species of sphingolipids in eukaryotes³³, and in *Arabidopsis* alone it has been reported that there are at least 168 different sphingolipids^{43, 44}. The structural variability found in plants arises from the diversity of the ceramide backbone and the high proportion of very long chain fatty acids (VLCFAs) found; whereby the fatty acid component can vary from C14-C26 and be saturated or monounsaturated. The fatty acid component can be an α hydroxylated VLCFA, which is predominantly found in maize root plasma membranes⁴⁵. The most abundant α -hydroxylated FA composed of saturated C₁₆, C₂₀, C₂₂ and C₂₄ long chains, in contrast there is a sparse population of ω 9-monounsaturated FAs composed of C₂₂-₂₆ carbon chains⁴⁶.

In plants there are four classes of sphingolipids: glycosyl inositol phosphoceramides (GIPCs), glycosylceramides, ceramides, and free long-chain bases (Figure 1-7)⁴⁷. IPC is a precursor for the synthesis of GIPCs, but in fungi is identified as one of the predominant sphingolipids, which includes the two mannose-containing derivatives:

mannosyl inositolphosphorylceramide (MIPC) and mannosyl diinositolphosphorylceramide (M(IP)₂C)⁴⁸.

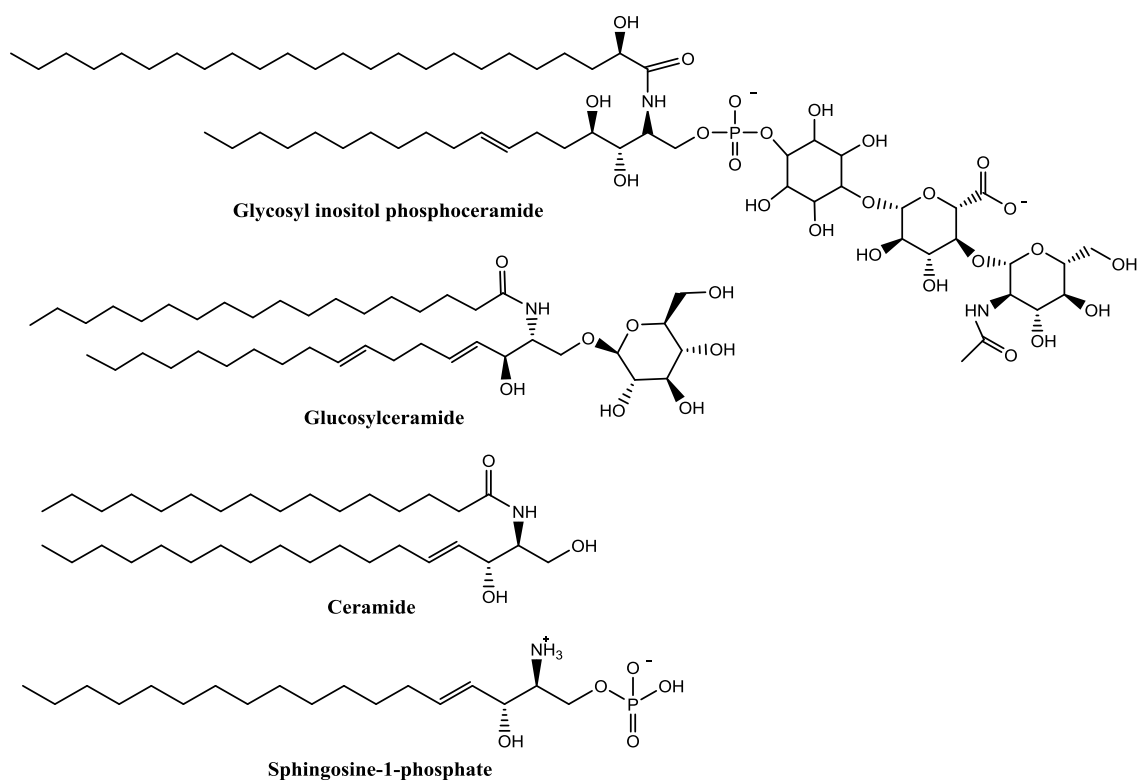


Figure 1-7: Structures of sphingolipids found in plants: Ceramide is the precursor to the more complex sphingolipids glucosylceramide (GlcCer) and glycosyl inositol phosphorylceramide (GIPC). Sphingosine-1-phosphate is a phosphorylated free LCB which has bioactive properties.

The deletion of inositol phosphorylceramide (IPC) synthase which synthesizes IPC has been found to be lethal in *Saccharomyces* and *Aspergillus* and therefore essential for survival⁴⁹. To date sphingomyelin, which is the major sphingolipid in animal tissues, has not been detected in plants.

1.3.2.1 Glycosyl inositol phosphoceramides

Glycosyl inositol phosphoceramides (GIPCs) are acidic glycosphingolipids (GSLs) and are the major sphingolipids in plants, accounting for 64% of total sphingolipids³⁷. IPC is the precursor for the synthesis of GIPCs, which consists of a polar head group linked to

C-1 of an *N*-acyl long-chain base that is attached to a fatty acid moiety⁵⁰ (Figure 1-8). The two GIPC core head groups, phytylglycerolipid (PGL) and ceramide phosphate polysaccharide (CPPS), were identified by Carter et. al, and found to be attached to different sugars⁵¹. The oligosaccharide chains of PGL consist of glucosamine, hexuronic acid and inositol linked to mannose. The core head groups, phosphosphingolipid II and glycoposphoceramide consist of oligosaccharide chains attached to C-2 and/or to C-6 of *myo*-inositol and contain either *N*-acetylglucosamine or glucosamine and glucuronic acid with differing amounts of the additional sugars, arabinose, galactose, mannose and fucose^{52, 53} (Table 2).

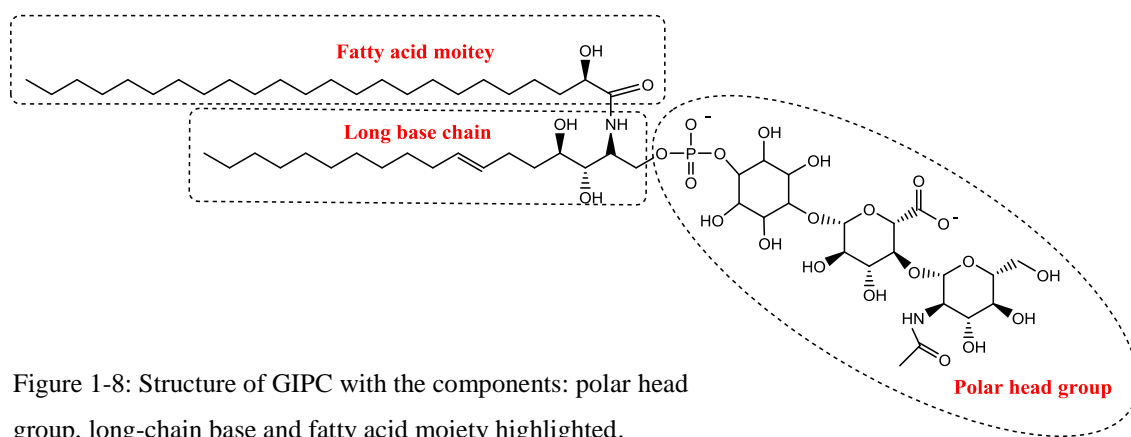


Figure 1-8: Structure of GIPC with the components: polar head group, long-chain base and fatty acid moiety highlighted.

Analysis of the LCB moiety of GIPCs located in leaf and endosperm tissue in the *Poaceae* family showed that t18:1 Δ 8(E/Z) and t18:0 LCBs are predominant^{47, 54}, with a high percentage of saturated and α hydroxylated very long fatty acid chains that are C24 or C25 long⁵⁵; 2-hydroxylignoceric acid (h24:0) being the most common⁴⁹.

The enzyme, inositol phosphorylceramide glucuronosyltransferase (IPUT) from *Arabidopsis* when introduced into yeast has been shown to transfer glucuronic acid to IPC which is the first step in IPC glycosylation pathway to produce GIPCs, with the silencing of *IPUT* resulting in the accumulation of IPC (30% increase)⁵⁶.

This class of sphingolipids have been to interact with the oligosaccharide, rhamnogalacturonan II (RG-II) via boron, suggesting it has a role to play in the dimerization of RG-II which maintains the mechanical properties of the cell wall⁵⁷.

Table 2: Core head group structure of GIPCs in different plant species. Adapted from Pata *et. al* 2009³³

| Family | Species | Tissue | Core head group | Core head group structure | Additional sugars |
|------------|--|----------|-----------------------------------|--|--|
| Linaceae | <i>Linum usitatissimum</i> | Seed | PGL | Glucosamine-hexuronic acid- inositol-P-Man | Ara, Gal, Man, Fuc |
| | | | CPPS | Hexuronic acid- inositol | |
| Poaceae | <i>Zea mays</i> | Seed | PGL | Glucosamine-hexuronic acid- inositol-P-Man | Ara, Gal |
| | | | CPPS | Hexuronic acid- inositol | |
| Asteraceae | <i>Triticum aestivum</i> | Seed | (Inositol-P, GlcN, Ara, Gal, Man) | - | |
| | <i>Helianthus annuus</i> | Seed | (Inositol-P, GlcN, Ara, Gal, Man) | - | |
| Solanaceae | <i>Carthamus tinctorius</i> | Seed | CPPS | Hexuronic acid- inositol | Ara, Gal, Fuc, Man |
| | <i>Nicotiana tabacum</i> | leaf | PSL-I | N-acetylglucosamine (α 1-4)-glucuronic acid (α 1-2)-myo- inositol-1-O-P | [Ara ₃ Gal ₂] [Ara ₂ Gal ₂] [Ara ₄ Gal ₂] |
| | | | PSL-II | Glucosamine-glucuronic acid- inositol-P | [Ara ₃ Gal] [Ara ₂ or3Gal ₂] [Ara ₂ Gal ₂ Man] |
| GPC | N-acetylglucosamine(α 1-4)-glucuronic acid(α 1-2)-myo- inositol-1-O-P Man | Ara, Gal | | | |

Table 2: The names of core head groups and their structures; PGL, phytyglycolipid; CPPS, ceramide phosphate polysaccharide; Ara, arabinose; Fuc, fucose; Gal, galactose; Man, mannose; CPPS, ceramide phosphate polysaccharide; GPC, glycoposphoceramide; PGL, phytyglycolipid; PSL, phosphosphingolipid. Adapted from Pata *et. al* (2009)³³.

In addition, GIPC has been found to be important for normal pollen development, with *iput1* mutants found to transmit the alleles through pollen at 1-2% compared to wild

type⁵⁶. GIPCs are not present in mammals but have been found in protozoa, plants, fungi, and nematodes⁵⁸.

A variant of GIPC are the glycosylphosphatidylinositol (GPI)-anchors. GPI-anchors in plants are linked to C-terminus of a protein via an ethanolamine phosphate linkage and a conserved oligosaccharide core to an inositol phospholipid moiety⁵⁹. Over 200 GPI-anchored proteins have been predicted to exist in the *A. thaliana* genome with functions including signaling, adhesion, response to stress, and cell-wall remodeling⁶⁰.

1.3.2.2 Glycosylceramides

Glycosylceramide is a ubiquitous sphingolipid found in animals, plants and fungi (but not *S. cerevisiae*). Glycosylceramide is often referred to as cerebroside due to its structural similarity with a compound found in the brain known as galactosylceramide⁴⁷. GlcCers are a component of the plasma membrane and the tonoplast of plant cells, accounting for about 5-30 mol % of total lipid in the plasma membrane^{61, 62} and constitute 34% of total sphingolipid content in plants³⁷. Their abundance in the plant plasma and vacuolar membranes, combined with the ease of extraction and purification, has led to extensive structural characterization⁶³.

Composed of a β -glucose or β -mannose head group, GlcCers consist of α hydroxylated short or long fatty acid chains attached at the C1 position to a hexose sugar⁶⁴. The exact nature of GlcCers structure varies for different plant species. GlcCers possessing VLCFAs are found to be predominant in the *Poaceae* family with a wide range of LCB variations; lower percentages of d18, d18:1 Δ^4E , d18:1 Δ^8E and d18:1 Δ^8Z LCBs are observed in leaf and endosperm tissues, with much higher percentages of LCBs found to be d18:2 $\Delta^4E\Delta^8E$, d18:2 $\Delta^4E\Delta^8Z$, t18:1 Δ^8E , t18:1 Δ^8E and t18:1 Δ^8Z ^{64, 65}. In the *Brassicaceae*

family, saturated α hydroxylated VLCFAs content in *Arabidopsis* are found to around 5%, with >80% of GlcCers possessing α hydroxylated short fatty acids⁶⁶.

GlcCers identified in *Oryza sativa* endosperm and seed bran were found to be enriched with the *cis* and *trans* isomers of 8-sphingenine and 4,8-sphingadienine with C₁₆-C₂₀ saturated hydroxyl fatty acids⁶⁷, contrasted with GlcCers identified in tobacco leaves which were enriched in trihydroxy long-chain bases (*cis* (*Z*) and *trans* (*E*) isomers of 4-hydroxy-8-sphingenine) with very long-chain (C₂₀- C₂₆) saturated⁵². The predominant fatty acid in both the leave and seed tissue in plants was found to be 2-hydroxypalmitic acid (h16:0)^{34, 68}. Monoenoic hydroxy fatty acids, such as 2-hydroxynervonic acid (h24:1) are prevalent in *Arabidopsis* GlcCers⁶⁹ and cold hardy cereals. The variation of the fatty acid moiety in GlcCers has been implicated in chilling tolerances of plants; glycosylceramides containing α -OH monounsaturated VLCFAs have been detected mainly in chilling-resistant plants⁶⁶ and in chilling-sensitive plants, the predominant GlcCers are α -OH FA⁷⁰.

1.3.2.3 Ceramide

Ceramides are the third class of plant sphingolipids and are formed by the *N*-acylation of an LCB and a FA. These compounds are bioactive and are known to induce apoptosis in animals and programmed cell death in plants⁷¹. In comparison to GIPCs and glycosylceramides, plant ceramides are less documented in literature⁴⁷; this can be attributed to their lower abundance in plant membranes making up 4-10% of plant glycosylceramides content and 1.7% of total sphingolipid content in *Arabidopsis*^{37, 72}.

Ceramide content in *Arabidopsis* is estimated to be 2-7 mol% and in rice leaf stems to be 6%^{37, 66}. The main FA moieties of plant ceramides are usually α hydroxylated VLCFAs especially in Poaceae, although high proportions of non-hydroxyl FAs have also been

found in rice and potato^{67, 73}. The predominant LCB in plant ceramides are the trihydroxy-LCBs; the main LCB in *Oryza sativa* and *Arabidopsis thaliana* is t18:0³⁷.

1.3.2.4 LCB

The fourth class of plant sphingolipids is the free LCB. LCBs have been shown to be mediators in cellular responses; a study conducted by Dharmawardhane *et al.* showed that free LCBs such as 4-sphingenine (d18:1^{Δ4E}), sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) modulated redox in oat mesophyll cells⁷⁴. These sphingoid bases are known to be the most active inhibitors of protein kinase C in animal cells, inhibit electron transport in the transplasmalemma of the mesophyll cells in the dark, and stimulate redox transport in light⁷⁴. Free LCBs such as 4-sphingenine (d18:1^{Δ4E}) regulate the vacuolar pyrophosphatase (V-PPase) proton pump in *Chenopodium rubrum* in order to maintain vacuolar and cellular acidity⁷⁵. Work done by Brodersen *et al.* has shown that the accumulation of sphingosine in *accelerated-cell-death11 Arabidopsis* mutant (*acd11*), a sphingosine transfer protein, results in cell death characteristic of animal apoptosis⁷⁶ with an elevation in ceramide-1-phosphate and phytoceramide levels⁷⁷.

Interestingly, the relative proportions of E and Z isomers of 4-hydroxy-8-sphingenines was found to contribute to freezing tolerance, with higher levels of the Z isomers found in twelve different species of chilling resistant plants⁷⁰.

1.4 Sphingolipid biosynthetic pathway

Sphingolipids can be formed via two pathways: the *de novo* pathway, starting with the condensation of a serine with an acyl-CoA and the salvage pathway whereby ceramide and LCBs are released from more complex sphingolipids, followed by channelling of the metabolites formed into the synthetic pathway²⁷.

1.4.1 The *de novo* pathway

The core biosynthetic pathway of sphingolipid synthesis in plants and animals (Figure 1-9) is complex, involving five different enzymes and with synthesis occurring in the endoplasmic reticulum (ER) and the Golgi apparatus. The first four enzymes in the biosynthetic pathway are located in the ER, whilst enzyme involved in the formation of complex sphingolipids are located in the Golgi apparatus, with the active site of most facing the cytoplasm, but some facing the lumen implying significant transbilayer and interbilayer movement²⁷ involving vesicular and non-vesicular trafficking⁷⁸. To date the known mode of transport between the ER and Golgi involves the cytoplasmic ceramide transport protein (CERT); this protein has a putative domain for catalysing lipid transfer, which enables it to specifically interact with ceramide in membranes where other complex sphingolipids are present^{79,80}.

Sphingolipid synthesis in plants and animals begins with the enzyme serine palmitoyl transferase (SPT), which was first identified in yeast and belonging to a family of pyridoxal 5'-phosphate-dependent α -oxoamine synthases. SPT converts serine and fatty acyl CoA into 3-ketosphinganine, CoA and CO₂⁸¹. Reduction of 3-ketosphinganine to dihydrosphingosine is accomplished by 3-ketosphinganine reductase (3KSR) using nicotinamide adenine dinucleotide phosphate (NADPH). After this reaction, there is a divergence in the sphingolipid biosynthetic pathway, whereby in mammals acylation of dihydrosphingosine by dihydroceramide synthase yields dihydroceramide²⁷, whereas in plants, dihydrosphingosine is hydroxylated to form phytosphingosine. Formation of varying acyl chain lengths results from the different substrate preference of dihydroceramide synthase isoforms.

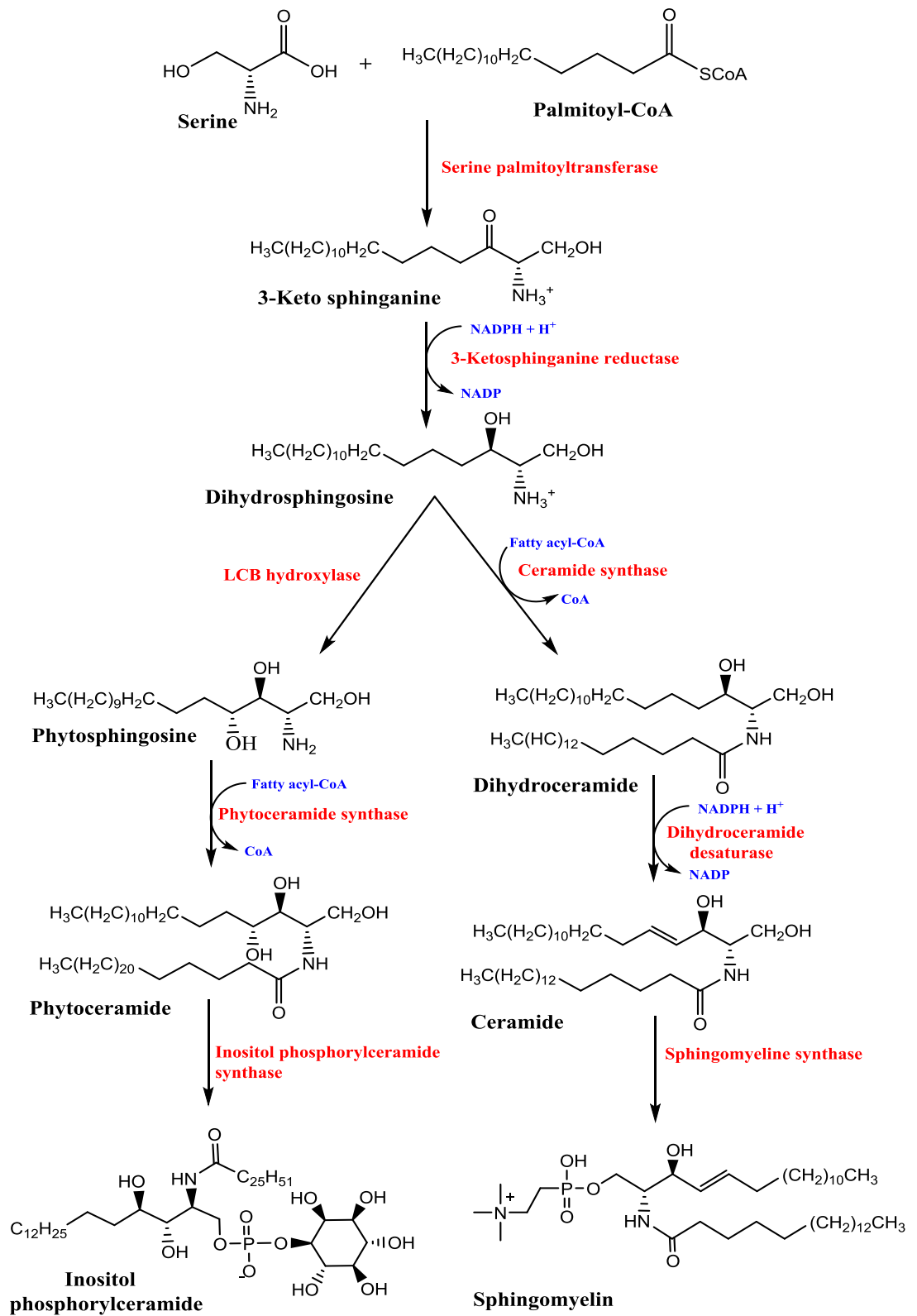


Figure 1-9: The biosynthetic pathway for the formation of IPC in plants and sphingomyelin in animals.

In mammals it has been found that there are six genes that encode ceramide synthase, known as the longevity-assurance gene (LAG1-6)^{82,83} which are located in the

endoplasmic reticulum. The ceramide synthase isoforms show specific substrate preference in terms of the fatty acyl CoAs chain length thereby generating distinct ceramide acyl chains⁸⁴. In plants, three ceramide synthase homologues termed LOH (LAG one homologue) have been identified, and like the mammalian orthologues show specific substrate preference, with LOH1 and 3 showing substrate specificity for trihydroxylated LCBs that have 20-26 carbon chains and LOH2 showing substrate specificity for LCB that are 16 carbon chain long and has a preference for dihydroxylated LCBs⁸⁵. In animals insertion of a cis-4,5 double bond is achieved by dihydroceramide desaturase (DHCD) thereby generating ceramide, which is a precursor of many complex sphingolipids found in mammals, yeasts and plants³⁶. In animals, ceramide is converted to sphingomyelin in the Golgi by sphingomyelin synthase (SMS), which involves transfer of the phosphorylcholine head group from phosphatidylcholine to ceramide yielding sphingomyelin and diacylglycerol²⁷.

Also, glucosylceramide synthase (GCS) converts ceramide to glucosylceramide, a precursor for the formation of complex GSLs; initial addition of glucose to ceramide occurs in the cytoplasmic side of the cis-Golgi and additional extension of the sugar chain occurs in the Golgi lumen as the GSLs are transported from the cis to the trans Golgi^{86,87}. IPC, which has not been identified in animals, is the precursor for the generation of GPICs, which are the major sphingolipids in plants, and is generated by the transfer of phosphoinositol to phytoceramide by IPCS.

1.4.2 Salvage pathway

To date the salvage pathway of sphingolipid synthesis has only been studied in mammals and is yet to be investigated in plants and yeast. The salvage pathway of long-chain sphingoid bases leading to the regeneration of sphingolipids has been estimated to

contribute 50% to 90% of sphingolipid biosynthesis⁸⁸. The degradation of sphingolipids occurs in the acidic subcellular compartments; the late endosomes and the lysosomes⁸⁹.

GSL degradation by exohydrolases results in the release of monosaccharide units from the end of the oligosaccharide chains thereby generating ceramide⁸⁹. Experiments conducted using astrocytes showed that the major sphingolipid in mammals, sphingomyelin, is converted to ceramide and phosphocholine by acid sphingomyelinase (SMase) which can be stimulated in response to oxidative stress and bioactive compounds such as tumour necrosis factor and γ -interferon^{20, 90}. Hydrolysis of ceramide by acid ceramidase forms sphingosine and free fatty acids in the lysosome²⁰; once released from the lysosome, sphingosine can re-enter the de novo pathway for the regeneration of ceramide and sphingosine-1-phosphate (Figure 1.10), whilst long chain sphingoid bases are recycled in to the synthetic pathway by ceramide synthases.

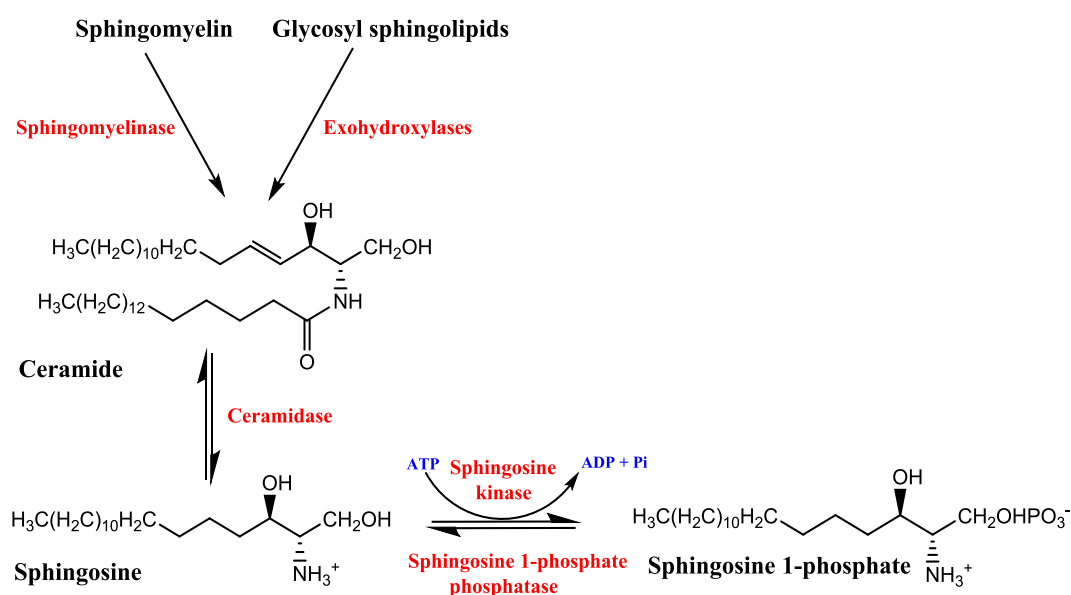


Figure 1-10: Schematic of the salvage pathway for the degradation of sphingolipids in animals.

1.5 Programmed cell death in plants

Programmed cell death (PCD) refers to the organised destruction of unwanted, infected and damaged cells⁹¹. This process is a genetically directed mechanism in response to

pathogen or abiotic stress. In addition it has a role in the maintenance of homeostasis in normal development such as senescence, vascular system formation and specification of unisexual floral organs⁹¹. The process known as apoptosis in animals, shares morphological characteristics with plant PCD such as condensation of nuclei, fragmentation of nuclear DNA, and cell shrinkage⁹².

In plants, the biosynthesis of sphingolipids has been linked to PCD. As mentioned earlier, ceramide induces apoptosis/programmed cell death in animals/plants⁷². The first documented evidence of ceramide involvement in PCD was by Liang *et al.* which showed that in *Arabidopsis* the ceramide kinase mutant known as accelerated cell death 5 (*acd5*) accumulates lipid substrates similar to those utilized by wild type ceramide kinase, and exhibited disease like symptoms and apoptotic-like cell death during pathogen attack⁹³. The mutant protein ceramidase activity was demonstrated in an assay which showed that the proteins had high specificity for synthetic C6 and C8 ceramides. The accumulation of ceramide in *acd5* mutant cells resulted in apoptotic like features which had regions of condensed chromatin in the nuclei, possessing large numbers of DNA strand breaks generated by endonucleolytic cleavage⁹³. The role of ceramide as an inducer of apoptosis was validated by treatment of wild type protoplasts with C2 ceramide which induced nuclear fragmentation characteristic of apoptotic cells⁹³. Interestingly, phosphorylated derivatives of ceramide were able to partially block plant PCD indicating the role of phosphorylated ceramides in modulating cell death in plants⁹³.

In tobacco (*Nicotiana tabacum*), cell death was found to be induced by palmitoleic acid (16:1), ceramide, and potassium cyanide; exhibiting features associated with PCD, including cell volume decrease, loss of membrane integrity, DNA damage, nuclear and plastid disorganization, and chromatin condensation⁹⁴. Cell volume decrease was found to be caused by loss of intracellular K⁺ channels; inhibition of K⁺ channel with blockers

such as Ba²⁺ and quinine have been found to prevent cell shrinkage and the subsequent biochemical events leading to PCD⁹⁴.

Research carried out by Wang *et al.* identified the *Arabidopsis* functional homolog of yeast and protozoan IPCS which enhanced RPW8 hypersensitive-like cell death⁹⁵. RPW8 is a resistance gene which confers broad-spectrum resistance to powdery mildew in *Arabidopsis*⁹⁶. RPW8 triggers the hypersensitive response (HR) to restrict powdery mildew infection via the salicylic acid–dependent signaling pathway; loss of function of *At*IPCS results in salicylic acid accumulation, RPW8-dependent spontaneous HR-like cell death (SHL) in leaf tissues, reduction in plant stature⁹⁵. This study showed that T-DNA insertional mutants of *At*IPCS in plants unchallenged with powdery mildew, had an observable phenotype of chlorotic lesions and sporadic cell death, thereby revealing the role of IPCS as a negative regulator of PCD in plants⁹⁵.

1.6 IPC Synthase

IPCS belongs to a superfamily called the ‘Sphingolipid Synthases’ which includes animal sphingomyelin synthase (SMS)⁹⁷, protozoan IPCS⁹⁸, fungal IPCS⁹⁹ and plant IPCS¹⁰⁰. Mutants *S. cerevisiae* strains defective in IPCS activity were found to accumulate ceramide which was accompanied by cell death, showing that IPCS is necessary for survival¹⁰¹; expression of a gene encoding AUR1 protein in these mutants was found to complement for the activity of IPCS¹⁰¹. Mutations in AUR1 have also been found to confer resistance to the antifungal IPCS inhibitor Aureobasidin A (AbA)^{101, 102}, showing that AUR1 is fungal IPCS.

The protozoan IPCS enzyme was identified in kinetoplastid *Leishmania major* by Denny *et al.* in 2006 after screening the genome database for the two conserved active site motifs (CGDX₃SGHT and HYTXDVX₃YX₆FX₂YH) shared by lipid phosphate phosphatase,

SMS and AUR1⁹⁸. The conserved motifs contain the HHD catalytic triad (underlined and bold) involved in the proposed double displacement mechanism of IPCS. This protein was shown to rescue an *AUR1* yeast mutant⁹⁸ and subsequent analyses of the genomes of related kinetoplastids, *Trypanosoma brucei* and *Trypanosoma cruzi* genome, revealed four IPCS orthologues in *T. brucei* and two in *T. cruzi*¹⁰³.

Plant IPCS was first identified in *Arabidopsis thaliana* by Wang *et al.*⁹⁵ and the three encoded isoforms *AtIPCS1-3* were shown to be AbA resistant AUR1p orthologues differentially expressed in various tissues¹⁰⁴. Across species, *AtIPCS1-3* and *LmjIPCS* showed the highest sequence identity (~30 to 45%), especially in the conserved domains, D3 and D4¹⁰⁴. A Basic Local Alignment Search Tool (BLAST) search of the *Oryza sativa* (rice) genome using the *AtIPCS1* protein sequence revealed three orthologues designated *OsIPCS1-3*.

1.6.1 IPCS topology

The first proposed model for IPCS topology was by Denny *et al.* based on based on the single sequence analysis of *LmjIPCS*⁹⁸. This model proposed that IPCS consists of seven transmembrane helices with the catalytic site facing the lumen of the Golgi apparatus and lacked the conserved domain D2 present in mammals; in addition, there is an extra helix between TM3 and TM4 consisting of 11 residues with the inversion of TM1-3 which preserves the orientation of the active. Another model has been proposed by Bangs *et al.*¹⁰⁵ based on the alignment of single sequences from three kinetoplastids species, suggests the presence of the D2 domain which is conserved in animal SM synthase⁹⁷, and discounts the existence of the extra helix (Figure 1-11). There has been no experimental evidence for either model, although the latter model is more in keeping with data from mammalian systems⁹⁷

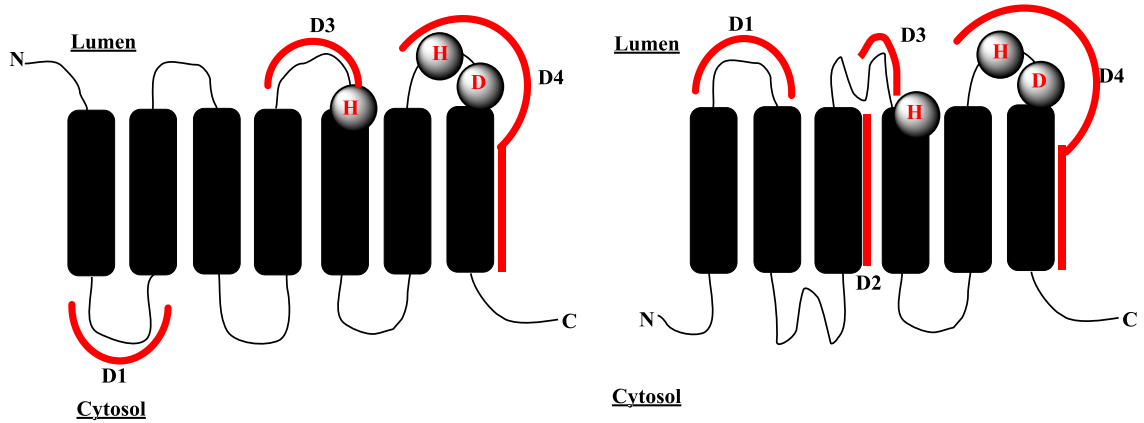


Figure 1-11: (A) Denny *et al.* model of *L. major* consisting of seven transmembrane helices and lacking D2 domain (B) Bang *et al.* model consisting of six helices and D2 domain.

1.6.2 Catalytic mechanism

The double displacement model is based on lipid phosphate phosphatase (LPP) hydrolysis of lipid phosphates. The domains designated D3 and D4 are similar to the C2 and C3 domains of LPPs; both enzymes have a catalytic triad consisting of two histidine residues and an aspartate residue⁹⁷. Hence it has been proposed that IPCS catalysis follows a similar mechanism to the LLPs which involve the activation of one of the histidine by aspartate via a charge-relay system resulting in the deprotonation of the histidine, the formation of an inositol phosphoryl histidine intermediate and the release of diacyl glycerol (DAG)⁹⁹.

Heiddler and Radding proposed an IPCS mechanism based on the catalytic model of lipid phosphatases, whereby ceramide C₁-hydroxyl nucleophilicity is increased through hydrogen bonding with the histidine in D3, and the inositol phosphoryl histidine intermediate is protected from hydrolysis by the increased hydrophobicity of the catalytic site⁹⁹. This sets up the conditions required for the nucleophilic attack on the inositol phosphoryl histidine intermediate by the C₁-hydroxyl of ceramide leading to the transfer of inositol phosphate to the C₁ of ceramide. Heiddler and Radding also postulate that the

role of domains D1 and D2 might be able to bind and stabilize ceramide, for a favourable positioning of the C₁-hydroxyl which facilitates the transfer of inositol phosphate at the surface of the membrane⁹⁹.

The catalytic phosphoinositol transfer has been proposed based on a catalytic mechanism of LPP activity by Sigal *et al.*, which involves the HHD catalytic triad, arginine, lysine, serine and glycine residues that stabilize the transition state¹⁰⁶. Across different organisms, the LPP family of phosphatase have been found to consist of three conserved motifs in which one motif has the nucleophilic histidine in close proximity to an arginine residue¹⁰⁷. This arginine residue has been proposed to be Arg262 in *Lmj*IPCS and found to be a conserved residue in all the identified orthologues of IPCs in *L. major*, *Trypanosoma brucei*, and *Trypanosoma cruzi* (Trityp) genomes¹⁰⁸. The amino acid residue Arg262 is hypothesised to be involved in the stabilisation of the transition state, with the protonation of the arginine group speculated to be affected by the protonation of the amino group of inhibitory sphingosines, resulting in inefficient stabilisation of the transition state and inhibition of IPCS activity¹⁰⁸.

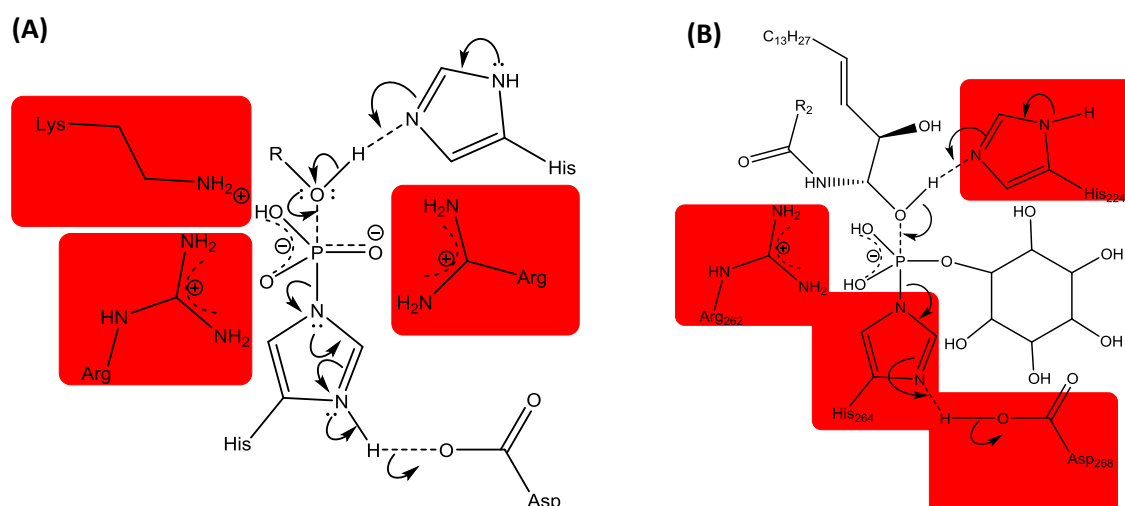


Figure 1-12: (A) Proposed mechanism of action of phosphoryl transferases (B) Proposed mechanism of action of *Lmj*IPCS, adapted from Sigal *et al.*

1.7 Targeting IPCS

As mentioned earlier, sphingolipids play an essential role in maintaining the integrity of the plasma membrane and are also involved in a plethora of biological pathways including signal transduction, cellular differentiation, and embryogenesis, as a result they are important for survival in organisms. The divergence in the sphingolipid biosynthetic pathway in animals, plants, fungi and protozoa post ceramide can be exploited in the development of herbicides, antifungal, and antiprotozoal drugs. (Figure 1-14).

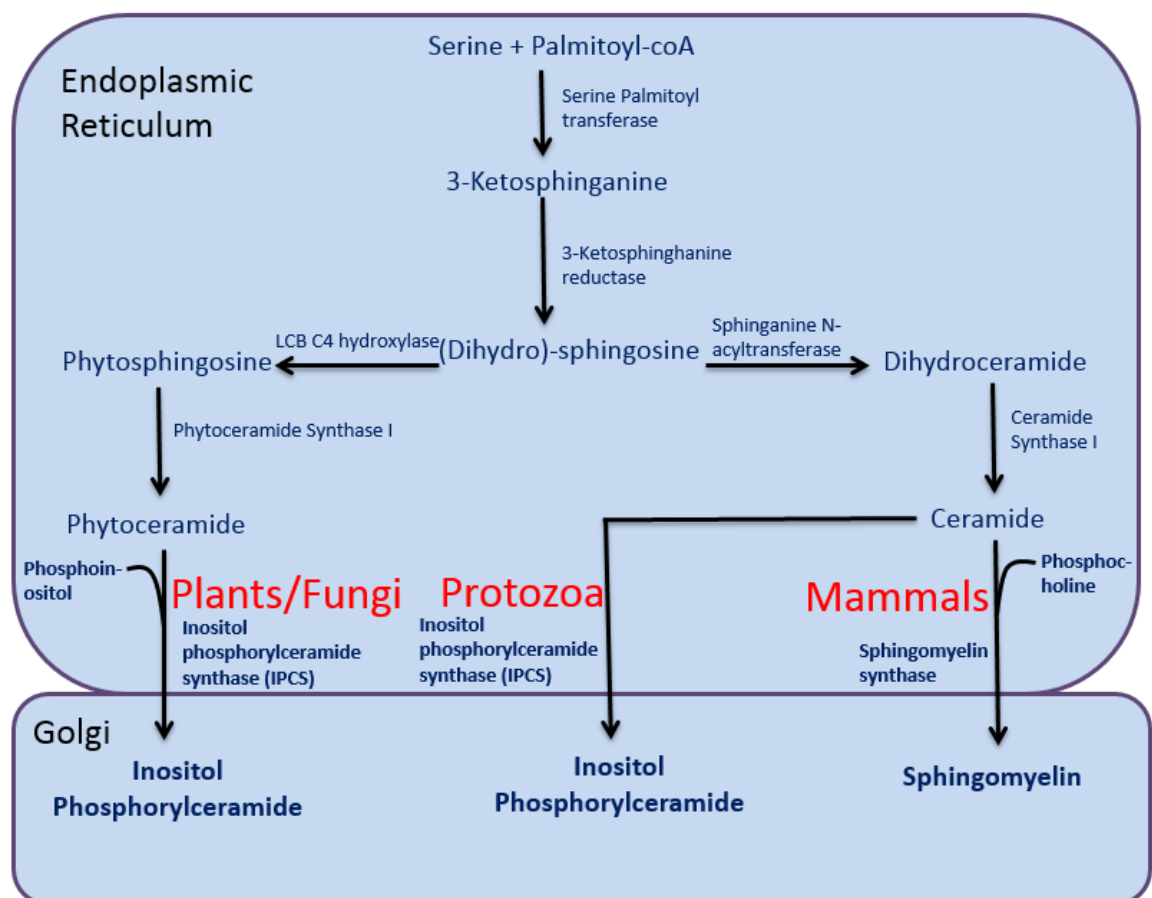


Figure 1-14: Schematic of the sphingolipid biosynthetic pathway: Fungi and higher plants IPCS converts phytoceramide into inositol phosphoryl ceramide, whilst kinetoplastids and mammals SMS converts ceramide to sphingomyelin (B) Inhibition of IPCS results in a decrease in mitogenic diacylglycerol, an accumulation of pro-apoptotic phytoceramide and a decrease in IPC, the precursor of the major plant sphingolipid GIPC.

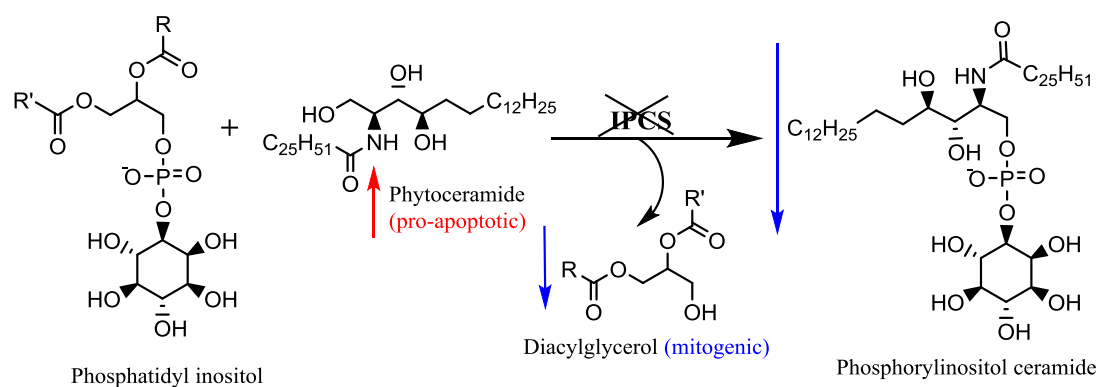


Figure 1-15: Inhibition of IPCS results in a decrease in mitogenic diacylglycerol, an accumulation of pro-apoptotic phytoceramide and a decrease in IPC, the precursor of the major plant sphingolipid GIPC.

The absence of sphingomyelin synthase in plants and the difference in enzyme substrate, i.e. phytoceramide and phosphatidylinositol for plant IPCS and ceramide and phosphatidylcholine for SMS, create the opportunity for the development of herbicides that specifically target plant enzyme with minimal toxicity to animals. Work done by Bi et al. showed that the loss of ceramide kinase results in ceramide accumulation and the formation of necrotic lesions associated with PCD, indicating that the accumulation of ceramide could trigger PCD¹⁰⁹. Similarly, inhibition¹⁰⁹ of IPCS should result in the accumulation of the pro-apoptotic phytoceramide, and a decrease in the turnover of the mitogenic compound diacylglycerol¹¹⁰ (Figure 1-15) thereby initiating PCD.

1.7.1 Anti-protozoal and anti-fungal applications

The most common human diseases caused by kinetoplastids protozoa include human African trypanosomiasis (HAT), caused by *Trypanosoma brucei*; Chagas disease caused by infection with *Trypanosoma cruzi*, and leishmaniasis caused by *Leishmania* species¹¹¹. Half a billion people are at risk of contracting these diseases, and it is estimated that more than 20 million people are infected with the pathogens that cause them, with more than 100,000 deaths recorded per year¹¹¹. Current treatment for these diseases involves the use of drugs which are toxic with variable efficacy, difficult administration

and lengthy periods of treatment¹¹². Research is currently being carried out to develop drugs against these diseases by the design of IPCS inhibitors.

The focus on IPCS inhibition has also been directed towards the development of anti-fungal drugs targeting phytopathogenic fungi which cause diseases in plants, animals and humans¹¹³.

Saprophytic fungi from the *Aspergillus* genus are found worldwide in soil, food products, and decomposing matter¹¹⁴. However, the two species *Aspergillus flavus* and *Aspergillus parasiticus* are found to produce potent toxins (aflatoxins) on certain crops including oilseeds, corn, cottonseed and peanuts, which have been linked to death in humans and animals; in humans, inhaled aflatoxin B1 can cause inflammation and irreversible pulmonary interstitial fibrosis¹¹⁵.

Four species, *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger*, are found to be the causative agents of fatal opportunistic infections such as invasive pulmonary aspergillosis¹¹⁵. Agricultural workers in developing countries whose eyes are injured with subsequent contamination by organic matter develop corneal infections by *Aspergillus* species¹¹⁶. Other phytopathogenic fungi species are part of the genus: *Fusarium*, *Alternaria*, *Curvularia*, *Cladosporium* and *Alternaria*; these produce toxins that affect humans and plant and create economic losses in agriculture and medicine.

There are a limited number of antifungals currently available for the treatment of life-threatening fungal infections¹¹⁷. These antifungal agents show some limitations, such as the significant nephrotoxicity of amphotericin B¹¹⁸ and emerging resistance to the azoles¹¹⁹. The development of new antifungal agents is needed in medicine and agriculture, so the inhibition of essential fungal IPCS, which differs from the mammalian SMS, is a promising approach.

1.7.2 Herbicide application

A close look at herbicide weed resistance worldwide revealed that *Lolium rigidum* remains the world's most herbicide-resistant weed, with resistance to 11 sites of action and 95% of populations resistant to at least two herbicide modes of action (especially ACCase and ALS inhibition)¹²⁰. *L. rigidum* affects 12 countries¹¹, especially southern Australia, and is found in crop fields growing wheat, barley, and oilseed rape. Other weeds that have a huge effect on agronomically important crops worldwide are the drought tolerant weed *Amaranthus palmeri* which affects cotton yields in north America, the weed *Avena fatua* which competes with wheat¹²¹, and *Echinochloa crus-galli* a noxious weed affecting a wide variety of crops including wheat, rice, and potatoes¹²².

Selectivity of herbicides for weeds over crops have been achieved in most cases because of differences in herbicide uptake, translocation and degradation. These differences have been found in some cases to be a result of structural differences between monocot and dicot plants. The classification of plants based on the monocot/dicot system is based on anatomical and developmental differences encompassing germination, root systems, leaf, stem, and vascular bundle architecture. The names are derived from the number of embryonic leaves found in the seed which is termed the cotyledon; monocots have one and dicots have two cotyledons. Difference in the germination process of seeds between the two classes refers to the location of the cotyledon below or above ground during plant development; monocots have the cotyledon below the ground whilst dicots have the cotyledon brought above the ground due to the elongation of hypocotyl¹²³. A detailed look at the structural differences show that monocots have a fibrous root system which is shallow in depth compared to the tap root system found in dicots which possess lateral roots that branch out into tertiary roots, providing depth in growth¹²⁴. In addition, the vascular architecture of the stem in dicots is arranged in the shape of a ring and the veins

of the leaves are reticulate¹²⁵, whilst in monocots the vascular bundles of the stem are highly complex¹²⁶ and the leaves have veins that are parallel in arrangement.

Discovering new herbicide modes of action is essential in the management of herbicide resistance worldwide. The study of IPCS as a new herbicide is promising because of the divergence in the latter stages of the sphingolipid biosynthesis in animals and plants, combined with the lack of IPCS in animals which would allow for the design of inhibitors/herbicides that should have no detrimental effect on animals and humans. The classification of plants based on the dicotyledon and monocotyledon system can be exploited to create IPCS inhibitors that would differentially inhibit monocotyledon weeds over dicotyledon crops or dicotyledon weeds over monocotyledon plants. For example it is well known that auxinic herbicides kill dicot weeds without killing acres of monocot crops such as corn, wheat, and barley¹²⁷.

1.7.3 Inhibitors of IPCS

To date there are five known potent inhibitors of fungal IPCS (Figure 1-16): AbA¹⁰², khafrefungin¹²⁸, rustimicin¹²⁹, pleofungin A¹³⁰ and haplofungin A¹³¹, with IC₅₀ values of 0.2 nM, 7nM, 20 nM, 40 μM and 1.5 μM against *Saccharomyces cerevisiae*.

The *T. brucei* IPCS which synthesises sphingomyelin and inositol phosphoryl ceramide is inhibited by AbA with an IC₅₀ of 0.42 nM¹³². In contrast the *L. major* IPCS enzyme is relatively insensitive to AbA. *T. cruzi* IPC has been shown to be insensitive to rustmicin and Aba¹³³, although IPC formation was indicated to be inhibited by AbA suggesting the compound is active against a different enzyme in the sphingolipid biosynthetic pathway¹³³.

Differential selectivity between dicotyledon species has been observed between *Arabidopsis* and *Phaseolus*. All *Arabidopsis* IPCS are AbA insensitive¹⁰³, whereas in

Phaseolus vulgaris, AbA and rustmicin are found to be potent inhibitors of IPCS exhibiting IC_{50} values of 0.4–0.8 and 16–20 nM respectively¹⁰⁰. The screening of a library of compounds against *L. major* IPCS revealed a class of inhibitors which possess a structural motif consisting of an electronegatively-substituted biaryl unit separated from a structural amine by an alkyl chain¹³⁴, such as clemastine (Figure 1-17).

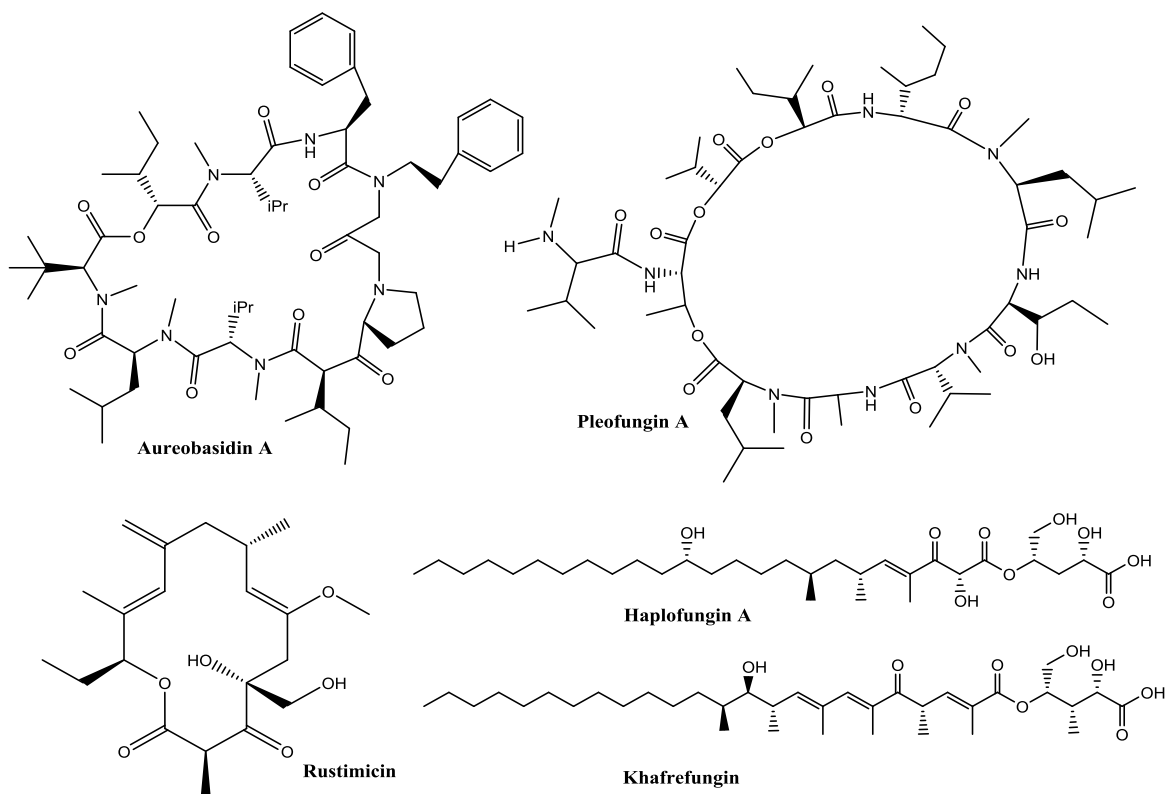


Figure 1-16: The structures of potent inhibitors of IPCS.

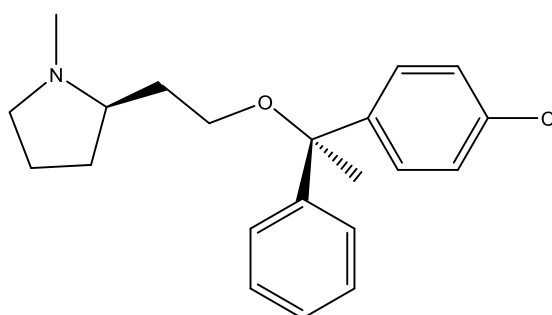


Figure 1-17: Structure of clemastine.

1.8 Project aims

The aim of this project was to identify and characterize IPCS orthologues from the agronomically important cereal crop, rice (*Oryza sativa*), and competing weeds, field poppy (*Papaver rhoeas*), a major persistent weed of cereal crops. However, due to lack of access to a weed genome to search for IPCS in *Papaver rhoeas* genome, *Arabidopsis thaliana* has been utilised as a model dicot weed in place of *Papaver rhoeas*. Mutant *S. cerevisiae* strains expressing functional plant IPCSs were formatted into an assay for the high throughput screening for plant IPCS inhibitors, that show selectivity for the monocot crop over the dicot weed (and vice versa). In tandem, transgenic lines of *Arabidopsis* with varying expression of IPCS were created and RNASeq carried out to identify genes/biological pathways that were altered in response to changes in IPCS expression; this gave an insight into the global function of IPCS in plants.

1.9 References

1. UN, FAO statistical year book 2013: World food and agriculture. http://reliefweb.int/sites/reliefweb.int/files/resources/FAO_2013_stats_yrbook.pdf, 2013.
2. Maxwell, S., World development report 2010: Development and climate change. Taylor & Francis Group: 2010.
3. Hallegatte, S.; Fay, M.; Bangalore, M.; Kane, T.; Bonzanigo, L., *Shock waves: managing the impacts of climate change on poverty*. World Bank Publications: 2015.
4. Arnell, N. W.; Cannell, M. G.; Hulme, M.; Kovats, R. S.; Mitchell, J. F.; Nicholls, R. J.; Parry, M. L.; Livermore, M. T.; White, A., The consequences of CO₂ stabilisation for the impacts of climate change. *Climatic Change* **2002**, 53 (4), 413-446.
5. Facts and figures 2013-2014 Bayer. <http://www.cropscience.bayer.com/en/Company/Facts-and-Figures.aspx>.
6. De, A.; Bose, R.; Kumar, A.; Mozumdar, S., *Targeted delivery of pesticides using biodegradable polymeric nanoparticles*. Springer: 2014.
7. Goldberg, R.; Rissler, J.; Shand, H.; Hassebrook, C., Biotechnology's bitter harvest: herbicide-tolerant crops and the threat to sustainable agriculture. *Biotechnology's bitter harvest: herbicide-tolerant crops and the threat to sustainable agriculture*. **1990**.
8. Flint, M. L.; Van den Bosch, R., *Introduction to integrated pest management*. Springer Science & Business Media: 2012.
9. Zimdahl, R. L., *A history of weed science in the United States*. Elsevier: 2010.
10. Quastel, J., 2, 4-dichlorophenoxyacetic acid (2, 4-D) as a selective herbicide. ACS Publications: 1950.
11. Heap, I., Global perspective of herbicide-resistant weeds. *Pest management science* **2014**, 70 (9), 1306-1315.
12. AN, Y., What Happens When Weed Killers Stop Killing?
13. Heap, I., The International Survey of Herbicide Resistant Weeds. www.weedscience.org **2017**.
14. Kettles, M.; Browning, S. R.; Prince, T. S.; Horstman, S. W., Triazine herbicide exposure and breast cancer incidence: an ecologic study of Kentucky counties. *Environmental health perspectives* **1997**, 105 (11), 1222.
15. Kogevinas, M.; Becher, H.; Benn, T.; Bertazzi, P. A.; Boffetta, P.; Bueno-de-Mesquita, H. B.; Coggon, D.; Colin, D.; Flesch-Janys, D.; Fingerhut, M., Cancer mortality in workers exposed to phenoxy herbicides, chlorophenols, and dioxins an expanded and updated international cohort study. *American journal of epidemiology* **1997**, 145 (12), 1061-1075.
16. Thurman, E. M.; Goolsby, D.; Meyer, M.; Kolpin, D., Herbicides in surface waters of the midwestern United States: The effect of spring flush. *Environmental Science and Technology* **1991**, 25 (10), 1794-1796.
17. Bieberich, E., Ceramide in stem cell differentiation and embryo development: novel functions of a topological cell-signaling lipid and the concept of ceramide compartments. *Journal of lipids* **2011**, 2011.
18. Schengrund, C.-L., Gangliosides: glycosphingolipids essential for normal neural development and function. *Trends in biochemical sciences* **2015**, 40 (7), 397-406.
19. Eich, C.; Manzo, C.; De Keijzer, S.; Bakker, G.-J.; Reinieren-Beeren, I.; García-Parajo, M. F.; Cambi, A., Changes in membrane sphingolipid composition modulate dynamics and adhesion of integrin nanoclusters. *Scientific reports* **2016**, 6, 20693.
20. Riboni, L.; Viani, P.; Bassi, R.; Prinetti, A.; Tettamanti, G., The role of sphingolipids in the process of signal transduction. *Progress in lipid research* **1997**, 36 (2-3), 153-195.

-
21. Merrill Jr, A. H.; Sullards, M. C.; Wang, E.; Voss, K. A.; Riley, R. T., Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental health perspectives* **2001**, *109* (Suppl 2), 283.
 22. Patton, J. L.; Lester, R. L., The phosphoinositol sphingolipids of *Saccharomyces cerevisiae* are highly localized in the plasma membrane. *Journal of Bacteriology* **1991**, *173* (10), 3101-3108.
 23. Skrzypek, M. S.; Nagiec, M. M.; Lester, R. L.; Dickson, R. C., Analysis of phosphorylated sphingolipid long-chain bases reveals potential roles in heat stress and growth control in *Saccharomyces*. *Journal of bacteriology* **1999**, *181* (4), 1134-1140.
 24. Schmelzle, T.; Helliwell, S. B.; Hall, M. N., Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast. *Molecular and Cellular Biology* **2002**, *22* (5), 1329-1339.
 25. Thudichum, J. L. W., *A Treatise on the Chemical Constitution of the Brain*. Archon Books: 1962.
 26. Rao, R. P.; Acharya, J. K., Sphingolipids and membrane biology as determined from genetic models. *Prostaglandins & other lipid mediators* **2008**, *85* (1), 1-16.
 27. Futerman, A. H.; Riezman, H., The ins and outs of sphingolipid synthesis. *Trends in cell biology* **2005**, *15* (6), 312-318.
 28. Huwiler, A.; Kolter, T.; Pfeilschifter, J.; Sandhoff, K., Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **2000**, *1485* (2), 63-99.
 29. Lochnit, G., Structural analysis and immunohistochemical localization of two acidic glycosphingolipids from the porcine, parasitic nematode, *Ascaris suum*. *Glycobiology* **1998**, *8* (9), 891-899.
 30. Berkey, R.; Bendigeri, D.; Xiao, S., Sphingolipids and plant defense/disease: the "death" connection and beyond. *Frontiers in plant science* **2012**, *3*, 68.
 31. Chalfant, C.; Del Poeta, M., *Sphingolipids as signaling and regulatory molecules*. Springer Science & Business Media: 2011; Vol. 688.
 32. Merrill, A.; Williams, R. D., Utilization of different fatty acyl-CoA thioesters by serine palmitoyltransferase from rat brain. *Journal of lipid research* **1984**, *25* (2), 185-188.
 33. Pata, M. O.; Hannun, Y. A.; Ng, C. K. Y., Plant sphingolipids: decoding the enigma of the Sphinx. *New Phytologist* **2010**, *185* (3), 611-630.
 34. Fujino, Y.; Ohnishi, M., Sphingolipids in wheat grain. *Journal of Cereal Science* **1983**, *1* (2), 159-168.
 35. Sperling, P.; Heinz, E., Plant sphingolipids: structural diversity, biosynthesis, first genes and functions. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **2003**, *1632* (1), 1-15.
 36. Dunn, T. M.; Lynch, D. V.; Michaelson, L. V.; Napier, J. A., A Post- genomic Approach to Understanding Sphingolipid Metabolism in *Arabidopsis thaliana*. *Annals of Botany* **2004**, *93* (5), 483-497.
 37. Markham, J. E.; Li, J.; Cahoon, E. B.; Jaworski, J. G., Separation and identification of major plant sphingolipid classes from leaves. *Journal of Biological Chemistry* **2006**, *281* (32), 22684-22694.
 38. Gurr, M. I., Harwood, J. L., & Frayn, K. N., *Lipid biochemistry*. Blackwell Science: 2002.
 39. Chitwood, D. J.; Lusby, W. R.; Thompson, M. J.; Kochansky, J. P.; Howarth, O. W., The glycosylceramides of the nematode *Caenorhabditis elegans* contain an unusual, branched-chain sphingoid base. *Lipids* **1995**, *30* (6), 567-573.
 40. Fyrst, H.; Zhang, X.; Herr, D. R.; Byun, H. S.; Bittman, R.; Phan, V. H.; Harris, G. L.; Saba, J. D., Identification and characterization by electrospray mass spectrometry

of endogenous *Drosophila* sphingadienes. *Journal of lipid research* **2008**, 49 (3), 597-606.

41. Seki, M.; Mori, K., Synthesis of a Prenylated and Immunosuppressive Marine Galactosphingolipid with Cyclopropane- Containing Alkyl Chains:(2S, 3R, 11S, 12R, 2''' R, 5''' Z, 11''' S, 12''' R)- Plakoside A and Its (2S, 3R, 11R, 12S, 2''' R, 5''' Z, 11''' R, 12''' S) Isomer. *European Journal of Organic Chemistry* **2001**, 2001 (20), 3797-3809.

42. Pruett, S. T.; Bushnev, A.; Hagedorn, K.; Adiga, M.; Haynes, C. A.; Sullards, M. C.; Liotta, D. C.; Merrill, A. H., Thematic Review Series: Sphingolipids. Biodiversity of sphingoid bases ("sphingosines") and related amino alcohols. *Journal of lipid research* **2008**, 49 (8), 1621-1639.

43. Futerman, A. H.; Hannun, Y. A., The complex life of simple sphingolipids. *EMBO reports* **2004**, 5 (8), 777-782.

44. Markham, J. E.; Jaworski, J. G., Rapid measurement of sphingolipids from *Arabidopsis thaliana* by reversed- phase high- performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **2007**, 21 (7), 1304-1314.

45. Bohn, M.; Heinz, E.; Lühje, S., Lipid composition and fluidity of plasma membranes isolated from corn (*Zea mays* L.) roots. *Archives of Biochemistry and Biophysics* **2001**, 387 (1), 35-40.

46. Imai, H.; Yamamoto, K.; Shibahara, A.; Miyatani, S.; Nakayama, T., Determining double-bond positions in monoenoic 2-hydroxy fatty acids of glucosylceramides by gas chromatography-mass spectrometry. *Lipids* **2000**, 35 (2), 233.

47. Lynch, D. V.; Dunn, T. M., An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function. *New phytologist* **2004**, 161 (3), 677-702.

48. Wells, G. B.; Dickson, R. C.; Lester, R. L., Isolation and composition of inositolphosphorylceramide-type sphingolipids of hyphal forms of *Candida albicans*. *Journal of bacteriology* **1996**, 178 (21), 6223-6226.

49. Kazuhiro, A.; Uchiyama, R.; Itonori, S.; Sugita, M.; Fang-Sik, C.; Isogai, A.; Noriyasu, H.; Junko, H.; Takeda, T.; Kumagai, H., Structural elucidation of novel phosphocholine-containing glycosylinositol-phosphoceramide in filamentous fungi and their induction of cell death of cultured rice cells. *Biochemical Journal* **2004**, 378 (2), 461-472.

50. Buré, C.; Cacas, J.-L.; Mongrand, S.; Schmitter, J.-M., Characterization of glycosyl inositol phosphoryl ceramides from plants and fungi by mass spectrometry. *Analytical and bioanalytical chemistry* **2014**, 406 (4), 995-1010.

51. Carter, H. E.; Celmer, W. D.; Galanos, D. S.; Gigg, R.; Lands, W. E.; Law, J. H.; Mueller, K. L.; Nakayama, T.; Tomizawa, H.; Weber, E., Biochemistry of the sphingolipides. X. Phytoglycolipide, a complex phytosphingosine-containing lipide from plant seeds. *Journal of the American Oil Chemists Society* **1958**, 35 (7), 335-343.

52. Kaul, K.; Lester, R. L., Characterization of Inositol-containing Phosphosphingolipids from Tobacco Leaves Isolation and Identification of Two Novel, Major Lipids: N-Acetylglucosamidoglucuronidoinositol Phosphorylceramide and Glucosamidoglucuronidoinositol Phosphorylceramide. *Plant physiology* **1975**, 55 (1), 120-129.

53. Kaul, K.; Lester, R. L., Isolation of six novel phosphoinositol-containing sphingolipids from tobacco leaves. *Biochemistry* **1978**, 17 (17), 3569-3575.

54. Carter, H. E.; Brooks, S.; Gigg, R.; Strobach, D. R.; Suami, T., Biochemistry of the Sphingolipids XVI. STRUCTURE OF PHYTOGLYCOLIPID. *Journal of Biological Chemistry* **1964**, 239 (3), 743-746.

-
55. Carter, H.; Kusic, A., Countercurrent distribution of inositol lipids of plant seeds. *Journal of lipid research* **1969**, *10* (4), 356-362.
56. Rennie, E. A.; Ebert, B.; Miles, G. P.; Cahoon, R. E.; Christiansen, K. M.; Stonebloom, S.; Khatab, H.; Twell, D.; Petzold, C. J.; Adams, P. D., Identification of a sphingolipid α -glucuronosyltransferase that is essential for pollen function in Arabidopsis. *The Plant Cell* **2014**, *26* (8), 3314-3325.
57. Voxeur, A.; Fry, S. C., Glycosylinositol phosphorylceramides from Rosa cell cultures are boron- bridged in the plasma membrane and form complexes with rhamnogalacturonan II. *The Plant Journal* **2014**, *79* (1), 139-149.
58. Simenel, C.; Coddeville, B.; Delepierre, M.; Latgé, J.-P.; Fontaine, T., Glycosylinositolphosphoceramides in Aspergillus fumigatus. *Glycobiology* **2008**, *18* (1), 84-96.
59. Spassieva, S.; Hille, J., Plant sphingolipids today-are they still enigmatic? *Plant Biology* **2003**, *5* (02), 125-136.
60. Borner, G. H.; Sherrier, D. J.; Stevens, T. J.; Arkin, I. T.; Dupree, P., Prediction of glycosylphosphatidylinositol-anchored proteins in Arabidopsis. A genomic analysis. *Plant Physiology* **2002**, *129* (2), 486-499.
61. Yoshida, S.; Uemura, M., Lipid composition of plasma membranes and tonoplasts isolated from etiolated seedlings of mung bean (*Vigna radiata* L.). *Plant physiology* **1986**, *82* (3), 807-812.
62. Rochester, C. P.; Kjellbom, P.; Andersson, B.; Larsson, C., Lipid composition of plasma membranes isolated from light-grown barley (*Hordeum vulgare*) leaves: identification of cerebroside as a major component. *Archives of biochemistry and biophysics* **1987**, *255* (2), 385-391.
63. Takakuwa, N.; Saito, K.; Ohnishi, M.; Oda, Y., Determination of glucosylceramide contents in crop tissues and by-products from their processing. *Bioresource technology* **2005**, *96* (9), 1089-1092.
64. Sullards, M.; Lynch, D.; Merrill, A.; Adams, J., Structure determination of soybean and wheat glucosylceramides by tandem mass spectrometry. *Journal of Mass Spectrometry* **2000**, *35* (3), 347-353.
65. Ohnishi, M.; Ito, S.; Fujino, Y., Structural characterization of sphingolipids in leafy stems of rice. *Agricultural and biological chemistry* **1985**, *49* (11), 3327-3329.
66. Uemura, M.; Joseph, R. A.; Steponkus, P. L., Cold acclimation of Arabidopsis thaliana (effect on plasma membrane lipid composition and freeze-induced lesions). *Plant physiology* **1995**, *109* (1), 15-30.
67. Fujino, Y.; Ohnishi, M.; Seisuke, I., Molecular Species of Ceramide and Mono-, Di-, Tri-, and Tetraglycosyl ceramide in Bran and Endosperm of Rice Grains. *Agricultural and biological chemistry* **1985**, *49* (9), 2753-2762.
68. Lester, R. L.; Wells, G. B.; Oxford, G.; Dickson, R. C., Mutant strains of Saccharomyces cerevisiae lacking sphingolipids synthesize novel inositol glycerophospholipids that mimic sphingolipid structures. *Journal of Biological Chemistry* **1993**, *268* (2), 845-856.
69. Cahoon, E. B.; Lynch, D. V., Analysis of glucocerebrosides of rye (*Secale cereale* L. cv Puma) leaf and plasma membrane. *Plant Physiology* **1991**, *95* (1), 58-68.
70. Imai, H.; Ohnishi, M.; Kinoshita, M.; Kojima, M.; Ito, S., Structure and distribution of cerebroside containing unsaturated hydroxy fatty acids in plant leaves. *Bioscience, biotechnology, and biochemistry* **1995**, *59* (7), 1309-1313.
71. Hannun, Y. A.; Obeid, L. M., Principles of bioactive lipid signalling: lessons from sphingolipids. *Nature reviews Molecular cell biology* **2008**, *9* (2), 139-150.

-
72. Wang, L.; Wang, T.; Fehr, W. R., HPLC quantification of sphingolipids in soybeans with modified palmitate content. *Journal of agricultural and food chemistry* **2006**, *54* (20), 7422-7428.
73. Bartke, N.; Fischbeck, A.; Humpf, H. U., Analysis of sphingolipids in potatoes (*Solanum tuberosum* L.) and sweet potatoes (*Ipomoea batatas* (L.) Lam.) by reversed phase high- performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC- ESI- MS/MS). *Molecular nutrition & food research* **2006**, *50* (12), 1201-1211.
74. Dharmawardhane, S.; Rubinstein, B.; Stern, A. I., Regulation of transplasmalemma electron transport in oat mesophyll cells by sphingoid bases and blue light. *Plant physiology* **1989**, *89* (4), 1345-1350.
75. Bille, J.; Weiser, T.; Bentrup, F. W., The lysolipid sphingosine modulates pyrophosphatase activity in tonoplast vesicles and isolated vacuoles from a heterotrophic cell suspension culture of *Chenopodium rubrum*. *Physiologia Plantarum* **1992**, *84* (2), 250-254.
76. Brodersen, P.; Petersen, M.; Pike, H. M.; Olszak, B.; Skov, S.; Ødum, N.; Jørgensen, L. B.; Brown, R. E.; Mundy, J., Knockout of Arabidopsis accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes & Development* **2002**, *16* (4), 490-502.
77. Simanshu, D. K.; Zhai, X.; Munch, D.; Hofius, D.; Markham, J. E.; Bielawski, J.; Bielawska, A.; Malinina, L.; Molotkovsky, J. G.; Mundy, J. W., Arabidopsis accelerated cell death 11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels. *Cell reports* **2014**, *6* (2), 388-399.
78. Fukasawa, M.; Nishijima, M.; Hanada, K., Genetic evidence for ATP-dependent endoplasmic reticulum-to-Golgi apparatus trafficking of ceramide for sphingomyelin synthesis in Chinese hamster ovary cells. *The Journal of cell biology* **1999**, *144* (4), 673-685.
79. Kumagai, K.; Yasuda, S.; Okemoto, K.; Nishijima, M.; Kobayashi, S.; Hanada, K., CERT mediates intermembrane transfer of various molecular species of ceramides. *Journal of Biological Chemistry* **2005**, *280* (8), 6488-6495.
80. Hanada, K.; Kumagai, K.; Yasuda, S.; Miura, Y.; Kawano, M.; Fukasawa, M.; Nishijima, M., Molecular machinery for non-vesicular trafficking of ceramide. *Nature* **2003**, *426* (6968), 803-809.
81. Hanada, K., Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **2003**, *1632* (1), 16-30.
82. Mizutani, Y.; Kihara, A.; Igarashi, Y., LASS3 (longevity assurance homologue 3) is a mainly testis-specific (dihydro) ceramide synthase with relatively broad substrate specificity. *Biochemical Journal* **2006**, *398* (3), 531-538.
83. Mizutani, Y.; Kihara, A.; Igarashi, Y., Mammalian Lass6 and its related family members regulate synthesis of specific ceramides. *Biochemical Journal* **2005**, *390* (1), 263-271.
84. Pewzner-Jung, Y.; Ben-Dor, S.; Futerman, A. H., When do Lasses (longevity assurance genes) become CerS (ceramide synthases)? Insights into the regulation of ceramide synthesis. *Journal of Biological Chemistry* **2006**, *281* (35), 25001-25005.
85. Markham, J. E.; Molino, D.; Gissot, L.; Bellec, Y.; Hématy, K.; Marion, J.; Belcram, K.; Palauqui, J.-C.; Satiat-JeuneMaître, B.; Faure, J.-D., Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in Arabidopsis. *The Plant Cell* **2011**, *23* (6), 2362-2378.

-
86. Futerman, A. H.; Pagano, R. E., Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver. *Biochemical Journal* **1991**, *280* (2), 295-302.
87. Gillard, B. K.; Clement, R. G.; Marcus, D. M., Variations among cell lines in the synthesis of sphingolipids in de novo and recycling pathways. *Glycobiology* **1998**, *8* (9), 885-890.
88. Tettamanti, G.; Bassi, R.; Viani, P.; Riboni, L., Salvage pathways in glycosphingolipid metabolism. *Biochimie* **2003**, *85* (3), 423-437.
89. Kitatani, K.; Idkowiak-Baldys, J.; Hannun, Y. A., The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cellular signalling* **2008**, *20* (6), 1010-1018.
90. Riboni, L.; Prinetti, A.; Bassi, R.; Tettamanti, G., Formation of bioactive sphingoid molecules from exogenous sphingomyelin in primary cultures of neurons and astrocytes. *FEBS letters* **1994**, *352* (3), 323-326.
91. Pennell, R. I.; Lamb, C., Programmed cell death in plants. *The Plant Cell* **1997**, *9* (7), 1157.
92. Collazo, C.; Chacón, O.; Borrás, O., Programmed cell death in plants resembles apoptosis of animals. *Biotechnología Aplicada* **2006**, *23* (1), 1-10.
93. Liang, H.; Yao, N.; Song, J. T.; Luo, S.; Lu, H.; Greenberg, J. T., Ceramides modulate programmed cell death in plants. *Genes & development* **2003**, *17* (21), 2636-2641.
94. Peters, J.; Chin, C.-k., Potassium loss is involved in tobacco cell death induced by palmitoleic acid and ceramide. *Archives of biochemistry and biophysics* **2007**, *465* (1), 180-186.
95. Wang, W.; Yang, X.; Tangchaiburana, S.; Ndeh, R.; Markham, J. E.; Tsegaye, Y.; Dunn, T. M.; Wang, G.-L.; Bellizzi, M.; Parsons, J. F., An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in Arabidopsis. *The Plant Cell* **2008**, *20* (11), 3163-3179.
96. Xiao, S.; Ellwood, S.; Findlay, K.; Oliver, R. P.; Turner, J. G., Characterization of three loci controlling resistance of Arabidopsis thaliana accession Ms-0 to two powdery mildew diseases. *The Plant Journal* **1997**, *12* (4), 757-768.
97. Huitema, K.; van den Dikkenberg, J.; Brouwers, J. F.; Holthuis, J. C., Identification of a family of animal sphingomyelin synthases. *The EMBO journal* **2004**, *23* (1), 33-44.
98. Denny, P. W.; Shams-Eldin, H.; Price, H. P.; Smith, D. F.; Schwarz, R. T., The protozoan inositol phosphorylceramide synthase a novel drug target that defines a new class of sphingolipid synthase. *Journal of Biological Chemistry* **2006**, *281* (38), 28200-28209.
99. Heidler, S. A.; Radding, J. A., Inositol phosphoryl transferases from human pathogenic fungi. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **2000**, *1500* (1), 147-152.
100. Bromley, P. E.; Li, Y. O.; Murphy, S. M.; Sumner, C. M.; Lynch, D. V., Complex sphingolipid synthesis in plants: characterization of inositolphosphorylceramide synthase activity in bean microsomes. *Archives of Biochemistry and Biophysics* **2003**, *417* (2), 219-226.
101. Nagiec, M. M.; Nagiec, E. E.; Baltisberger, J. A.; Wells, G. B.; Lester, R. L.; Dickson, R. C., Sphingolipid synthesis as a target for antifungal drugs complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the AUR1 gene. *Journal of Biological Chemistry* **1997**, *272* (15), 9809-9817.

-
102. Heidler, S. A.; Radding, J. A., The AUR1 gene in *Saccharomyces cerevisiae* encodes dominant resistance to the antifungal agent aureobasidin A (LY295337). *Antimicrobial agents and chemotherapy* **1995**, *39* (12), 2765-2769.
103. Mina, J., Thesis: Exploring IPC synthase: a potential anti-leishmanial drug target. **2010**.
104. Mina, J.; Okada, Y.; Wansadhipathi-Kannangara, N.; Pratt, S.; Shams-Eldin, H.; Schwarz, R.; Steel, P.; Fawcett, T.; Denny, P., Functional analyses of differentially expressed isoforms of the Arabidopsis inositol phosphorylceramide synthase. *Plant molecular biology* **2010**, *73* (4-5), 399-407.
105. Sutterwala, S. S.; Hsu, F. F.; Sevova, E. S.; Schwartz, K. J.; Zhang, K.; Key, P.; Turk, J.; Beverley, S. M.; Bangs, J. D., Developmentally regulated sphingolipid synthesis in African trypanosomes. *Molecular microbiology* **2008**, *70* (2), 281-296.
106. Sigal, Y. J.; McDERMOTT, M. I.; Morris, A. J., Integral membrane lipid phosphatases/phosphotransferases: common structure and diverse functions. *Biochemical Journal* **2005**, *387* (2), 281-293.
107. Stuke, J.; Carman, G. M., Identification of a novel phosphatase sequence motif. *Protein science* **1997**, *6* (2), 469-472.
108. Mina, J. G.; Mosely, J. A.; Ali, H. Z.; Denny, P. W.; Steel, P. G., Exploring Leishmania major Inositol Phosphorylceramide Synthase (LmjIPCS): Insights into the ceramide binding domain. *Organic & Biomolecular Chemistry* **2011**, *9* (6), 1823-1830.
109. Bi, F.-C.; Liu, Z.; Wu, J.-X.; Liang, H.; Xi, X.-L.; Fang, C.; Sun, T.-J.; Yin, J.; Dai, G.-Y.; Rong, C., Loss of ceramide kinase in Arabidopsis impairs defenses and promotes ceramide accumulation and mitochondrial H₂O₂ bursts. *The Plant Cell* **2014**, *26* (8), 3449-3467.
110. Tafesse, F. G.; Ternes, P.; Holthuis, J. C., The multigenic sphingomyelin synthase family. *Journal of Biological Chemistry* **2006**, *281* (40), 29421-29425.
111. Stuart, K.; Brun, R.; Croft, S.; Fairlamb, A.; Gürtler, R. E.; McKerrow, J.; Reed, S.; Tarleton, R., Kinetoplastids: related protozoan pathogens, different diseases. *The Journal of clinical investigation* **2008**, *118* (4), 1301-1310.
112. Croft, S. L.; Barrett, M. P.; Urbina, J. A., Chemotherapy of trypanosomiasis and leishmaniasis. *Trends in parasitology* **2005**, *21* (11), 508-512.
113. De Lucca, A. J., Harmful fungi in both agriculture and medicine. *Revista iberoamericana de micología* **2007**, *24* (1), 3.
114. Gugnani, H. C., Ecology and taxonomy of pathogenic aspergilli. *Front Biosci* **2003**, *8* (1-3), 346.
115. Dvoráčková, I.; Pichova, V., Pulmonary interstitial fibrosis with evidence of aflatoxin B1 in lung tissue. **1986**.
116. Thomas, P., Fungal infections of the cornea. *Eye* **2003**, *17* (8), 852-862.
117. Vicente, M.; Basilio, A.; Cabello, A.; Peláez, F., Microbial natural products as a source of antifungals. *Clinical Microbiology and Infection* **2003**, *9* (1), 15-32.
118. Deray, G., Amphotericin B nephrotoxicity. *Journal of Antimicrobial Chemotherapy* **2002**, *49* (suppl 1), 37-41.
119. Snelders, E.; van der Lee, H. A.; Kuijpers, J.; Rijs, A. J. M.; Varga, J.; Samson, R. A.; Mellado, E.; Donders, A. R. T.; Melchers, W. J.; Verweij, P. E., Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med* **2008**, *5* (11), e219.
120. Han, H.; Yu, Q.; Owen, M. J.; Cawthray, G. R.; Powles, S. B., Widespread occurrence of both metabolic and target-site herbicide resistance mechanisms in *Lolium rigidum* populations. *Pest management science* **2016**, *72* (2), 255-263.
121. Carlson, H. L.; Hill, J. E., Wild oat (*Avena fatua*) competition with spring wheat: plant density effects. *Weed Science* **1985**, *33* (2), 176-181.
-

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122. Bajwa, A. A.; Jabran, K.; Shahid, M.; Ali, H. H.; Chauhan, B. S., Eco-biology and management of *Echinochloa crus-galli*. *Crop Protection* **2015**, *75*, 151-162.
123. Chandler, J. W., Cotyledon organogenesis. *Journal of Experimental Botany* **2008**, *59* (11), 2917-2931.
124. Taiz, L.; Zeiger, E.; Møller, I. M.; Murphy, A., *Plant physiology and development*. Sinauer Associates, Incorporated: 2015.
125. Nelson, T.; Dengler, N., Leaf vascular pattern formation. *The Plant Cell* **1997**, *9* (7), 1121.
126. Zimmermann, M. H.; Tomlinson, P. B., The vascular system of monocotyledonous stems. *Botanical Gazette* **1972**, *133* (2), 141-155.
127. McSteen, P., Auxin and monocot development. *Cold Spring Harbor perspectives in biology* **2010**, *2* (3), a001479.
128. Mandala, S. M.; Thornton, R. A.; Rosenbach, M.; Milligan, J.; Garcia-Calvo, M.; Bull, H. G.; Kurtz, M. B., Khafrefungin, a novel inhibitor of sphingolipid synthesis. *Journal of Biological Chemistry* **1997**, *272* (51), 32709-32714.
129. Mandala, S. M.; Thornton, R. A.; Milligan, J.; Rosenbach, M.; Garcia-Calvo, M.; Bull, H. G.; Harris, G.; Abruzzo, G. K.; Flattery, A. M.; Gill, C. J., Rustmicin, a potent antifungal agent, inhibits sphingolipid synthesis at inositol phosphoceramide synthase. *Journal of Biological Chemistry* **1998**, *273* (24), 14942-14949.
130. Yano, T.; Aoyagi, A.; Kozuma, S.; Kawamura, Y.; Tanaka, I.; Suzuki, Y.; Takamatsu, Y.; Takatsu, T.; Inukai, M., Pleofungins, novel inositol phosphorylceramide synthase inhibitors, from *Phoma* sp. SANK 13899. *Journal of Antibiotics* **2007**, *60* (2), 136.
131. Ohnuki, T.; Yano, T.; Takatsu, T., Haplofungins, new inositol phosphorylceramide synthase inhibitors, from *Lauriomyces bellulus* SANK 26899 II. Structure elucidation. *Journal of Antibiotics* **2009**, *62* (10), 551-557.
132. Mina, J. G.; Pan, S.-Y.; Wansadhipathi, N. K.; Bruce, C. R.; Shams-Eldin, H.; Schwarz, R. T.; Steel, P. G.; Denny, P. W., The *Trypanosoma brucei* sphingolipid synthase, an essential enzyme and drug target. *Molecular and biochemical parasitology* **2009**, *168* (1), 16-23.
133. Figueiredo, J. M.; MENDONÇA-PREVIATO, L.; PREVIATO, J. O.; HEISE, N., Characterization of the inositol phosphorylceramide synthase activity from *Trypanosoma cruzi*. *Biochemical Journal* **2005**, *387* (2), 519-529.
134. Brown, C. Inhibitors of *Leishmania major* Inositol Phosphorylceramide Synthase: New Therapies for Leishmaniasis. Durham University, 2016.

CHAPTER 2

CONSTRUCTION OF PLANT IPCS COMPLEMENTED YEAST STRAINS AND
ASSAY VALIDATION

2.1 Chapter synopsis

In this chapter, bioinformatics analysis of the plant IPCS was carried out. Phylogenetic analyses of IPCS revealed a divide in clustering between monocots and dicots. Conserved domains previously identified in animal sphingomyelin synthases and protozoan IPCSs were also identified among the plant IPCSs. Analyses of the promoter region of the three IPCS isoforms in *Arabidopsis thaliana* (*At*IPCS1-3) and *Oryza sativa* (*Os*IPCS1-3) were carried out to identify binding sites of transcription factors. The three IPCS isoforms in *A. thaliana* and *O. sativa* were cloned into an expression vector in *Saccharomyces cerevisiae* and their activities characterized. These yeast strains were formatted into an assay and the assay conditions were validated to screen for inhibitors of plant IPCSs.

2.2 Bioinformatics analysis of plant IPCS

Identification of inhibitors that show differential selectivity for *At*IPCSs over *Os*IPCSs (vice versa) is possible if there are structural differences between the orthologues which arise from differences in the amino acid sequence. To find out these differences, structural analysis of *At*IPCS1-3 and *Os*IPCS1-3 was carried out.

The identification of functional IPCS orthologues in *Oryza sativa* was achieved by a BLAST search of phytozyme database using the coding sequence of *At*IPCS1 which yielded three homologues. Analyses of the coding sequence for the three isoforms found in *Arabidopsis thaliana* and *Oryza sativa*, showed a difference in the number and length of exons and introns (Figure 2-1). *At*IPCS1 possesses 11 exons and 10 introns, whilst *At*IPCS2 has 12 exons and 11 introns and *At*IPCS3 possesses 11 exons and 10 introns. The analogous orthologues in *O. sativa* are approximately two times longer and are composed of 12 exons and 11 introns.

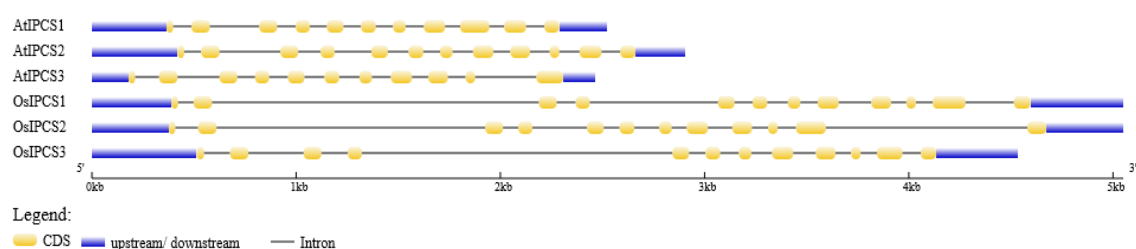


Figure 2-1: Schematic representation of exon and intron organisation of IPCS in *O. sativa* and *A. thaliana*; black line and yellow boxes indicates introns and exon respectively, with the blue boxes representing the 5' and 3' UTRs.

From work done by Mina *et al.* looking at the differential expression of the three homologues of IPCS in *A. thaliana*, it is known that the second orthologue of IPCS is the most highly expressed by a log 2-fold difference per μg RNA extracted¹. It is highly expressed in stem, flower and siliques and to an even greater extent (one log fold increase per μg of RNA extracted) in roots, cauline leaf, and rosette leaf², pointing to a key role in these tissues.

2.2.1 Phylogenetic analysis

With IPCS2 being the most highly expressed isoform in *A. thaliana*, a phylogenetic analysis of the evolutionary distance of this particular isoform in different plant species, protozoa and fungi was carried out to gain an insight into how closely related the orthologues were to each other. Unsurprisingly, the result shows that the plant IPCS are clustered together and are independent of the protozoan and fungi IPCS (Figure 2-2). Interestingly, the clustering of IPCS2 in plants predominantly shows a divide between monocots and dicots, which is promising in the aim to identify inhibitors that show differential selectivity for either dicots over monocots or monocots over dicots.

An examination of the phylogenetic relationship between the three isoforms in *A. thaliana* and *O. sativa* show that IPCS1 and IPCS2 in both plants are more closely related compared to each other than IPCS3. Access was given by Bayer Crop Science to the genome of the noxious weed *Echinochloa crus-galli*, known to cost billions of dollars of losses to rice production, but the sequence was not complete and attempts to reconstruct the sequence by primer walking failed. However, analyses were carried out to see how closely related it is to the IPCS found in *O. sativa* and *A. thaliana* (Figure 2-3). From the phylogenetic tree it was clear that the *E. crus-galli* IPCS is distantly related and forms a cluster separate from *At*IPCS1-3, *Os*IPCS1 and 2. Interestingly, *Os*IPCS3 is placed on a separate evolutionary branch from *Os*IPCS1 and 2, which indicates that it is very different from these two isoforms and is shown to be more closely related to *Ech*IPCS1. The reason for this is not immediately clear from differences in the amino acid sequence between *Os*IPCS1, 2 and 3. However, the evolutionary distance between *O. sativa* isoforms and *Ech*IPCS1 is particularly promising, as it indicated that in the search for new herbicide mode of action, selective IPCS inhibitors targeting *E. crus-galli* over *O. sativa* IPCS could potentially be developed.

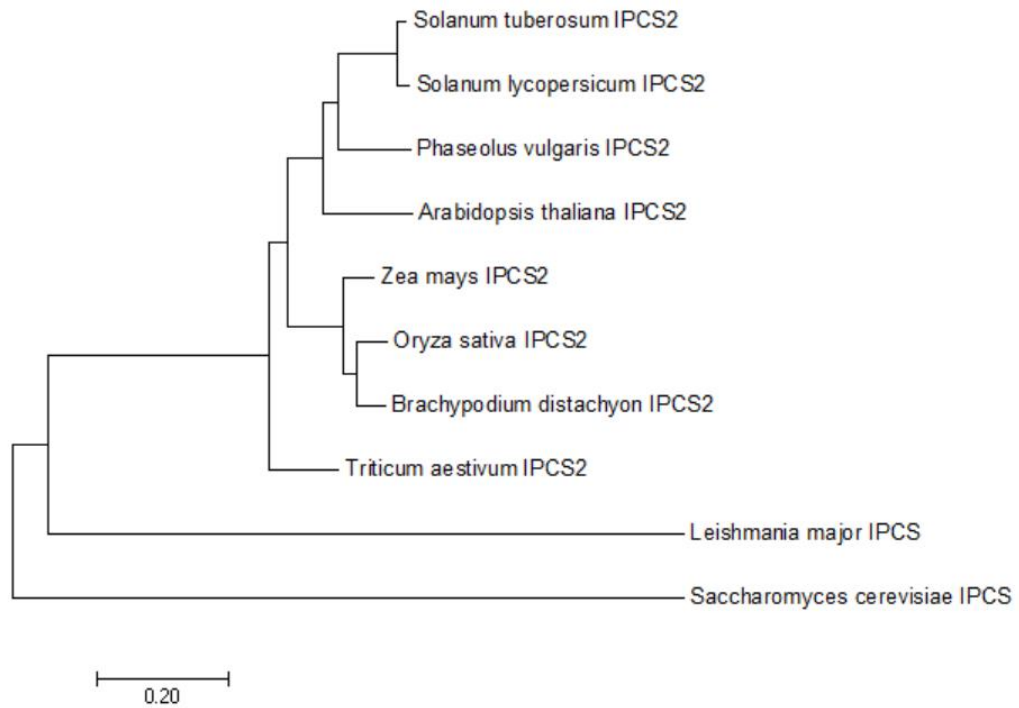


Figure 2-2: Phylogenetic tree generated from the amino acid sequence of IPCS from plant, protozoa and fungi. The evolutionary distances were calculated using the neighbourhood joining method and analysed in MEGA7; drawn to scale with branch lengths in the same units as those of the evolutionary distances used to construct the phylogenetic tree in units of the number of amino acid substitution per site.

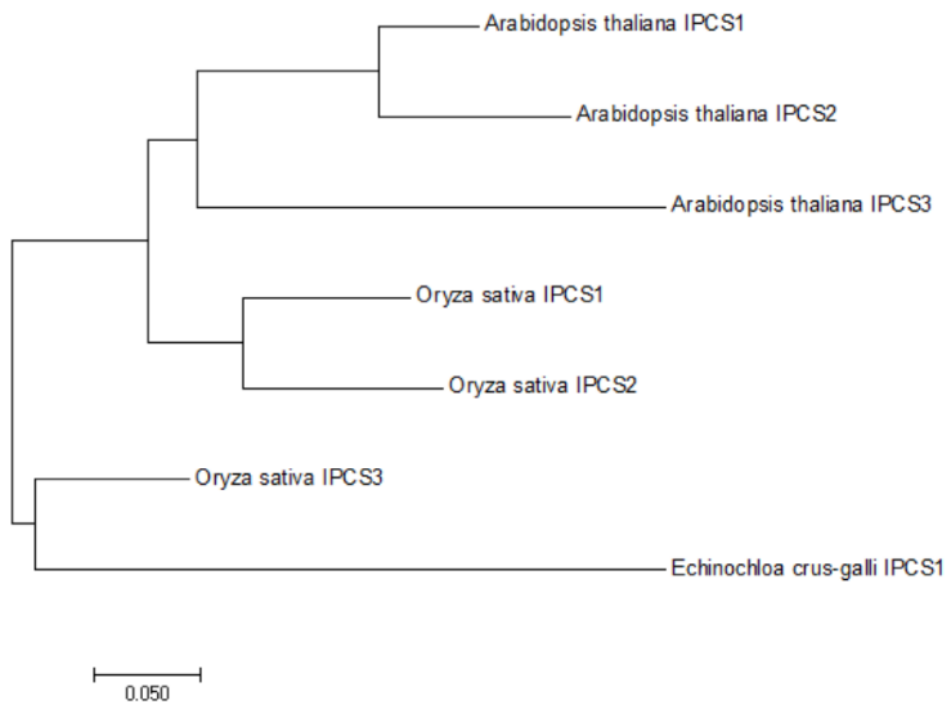


Figure 2-3: Phylogenetic tree constructed using IPCS amino acid sequence from *A. thaliana*, *O. sativa* and *E. crus-galli*. The evolutionary distances were calculated using the neighbourhood joining method and analysed in MEGA7; drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to construct the phylogenetic tree. The unit of measurement quantifies the number of amino acid substitutions per site.

2.2.2 Prediction of transmembrane topology

Using the transmembrane hidden Markov model³ (TMHMM) server, the number, location of the transmembrane regions and protein topology were identified for *Os*IPCS1-3, *At*IPCS1-3 and *Ech*IPCS. It was found that *At*IPCS1, *At*IPCS2, *At*IPCS3 and *Os*IPCS3 were predicted to have five transmembrane regions as opposed to six, because the probability of the first transmembrane region was below 0.5 so was not significant enough to use as a reliable indication of its presence (Figure 2-4). *Ech*IPCS was predicted to have three transmembrane regions because of the low probability of the other three transmembrane regions (data not shown). However, *Os*IPCS1 and *Os*IPCS2 were both predicted to have six transmembrane regions with a probability of the prediction being between 0.8 and 1 (Figure 2-3). The defined transmembrane regions are also in agreement with the predicted transmembrane region and topology carried out using predictprotein⁴ server, with an inside-inside orientation of the N and C terminal which is in agreement with Bang's model and the luminal topology of the active site in the Golgi⁵.

From the structural analysis, it seems that there is a structural difference between the plant IPCS from *A. thaliana*, *O. sativa* and *E. crus-gali*, with *At*IPCS1-3 and *Os*IPCS3 predicted to have five transmembranes and an extracellular domain, whilst *Os*IPCS1 and 2 have six transmembrane regions. The differences in the structural predictions particularly in regard to *At*IPCS2 and *Os*IPCS2 arise due to the presence of hydrophobic residues such as phenylalanine (Phe¹⁶) and tyrosine (Tyr²² and Tyr³³) at the N-terminus of *At*IPCS2 in place of valine (Val¹⁶ and Val²²) and leucine (Leu³³) at the N-terminus of *Os*IPCS2. Additional variation in amino acid sequence at the N-terminus include the presence of serine (Ser⁹), cysteine (Cys¹⁷) and asparagine Asn³⁰ (*At*IPCS2) in place of alanine (Ala⁹), tyrosine (Tyr¹⁷) and lysine (Lys³⁰) respectively (*Os*IPCS2). These differences affect topology prediction using TMHMM because the prediction is based on

the positive-inside rule with positively charged residues positioned in the cytosol and hydrophobic residues located in the transmembrane region. For *AtIPCS2* the existence of hydrophobic residues makes it difficult for the TMHMM programme to ascribe the location of the N-terminus and subsequently affects the topology prediction of the first transmembrane. Topology prediction using MEMSAT3 a program that relies on a neural network to determine the location of amino acid residues in a protein structure⁶. Utilising MEMSAT3, *EchIPCS*, *AtIPCS2* and *OsIPCS2* (data not shown) were predicted to be six transmembrane spanning proteins, however, with no experimental evidence of IPCS structure available at the moment, there is no way of knowing if the variation in amino acid sequence results in a difference in structure between the orthologues. If the structural differences predicted by TMHMM are correct, then the possibility of the identification of selective inhibitors is much more likely.

Using the structural prediction of *OsIPCS1* and 2 which is in agreement with Bang's model⁷, and the identified conserved domains identified in yeast⁵, and human sphingomyelin synthase⁷, the transmembrane regions, domains, and catalytic triad (His, His and Asp) were identified from the multiple sequence alignment of plant IPCSs amino acid coding sequence (Figure 2-4).

From the alignment, the plant IPCSs are shown to have highly conserved regions at the amino acid level in the transmembrane regions and domains, however, it is clear that at the N-terminal, there is variation at the amino acid level with fewer amino acids observed to be identical but do share similar chemical properties. This difference could account for the observed difference in the structural prediction. *EchIPCS* does not align with the other IPCSs, and the catalytic triad is unidentified upon alignment, which is probably more likely to be due to the sequence being incomplete, than to a real structural difference at the active site.

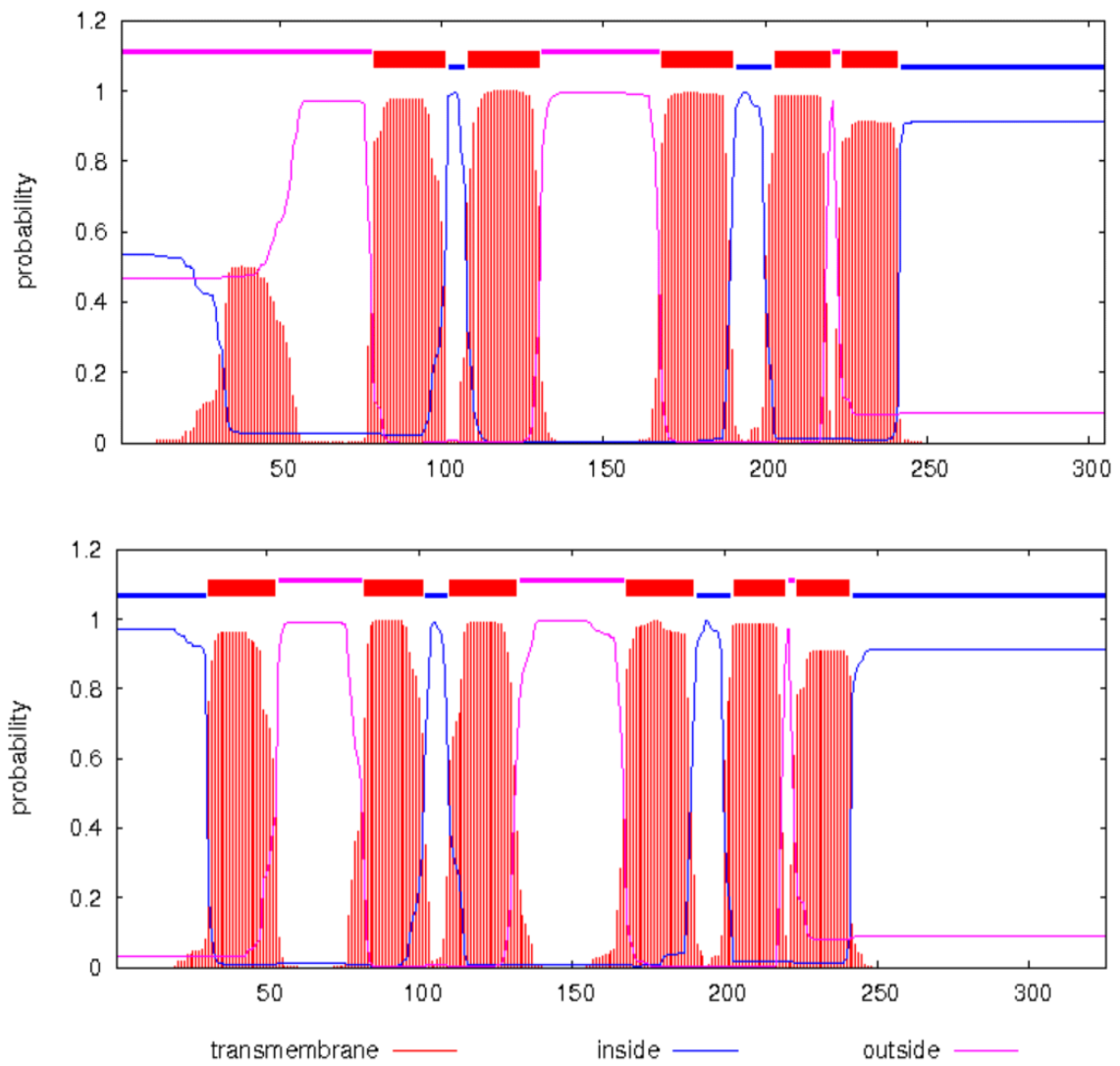


Figure 2-3: TMHMM posterior probabilities for (A) *OsIPCS2* and (B) *AtIPCS2* showing the six predicted transmembranes and their topologies.

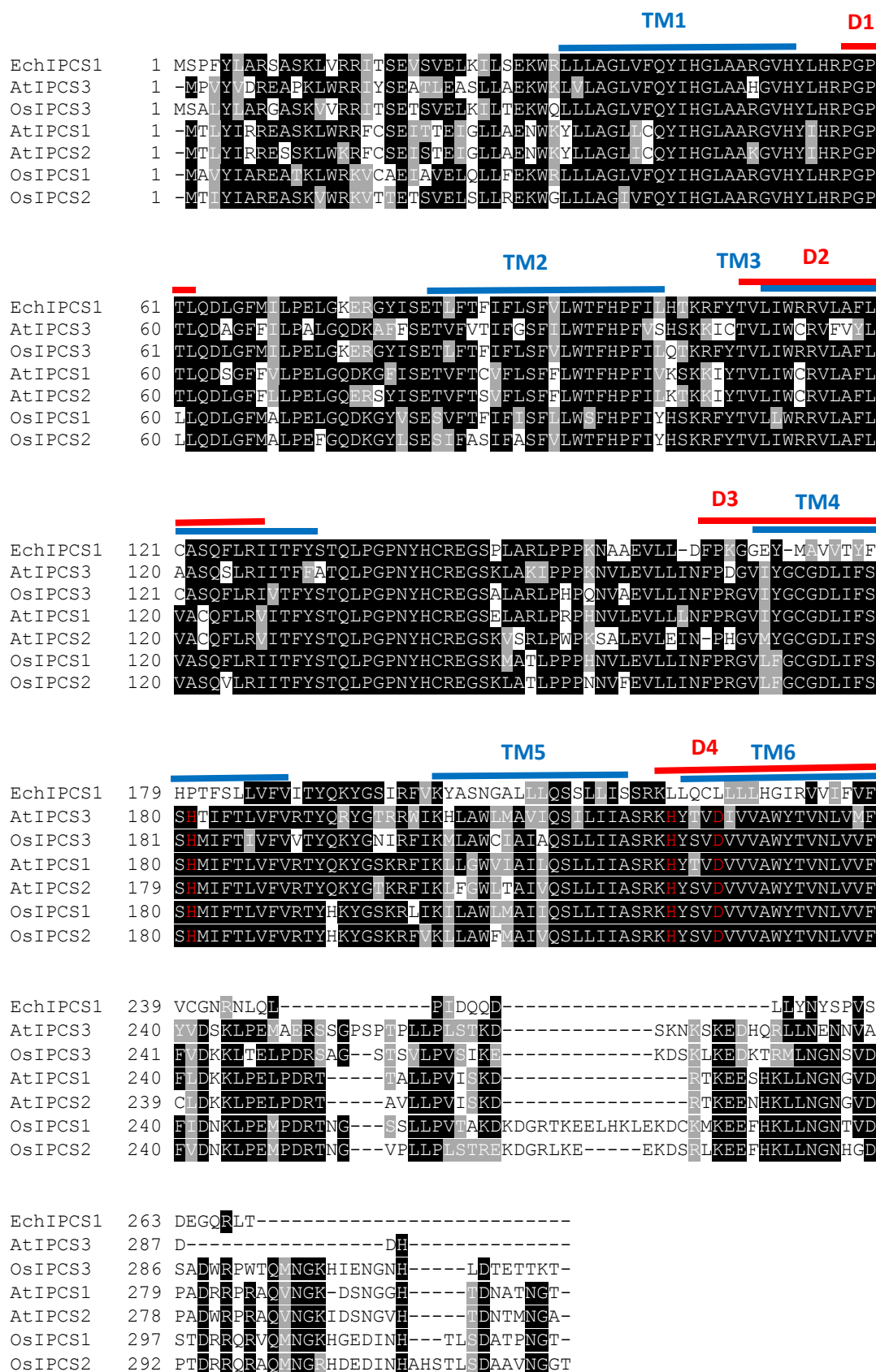


Figure 2-4: Alignment of *AtIPCS1-3*, *OsIPCS1-3* and *EchIPCS* with transmembrane regions and conserved domains highlighted; catalytic residues are also highlighted in red.

2.2.3 Identification of cis-elements and transcription factors upstream of *AtIPCS* promoter

To get an insight into the biological processes that IPCS might be involved in, an investigation of the promoter region was carried out to identify cis-regulatory elements to which transcription factors can bind, eliciting a response in plants. The IPCS promoter region (1000 bp upstream of transcription initiation) was examined using PlantPAN2 to identify motifs and transcription factors involved in the response to biotic and abiotic stress (Figure 2-5). A number of WRKY transcriptional factors binding sites were identified in the promoter region of *AtIPCS*1-3, and of particular interest was WRKY4 which is involved in plant pathogen response, having a negative effect on plant response to biotrophic pathogens, and a positive effect on plant resistance to necrotrophic pathogens⁸. Also identified were WRKY18 and WRKY60, which are known to enhance plant sensitivity to abscisic acid in relation to root development, whilst also enhancing sensitivity to salt and osmotic stress⁹.

The binding site of the *cis*-regulatory elements LS5ATPR1 and LS7ATPR1 were identified and are well characterized positive salicylic acid-inducible elements recognized by TGA factors¹⁰ (Figure 2-5). Binding sites for TGA factors are frequently found in promoters of stress-regulated plant genes¹¹; TGA2 and TGA3 interaction with LS7 is enhanced by binding with non-expresser of pathogenesis related genes protein (NPR1)¹²,¹³, a defence response gene associated with the induction of systematic acquired resistance in plant defence response to pathogen¹⁴.

The same analysis was carried out for *OsIPCS*1-3 and no WRKY transcriptional factor binding sites were found, but the search did yield different basic region leucine zipper binding sites with unknown targets. Expansion of the region upstream the transcription start site to 1500 bp and subsequent analyses using the PlantCARE database, generated

several motifs which are shared between *A. thaliana* and *O. sativa* IPCSs; *cis* acting elements involved in stress and pathogen response were identified, as were *cis*-regulatory elements inducible in response to the hormones abscisic acid and gibberellin (Table 2-1) indicating that the modulation of plant IPCSs expression is involved in plant response to abiotic and biotic stress.



Figure 2-5: Transcription factor binding motif sequence located 1000bp upstream of the transcription start site; the transcription factor binding sites highlighted are conserved in *At*IPCS1-3; shown above is the promoter region of *At*IPCS1; table of motifs can be found in Appendix A.

It is clear from the analyses that the IPCS from *A. thaliana* and *O. sativa* share the same transcriptional binding elements, and at the amino acid level the sequence is highly conserved. *Os*IPCS1 shares 70% sequence identity and 81% similarity to *At*IPCS1; *At*IPCS1 sequence identity and similarity was between 69-70% and 79-80% for *Os*IPCS

2 and 3 respectively. Sequence identity between *OsIPCS1* and *OsIPCS2* is 82% and the sequence similarity is 89%; for *OsIPCS3* the sequence identity and similarity to *OsIPCS1* is 70% and 84% respectively. The sequences identify between *AtIPCS1* and *AtIPCS2* is 86% and the similarity is 93%, whilst *AtIPCS3* shares a lower sequence identity with *AtIPCS1* of 69% and a sequence similarity of 82%.

Mutagenesis studies of fungal IPC (*AUR1p*) have shown that a single amino acid mutation is enough to alter the sensitivity of the enzyme to Aureobasidin A (AbA). Mutation of Leu-137 to phenylalanine and His-157 to tyrosine in the *AUR1* gene sequence resulted in AbA resistance in *S. cerevisiae*¹⁵. This is corroborated by a similar study carried out by Okado *et al.* which revealed the ability of *AUR1* to become insensitive to AbA upon the mutation of Phe-158 to a tyrosine¹⁶. In addition, a mutation changing glycine-240 to a cysteine residue in the *AUR1* homologue in *Schizosaccharomyces pombe* has been found to be responsible for the acquisition of AbA resistance in both fungal species^{17, 18}. Likewise, the corresponding residue 275 in the *aurA* gene of *Aspergillus nidulans*, a homolog of yeast *AUR1* has been found to confer a high level of AbA resistance¹⁹. These studies show that IPCS sensitivity to an inhibitor can be altered by a single change of an amino acid residue and stimulates the consideration of an inhibitor being selective for different orthologues of IPCS in the same plant, which could open up the possibility of creating a cocktail of herbicides that targets two out of the three orthologues, thereby reducing the likelihood of herbicide resistance. The possibility of selective inhibition between the very closely related IPCS isoforms is possible, and has been shown to be the case by the selective inhibition of MAP Kinase p38 α over p38 β , which share a sequence identity of 75% at the amino acid level²⁰.

In the next section, work carried out to express *AtIPCS1-3* and *OsIPCS1-3* and characterise the activity of plant IPCS in a *S. cerevisiae* mutant line is presented, with the

aim of utilising the transgenic yeast lines in a high throughput screen for inhibitors. This will enable the identification of compounds which show differential selectivity for either IPCS from *A. thaliana* or *O. sativa*.

Table 2-1: Identification of conserved cis-elements located upstream of the transcription start site of *At*IPCS1-3 and *Os*IPCS1-3

| Motif name | Sequence function |
|------------------------|---|
| ABRE | <i>Cis</i> -acting element involved in abscisic acid responsiveness |
| Box W1 | Fungal elicitor responsive element |
| MBS | MYB binding site involved in drought-inducibility |
| TCA element | <i>Cis</i> -acting regulatory element involved in salicylic acid responsiveness |
| Circadian (CBS) | <i>Cis</i> -acting regulatory element involved in circadian control |
| ARE | <i>Cis</i> -acting regulatory element essential for anaerobic induction |
| P-Box | Gibberellin responsive element |
| TC-rich repeats | <i>Cis</i> -acting element involved in defence and stress responsiveness |
| RY-element | <i>Cis</i> -acting regulatory element involved in seed-specific regulation |
| HSE | Heat responsive elements |

2.3 Cloning and expression of plant IPCS to complement a mutant yeast strain

To analyse the multi-transmembrane plant IPCS and format a high throughput screening (HTS) assay, the coding sequences were cloned into suitable expression vectors to complement a yeast mutant as described below.

Saccharomyces cerevisiae pdr genes encode extrusion pumps responsible for pleiotropic drug resistance²¹. Fungi invoke resistance by the up-regulation of these genes to facilitate the efflux of cytotoxic drugs, thus leading to decreased drug accumulation and diminished concentrations²². In order to maximize the possibility of identifying IPCS inhibitors, it was necessary to prepare a suitable *S. cerevisiae* strain lacking these efflux pumps.

A mutant yeast strain lacking fungal IPCS, AUR1 (MATa his3Δ1leu2Δ0ura3Δ0 aur1::HIS3) and four genes conferring drug hypersensitivity (*pdr1Δ::KanMX4*, *pdr3Δ::KanMX4*, *pdr16Δ::KanMX4* and *pdr17Δ::KanMX4*) were created by Prof. Michael Stark (University of Dundee). This strain can survive by the introduction of the pRS316-AUR1 plasmid, (Prof. Michael Stark), resulting in the expression AUR1 under a URA3 selectable marker. This yeast strain (the parental line) can be maintained on media lacking histidine and uracil.

For the cloning of *At*IPCS1-3, *Os*IPCS1-3 into the pESC-LEU expression vector at multiple cloning site 2, primers were designed for each IPCS insert and amplified by proofreading PCR from the pRS426MET plasmid. Subsequently, infusion® cloning was carried out, followed by transformation in D-H5α *E. coli* to isolate high yields of the purified plasmid. These plasmids were then used to transform the parental yeast strain using the lithium acetate/single carrier DNA/polyethylene glycol method^{23, 24}.

To obtain the yeast strains containing only the pESC-LEU vector with the IPCS of interest, and lacking pRS316-AUR1, the mutant strains were grown on media containing 5-fluoroorotic acid (5-FOA). 5-FOA is an extremely useful reagent for the selection of ura⁻ cells amid a population of ura⁺ cells; this selection is effective in studies where loss of *URA3* is desired²⁵. *URA3* encodes for orotidine-5'-monophosphate decarboxylase (ODcase), an enzyme responsible for catalysing the last step in the de novo synthesis of

pyrimidines by the decarboxylation of orotidine monophosphate to generate uridine monophosphate²⁶. In cells maintaining the pRS316-AUR1 URA3 plasmid, ODcase is expressed and converts 5-FOA to fluorouracil (Figure 2-6), a toxic analogue of uracil, resulting in cell death. This allows selection of yeast strain lacking this plasmid, a process termed ‘plasmid shuffle’ (Figure 2-7)²⁵.

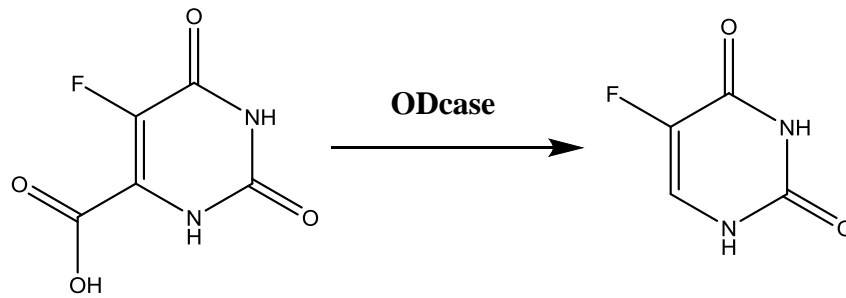


Figure 2-6: Conversion of 5-fluoroorotic acid to fluorouracil is used to select against yeast harbouring the AUR1 plasmid under the URA3 marker.

Yeast cells without either the pRS316-AUR1 URA3 plasmid or an introduced plasmid encoding a complementing enzyme, have no IPCS, and so cannot synthesise essential sphingolipids. The plant IPCSs cloned into the pESC-LEU vector are under the influence of the GAL1 promoter, therefore yeast dependent on IPCS expression from these vectors can only grow in media/agar containing galactose (SGR-H-L).

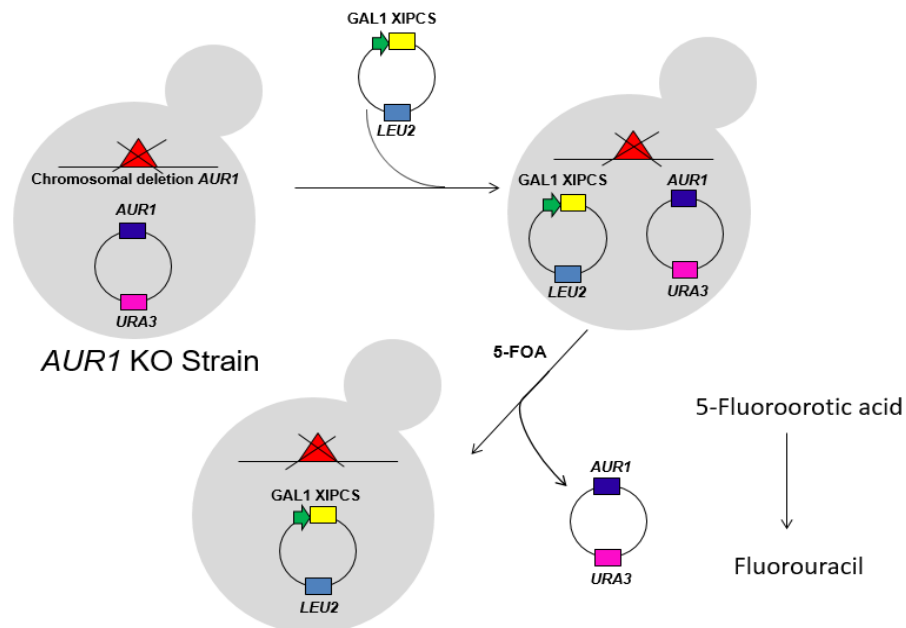


Figure 2-7: Plasmid shuffle; transformation with pESC-LEU plant IPCS plasmid and elimination of pRS426-AUR1 plasmid in yeast.

*At*IPCS1-3 and *Os*IPCS1-3 complemented yeast strains were taken through two rounds of 5FOA selection. Using PCR, yeast colonies that grew on SGR-H-L+FOA agar were verified for the presence of the pESC-LEU IPCS plasmid and the absence of the pRS316 AUR1 plasmid; these colonies were then used in subsequent experiments. It was noted that *Os*IPCS3 complemented strains had few colonies following FOA selection and required an extended period of incubation at 30°C (10 days) for colony formation.

2.3.1 Complementation Assay

All six complemented strains were grown on SGR-H-L agar and on glucose agar without histidine and leucine (SD-H-L) agar respectively. As expected, all the strains grew only on SGR-H-L due to the IPCS being under the influence of the galactose promoter (Figure 2-8). This indicated that the encoded plant enzymes complemented for the absence of the yeast AUR1p. The parental strain can grow in glucose and galactose because AUR1, which is in the pRS316 plasmid is not under the influence of the GAL1 promoter.

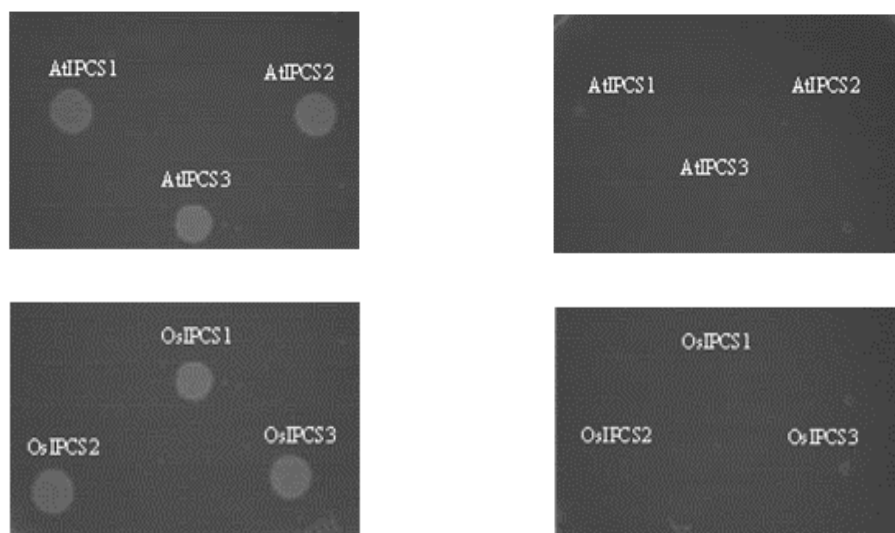


Figure 2-8: Complementation Assay - SGR-H-L plate (left) shows that all yeast strains grew but in SD-H-L plate (right) none of the strains grew because all plant IPCSs are under the influence of the GAL1 promoter.

2.3.2 Diffusion Assay

A diffusion assay is a simple and low-cost technique that has been previously used to characterise the selectivity and sensitivity of AUR1p to AbA^{26, 27}. Diffusion assays involve embedding the yeast strain in the required media with 0.8% agarose. The inhibitor is then applied to the surface and diffuses through the agarose; a zone of clearance around the point of application is formed, where the yeasts are unable to grow. The zone of clearance is dictated by how active the compound is against the yeast strains.

As mentioned previously, AbA is a cyclic peptide that is a well-known inhibitor of fungal AUR1 and is toxic against various *Leishmania* species²⁸. However, the concentration of AbA needed to inhibit the growth of yeast strains harbouring *Lmj*IPCS was found to be 100 μ M, which is so high that the yeast strains can be considered as insensitive to the compound²⁷. The sensitivity to AbA of all six yeast strains rescued with the respective plant IPCSs, *At*IPCS1-3 and *Os*IPCS1-3 were tested in a diffusion assay as was the sphingolipid biosynthesis inhibitor myriocin²⁹. Myriocin inhibits the enzyme SPT thereby inhibiting sphingolipid formation and hence, membrane formation, therefore myriocin should inhibit all strains of yeast in this experiment. The sensitivity of the complemented, *pdr/aur1* mutant strain to AbA and myriocin was tested, with the vehicle dimethyl sulphoxide (DMSO) as the negative control (Figure 2-9).

All plant IPCS complemented yeast strains showed a zone of clearance for myriocin (1 mM), and, interestingly, all but *Os*IPCS3 showed insensitivity to AbA at a concentration of 100 μ M (Figure 2-9) implying differential selectivity of the compound for *Os*IPCS3. The same assay carried out for *Lmj*IPCS and AUR1 showed contrary to results from Denny et al. 2006, yeast strains complemented with *Lmj*IPCS being insensitive to AbA at 100 μ M (Figure 2-10) which indicated differential selectivity between protozoan and

fungal IPCS. However, it is possible that insensitivity of *Lmj*IPCS complemented yeast at 100 μ M might be due to a higher number of cell used in the assay. Based on the findings of Denny *et al.*, *Lmj*IPCS and *Os*IPCS3 are sensitive to AbA at 100 μ M which indicates that they share a structural similarity. Analysis of the amino acid sequence shows that two IPCs share a percentage identity of 25% which is quite low and alignment of the two sequences revealed no identifiably conserved region.

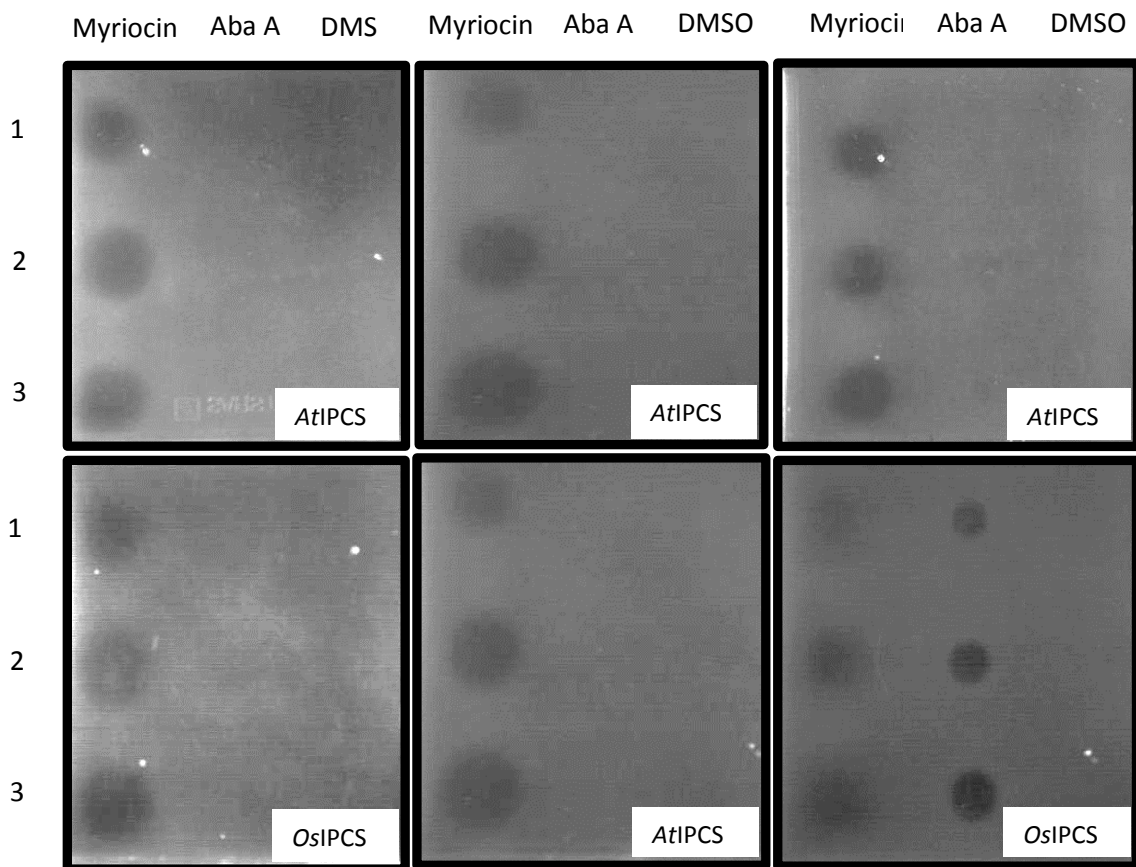


Figure 2-9: Diffusion assay plates for (top, left to right) *At*IPCS31-3 and (bottom, left to right) *Os*IPCS1-3 mutant yeast strains embedded in SGR-H-L + 0.8% agarose media. 1 mM Myriocin and 100 μ M AbA in DMSO and the negative control DMSO, were pipetted in 1 μ l, 2 μ l and 3 μ l aliquots onto the plates and left to grow at 30°C for 5 days.

To confirm the loss of pRS426 AUR1 and the gain of pESC-LEU IPCS, specific oligonucleotide primers were designed to amplify a region of about 300bp of each plant IPCS and 500bp from AUR1 by PCR. Two yeast colony PCR reactions were carried out for each yeast strain; one with the plant IPCS and the other with the AUR1 primers. The

results demonstrated that each complemented strain contained the expected *At*IPCS or *Os*IPCS orthologue in the absence of AUR1 (Figure 2-11A and Figure 211B); confirming that the yeast strains lacked AUR1 and were expressing the respective plant IPCS isoforms.

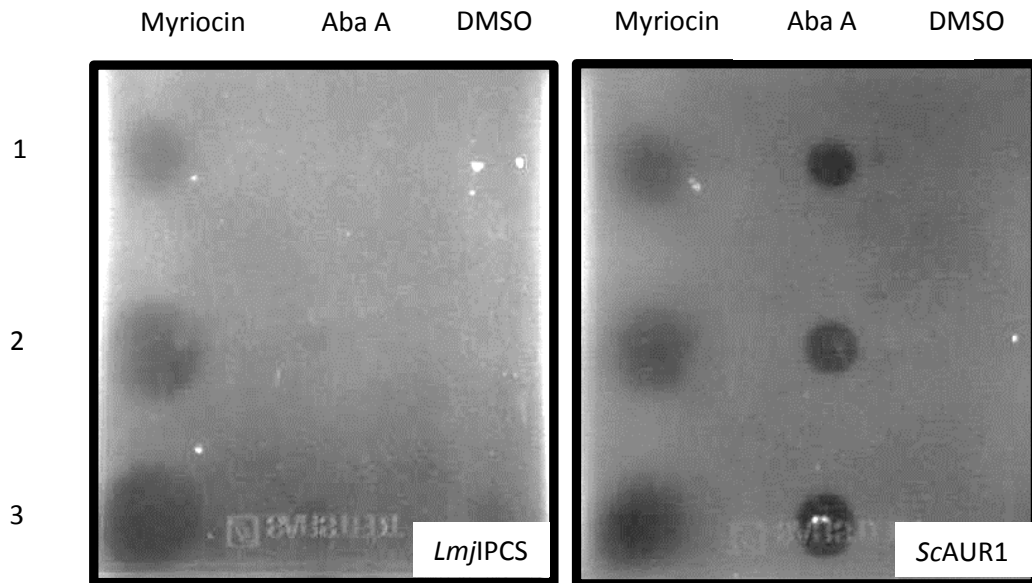


Figure 2-10: Diffusion assay plates for (left to right) *Lmj*IPCS and *ScAUR1* mutant yeast strains embedded in SGR-H-L + 0.8% agarose media. 1 mM Myriocin, 100 μ M Ab A in DMSO, and the negative control DMSO, were pipetted in 1 μ l, 2 μ l and 3 μ l aliquots onto the plates and left to grow at 30°C for 5 days.

2.3.3 Verification of *At*IPCS and *Os*IPCS3 activity

Following verification of the successful complementation of the plant IPCS yeast strain, microsomal preparation was undertaken to isolate microsomes containing *At*IPCS1-3, *Os*IPCS1-3 and AUR1. To measure the activity of IPCS, the turnover of NBD-C₆-ceramide into the fluorescent product NBD-C₆-IPC (Figure 2-12) was quantified by fluorescence scanning following high-performance thin layer chromatography (HPTLC) fractionation. NBD-C₆-ceramide has an R_f value of 0.96 and NBD-C₆-IPC has an R_f value of 0.57³⁰. This results to a clear resolution between the substrate and the product bands on the TLC plate, allowing for the quantification of IPC turnover.

The activity of *At*IPCS1-3 and *Os*IPCS1-3 were tested in the presence and absence of the known fungal IPCS inhibitor AbA at a concentration of 5 μ M. The result demonstrated that *At*IPCS1-3 and *Os*IPCS1-2 were insensitive to AbA, whilst *Os*IPCS3 was sensitive to the inhibitor (Figure 2-13 and 2-14) in agreement with the diffusion assay data (Figure 2-9). The results for *At*IPCS1-3 and AUR1 are as expected; work previously done by Mina *et al.* had shown that the three *A. thaliana* IPCS isoforms are insensitive to Aba¹ and earlier work by Heidler *et al.*, as mention in chapter 1 demonstrated that AUR1 is sensitive to the inhibitor¹⁵.

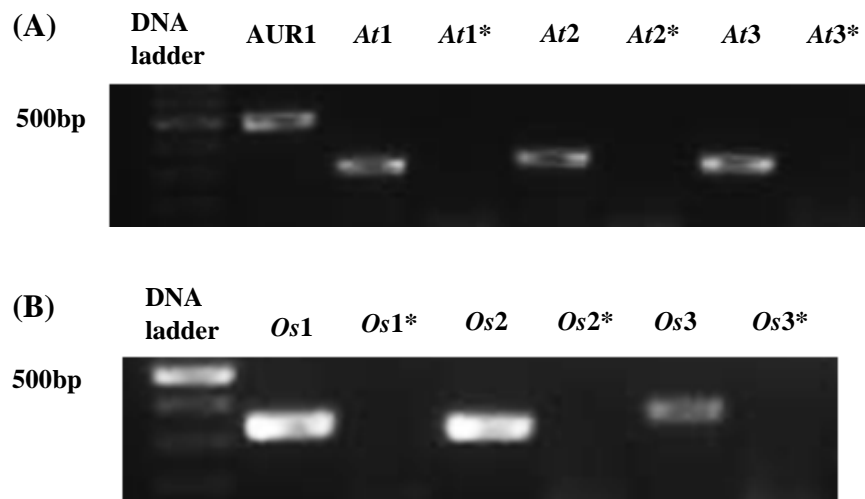


Figure 2-11: Transformed yeast colony PCR for (A) AUR1 and *At*IPCS1-3 (B) *Os*IPCS1-3 with specific primers for AUR1 (*) and each insert.

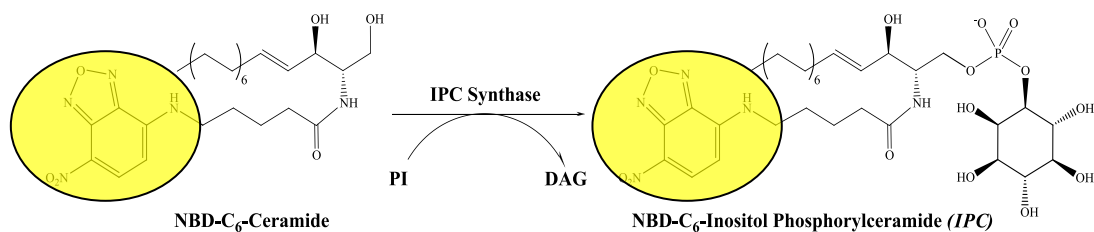


Figure 2-12: Conversion of NBD-C₆-ceramide to NBD-C₆-IPC.

To facilitate further analysis, the enzyme activity for each IPCS of interest was deduced using an NBD-C₆-ceramide standard curve. It was assumed that the fluorescence of the

substrate NBD-ceramide is equal to the fluorescence of NBD-IPC, which allowed for the quantification of IPC turnover in a reaction and was subsequently used to deduce the concentration of active IPCS in the microsome mixture. Active IPCS concentration was quantified in ‘Unit/ μ l’, where one Unit is defined as the amount of enzyme required to catalyse the formation of 1 picomol of product in 1 minute³¹.

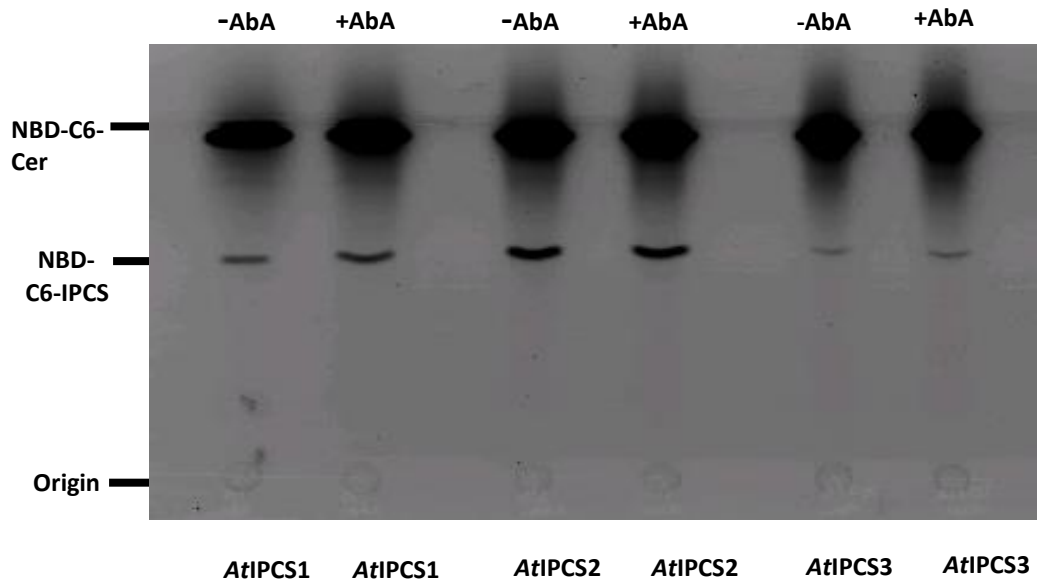


Figure 2-13: HPTLC assay *AtIPCS1-3* in the absence and presence of AbA

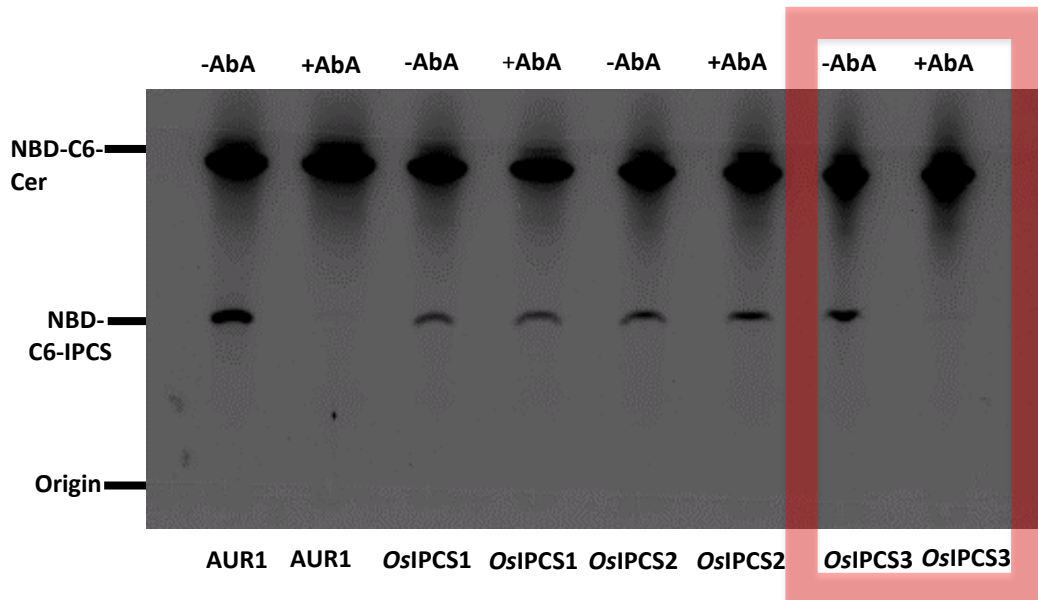


Figure 2-14: HPTLC assay *OsIPCS1-3* in the absence and presence of AbA with the inhibition of *OsIPCS3* highlighted in the red box showing the lack of IPC turnover in the presence of the compound.

To establish the IC_{50} of AbA against AUR1 and *OsIPCS3*, it was necessary to quantify

the enzyme activity; the activity of the *OsIPCS3* and AUR1 samples were deduced to be 5.32 Unit/ μ l and 14.68 Unit/ μ l respectively (Table 2-2). By quantifying the enzyme activity, it was possible to ensure that the enzyme concentration of *OsIPCS3* and AUR1 used in the assay was the same.

Table 2-2: IPCS activity for AUR1, *AtIPCS1-3* and *OsIPCS1-3* quantified

| Microsomes | Fluorescence at 473nm | pmol | pmol/min (Unit) | Unit/ μ l |
|-----------------------|-----------------------|-------|-----------------|---------------|
| AUR1 | 96150 | 11000 | 183.3 | 14.68 |
| <i>AtIPCS1</i> | 18123 | 1400 | 23.3 | 1.88 |
| <i>AtIPCS2</i> | 38985 | 3200 | 53.3 | 4.28 |
| <i>AtIPCS3</i> | 6902 | 480 | 8.0 | 0.64 |
| <i>OsIPCS1</i> | 16934 | 1240 | 20.7 | 1.64 |
| <i>OsIPCS2</i> | 16057 | 1200 | 20.0 | 1.6 |
| <i>OsIPCS3</i> | 46541 | 4000 | 66.7 | 5.32 |

Equivalent active enzyme concentration of AUR1 and *OsIPCS3* was assayed in the presence of AbA at concentrations ranging from 0.05-5 nM. The IC₅₀ of AUR1 was found to be 0.6 nM compared to the literature value of 0.89 nM²⁶ and the IC₅₀ of *OsIPCS3* was found to be 2.62 nM. The IC₅₀ values obtained show that there is a more than 2-fold difference in sensitivity to AbA between AUR1 and *OsIPCS3*; AUR1 is much more sensitive to Aureobasidin A than *OsIPCS3* (Figure 2-15).

To ensure that all complemented strains were still fully dependent on the specific plant IPCSs, another colony PCR experiment was carried out five months later and it was found that all yeast strains had retained their specific IPCS apart from yeast strains complemented with *OsIPCS3* (Figure 2-16) which showed the presence of the fungal IPCS signifying reversion to dependence on the fungal orthologue. There is currently no known mechanism for how this type of gene reversion occurs but work done by Schiestel *et al.*³² show that reversion of a deleted gene results from the excision of plasmid

harbouring the dominant marker (usually HIS3) which replaces the target gene and integrated into the genome.

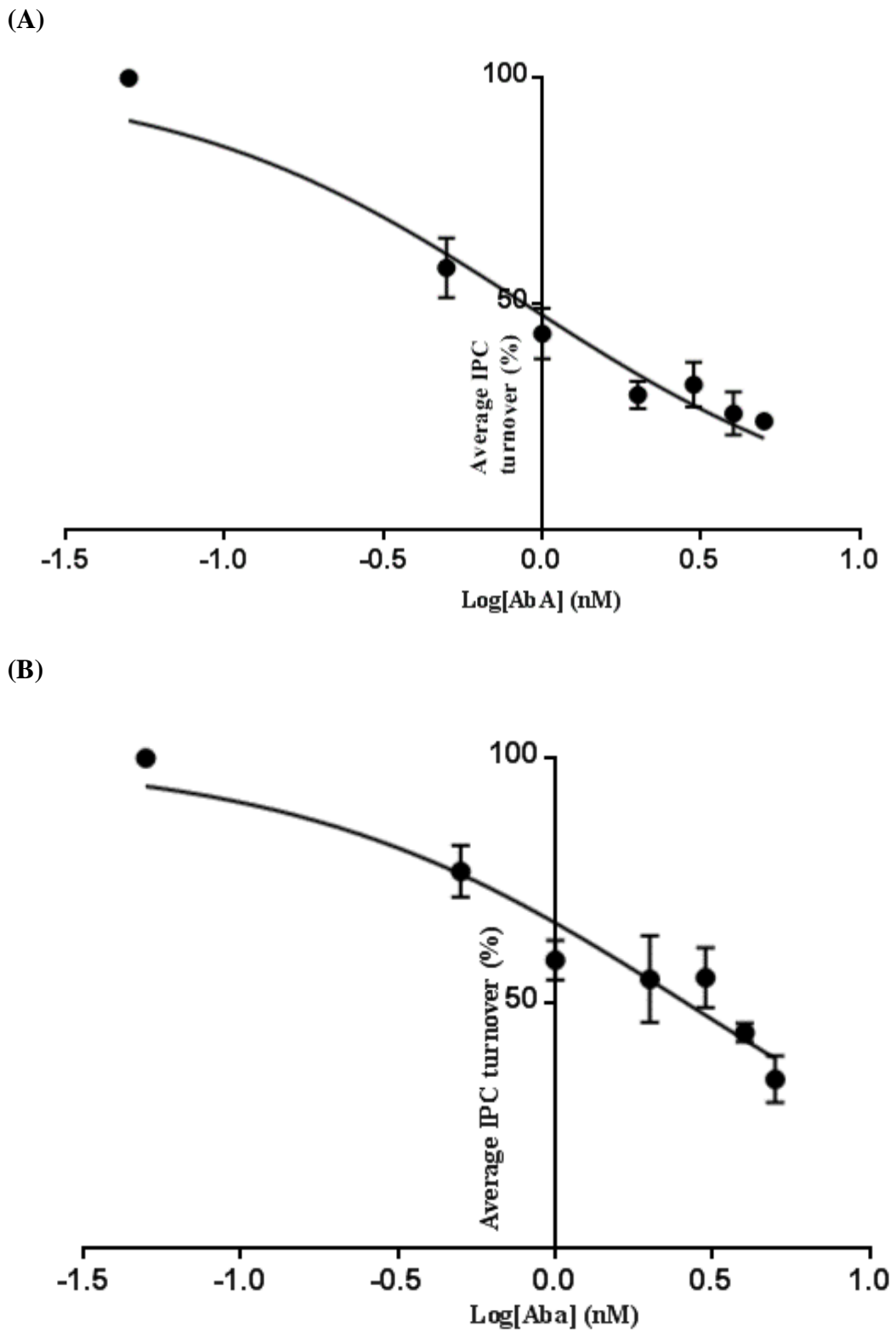


Figure 2-15: IC₅₀ graph showing the percentage turnover of IPC upon addition of different concentration of AbA for (A) AUR1 (B) *OsIPCS3*.

For successful integration into the genome, the plasmid usually has ends flanked with areas homologous to the target gene; when reversion occurs, this is because these ends are retained upon excision of the plasmid, which is repaired by sister chromatid conversion, resulting in the reconstruction of the wildtype gene that is fully functional. In this case, although *OsIPCS3* is expressed in the yeast strains, the deleted AUR1 gene in the genome has undergone reversion, resulting in the expression of the fungal IPCS.

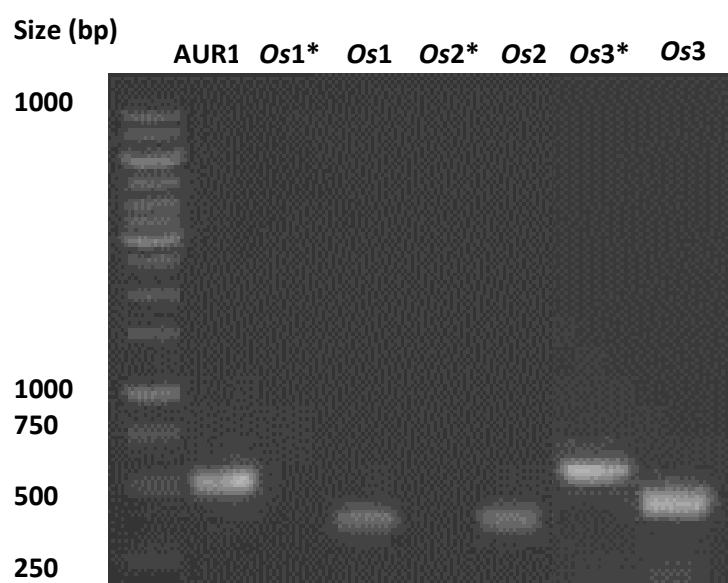


Figure 2-16: Agarose gel (0.8%) showing bands amplified for *OsIPCS1-3* with respective specific primers and AUR1 primers as control (* denotes PCR carried out with AUR1 primers); *OsIPCS3* shows a product for *OsIPCS3* and AUR1 primers.

2.4 Assay development

Following the verification of *AtIPCS1-3* and *OsIPCS1-2* yeast complemented strains, an assay to test compounds against the different yeast strains was developed and formatted in a 96 well plate. To set the assay parameters for HTS, the growth of all yeast strains with the IPCS of interest was monitored by optical density (OD₆₀₀) at different starting concentrations, over 72 hours at 24-hour intervals. From the results, it was clear that yeast growth plateaus after 48 hours. At the highest starting concentration in some cases, there

was a decrease in the OD₆₀₀, presumably, due to cell death as nutrients become a limiting factor.

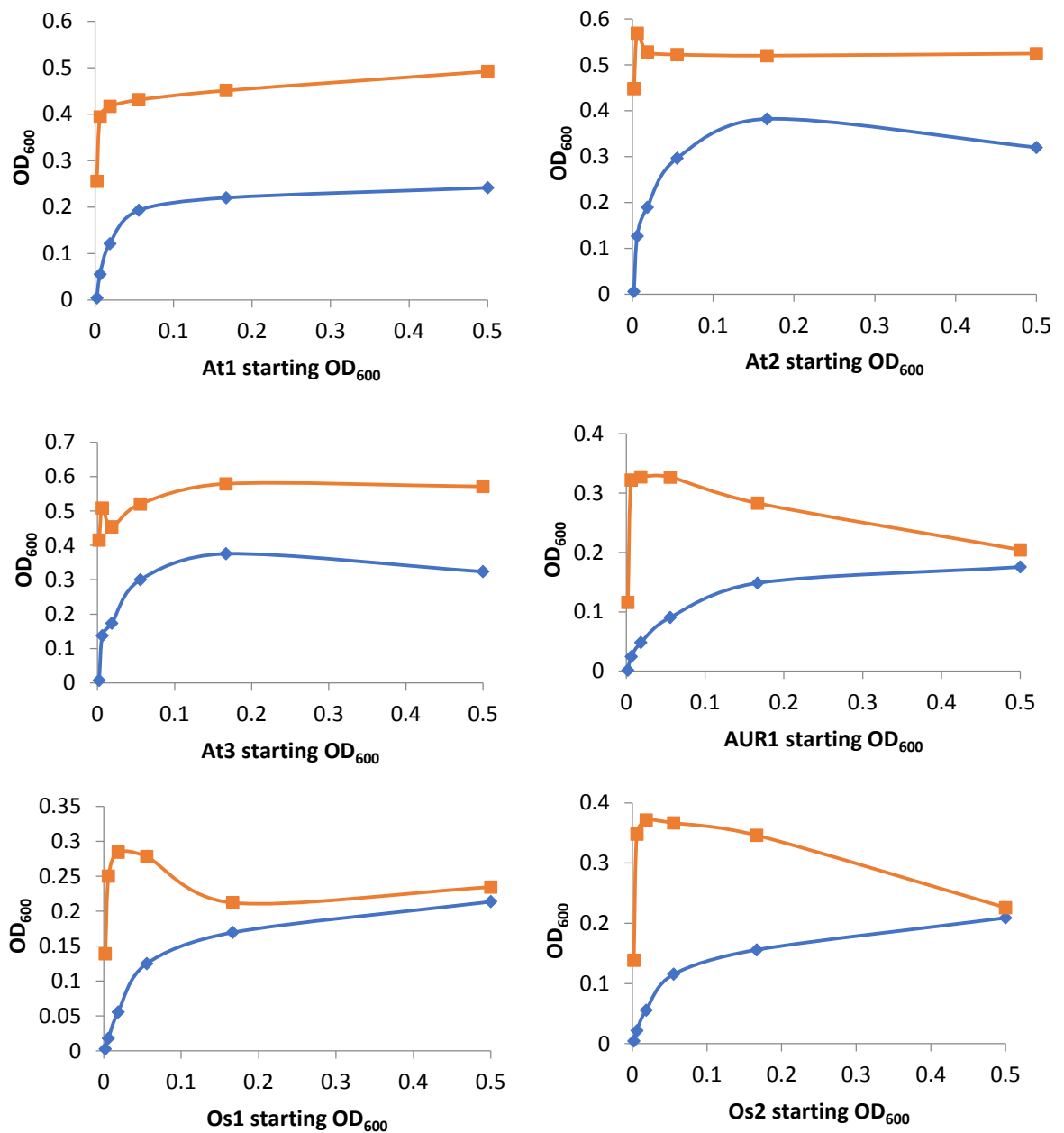


Figure 2-17: Graphs showing the relationship between OD₆₀₀ starting cell concentrations and AUR1, *At*IPCS1-3, *Os*IPCS1 and 2 cell concentrations measured after 24 (blue line) and 48 hours (orange line) growth respectively (starting OD₆₀₀ from highest to lowest corresponds to dilutions of 0, 1:3, 1:6, 1:9, 1:12 and 1:16 respectively).

For an assay (e.g. HTS) the conditions must ensure a direct linear correlation between OD₆₀₀ read out and cell number over a defined time period. This creates well defined

assay parameters whereby the efficacy of a compound can be directly measured by monitoring yeast growth. Similar assay parameters have been applied for the testing of antimicrobial peptides against *Leishmania*³³. As the results show, a consistent linear correlation was observed at 24 hours for 1:9 (OD₆₀₀= 0.018) and 1:12 (OD₆₀₀=0.006) dilutions (Figure 2-17). To increase the signal to noise ratio, the assay starting cell concentration was set at an OD₆₀₀ = 0.018 corresponding to a 1:9 dilution.

HTS usually involve the screening of compounds once or twice for the identification of hit compounds and requires that the assay has high sensitivity and accuracy. Due to the perturbations introduced in the assay resulting from human and instrumental error, assay measurements can contain high degrees of variation which need to be considered. For the identification of ‘real’ hits, it is necessary that variations are low which can be measured by standard deviation from the mean and by the coefficient of variation³³.

The Z'-factor³⁴ is a quantitative assessment of the quality of the assay that can be calculated using the standard deviation and the mean of the positive and negative control (Equation 1.1). An ideal assay would have a Z' factor value equal to 1, whilst an ‘excellent assay’ has a Z' factor value < 1 and ≥ 0.5.

$$Z\text{-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{(\mu_p - \mu_n)} \quad \text{[Equation 1.1]}$$

σ is the standard deviation and μ is the mean of the positive (p) and negative (n) control

To test the suitability of the assay in identifying hit compounds, Z' factor values were calculated for AtIPCS1-3 and AUR1 (Table 2-3). DMSO was used as the negative control and cycloheximide (1 mM), a known inhibitor of cytoplasmic protein synthesis in yeast and other eukaryotes³⁵ was used as the positive control in the assay. The assay was set up in 96 well plates, incubated with shaking over 24 hours and yeast cell viability quantified

by measuring cell density at OD₆₀₀.

A trial of the assay's reproducibility and hit identification was carried out by testing 15 compounds against *At*IPCS1 complemented yeast strain. The 15 compounds included Aba, clemastine analogues³⁶, khafrefungin analogues and the known sphingolipid biosynthesis inhibitor, myriocin. Results obtained from the assay were reproducible and identified myriocin, khafrefungin and the khafrefungin analogues: W2345 and ND8 as hits (Figure 2-18, structures of compounds tested are in Appendix D). Interestingly, the khafrefungin analogue, W2345, has been shown to have no activity against *S. cerevisiae* (work done by Kentaro Hanada, unpublished), indicating differential selectivity for the plant IPCS over fungal IPCS. However, the khafrefungin analogues are not attractive as inhibitors of IPCS because these compounds are not easily synthesised and required several synthetic steps to produce the final product. In this study, the aim is to find small molecule inhibitors that can be easily modified for use as herbicides, so the selectivity of the khafrefungin analogue, W2345, although interesting was not pursued further.

Table 2-3: Calculated Z' factor value to validate assay

| | DMSO OD ₆₀₀ average | Cycloheximide OD ₆₀₀ average | Z' factor |
|-----------------|-----------------------------------|--|--------------|
| <i>At</i> IPCS1 | 1.54 | 0.07 | 0.9 |
| <i>At</i> IPCS2 | 1.47 | 0.15 | 0.7 |
| <i>At</i> IPCS3 | 1.46 | 0.14 | 0.6 |
| AUR1 | 1.26 | 0.21 | 0.6 |

Having established that the assay was suitable for the identification of hit compounds, yeast strains were grown to log phase (OD₆₀₀ = 0.5-0.7) and frozen to screen the 11,440

compound library for inhibitors of plant IPCSs which was carried out at Bayer Crop Sciences, Frankfurt Germany.

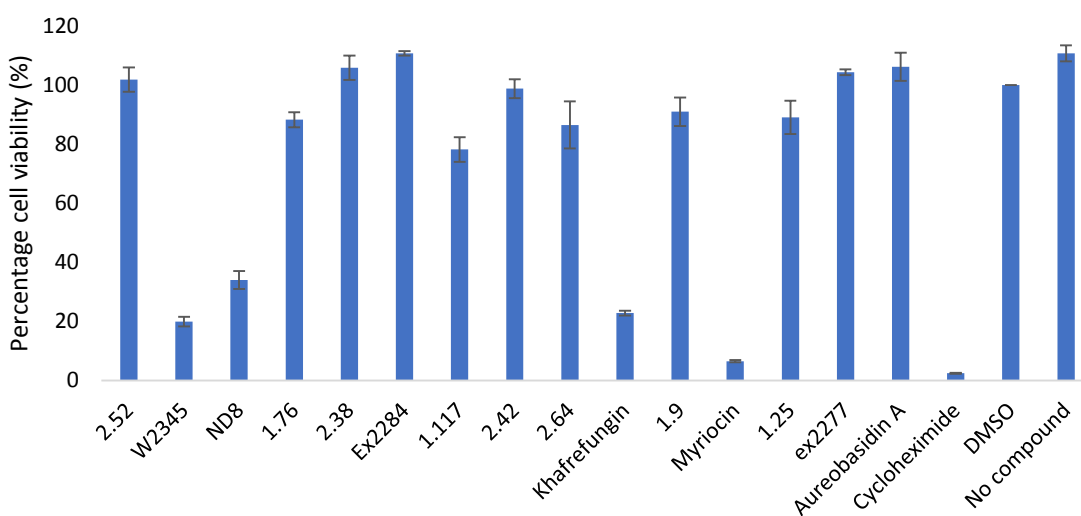


Figure 2-18: Percentage cell viability of *AtIPCS1* complemented yeast cells upon addition of test compounds and incubation for 24 hours; each compound was tested in triplicate at 10 μ M.

2.5 Conclusion

Cloning of *OsIPCS1-3* and *AtIPCS1-3* was achieved and complementation of plant IPCS yeast strains was successful for all but *OsIPCS3*. It was shown that plant IPCS unlike fungal IPCS, were insensitive to Aba. The yeast strains were formatted into an assay which requires quantifying the growth of the yeast strains by measuring OD₆₀₀ after a 24 hours incubation period. The assay conditions were validated for the screening of compounds by quantifying the Z' factor value of an assay with DMSO as negative control and cycloheximide as positive control. Initial screening of a compound library consisting of clemastine analogues, and known inhibitors of sphingolipid biosynthesis, were tested on *AtIPCS1* complemented yeast strains. Myriocin and khafrefungin were identified as hits, and more importantly the assay showed that the *AtIPCS1* complemented yeast strains were insensitive to Aba, which supports the biochemical assay results of plant IPCSs being insensitive to the compound. Other identified hits were the khafrefungin analogues, W2345 and ND8. The activity of W2345 against *AtIPCS1* complemented yeast indicates

differential selectivity for plant IPCS over fungal IPCS, which was a promising indication of the ability of the assay to identify hits which show differential selectivity. Following the validation of assay conditions, the screening of a library of bioactive compounds against the plant IPCSs and AUR1 was carried out; this is presented in the next chapter.

2.6 References

1. Mina, J. G.; Okada, Y.; Wansadhipathi-Kannangara, N. K.; Pratt, S.; Shams-Eldin, H.; Schwarz, R. T.; Steel, P. G.; Fawcett, T.; Denny, P. W., Y Functional analyses of differentially expressed isoforms of the Arabidopsis inositol phosphorylceramide synthase. *Plant Molecular Biology* **2010**, *73* (4-5), 399-407.
2. Mina, J.; Okada, Y.; Wansadhipathi-Kannangara, N.; Pratt, S.; Shams-Eldin, H.; Schwarz, R.; Steel, P.; Fawcett, T.; Denny, P., Functional analyses of differentially expressed isoforms of the Arabidopsis inositol phosphorylceramide synthase. *Plant molecular biology* **2010**, *73* (4-5), 399-407.
3. Krogh, A.; Larsson, B.; Von Heijne, G.; Sonnhammer, E. L., Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology* **2001**, *305* (3), 567-580.
4. Yachdav, G.; Kloppmann, E.; Kajan, L.; Hecht, M.; Goldberg, T.; Hamp, T.; Hönigschmid, P.; Schafferhans, A.; Roos, M.; Bernhofer, M., PredictProtein—an open resource for online prediction of protein structural and functional features. *Nucleic acids research* **2014**, *42* (W1), W337-W343.
5. Levine, T. P.; Wiggins, C. A.; Munro, S., Inositol phosphorylceramide synthase is located in the Golgi apparatus of *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **2000**, *11* (7), 2267-2281.
6. Jones, D. T., Improving the accuracy of transmembrane protein topology prediction using evolutionary information. *Bioinformatics* **2007**, *23* (5), 538-544.
7. Sutterwala, S. S.; Hsu, F. F.; Sevova, E. S.; Schwartz, K. J.; Zhang, K.; Key, P.; Turk, J.; Beverley, S. M.; Bangs, J. D., Developmentally regulated sphingolipid synthesis in African trypanosomes. *Molecular microbiology* **2008**, *70* (2), 281-296.
8. Lai, Z.; Vinod, K.; Zheng, Z.; Fan, B.; Chen, Z., Roles of Arabidopsis WRKY3 and WRKY4 transcription factors in plant responses to pathogens. *BMC plant biology* **2008**, *8* (1), 68.
9. Pandey, S. P.; Roccaro, M.; Schön, M.; Logemann, E.; Somssich, I. E., Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. *The Plant Journal* **2010**, *64* (6), 912-923.
10. Lebel, E.; Heifetz, P.; Thorne, L.; Uknes, S.; Ryals, J.; Ward, E., Functional analysis of regulatory sequences controlling PR- 1 gene expression in Arabidopsis. *The Plant Journal* **1998**, *16* (2), 223-233.
11. Strompen, G.; Grüner, R.; Pfitzner, U. M., An as-1-like motif controls the level of expression of the gene for the pathogenesis-related protein 1a from tobacco. *Plant molecular biology* **1998**, *37* (5), 871-883.
12. Zhou, J.-M.; Trifa, Y.; Silva, H.; Pontier, D.; Lam, E.; Shah, J.; Klessig, D. F., NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Molecular Plant-Microbe Interactions* **2000**, *13* (2), 191-202.

13. Johnson, C.; Boden, E.; Arias, J., Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in Arabidopsis. *The Plant Cell* **2003**, *15* (8), 1846-1858.
14. Cao, H.; Glazebrook, J.; Clarke, J. D.; Volko, S.; Dong, X., The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **1997**, *88* (1), 57-63.
15. Heidler, S. A.; Radding, J. A., The AUR1 gene in *Saccharomyces cerevisiae* encodes dominant resistance to the antifungal agent aureobasidin A (LY295337). *Antimicrobial agents and chemotherapy* **1995**, *39* (12), 2765-2769.
16. Wang, X. H.; Guo, X. J.; Li, H. Y.; Gou, P., Characteristics of inositol phosphorylceramide synthase and effects of aureobasidin A on growth and pathogenicity of *Botrytis cinerea*. *Journal of General and Applied Microbiology* **2015**, *61* (4), 108-116.
17. Hashida-Okado, T.; Yasumoto, R.; Endo, M.; Takesako, K.; Kato, I., Isolation and characterization of the aureobasidin A-resistant gene, *aur1R*, on *Schizosaccharomyces pombe*: roles of *Aur1p+* in cell morphogenesis. *Current genetics* **1998**, *33* (1), 38-45.
18. Hashida-Okado, T.; Ogawa, A.; Kato, I.; Takesako, K., Transformation system for prototrophic industrial yeasts using the AUR1 gene as a dominant selection marker. *FEBS letters* **1998**, *425* (1), 117-122.
19. Kuroda, M.; Hashida-Okado, T.; Yasumoto, R.; Gomi, K.; Kato, I.; Takesako, K., An aureobasidin A resistance gene isolated from *Aspergillus* is a homolog of yeast AUR1, a gene responsible for inositol phosphorylceramide (IPC) synthase activity. *Molecular and General Genetics MGG* **1999**, *261* (2), 290-296.
20. Xing, B.; Bachstetter, A. D.; Van Eldik, L. J., Inhibition of neuronal p38 α , but not p38 β MAPK, provides neuroprotection against three different neurotoxic insults. *Journal of Molecular Neuroscience* **2015**, *55* (2), 509-518.
21. Balzi, E.; Chen, W.; Ulaszewski, S.; Capieaux, E.; Goffeau, A., The multidrug resistance gene PDR1 from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **1987**, *262* (35), 16871-16879.
22. Georgiev, V. S., Membrane transporters and antifungal drug resistance. *Current drug targets* **2000**, *1* (3), 261-284.
23. Schiestl, R. H.; Gietz, R. D., High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Current genetics* **1989**, *16* (5), 339-346.
24. Hill, J.; Donald, K. I. G.; Griffiths, D. E., DMSO-enhanced whole cell yeast transformation. *Nucleic acids research* **1991**, *19* (20), 5791-5791.
25. Boeke, J. D.; Trueheart, J.; Natsoulis, G.; Fink, G. R., [10] 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods in enzymology* **1987**, *154*, 164-175.
26. Nagiec, M. M.; Nagiec, E. E.; Baltisberger, J. A.; Wells, G. B.; Lester, R. L.; Dickson, R. C., Sphingolipid synthesis as a target for antifungal drugs complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the AUR1 gene. *Journal of Biological Chemistry* **1997**, *272* (15), 9809-9817.
27. Denny, P. W.; Shams-Eldin, H.; Price, H. P.; Smith, D. F.; Schwarz, R. T., The protozoan inositol phosphorylceramide synthase a novel drug target that defines a new class of sphingolipid synthase. *Journal of Biological Chemistry* **2006**, *281* (38), 28200-28209.
28. Tanaka, A. K.; Valero, V. B.; Takahashi, H. K.; Straus, A. H., Inhibition of *Leishmania (Leishmania) amazonensis* growth and infectivity by aureobasidin A. *Journal of Antimicrobial Chemotherapy* **2007**, *59* (3), 487-492.

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29. Hanada, K., Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **2003**, *1632* (1), 16-30.
 30. Mina, J. G.; Mosely, J. A.; Ali, H. Z.; Shams-Eldin, H.; Schwarz, R. T.; Steel, P. G.; Denny, P. W., A plate-based assay system for analyses and screening of the *Leishmania major* inositol phosphorylceramide synthase. *International Journal of Biochemistry & Cell Biology* **2010**, *42* (9), 1553-1561.
 31. Loening, K. L., ACS Committee on Nomenclature, Annual Report, 1979. *Journal of Chemical Information and Computer Sciences* **1980**, *20* (2), 61-64.
 32. Schiestl, R. H.; Igarashi, S.; Hastings, P., Analysis of the mechanism for reversion of a disrupted gene. *Genetics* **1988**, *119* (2), 237-247.
 33. Chadbourne, F. L.; Raleigh, C.; Ali, H. Z.; Denny, P. W.; Cobb, S. L., Studies on the antileishmanial properties of the antimicrobial peptides temporin A, B and 1Sa. *Journal of Peptide Science* **2011**, *17* (11), 751-755.
 34. Zhang, J.-H.; Chung, T. D.; Oldenburg, K. R., A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *Journal of biomolecular screening* **1999**, *4* (2), 67-73.
 35. Boulton, C.; Quain, D., *Brewing yeast and fermentation*. John Wiley & Sons: 2008.
 36. Brown, C. Inhibitors of *Leishmania major* Inositol Phosphorylceramide Synthase: New Therapies for Leishmaniasis. Durham University, 2016.

CHAPTER 3

SCREENING FOR INHIBITORS OF PLANT IPCS

3.1 Chapter synopsis

In this chapter, the screening strategies utilized to identify herbicidal leads or chemical probes are outlined, and results from the screening of a compound library of 11,440 bioactive compounds are presented. Following the identification of hits in the primary screen, assay conditions for the secondary biochemical screen was carried out. Variants of phosphatidyl inositol were tested to see which gave maximal activity in terms of plant IPCS turnover. In addition various substrate concentration were tested to ensure the identification of hits was not hindered by saturation of the enzyme with substrate. From the secondary screen 15 compounds were found to show differential selectivity for plant IPCS over AUR1. In addition, compounds were identified that exhibited differential selectivity for *At*IPCS2 over *Os*IPCS2. The top two compounds, showing the most activity against plants IPCS were tested *in vivo* and found to have herbicidal effects that point to the biological pathway targeted, affecting root development and involving PCD.

3.2 Approaches to screening for inhibitors

In agrochemical discovery, two approaches are employed: a phenotypic chemical screen or a target-based chemical screen. In a phenotypic based screen also termed a forward chemical screen, chemicals are tested on plants in a microplate format, and bioactive compounds discovered by identifying an alteration in phenotype upon application of the compound². Following this, compounds are selected and tested against plausible targets (phenotype can give an idea of mode of action) in an assay or a biochemical purification strategy is employed to identify targets. The second approach which is a reverse chemical screen (target-based chemical screen), involves the testing of a compound library against a specific target, with the identified hits then tested *in vivo* to see if the compound remains active which is deduced by a phenotypic alteration in planta³.

The forward chemical screening approach has been utilized in numerous studies for the identification of bioactive compounds. For example, the screening of a library of compounds against *A. thaliana* seedlings, identified hits that either enhanced or inhibited gravitropic response, ascertained by the presence or absence of root reorientation. From the hits identified, compounds were discovered that also affected the endomembrane system, discovered by observation of aberrant vacuoles displaying a vesiculated morphology⁴. This phenotypic screening strategy to identify inhibitors or enhancers of plant development has also been used to identify auxin analogues that enhance hypocotyl growth⁵ and the compound hyperphyllin which enhances leaf formation and shoot meristem enlargement⁶. Chung-lin et al. identified compounds that interfered with *A. thaliana* response to ethylene by screening for the suppression of triple response phenotypic read out in response to ethylene⁷. Hits were subsequently validated as inhibitors of 1-aminocyclopropane-1-carboxylic acid synthase. The discovery of a class of sulfanilamide compounds able to protect wheat crops from the fungal pathogen,

Fusarium graminearum, was also facilitated by an *in vivo* screen for the attenuation of dark lesion-like spots resulting from fungal infection⁸.

Following the identification of a compound that has a particular effect of interest on the plant, the compounds are usually then either tested in cellular or enzymatic assays against a series of possible targets in order to facilitate the identification of the compound's mode of action. The identification of small molecule inhibitors can be coupled with omics tools, by utilizing reverse genetics and next generation sequencing to identify changes in the transcriptome or metabolome which may have a corresponding phenotype; this approach to the identification of bioactive compounds and their targets is termed chemical genomics⁹.

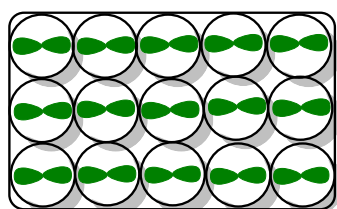
In this study, a combination of both phenotypic and target-based chemical screening has been utilised for the identification of IPCS inhibitors. A compound library of 11,440 compounds, which have exhibited herbicidal or fungicidal activity in *in vivo* screens were tested against plant IPCS.

3.3 Hypothetical effects of an IPCS inhibitor

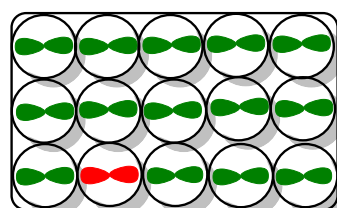
To understand the possible impacts of IPCS inhibition, a review of the ramifications of sphingolipid biosynthesis dysfunction is necessary. Various studies have linked the sphingolipid biosynthetic pathway to programmed cell death (PCD) in plants. Initial work carried out by Saucedo- García and colleagues showed that inhibition of phytoceramide synthase in plants treated with fumosin B results in PCD phenotypes at a cellular level, including interiorized and rounded chloroplasts, the disintegration of thylakoids vesicles and DNA fragmentation¹. This cell death phenotype was found to be attenuated by pre-treatment of plants with myriocin which targets serine palmitoyl transferase (SPT); the inhibition of SPT results in a decrease in 3-ketosphinganine which affects the synthesis

of other compounds downstream in the biosynthetic pathway. This results in a decrease in phytosphingosine, a substrate for phytoceramide synthase, which is inhibited by fumosin B (Figure 3-2). This indicated that the accumulation of the dihydroxylated long chain bases in the form of phytosphingosine is responsible for PCD in plants.

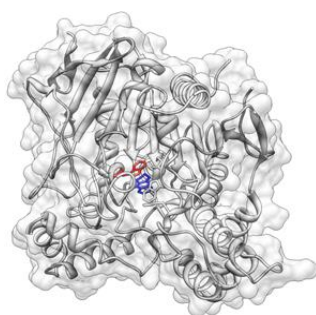
Phenotypic chemical screen



Phenotypic screen resulting in the identification of bioactive compound



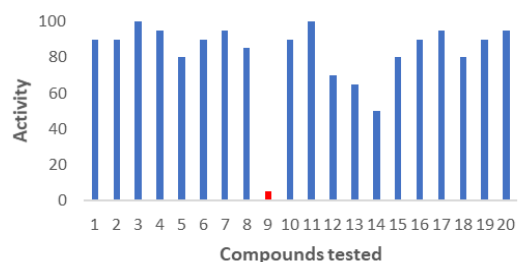
Target identification



Target-based chemical screen



Activity screen resulting in the identification of protein inhibitor



Phenotypic analysis

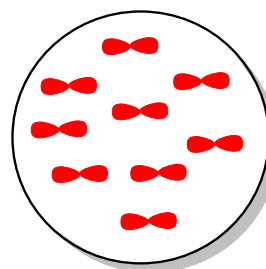


Figure 3-2: Schematic showing the two approaches for hit identification (A) Phenotypic chemical screen which involves *in vivo* testing to identify bioactive compounds which can then be screened against a number of targets to find the compound's target (B) Target-based screening which involves testing a compound library against a specific target to identify compounds that show activity against the target; the identified compound is subsequently tested *in vivo* to see if it results in any phenotypic alterations in planta.

This finding was corroborated was corroborated by Shi *et al.* who showed that treatment of *A. thaliana* with the sphingoid bases, dihydrosphingosine, phytosphingosine and sphingosine, resulted in cell death, which was preceded by the generation of reactive oxygen intermediates that are linked to PCD in response in abiotic stress¹⁰.

Gene silencing of the second subunit of SPT (LCB2) in *Nicotiana benthamiana* was shown to result in an approximate 50% reduction in LCB2 transcript, and induced cell death, with a decrease in levels of phytosphingosine and an increase in the levels of dihydro-sphingosine and sphingosine, which were also associated with elevated SA levels and constitutive expression of the gene pathogenesis-related protein 1 (*PR1*) expression¹¹, both markers of PCD. The elevation in dihydro-sphingosine levels upon deletion of the first SPT subunit (LCB1) is corroborated by work done by Chen et al.¹² in *Arabidopsis*, and indicates control of the levels of the proapoptotic compound, phytoceramide, by downregulation of LCB C4 hydroxylase resulting in the build-up of dihydroshpingosine, and the subsequence reduction in phytosphingosine and phytoceramide generated; both are bioactive compounds known to trigger PCD. The elevation of sphingosine levels is also as a result of keeping phytoceramide levels down, by breaking down phytoceramide to phytosphingosine, which is achieved by ceramidase.

Disruption of phytoceramide synthesis in *Arabidopsis* by mutation of one of phytoceramide synthase genes, *LOH1*, results in spontaneous cell death, concomitant with a 160-fold increase in *PR1* transcription¹³. Similarly, RNAi suppression of LCB-C4 hydroxylase resulted in the formation of necrotic lesions and constitutive *PR1* expression¹⁴. Mutation of ceramide kinase, *ACD5*, in *Arabidopsis* resulted in the accumulation of phytoceramide, with constitutive *PR1* expression, the accumulation of free SA and conjugate SA, and necrotic lesions¹⁵; markers associated with PCD in plants.

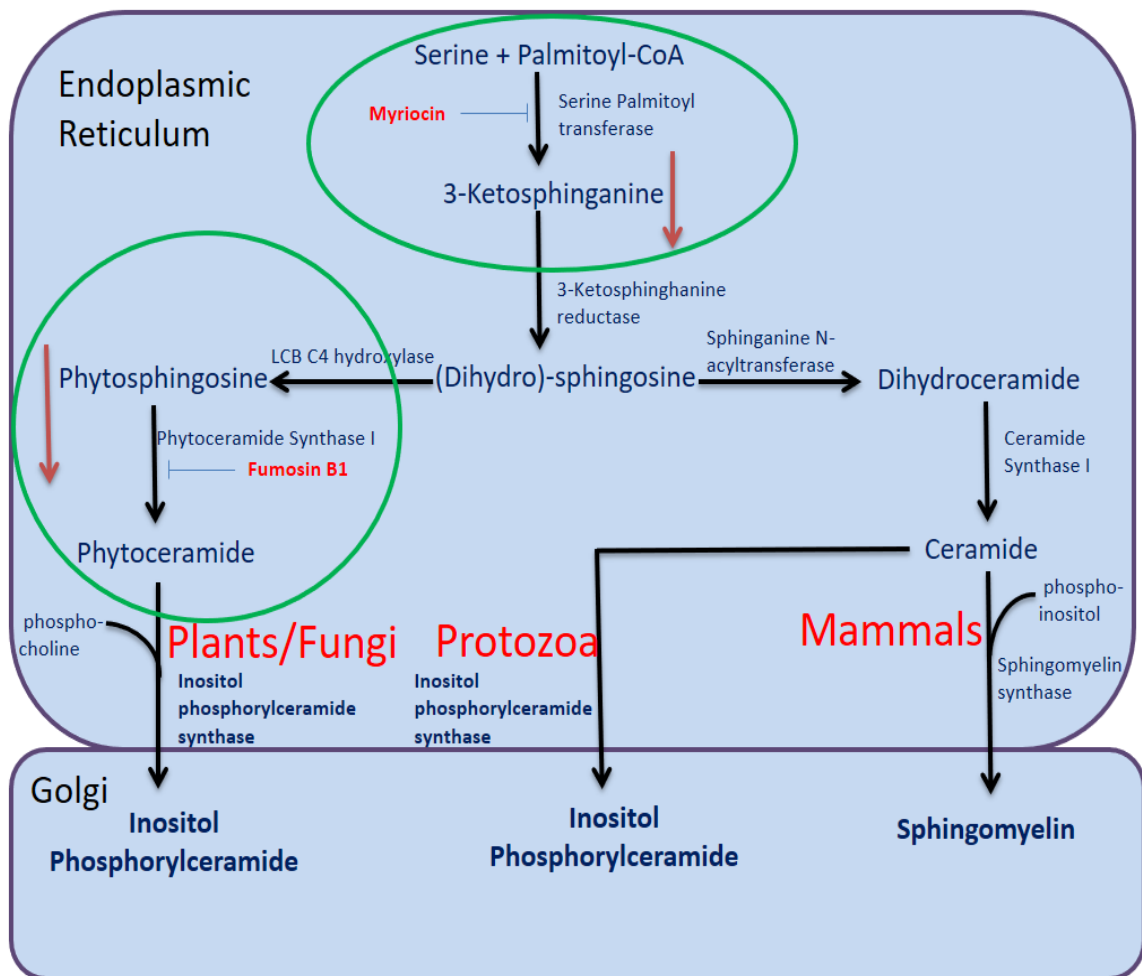


Figure 3-2: Inhibition of phytoceramide synthase I by fumosin B1 results in an increase of phytosphingosine with plants displaying PCD phenotypes¹ which are attenuated by the inhibition of SPT by myriocin, leading to a decrease in 3-ketosphinganine and phytosphingosine.

The findings of the studies outlined above, show that there is a consistent link between the disruption of sphingolipid synthesis, the buildup of intermediates in the biosynthetic pathway and the initiation of PCD. This biosynthetic pathway is clearly tightly regulated and inextricably linked to biological pathways/signaling involved in plant survival.

A homozygous knockout of *IPCS2* in *Arabidopsis* was found to lead to elevated levels of SA and phytoceramide, HR-like lesion formation on leaves associated with PCD, and a reduction in plant stature¹⁶. A working hypothesis for the inhibition of plant *IPCS* involves an offset in the balance between the pro-apoptotic compound, phytoceramide (substrate of plant *IPCS*) and the mitogenic compound, diacylglycerol (by-product), with

inhibition resulting in an accumulation of the former, with a concomitant decrease in the latter¹⁷. In addition, it would result in the decrease of the product inositol phosphorylceramide (IPC), which is attached to a number of oligosaccharides to generate GIPC, a major class of sphingolipids in plants that make up a quarter of the total lipids in the plasma membrane¹⁸.

GIPCs have been found to be essential for normal growth and reproduction in *Arabidopsis*; homozygous knockout mutants of IPC glucuronosyltransferase 1 (IPUT1) are non-viable. To study the developmental effects of reduced GIPCs in *Arabidopsis*, a pollen specific rescue construct was used to select for homozygous knockout lines of *iput*, and these were found to display phenotypes of severe reduction in growth, increased levels of SA and lesions characteristic of PCD¹⁹. From these studies, it can be hypothesised that the inhibition of IPCS would result in a decrease in the sphingolipid precursor, IPC resulting in a decrease in GIPC which is essential for plant development. In addition, the build-up of the bioactive compound phytoceramide would initiate PCD, making IPCS a promising herbicidal target.

3.4 Primary screening

To identify putative inhibitors of IPCS, the focused library consisting of 11,440 bioactive compounds was screened against *At*IPCS2, *Os*IPCS2 and AUR1 formatted into the yeast-based assay described in Chapter 2. The IPCS2 isoform was selected for screening due to its well characterised role in plant defense and PCD^{16, 20} and the fact that it is the most highly expressed orthologue being log 2-fold greater than both IPCS1 and IPCS3 in *A.thaliana*²¹. Screening was also carried out against the AUR1 complemented yeast strain as a control to filter out compounds that are generically cytotoxic to yeast cells rather than on target.

In the pharmaceutical industry the Lipinski rule of 5 is used as a gold standard for the selection of compounds that have orally-active drug like features²². The physico-chemical rules require that the compound has a molecular weight ≤ 500 , $\text{clogP} \leq 5$, no more than 5 H-bond donors and 10 H-bond acceptors. In the agrochemical industry there is a need to have compounds that not only trigger cell death at a biochemical level but also have the right physico-chemical properties to penetrate the waxy cuticle and cell membranes of plants. Analyses of commercial herbicides and pesticides carried out by Tice, revealed that most have a molecular weight between 300-400 with an average of 329 for herbicides and 324 for insecticides²³. In addition, the clogP values of herbicides were in the range 1.2-2.4 with an average of 1.7, which means that most herbicides are hydrophobic in nature which is necessary for the compounds to enter the cells and reach their target. According to Briggs rule of three, for a compound to show activity when sprayed on plants, it must have a molecular weight of 300 ± 100 , $\text{clogP} = 3 \pm 3$, melting point $\leq 300^\circ\text{C}$ and no more than 3 H-donors²⁴. Based on these parameters, 50% of the compounds selected for screening had a molecular weight between 300-400 (Figure 3-3A), with 45% possessing a clogP of 3-5 (Figure 3-3B).

In the primary screen against *At*IPCS2, *Os*IPCS2 and AUR1, due to the configuration of the compound plates, the positive and negative control were repeatedly tested in the first row and alternated in terms of placement in these wells. At this stage, the library of compounds was tested in duplicate at 10 μM with the inhibition of yeast growth measured after 24 hours.

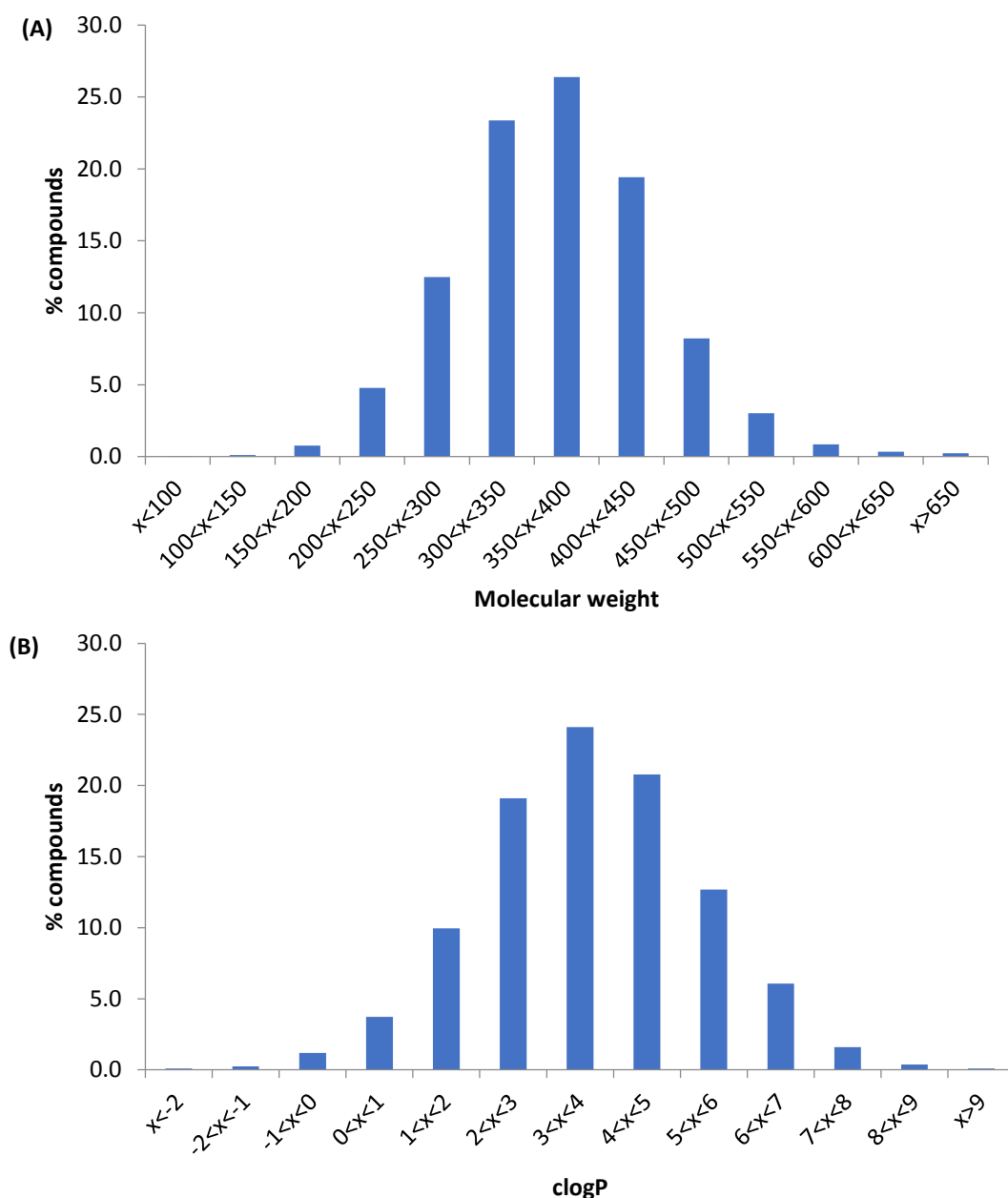


Figure 3-3: (A) Molecular weight and (B) clogP distribution of the library compounds screened.

To ensure reproducibility and reduce well to well variation, the plates were sealed with transparent breathable membranes which eliminated the ‘edge effect’²⁵ seen in assays incubated over long periods of time. For quality control, each plate in the yeast cell assay was required to have a calculated Z' factor of ≥ 0.5 in order for the assay results to be deemed as valid (Figure 3-4). Compounds exhibiting $\geq 80\%$ inhibition (the threshold) were selected to be taken forward. From the 11,440 compounds screened, 1164 compounds showed $\geq 80\%$ inhibition of *AtIPCS2*, 986 compounds showed $\geq 80\%$

inhibition of *OsIPCS2*, and 854 compounds had $\geq 80\%$ activity against AUR1 (Figure 3-5). The false positive hit rate was 2.6%, and these compounds were excluded leaving a final hit rate of 1.5%.

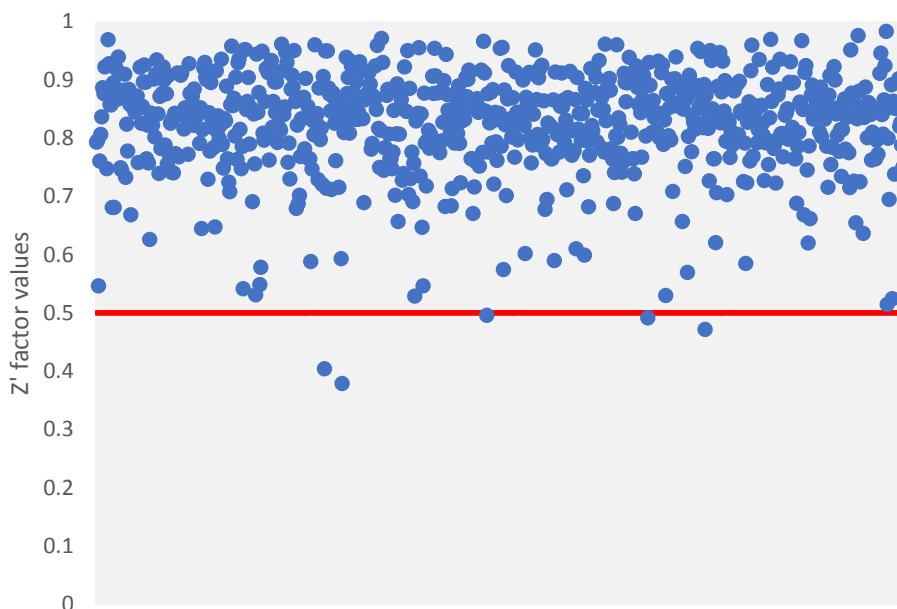


Figure 3-4: Z' factor of plates screened; cut off of 0.5 (red line) for a robust assay. Three plates fell below this value but were found to replicate the data in the n=2 screen and were accepted for further analyses.

To progress to secondary screening the compounds also had to show $\geq 50\%$ differential selectivity for the plant IPCS over the yeast AUR1 orthologue. From the approximately 1000 compounds that showed selectivity, 126 met the criteria above, 40 compounds showed selectivity for AUR1 over the plant IPCS (Figure 3-6) which would be of interest to study for use as antifungals in future work.

Of the 126 plant selective compounds, 4 were compounds were of particular interest as they were known to be pro-apoptotic, and as discussed above, *AtIPCS2* is associated with PCD. One of these compounds showed inhibition of both *AtIPCS2* and *OsIPCS2* in the yeast-based assay, whilst the other three exhibited selective inhibition of *OsIPCS2*. Using the same assay platform, dose response experiments (50 μM to 68 nM) were carried out in duplicate for the *AtIPCS2* and *OsIPCS2* hit compounds; 89 of the 105 *AtIPCS2*

inhibitors, and 35 of the 39 *Os*IPCS2 hits had IC₅₀ values $\leq 10 \mu\text{M}$ (Figure 3-7 A-C). The four pro-apoptotic compound hits are highlighted in red in Figure 3-6B and C.

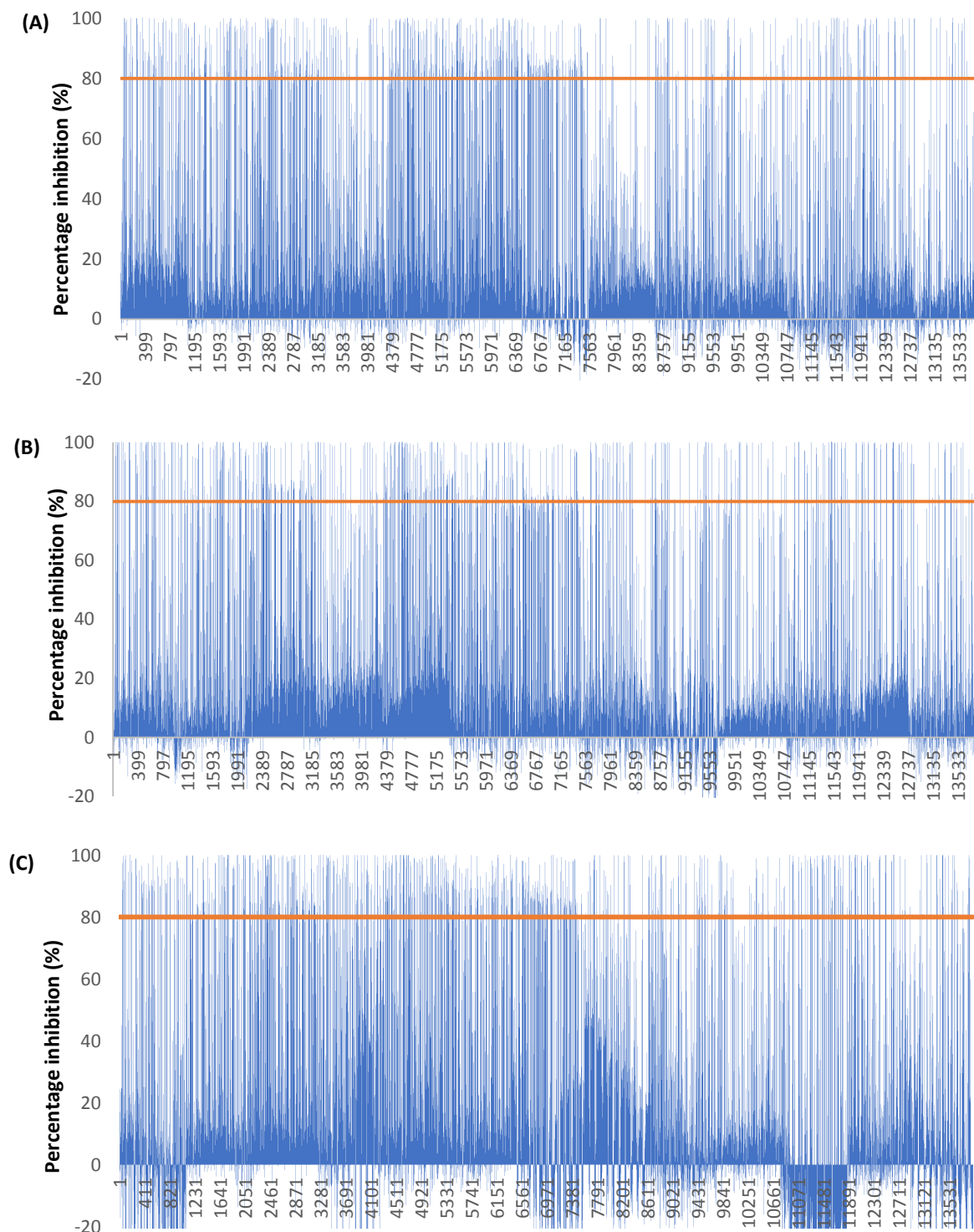


Figure 3-5: Plots showing percentage inhibition for compounds tested against (A) *At*IPCS2 (B) *Os*IPCS2 (C) *AUR1* complemented yeast strains.

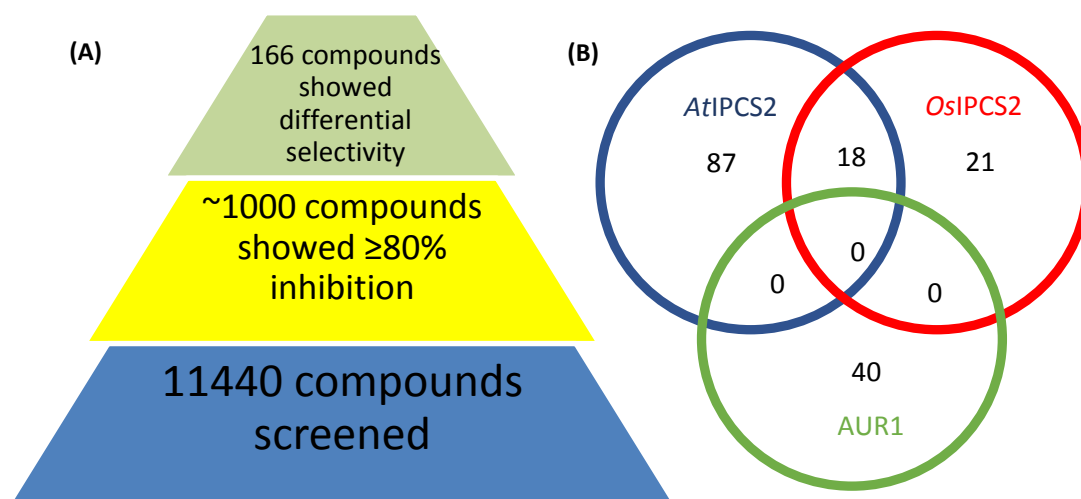


Figure 3-6: (A) A screening pyramid showing the number of compounds selected to progress to each stage of the screening campaign (B) Venn diagram of the 166 hits showing the differential selectivity for *AtIPCS2*, *OsIPCS2* and AUR1.

Interestingly one of the pro-apoptotic compounds showed activity against both *AtIPCS2* and *OsIPCS2* (6/12b; Figure 3-8A and B) whilst the other 3 hit only *OsIPCS2*. However, a closer inspection of the primary screen results shows that these compounds also targeted *AtIPCS2* but fell below the inhibition threshold of $\geq 80\%$ required for the compounds to be considered hits. Of the 4 pro-apoptotic compounds identified as hits, 3 share a common core and exhibit low μM activity in the assay; compound 6/12b is a dihydropyridazinone, compound 6/8c is an isothiazole carboxamide, and compounds 6/12a and 62/10d are dihydrotriazinones (Figure 3-7). For the biochemical screen, all 126 hits were carried forward in order to prevent the loss of active compounds.

Structures redacted

6/12b – $\text{IC}_{50} = 3.1\mu\text{M}$ 6/8c – $\text{IC}_{50} = 35.8\mu\text{M}$ 6/12a – $\text{IC}_{50} = 2.3\mu\text{M}$ 62/10d – $\text{IC}_{50} = 4.5\mu\text{M}$
 6/12b – $\text{IC}_{50} = 6.8\mu\text{M}$

Figure 3-7: Core structures of the four apoptotic inducer compounds with IC_{50} values – highlighted in red are compounds which showed activity against *OsIPCS2* and in blue those showing activity against *AtIPCS2* in the yeast-based primary assay.

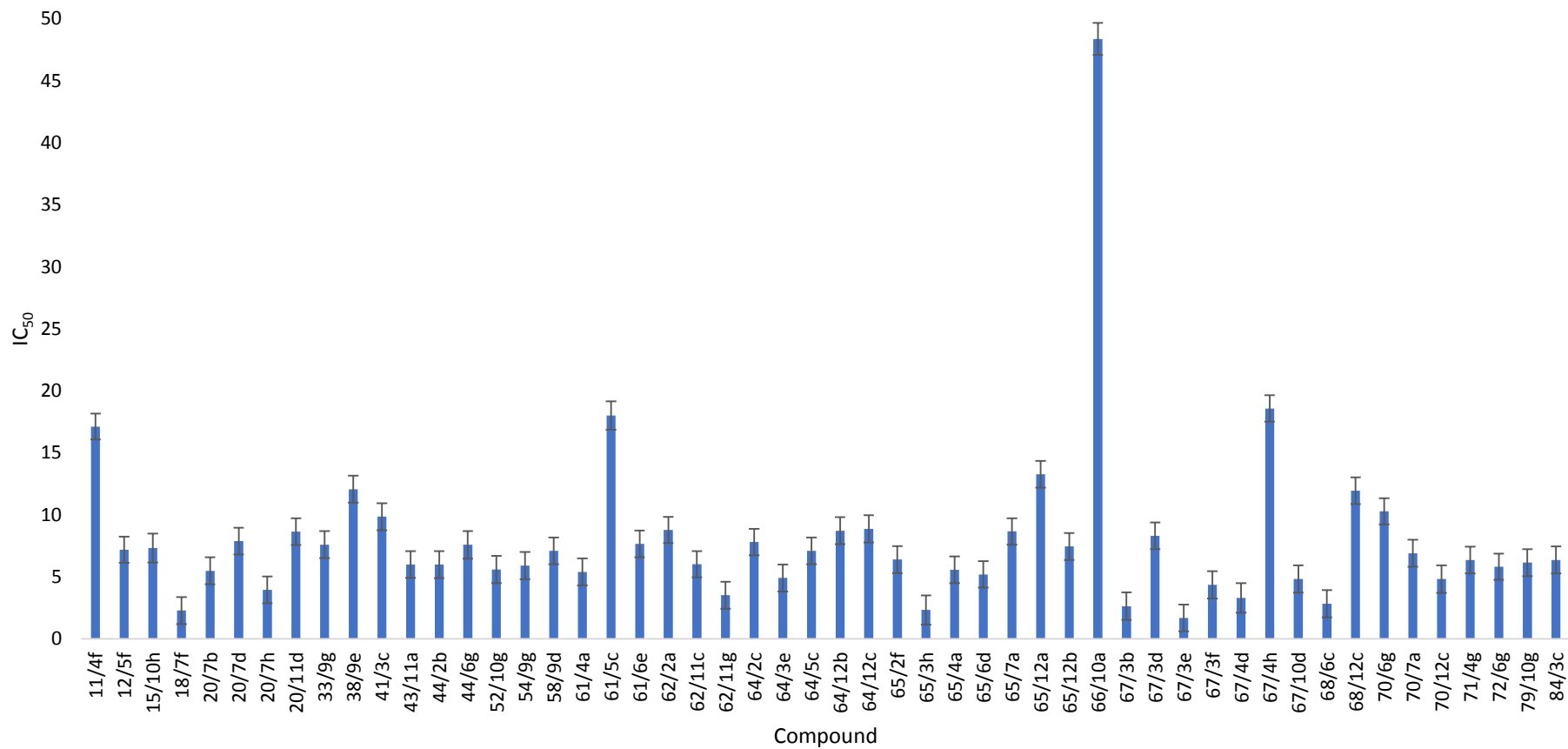


Figure 3-8: (A) IC₅₀ values for compounds tested against *AtIPCS2*. Each value is representative of the mean of two independent biological replicates.

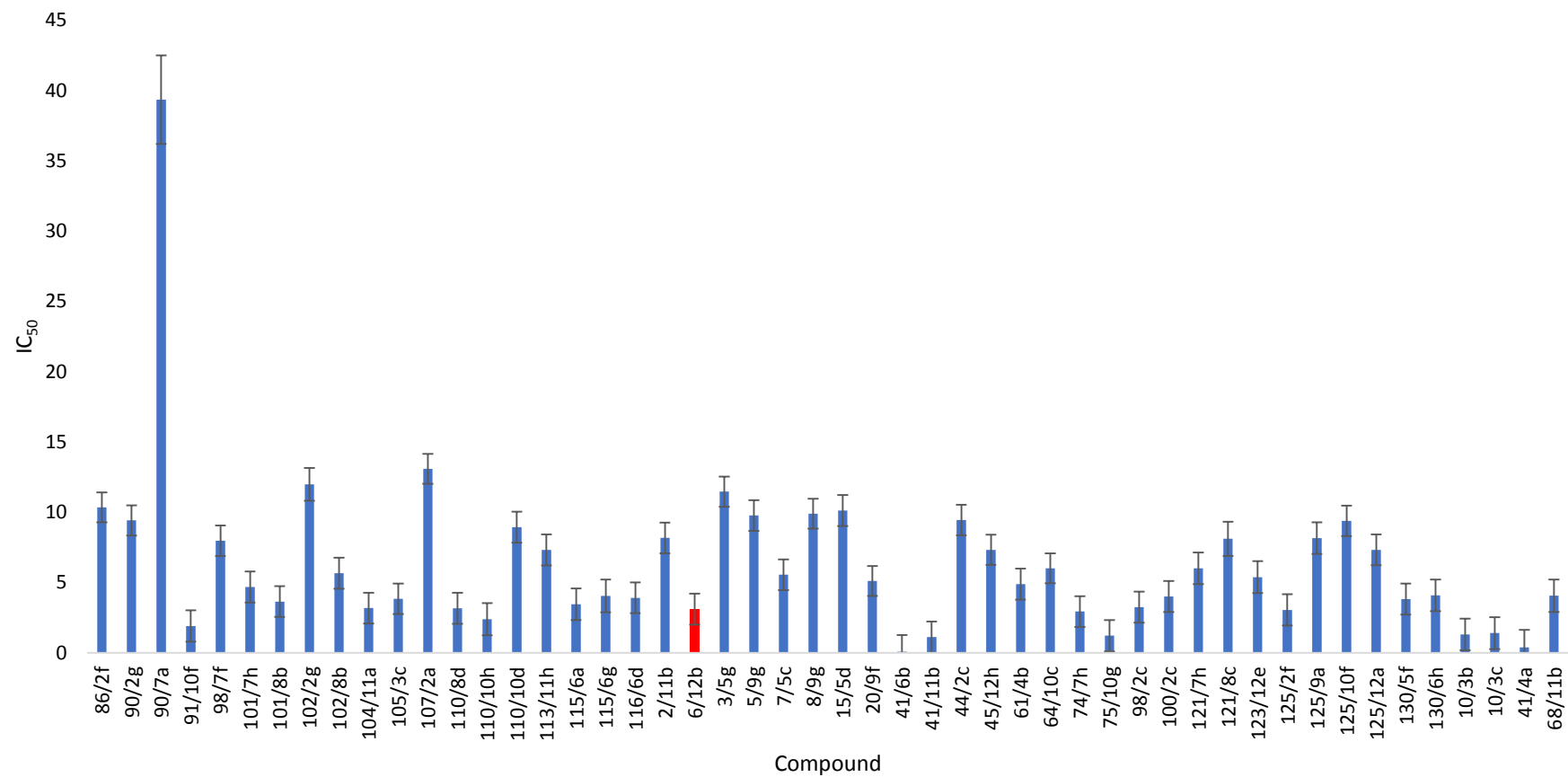


Figure 3-8: (B) IC₅₀ values for compounds tested against *AtIPCS2*, with known PCD inducer in red. Each value is representative of the mean with standard error calculated for two independent biological replicates.

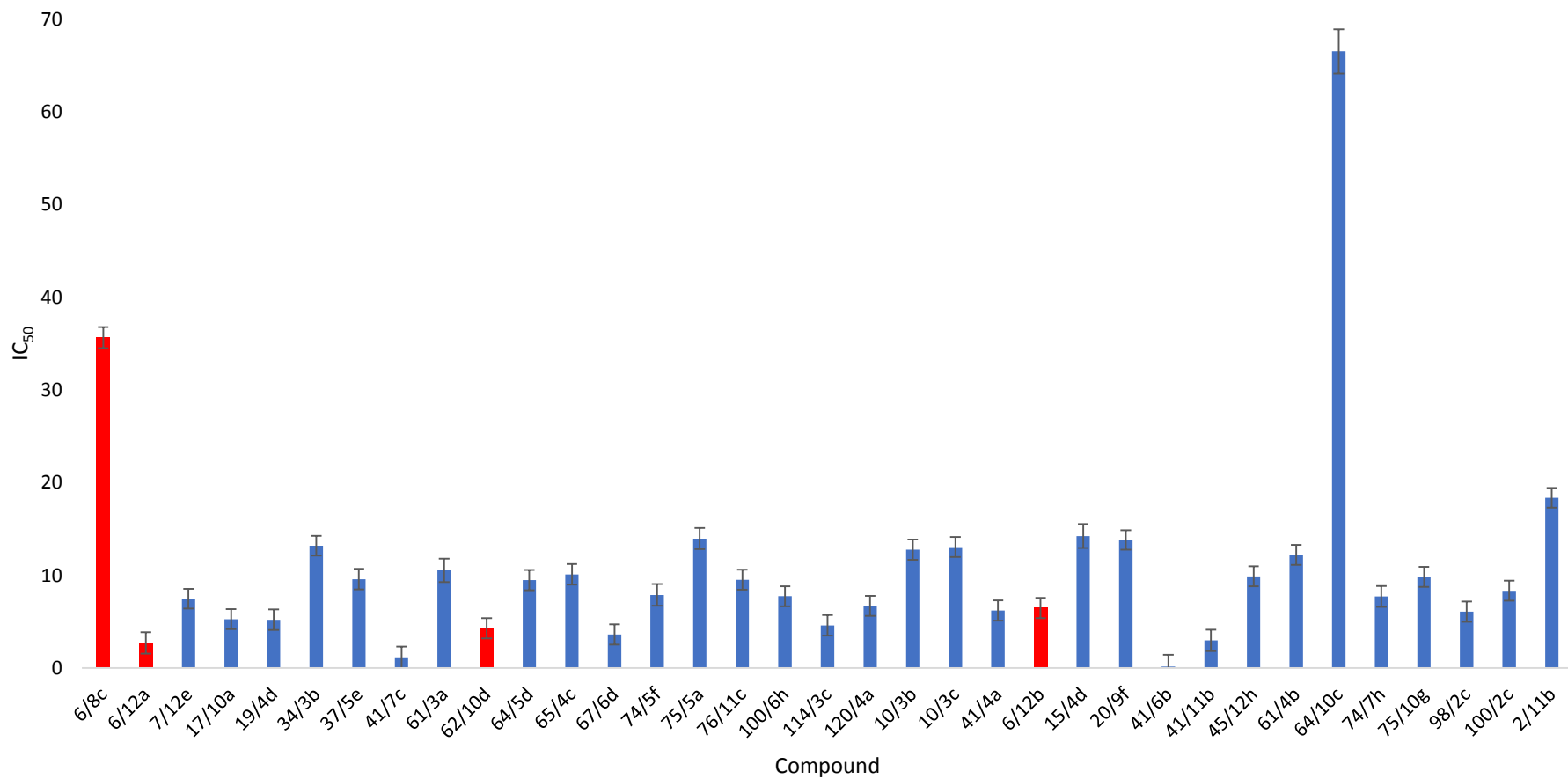


Figure 3-8: (C) IC₅₀ values for compounds tested against *OsIPCS2*, with known PCD inducer in red. Each value is representative of the mean with standard error calculated for two independent biological replicates.

3.5 Secondary screening

A 96-well plate assay to screen for inhibitors of IPCS had already been developed within our group²⁶. This assay allows for the separation of the fluorescent product IPC from the fluorescent substrate NBD-ceramide/NBD-phytoceramide using ion exchange chromatography. Assay parameters for secondary screening were first established to determine the initial velocity of the reaction so that the optimal concentrations of substrates could be chosen to ensure that the enzyme is not saturated, and the reaction not limited by substrate concentrations. The linear range was used in the enzyme assay to allow accurate quantitation.

Following yeast microsomes preparation as described, the activity of *At*IPCS2 and *Os*IPCS2 in the presence and absence of exogenous phosphatidylinositol (PI, soy or bovine) was quantified. The result shows that there is a 330% increase in *At*IPCS2 activity following the addition of exogenous soy PI. Bovine PI results in an increase of activity by about 200%, therefore unsurprisingly the *Arabidopsis* enzyme has a preference for the plant PI. However, *Os*IPCS2 lacks such a marked difference in activity in the presence

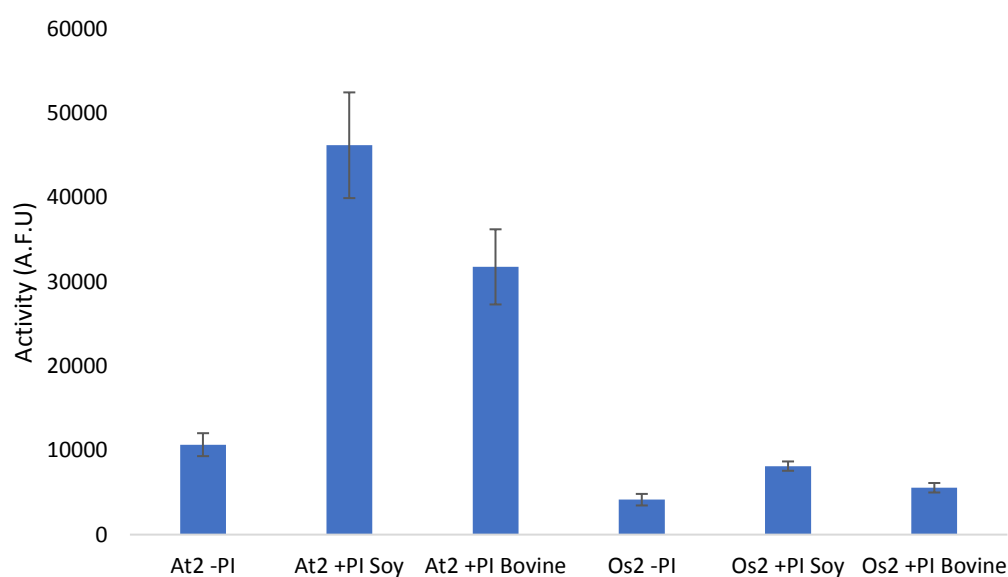


Figure 3-9: *At*IPCS2 and *Os*IPCS2 activity assayed in the presence and absence of exogenous PI variants (1 mM) and NBD ceramide (4 μM). Each value is representative of the mean with standard error calculated for three independent biological replicates.

and absence of exogenous PI from either source (Figure 3-9). The reasons for this are unclear, it is possible that rice preferentially utilises a different PI species.

In addition, to establish if the concentration of NBD-ceramide was limiting due to the much lower activity of *OsIPCS2* microsomes, NBD-ceramide concentration was increased 3-fold to 12 μ M. This led to an increase in assayed *OsIPCS2* activity (Figure 3-10), particularly the response to soy PI (2-fold compared to -PI), indicating that NBD-ceramide concentration had been limiting. In plants phytoceramide rather than ceramide is used as a natural substrate. To test if this is a preferred substrate, *AtIPCS2* and *OsIPCS2* were assayed with 4 μ M NBD-phytoceramide in the presence and absence of PI (soy and bovine). In the presence of NBD-phytoceramide, *AtIPCS2* activity was similar when assayed with NBD-ceramide, although for reasons unknown, the enzyme now demonstrated a slight preference for bovine PI (Figure 3-11).

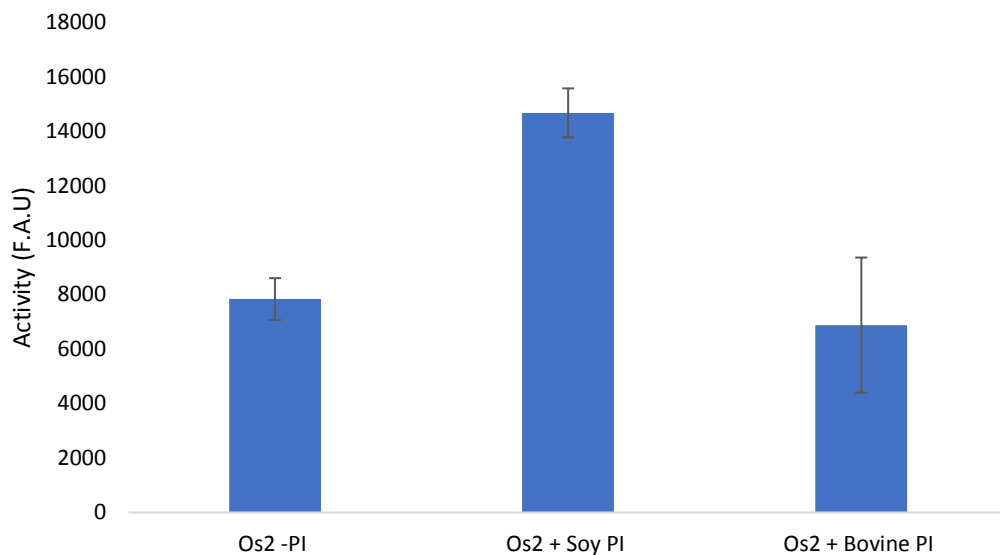


Figure 3-10: *OsIPCS2* activity increased upon a 3-fold increase in NBD-ceramide to 12 μ M and became more responsive to soy PI (1 mM). Each value is representative of the mean and the standard error calculated for three independent biological replicates.

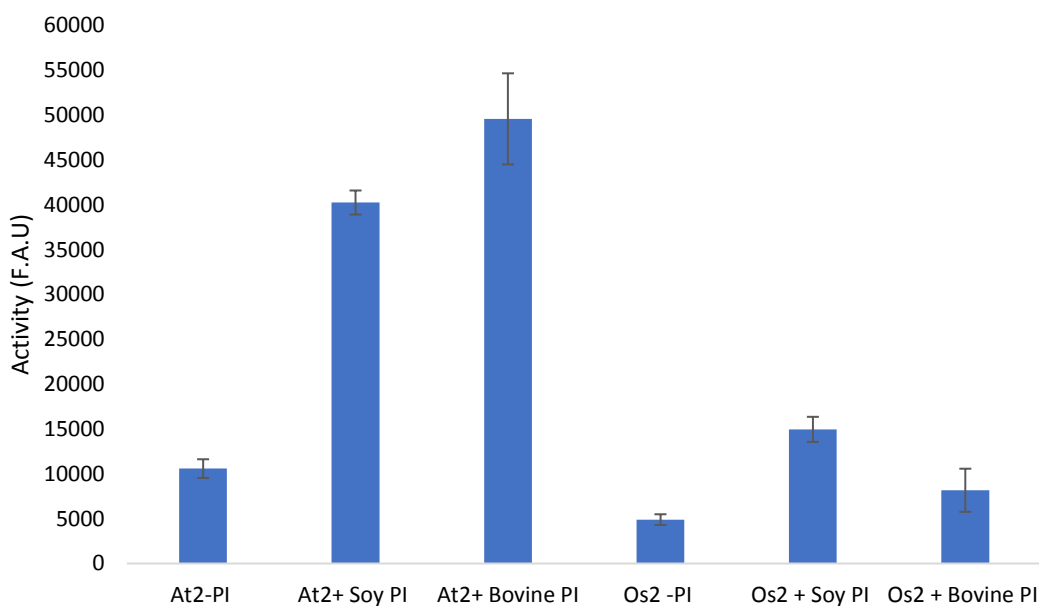


Figure 3-11: *AtIPCS2* and *OsIPCS2* activity assayed with NBD-phytoceramide (4 μ M) in the presence and absence of exogenous PI (1 mM). As with the increased concentration of NBD-ceramide, the activity *OsIPCS2* is increased when using NBD-phytoceramide (4 μ M), and more responsive to soy PI. Each value is representative of the mean and standard error calculated for three independent biological replicates.

In contrast, the activity of *OsIPCS2* increased markedly (Figure 3-10) showing that phytoceramide is a better substrate for IPCS synthesis in plants. These data established that the optimum substrates were NBD-phytoceramide and soy PI; these were then utilised in all subsequent experiments.

To determine the enzyme concentration required for the assay to achieve a high background to noise ratio, different amounts of *AtIPCS2* (Figure 3-12) were tested in the 96-well plate assay. Following incubation for 60 minutes, the product was quantified, and it was clear that an increase in enzyme concentration resulted in a likewise increase in IPC turnover.

For *OsIPCS2*, increased quantities of microsomes were assayed to compensate for the relatively low activity, and a likewise increase in IPC turnover was observed which was associated with increasing enzyme concentration (Figure 3-13).

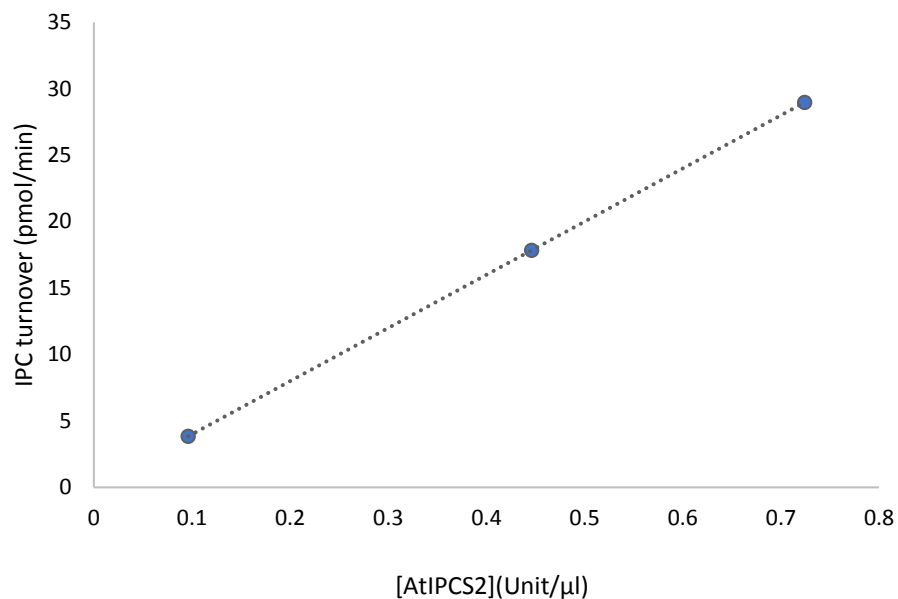


Figure 3-12: *AtIPCS2* concentration against IPC turnover. Each value is representative of the mean and standard error calculated for three independent biological replicates.

On the basis of these results (Figure 3-12 and 3-13), it was decided that 0.44 Unit/μl of *AtIPCS2* and 0.07 Unit/μl of *OsIPCS2* would be utilised in the secondary screen. In addition to producing a good level of signal, this also meant that the limited amount of microsomes available for screening would be sufficient.

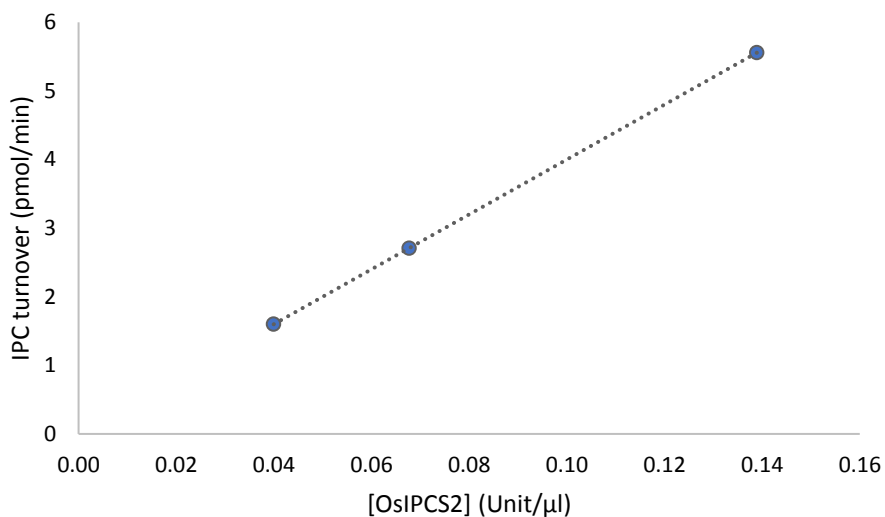


Figure 3-13: *OsIPCS2* concentration against IPC turnover. Each value is representative of the mean and standard error calculated for three independent biological replicates.

Subsequently, various concentrations of soy PI (with NBD-phytoceramide at 4 μM) were tested to determine the amount necessary to achieve a high signal without jeopardising

the activity of the enzyme. *At*IPCS2 activity peaks at 100 μ M PI, and at higher concentrations there was a considerable decrease in activity (Figure 3-14). The observed decrease in *At*IPCS2 activity resembled that seen for the *Leishmania* orthologue, *Lmj*IPCS, which was established as being due to surface dilution kinetics²⁷. In comparison, the activity profile for *Os*IPCS2 shows a peak in activity at 200 μ M, after which there is a decrease in enzyme turnover (Figure 3-15).

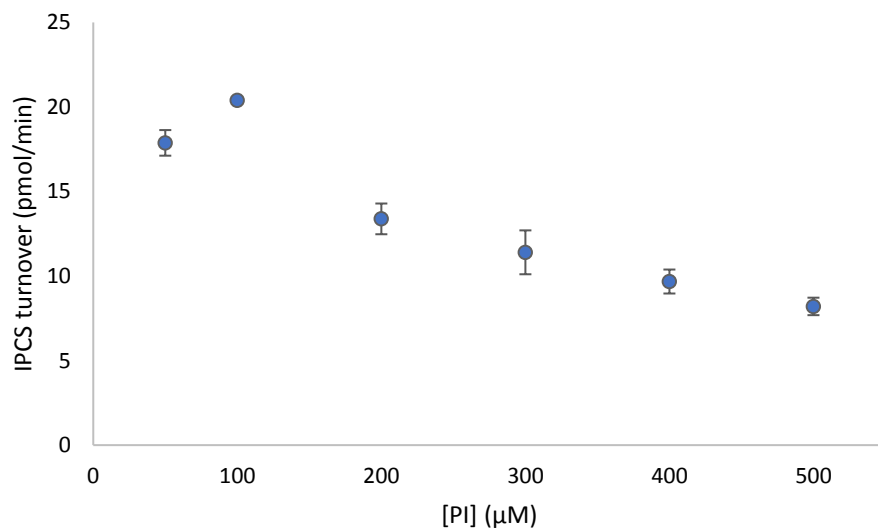


Figure 3-14: Soy PI concentration against *At*IPCS2 IPC turnover. Each value is representative of the mean and standard error calculated for three independent biological replicates.

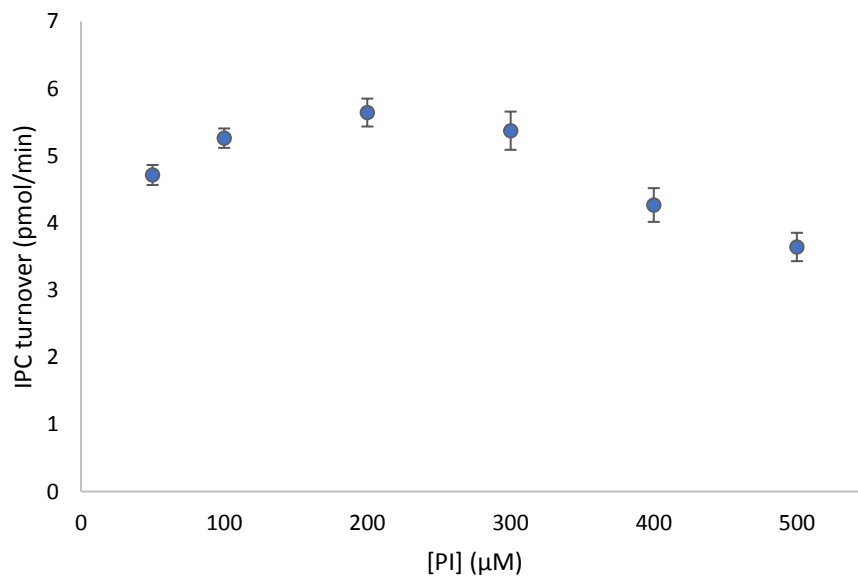


Figure 3-15: Soy PI concentration against *Os*IPCS2 IPC turnover. Each value is representative of the mean and standard error calculated for three independent biological replicates.

Based on these findings it was decided that 100 μM PI should be assayed against varying concentrations of NBD-phytoceramide to identify the optimum amount of NBD-phytoceramide for the assay (Figure 3-16).

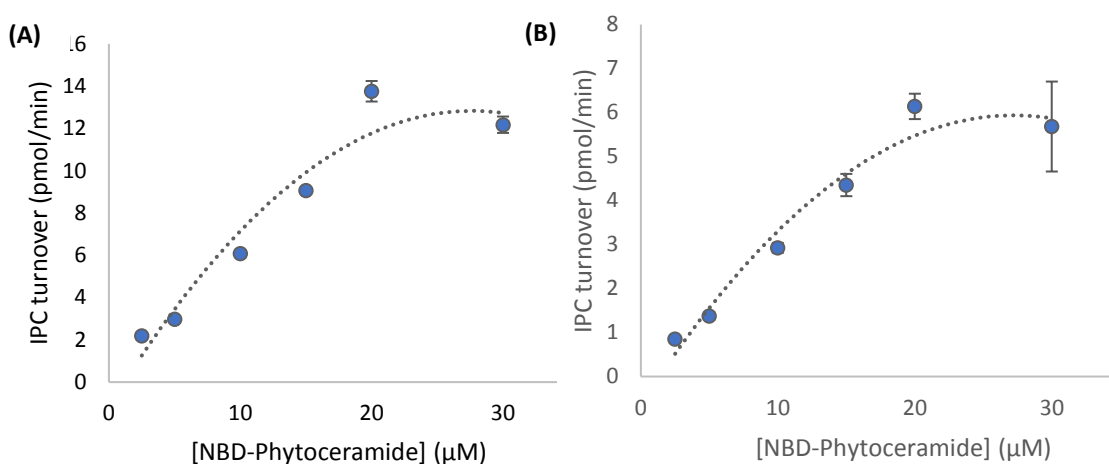


Figure 3-16: NBD-phytoceramide concentration against (A) *AtIPCS2* (B) *OsIPCS2* IPC turnover. Each value is representative of the mean and standard error calculated for three independent biological replicates.

From this experiment carried out it was determined that 15 μM NBD-phytoceramide was the optimal concentration of NBD-phytoceramide, giving a high signal and being in the linear range. Therefore, the assay conditions were: 0.44 Unit/ μl of *AtIPCS2* or 0.07 Unit/ μl of *OsIPCS2* 100 μM soy PI and 15 μM NBD-phytoceramide, incubated at 30 $^{\circ}\text{C}$ for 60 minutes.

With AUR1 used as a control in the screening it was necessary to check that the set of conditions defined for assaying *AtIPCS2* and *OsIPCS2* were suitable for this enzyme. Using the predetermined conditions (100 μM soy PI and 15 μM NBD-phytoceramide) the AUR1 reaction needed to be incubated for a maximum of 40 rather than 60 minutes to achieve a linear range (Figure 3-17).

With the assay parameters validated, screening commenced with 10 μM of each of the 126 compounds tested in duplicate. A threshold of $\geq 30\%$ percentage inhibition was set to identify hits to progress to dose response testing for IC_{50} determination.

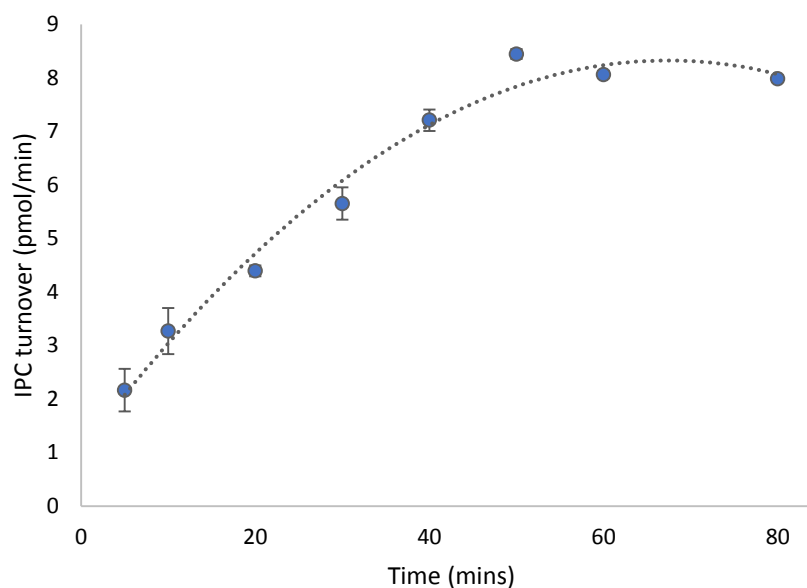


Figure 3-17: Incubation time against AUR1 reaction rate with 100 μ M PI and 15 μ M NBD-phytoceramide. Each value is representative of the mean and standard error calculated for three independent biological replicates.

Of the 126 compounds screened, 16 compounds were identified as hits; 14 were hits against *At*IPCS2 and 6 against *Os*IPCS2, 5 of these inhibited both enzymes. Dose response analyses was carried out for these compounds in triplicate against *At*IPCS2, *Os*IPCS2 and AUR1 to determine the IC_{50} for each hit against the 3 enzymes. Of the 16 compounds tested only 2 showed activity against AUR1 at the highest concentration only (100 μ M): compound 2 and 4. There was no activity at lower concentrations. In contrast, all compounds showed an inhibitory effect against the plant IPCSs.

Of the 4 compounds with an $IC_{50} \leq 10 \mu$ M, 2 are phenylamidines (Compound 1 and 2) and the other 2 compounds are triazinone (Compound 3 and 4). These 4 compounds all showed selective inhibition of *At*IPCS2 over *Os*IPCS2 in the yeast-based assay, with IC_{50} values ranging from 3-5 μ M for *At*IPCS2 compared to 15-31 μ M for *Os*IPCS2. The other three pro-apoptotic compounds (Table 3-1, structures highlighted in red) also share the same triazinone core but had IC_{50} values 15-47 μ M for the plant IPCSs and showed differential selectivity for *At*IPCS2 over *Os*IPCS2.

Table 3-1: IC₅₀ determination and chemical structures of the 16 compounds identified as hits in the biochemical assay; highlighted in blue are the compounds that exhibited an IC₅₀ value ≤ 10 μM, in red are the pro-apoptotic compounds and in purple is one of the proapoptotic compounds that also had an IC₅₀ ≤ 10 μM against *At*IPCS2

| Compound ID | <i>At</i> IPCS2 IC ₅₀ (μM) | <i>At</i> IPCS2 IC ₅₀ 95% confidence interval (μM) | R square | <i>Os</i> IPCS2 IC ₅₀ (μM) | <i>Os</i> IPCS2 IC ₅₀ 95% confidence interval (μM) | R square | Compound structure |
|--------------------|---------------------------------------|---|----------|---------------------------------------|---|----------|--------------------|
| 1 (15/10h) | 4.7 | 3.5 to 7.3 | 0.99 | 13.9 to ??? | 13.9 to ??? | 0.77 | Structure redacted |
| 2 (113/11h) | 4.0 | 3.3 to 4.9 | 0.99 | 61.9 | 33.6 to ??? | 0.94 | Structure redacted |
| 3 (6/12b) | 8.4 | 5.2 to 22.0 | 0.94 | 43.8 | undetermined | 0.40 | Structure redacted |
| 4 (101/7h) | 9.8 | 8.7 to 11.3 | 0.99 | 24.8 | 21.5 to ??? | 0.98 | Structure redacted |

| | | | | | | | |
|-------------------|------|---------------|------|--------|--------------|------|---------------------------|
| | | | | | | | Structure redacted |
| 5 (6/12a) | 15.1 | 11.1 to 25.2 | 0.99 | ~ 38.6 | undetermined | 0.78 | |
| | | | | | | | Structure redacted |
| 6 (7/4c) | 22.5 | 13.6 to 98.4 | 0.97 | ~ 35.3 | undetermined | 0.91 | |
| | | | | | | | Structure redacted |
| 7 (62/10d) | 30.7 | 15.9 to 310.9 | 0.98 | ~ 46.6 | undetermined | 0.83 | |
| | | | | | | | Structure redacted |
| 8 (101/8b) | 53.0 | 25.1 to ??? | 0.96 | ~ 44.2 | undetermined | 0.5 | |

| | | | | | | | |
|--------------------|---------|---------------|------|---------|--------------|------|---------------------------|
| | | | | | | | Structure redacted |
| 9 (121/7h) | 44.2 | 25.9 to 69712 | 0.96 | ~ 37.6 | undetermined | 0.91 | |
| | | | | | | | Structure redacted |
| 10 (120/4a) | 56.2 | 35.5 to 96.8 | 0.97 | ~ 54633 | undetermined | 0.91 | |
| | | | | | | | Structure redacted |
| 11 (15/4d) | 59.8 | 22.9 to ??? | 0.95 | ~ 28.9 | undetermined | 0.11 | |
| | | | | | | | Structure redacted |
| 12 (3/5g) | ~0.0002 | undetermined | 0.14 | ~ 200.7 | undetermined | 0.31 | |

| | | | | | | | |
|--------------------|------|---------------|------|--------------|--------------|--------------|---------------------------|
| | | | | | | | Structure redacted |
| 13 (41/4a) | 53.9 | 27.1 to 132.1 | 0.94 | undetermined | undetermined | undetermined | |
| | | | | | | | Structure redacted |
| 14 (74/7h) | 16.1 | 11.2 to 32.6 | 0.98 | 69.8 | 29.61 to ??? | 0.97 | |
| | | | | | | | Structure redacted |
| 15 (66/10a) | 1456 | undetermined | 0.71 | undetermined | undetermined | undetermined | |
| | | | | | | | Structure redacted |
| 16 (84/7a) | 1251 | 258.1 to ??? | 0.98 | ~ 64027 | undetermined | 0.95 | |

A literature search reveals that these compounds fall in to two compound classes: 1,2,4 triazin-6-ones (compounds 3-6) and phenylamidines (compounds 1 and 2). The 1,2,4 triazin-6-ones are fungicidal compounds which were filed for patent in 2001 by Dow Agrosciences²⁸. Variants of this class have exhibited activity against the pathogenic fungi, *L. nodorum*, *M. grisea*, *U. maydis* and *Z. tritici*²⁹. The phenylamidines are active pesticides which were first discovered in the 1960s by Bayer for control of plant fungi³⁰, with variants of these later patented for use as herbicides³¹.

Structural analysis of the hits identified show that an aryl group attached to both nitrogens is necessary for enhanced activity against the plant IPCS; compound 8 which has an alkyne substituent attached to one of the nitrogens as opposed to an aryl group shows the lowest activity of the triazinones identified. The presence of thio or ether linker does not have much of an effect on plant IPCS activity and it seems that the presence of the more electron withdrawing fluorine attached to both aryl rings as opposed to chlorine and perhaps the presence of the fluorine at the meta position doubles the activity of the compound against *At*IPCS2 (deduction made by comparing compound 3 and 5). Addition of a naphthalene substituent attached to sulphur as opposed to an aryl ring also seems to decrease activity due to sterics. Interestingly, compound 7 showed lowest activity of the triazinones possessing an aryl group attached to both nitrogens; the drop in activity is probably due to the change in configuration of all the substituents (structure has been flipped in compound 7) which shows that for good activity it is essential that the configuration of the substituents be maintained for a specific interaction between the electron withdrawing groups and a binding site in *At*IPCS2.

3.6 *In vivo* screening

In vivo testing of the two compounds (compounds 1 and 2) that had IC₅₀ values below 5 µM was undertaken against 7 days old *A. thaliana* seedlings. Seedlings grown were first

grown on agar containing no compound and then transferred to agar containing either compound 1 or 2. Seedling transferred to agar containing 10 μM of compound 2 had purple patches associated with anthocyanine biosynthesis in plant response to stress³², and no lateral roots compared to seedlings grown on agar containing DMSO (Figure 3-18A), whilst seedlings grown on agar containing 10 μM of compound 1, showed no obvious difference compared to wildtype (Figure 3-18B). Seedlings grown on 40 μM of compound 2 exhibited the same phenotypes of purple patches on leaves and no lateral root development. However, seedlings grown on 40 μM of compound 1, had chlorotic phenotype usually associated with PCD³³ and no lateral root development compared to controls (Figure 3-18B). These results indicated that both compounds affected root development.

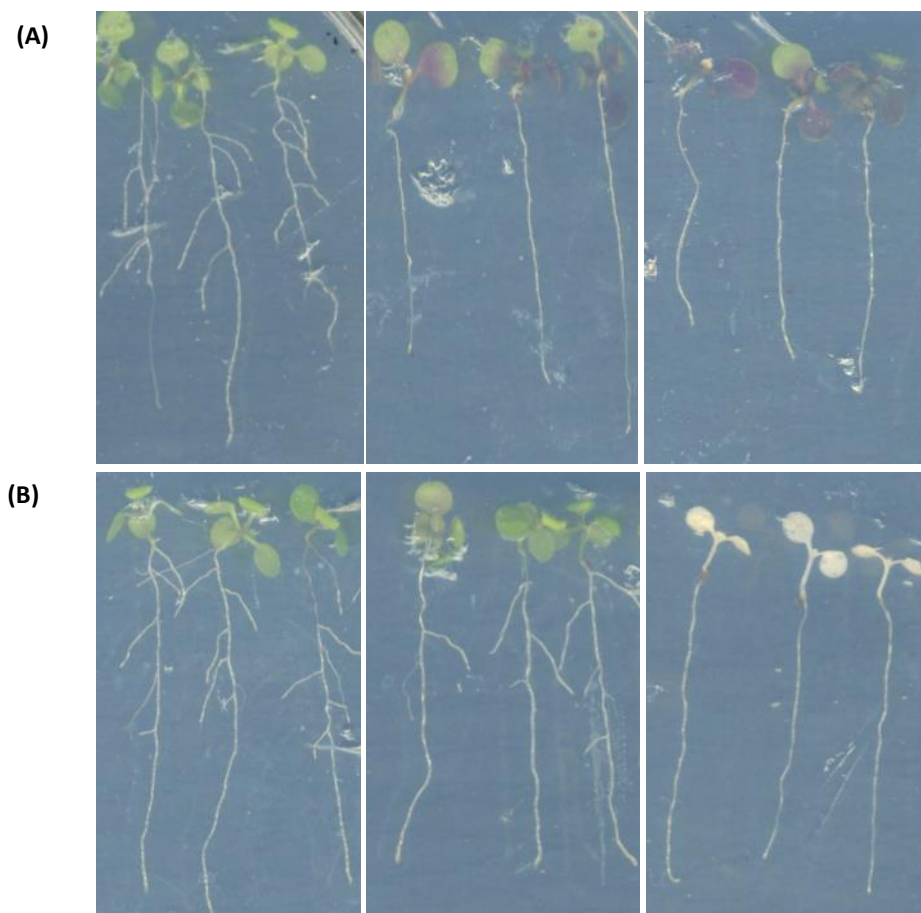


Figure 3-18: From left to right, 10 days old *A. thaliana* seedlings transferred to agar containing (A) 10 μL DMSO, 10 μM and 40 μM compound 2 (B) 40 μL DMSO, 10 μM and 40 μM of compound 1. Image taken 7 days after transfer of seedlings to agar containing the respective compounds.

Root growth was assessed by marking the root length after 4 days growth on the plates, then the changes in root length were measured after 3 days. No change in root length was observed in seedlings grown on 10 μ M or 40 μ M of compound 2. However, a significant difference was found between the primary root length of seedling grown on 10 μ M of compound 1, compared to wildtype, with a reduction in root length by 30% (Figure 3-19) and seedlings grown on 40 μ M of compound 1 showing no root development.

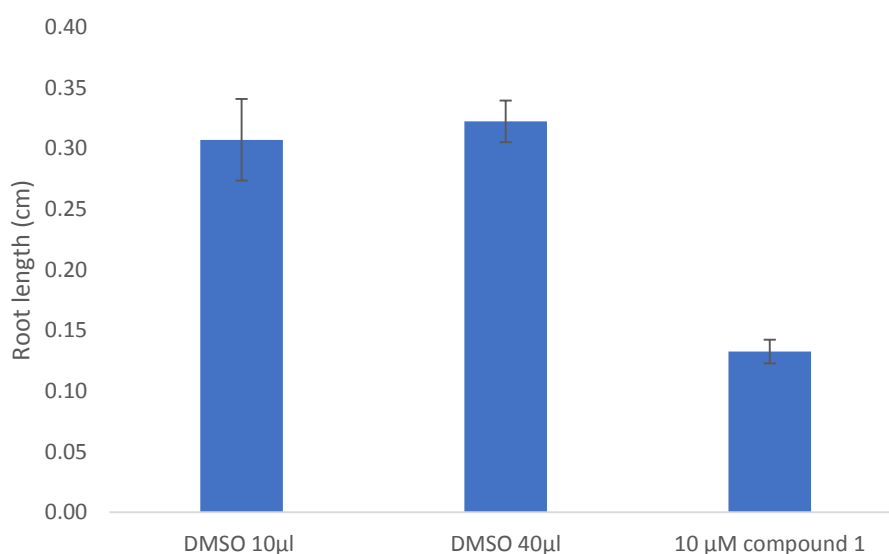


Figure 3-19: Root length measurements of seedlings grown in DMSO and in the presence of 10 μ M of compound 1. Each value is representative of the mean of six replicates.

3.7 Conclusion

The screening campaign to identify inhibitors of the plant IPCS led to the discovery of the phenylamidine and triazinone class of compounds as selective inhibitors of *At*IPCS over AUR1, with the phenylamidines validated in an in vivo screen against *A. thaliana* seedlings. The phenylamidines exhibit low μ M activity against *At*IPCS2 in vitro causing chlorotic leaves, stunted development of lateral roots and primary root length in treated seedlings. These findings are compelling and raise the question of an interplay between sphingolipid biosynthesis and root development. The differential selectivity of these compounds for *At*IPCS2 over *Os*IPCS2 and AUR1 in the yeast-based assay and the

enzyme assay, also raises the question as to how the compounds are interacting with the target; there is a high degree of sequence identity between *At*IPCS2 and *Os*IPCS2 (68%), both sharing well conserved domains, so it would be interesting to explore how these compounds are able to selectively inhibit one over the other. The differential selectivity of the inhibitors identified for the dicot (*A. thaliana*) over the monocot (*O. sativa*) IPCS can be utilized to create herbicide formulations that selectively kill dicot weeds without harming monocot crops.

3.8 References

1. Saucedo - García, M.; Guevara - García, A.; González - Solís, A.; Cruz - García, F.; Vázquez - Santana, S.; Markham, J. E.; Lozano - Rosas, M. G.; Dietrich, C. R.; Ramos - Vega, M.; Cahoon, E. B., MPK6, sphinganine and the LCB2a gene from serine palmitoyltransferase are required in the signaling pathway that mediates cell death induced by long chain bases in Arabidopsis. *New Phytologist* **2011**, *191* (4), 943-957.
2. Choi, H.; Kim, J.-Y.; Chang, Y. T.; Nam, H. G., Forward chemical genetic screening. In *Arabidopsis Protocols*, Springer: 2014; pp 393-404.
3. Serrano, M.; Kombrink, E.; Meesters, C., Considerations for designing chemical screening strategies in plant biology. *Frontiers in plant science* **2015**, *6*.
4. Surpin, M.; Rojas-Pierce, M.; Carter, C.; Hicks, G. R.; Vasquez, J.; Raikhel, N. V., The power of chemical genomics to study the link between endomembrane system components and the gravitropic response. *Proceedings of the National Academy of Sciences of the United States of America* **2005**, *102* (13), 4902-4907.
5. Savaldi-Goldstein, S.; Baiga, T. J.; Pojer, F.; Dabi, T.; Butterfield, C.; Parry, G.; Santner, A.; Dharmasiri, N.; Tao, Y.; Estelle, M., New auxin analogs with growth-promoting effects in intact plants reveal a chemical strategy to improve hormone delivery. *Proceedings of the National Academy of Sciences* **2008**, *105* (39), 15190-15195.
6. Poretska, O.; Yang, S.; Pitorre, D.; Rozhon, W.; Zwerger, K.; Uribe, M. C.; May, S. T.; McCourt, P.; Poppenberger, B.; Sieberer, T., The small molecule hyperphyllin enhances leaf formation rate and mimics shoot meristem integrity defects associated with AMP1 deficiency. *Plant physiology* **2016**, pp. 01633.2015.
7. Lin, L.-C.; Hsu, J.-H.; Wang, L.-C., Identification of novel inhibitors of 1-aminocyclopropane-1-carboxylic acid synthase by chemical screening in Arabidopsis thaliana. *Journal of Biological Chemistry* **2010**, *285* (43), 33445-33456.
8. Schreiber, K. J.; Nasmith, C. G.; Allard, G.; Singh, J.; Subramaniam, R.; Desveaux, D., Found in translation: high-throughput chemical screening in Arabidopsis thaliana identifies small molecules that reduce Fusarium head blight disease in wheat. *Molecular plant-microbe interactions* **2011**, *24* (6), 640-648.
9. Hicks, G. R.; Raikhel, N. V., Small molecules present large opportunities in plant biology. *Annual review of plant biology* **2012**, *63*, 261-282.
10. Brodersen, P.; Petersen, M.; Pike, H. M.; Olszak, B.; Skov, S.; Ødum, N.; Jørgensen, L. B.; Brown, R. E.; Mundy, J., Knockout of Arabidopsis accelerated-cell-

death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes & Development* **2002**, *16* (4), 490-502.

11. Rivas-San Vicente, M.; Larios-Zarate, G.; Plasencia, J., Disruption of sphingolipid biosynthesis in *Nicotiana benthamiana* activates salicylic acid-dependent responses and compromises resistance to *Alternaria alternata* f. sp. *lycopersici*. *Planta* **2013**, *237* (1), 121-136.

12. Chen, M.; Han, G.; Dietrich, C. R.; Dunn, T. M.; Cahoon, E. B., The essential nature of sphingolipids in plants as revealed by the functional identification and characterization of the *Arabidopsis* LCB1 subunit of serine palmitoyltransferase. *The Plant Cell* **2006**, *18* (12), 3576-3593.

13. Ternes, P.; Feussner, K.; Werner, S.; Lerche, J.; Iven, T.; Heilmann, I.; Riezman, H.; Feussner, I., Disruption of the ceramide synthase LOH1 causes spontaneous cell death in *Arabidopsis thaliana*. *New Phytologist* **2011**, *192* (4), 841-854.

14. Chen, M.; Markham, J. E.; Dietrich, C. R.; Jaworski, J. G.; Cahoon, E. B., Sphingolipid long-chain base hydroxylation is important for growth and regulation of sphingolipid content and composition in *Arabidopsis*. *The Plant Cell* **2008**, *20* (7), 1862-1878.

15. Bi, F.-C.; Liu, Z.; Wu, J.-X.; Liang, H.; Xi, X.-L.; Fang, C.; Sun, T.-J.; Yin, J.; Dai, G.-Y.; Rong, C., Loss of ceramide kinase in *Arabidopsis* impairs defenses and promotes ceramide accumulation and mitochondrial H₂O₂ bursts. *The Plant Cell* **2014**, *26* (8), 3449-3467.

16. Wang, W.; Yang, X.; Tangchaiburana, S.; Ndeh, R.; Markham, J. E.; Tsegaye, Y.; Dunn, T. M.; Wang, G.-L.; Bellizzi, M.; Parsons, J. F., An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*. *The Plant Cell* **2008**, *20* (11), 3163-3179.

17. Cerbón, J.; del Carmen López-Sánchez, R., Diacylglycerol generated during sphingomyelin synthesis is involved in protein kinase C activation and cell proliferation in Madin-Darby canine kidney cells. *Biochemical Journal* **2003**, *373* (3), 917-924.

18. Markham, J. E.; Li, J.; Cahoon, E. B.; Jaworski, J. G., Separation and identification of major plant sphingolipid classes from leaves. *Journal of Biological Chemistry* **2006**, *281* (32), 22684-22694.

19. Tartaglio, V.; Rennie, E. A.; Cahoon, R.; Wang, G.; Baidoo, E.; Mortimer, J. C.; Cahoon, E. B.; Scheller, H. V., Glycosylation of inositol phosphorylceramide sphingolipids is required for normal growth and reproduction in *Arabidopsis*. *The Plant Journal* **2017**, *89* (2), 278-290.

20. Mangwanda, R.; Myburg, A. A.; Naidoo, S., Transcriptome and hormone profiling reveals *Eucalyptus grandis* defence responses against *Chrysoporthe austroafricana*. *BMC genomics* **2015**, *16* (1), 319.

21. Mina, J.; Okada, Y.; Wansadhipathi-Kannangara, N.; Pratt, S.; Shams-Eldin, H.; Schwarz, R.; Steel, P.; Fawcett, T.; Denny, P., Functional analyses of differentially expressed isoforms of the *Arabidopsis* inositol phosphorylceramide synthase. *Plant molecular biology* **2010**, *73* (4-5), 399-407.

22. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J., Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews* **1997**, *23* (1-3), 3-25.

23. Tice, C. M., Selecting the right compounds for screening: does Lipinski's Rule of 5 for pharmaceuticals apply to agrochemicals? *Pest management science* **2001**, *57* (1), 3-16.

24. Briggs, G. In *Predicting uptake and movement of agrochemicals from physical properties*, Presentation at SCI Meeting, in: Uptake of Agrochemicals and Pharmaceuticals, Belgrave Square, London, England, 9th December, 1997.

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25. Maddox, C. B.; Rasmussen, L.; White, E. L., Adapting cell-based assays to the high-throughput screening platform: problems encountered and lessons learned. *JALA: Journal of the Association for Laboratory Automation* **2008**, *13* (3), 168-173.
 26. Mina, J., Thesis: Exploring IPC synthase: a potential anti-leishmanial drug target. **2010**.
 27. Mina, J. G.; Mosely, J. A.; Ali, H. Z.; Shams-Eldin, H.; Schwarz, R. T.; Steel, P. G.; Denny, P. W., A plate-based assay system for analyses and screening of the Leishmania major inositol phosphorylceramide synthase. *International Journal of Biochemistry & Cell Biology* **2010**, *42* (9), 1553-1561.
 28. Kelly, M. J.; Evans, K. A.; Gallagher, J. J.; Mulvihill, M. J., Substituted 4, 5-dihydro-1, 2, 4-triazin-6-ones, 1, 2, 4-triazin-6-ones and their use as fungicides and insecticides. Google Patents: 2009.
 29. Owen, W. J.; Sullenberger, M. T.; Loso, M. R.; Meyer, K. G.; Slanec, T. J., Synthesis and antifungal activity of 3 - aryl - 1, 2, 4 - triazin - 6 - one derivatives. *Pest management science* **2015**, *71* (1), 83-90.
 30. Dieter, D.; Hans, A.; Ludwig, E., Method for protecting plants from fungi. Google Patents: 1966.
 31. Kuhn, B.; Jakobi, H.; Mueller, T.; Tiebes, J.; Kehne, H.; Schmutzler, D.; Hills, M.; Rosinger, C.; Kunz, K.; Drewes, M. W., Use of n2-phenylamidines as herbicides. Google Patents: 2008.
 32. Chalker - Scott, L., Environmental significance of anthocyanins in plant stress responses. *Photochemistry and photobiology* **1999**, *70* (1), 1-9.
 33. Lam, E.; Fukuda, H.; Greenberg, J., *Programmed cell death in higher plants*. Springer Science & Business Media: 2000; Vol. 44.

CHAPTER 4

VALIDATION OF IPCS TRANSGENIC LINES FOR FUNCTIONAL GENOMIC ANALYSES

4.1 Chapter synopsis

In this chapter, the creation and characterisation of *Arabidopsis thaliana* transgenic lines over-expressing *AtIPCS1-3*, and knock-down lines of *AtIPCS1-3* are presented. Transgenic lines of 35s::GFP.*AtIPCS1*, 35s::GFP.*AtIPCS2* and 35s::GFP.*AtIPCS3* were also created for the analysis of protein localisation in *Arabidopsis thaliana* and *Nicotiana benthamiana*. These transgenic lines have been created to probe the changes in the transcriptome of knock-down/over-expression lines in response to a change in the expression of each orthologue. Also described in this chapter is the phenotypic screen of the transgenic lines, which identified an early flowering phenotype indicating a link between IPCS and vernalisation.

4.2 RNA interference, over-expression and localisation of *AtIPCS1-3* using gateway cloning

Creating multiple expression constructs for the target gene of interest (*AtIPCS1-3*) using traditional ligase-mediated cloning is time-consuming and laborious, hence the use of gateway cloning. Gateway cloning exploits the bacteriophage lambda recombination system, bypassing the use of ligase-mediated cloning¹.

For RNA interference (RNAi) knock-down of a target of interest, double stranded RNA from an inverted repeat of the target sequence was expressed, resulting in post-transcriptional gene silencing (PTGS). The classical understanding of RNAi triggered by the presence of an inverted repeat gene in plants involves transcription of the DNA sequence resulting in the formation of double stranded RNA (dsRNA). Following this, cleavage of dsRNA is mediated by the Dicer enzymes producing 21-25 nucleotides long siRNAs, which were first identified in plant lines harbouring a transgene that induced PTGS². RNAi is carried out by the RNA induced silencing complex (RISC) a protein-RNA effector complex, that utilizes the complementarity of the siRNA sequences to their target mRNA, resulting in degradation of the target mRNA mediated by the Argonaut proteins³. This leads to silencing or expression knock-down of the target.

Knock-down or silencing of an IPCS gene can be achieved by the introduction of exogenous miRNAs designed to specifically target one of the three IPCS isoforms. The system utilized in this study is attractive as an alternative to the use of miRNAs, in that the siRNA produced from the dsRNA could potentially target the expression of one or more of the IPCS isoforms because of the high sequence identity shared; making it possible to observe the cumulative effect of differential expression of each IPCS isoform on plant development.

The Gateway ‘destination’ vectors used in this study were designed by Karimi *et al.*⁴; the pK7WG2 vector was used for over-expression, the pK7GWIWG2 (I) for silencing and the pK7FWG2 for localisation by N-terminal tagging of each IPCS orthologue with green fluorescent protein (GFP).

Each IPCS orthologue (*AtIPCS1-3*) was cloned into the over-expression vector (pK7WG2), the silencing vector (pK7GWIWG2) and pK7FWG2 for GFP N-terminal tagging. The clones were verified as described in Materials and Methods. Subsequently, *Agrobacterium tumefaciens* (strain GV1301) mediated transformation of wild-type *Arabidopsis* (Col0) with the constructs was carried out to generate the T0 generation.

4.2.1 *AtIPCS1-3* localisation

For preliminary localisation of *AtIPCS1-3*, the N-terminal GFP-tagged IPCSs constructs were used to transform *Nicotiana benthamiana* using agrobacterium strain GV1301 for transient expression of the protein. The 35s::GFP cytosolic marker was also transiently expressed as a control.

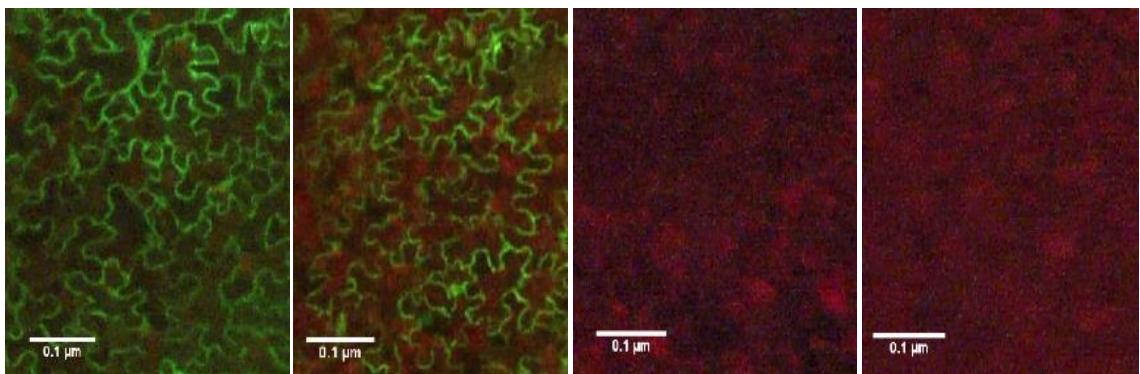


Figure 4-1: From left to right, expression of 35s::GFP cytosolic marker, 35s::GFP.*AtIPCS1*, 35s::GFP.*AtIPCS2* and 35s::GFP.*AtIPCS3* imaged 48 hours after infiltration of *Nicotiana benthamiana* with agrobacterium harbouring respective constructs for each isoform.

The first attempts at the transient expression of 35s::GFP.*AtIPCS2* and 35s::GFP.*AtIPCS3* failed with no fluorescence observed (Figure 4-1). However, 35s::GFP.*AtIPCS1* exhibited high levels of expression (Figure 4-1). A closer look using confocal microscopy revealed

that the expression pattern was indicative of cytosolic expression (Figure 4-2).

The cytosolic localisation of a multi-transmembrane protein such as *AtIPCS1* was surprising given previous work by Wang et al. which had demonstrated that *AtIPCS2* is localised to the trans Golgi network⁵. The results from 35s::GFP.*AtIPCS1*, 35s::GFP.*AtIPCS2* and 35s::GFP.*AtIPCS3* transient expression suggested that the plant had pre-empted the expression of GFP tagged IPCS, resulting in free cytosolic GFP expression for the 35s::GFP.*AtIPCS1* construct and no expression for 35s::GFP.*AtIPCS2* and 35s::GFP.*AtIPCS3* constructs. The function of *AtIPCS2* as a negative regulator of PCD is well characterized⁵; *AtIPCS* role in defence response could result in the silencing of 35s::GFP.*AtIPCS* expression in planta to pre-empt an incompatible reaction or could be due to the instability of the fusion protein.

To ensure that the cytosolic expression of 35s::GFP.*AtIPCS1* was not as a result of over-expression due to multiple t-DNA insertions in a cell which is a consequence of high numbers of bacteria, the optical density of agrobacterium used to infiltrate *N. benthamiana* with the localisation constructs was reduced by 1/3.

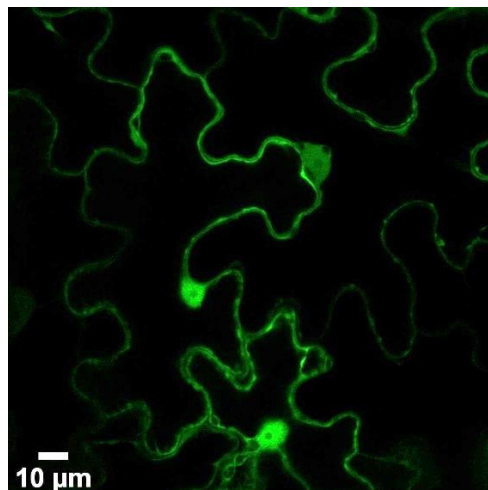


Figure 4-2: Subcellular localisation of 35s::GFP.*AtIPCS1* imaged 48 hours after tobacco infiltration with agrobacterium harbouring 35s::GFP.*AtIPCS1* expression construct.

This was also beneficial for testing 35s::GFP.*AtIPCS1* and 2 to find out if suppression of expression was a result of the detection of high levels of *AtIPCS* transcript. 35s::GFP.*AtIPCS1*, 2 and 3 were co-infiltrated respectively with RFP tagged sialyl transferase (ST) which is localised to the Golgi⁶. From the results (Figure 4-3) it was clear that the expression and localisation of ST RFP to the Golgi was successful, however, no expression could be seen for 35s::GFP.*AtIPCS1-3*. With the unsuccessful expression of 35s::GFP.*AtIPCS1-3* in *N. benthamiana*, 35s::GFP.*IPCS* expression was checked in *A. thaliana* transgenic lines. From the T2 generation; RNA was isolated from the 10 day old seedlings, and following cDNA synthesis, real-time PCR was used to quantify 35s::GFP.*AtIPCS1*, 2 and 3 expression.

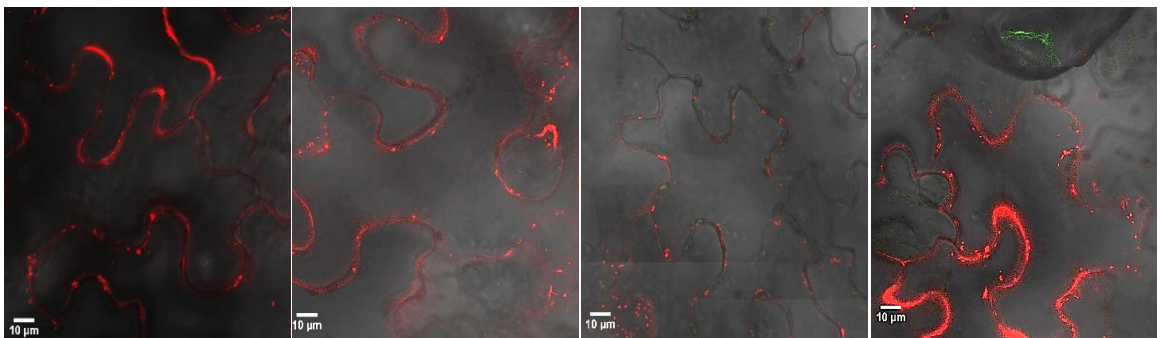


Figure 4-3: left to right, expression of the Golgi marker ST RFP, merged image of GFP tagged *AtIPCS1* and ST RFP, merged image of GFP tagged *AtIPCS2* and ST RFP, merged image of GFP tagged *AtIPCS3* and ST RFP. Plasmid DNA of ST RFP and 35s::GFP.*AtIPCS1*, 35s::GFP.*AtIPCS2* and 35s::GFP.*AtIPCS3* respectively was introduced into tobacco cells by infiltration with agrobacterium and imaged with a confocal microscope 72 hours after infiltration.

Analyses were carried out using the comparative cycle threshold (C_t) method which is based on the fold difference in gene expression between the target and an internal standard gene⁷. In this study, the internal standard was *PEX4*, which encodes a ubiquitin-conjugating enzyme-like protein involved in transporting proteins targeted to the peroxisome⁸.

Transgenic lines harbouring 35s::GFP.*AtIPCS2* and 3 were lost due to a viral infection, however, 35s::GFP.*AtIPCS1* transgenic plants were retrieved, and seeds from this line were grown and checked for expression of *IPCS1* using real-time PCR and gene specific primers. As expected these transgenic lines had higher expression of *AtIPCS1* compared to Col0 (Figure 4-4).

However, under the confocal microscope, only low levels of fluorescence were observed, surrounded by dark regions that were indicative of dead cells (data not shown). These and the data above indicated that 35s::GFP.*AtIPCS* constructs were not suitable for localisation analyses.

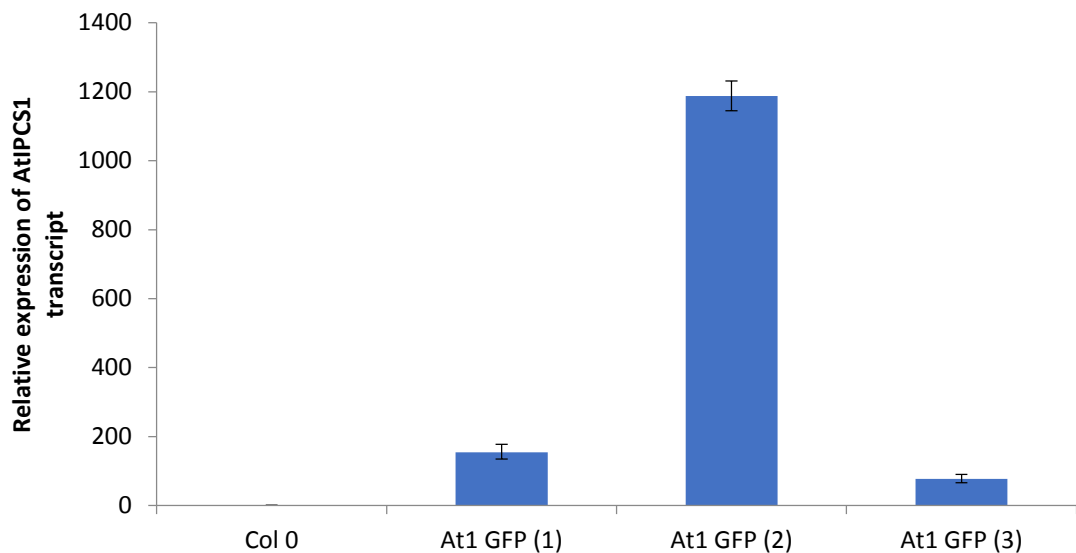


Figure 4-4: Relative transcript levels of *AtIPCS1* in *Arabidopsis* transgenic lines compared to wild-type: ten-week -old seedlings were grown on MS agar. Nomenclature of transgenic line is as follows: the first number identifies the *IPCS* isoform and the second number in brackets refers to the clonal line. Total RNA prepared from these seedlings and cDNA synthesised from 2 µg of total RNA, then used for transcript analysis using SYBR green RT-PCR. Results are an average with standard error calculated for three technical replicates in each clonal line.

4.2.2 *AtIPCS1-3* RNAi transgenic lines

In a similar manner, expression levels in RNAi *AtIPCS1*, 2 and 3 *Arabidopsis* knock-down lines were also quantified by real time PCR. To circumvent the detection of

accumulating *IPCS* mRNA in the cytoplasm destined for degradation, primers were designed to detect the 3' untranslated region (UTR) so that only the detection of mRNA destined for translation would be quantified.

Quantification of each *IPCS* isoform was probed with three specific primers pairs for each isoform to establish how the knock-down of one of the isoform would affect the expression pattern of the other (Figure 4-5). *AtIPCS1* transcript levels were 57 and 77% relative to Col0 for At1 RNAi 5 and 6 transgenic lines respectively. Interestingly *AtIPCS1* levels were also reduced in At2 RNAi 3, At2 RNAi 4 and At3 RNAi 2 (45, 85 and 46% respectively); these also exhibited the expected reduction of *AtIPCS2* transcript levels (61, 86 and 56% respectively). The transgenic lines At2 RNAi 1 and At2 RNAi 7 had *AtIPCS2* transcript levels of 53% and 61% relative to Col0, and At3 RNAi 2 exhibiting *AtIPCS2* levels of 56%.

Quantification of *AtIPCS3* transcripts in the transgenic lines, revealed that At2 RNAi 3 and At3 RNAi had significantly less *AtIPCS3* transcript compared to Col0 (19 and 45% respectively). Of all the transgenic lines tested, At2 RNAi 3 showed reduction in the expression of all three *IPCS* isoforms. This was of particular interest, for the further analyses of the global effects of sphingolipid dysfunction.

To understand the modulation of the three *IPCS* isoform in relation to one another, scatter graphs showing the transcript levels detected for one isoform plotted against the transcript levels detected for another isoform in the same transgenic lines were drawn with 85% reduction in *IPCS* transcript level set as the cut off value (Figure 4-6A-C). No correlation was found between *AtIPCS1* and *AtIPCS2* expression levels, however lines that had shown a decrease in *AtIPCS2* transcripts levels had an observable increase in *AtIPCS3* expression (Figure 4-6C – points highlighted in yellow).

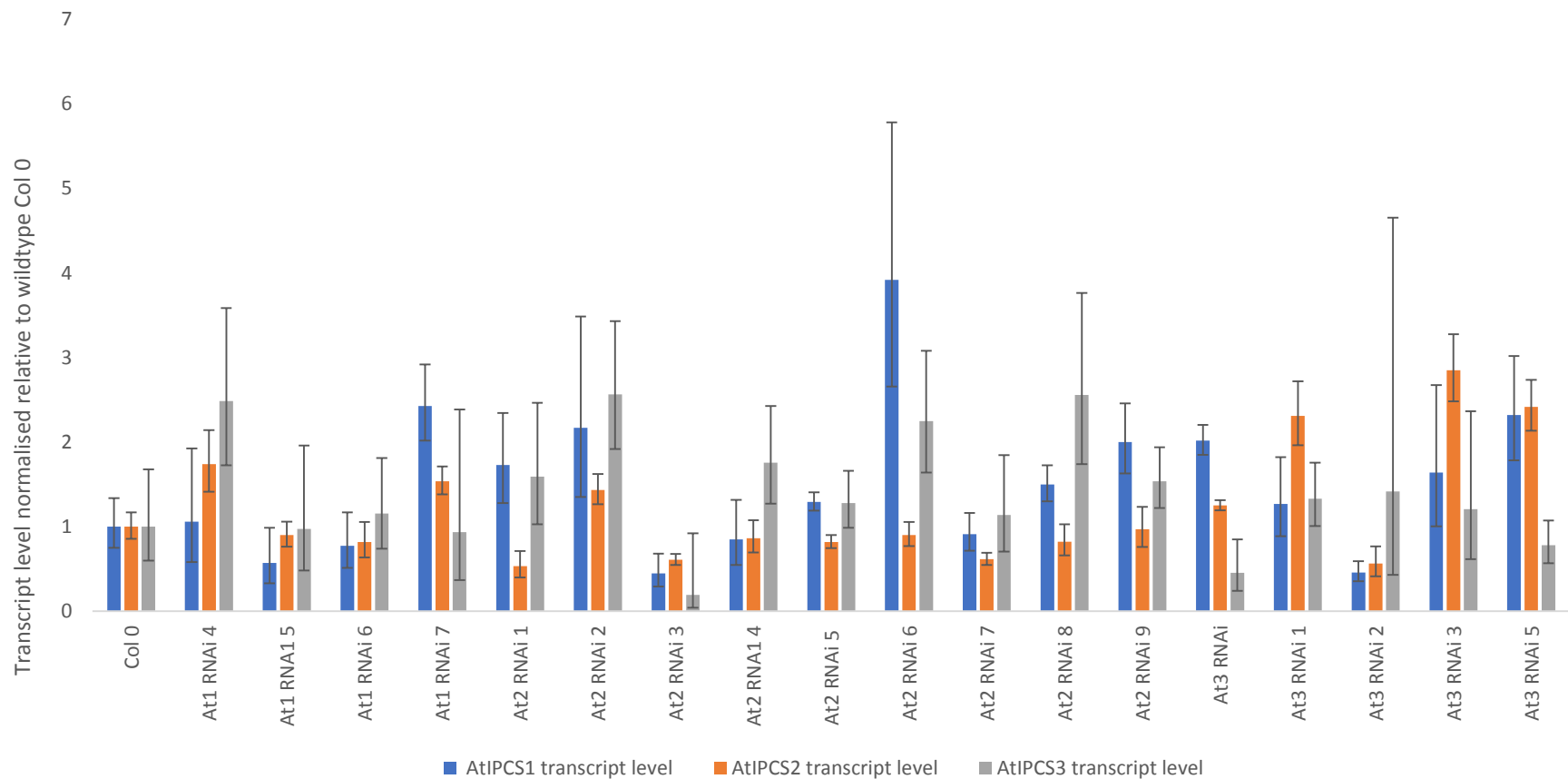


Figure 4-5: Relative transcript levels of *AtIPCS1-3* in *Arabidopsis* transgenic lines compared to wildtype using specific primers designed for each isoform and designed to detect the 3' UTR region of each isoform. Nomenclature of transgenic line is as follows: the first number identifies the IPCS isoform and the second number refers to the clonal line. Total RNA was extracted from ten-day-old seedlings grown on MS agar and cDNA synthesised from 2 µg of total RNA. Transcript levels were analysed using SYBR green RT-PCR and are the average with standard error calculated for three technical replicates in each clonal line.

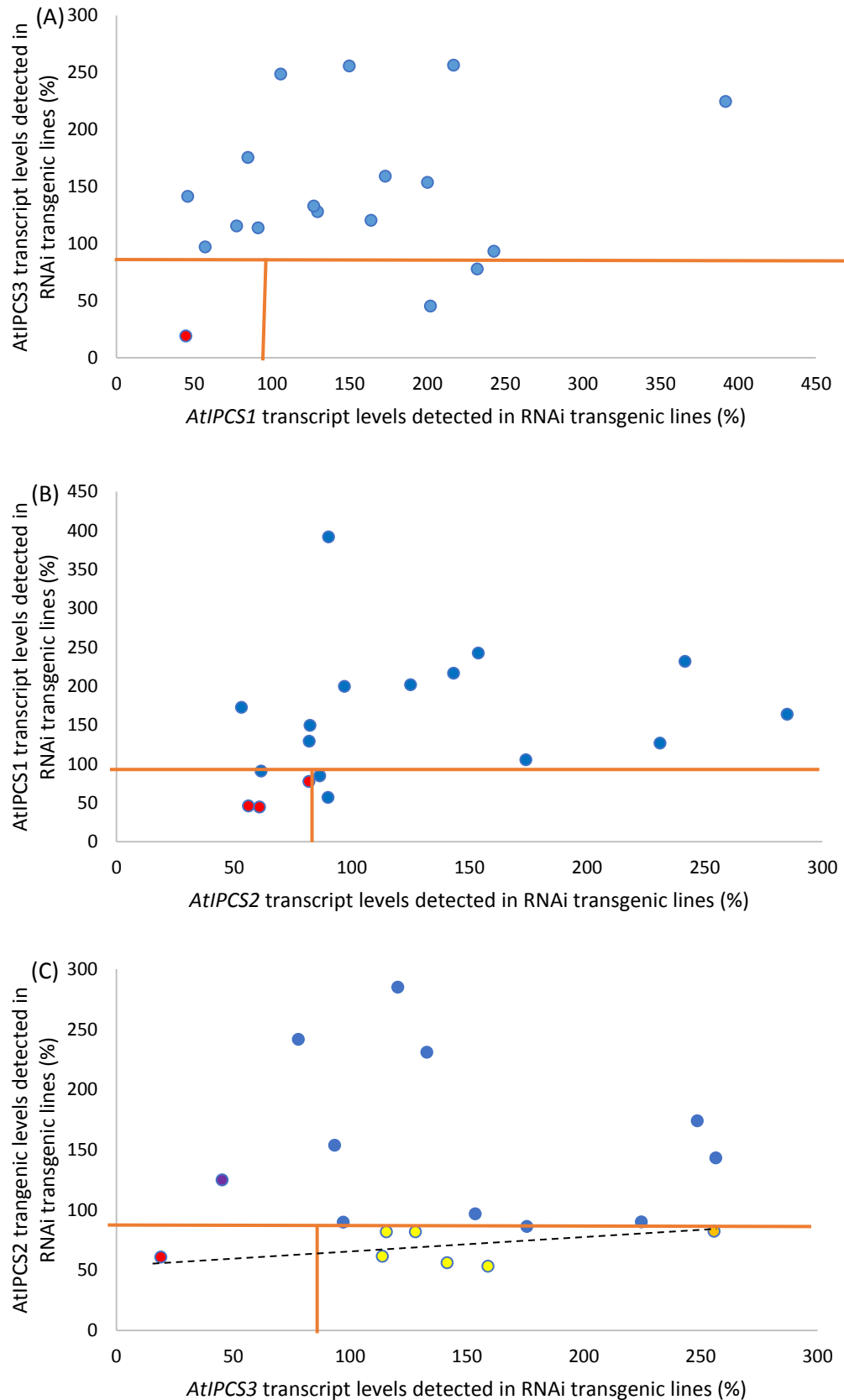


Figure 4-6: Percentage transcript levels of *AtIPCS1-3* in the transgenic lines relative to Col0 (A) *AtIPCS1* vs *AtIPCS3* levels (B) *AtIPCS2* vs *AtIPCS1* levels (C) *AtIPCS3* vs *AtIPCS2*. Highlighted in red are transgenic lines that showed significant reduction in transcript level for both IPCS isoforms and highlighted in yellow are transgenic lines which showed a relationship between the differential expression of *AtIPCS2* and *AtIPCS3*.

Based on the initial results, three transgenic lines were selected for each isoform of *AtIPCS* which showed a reduction in transcript levels: At1RNAi 5, At1 RNAi 6, At1 RNAi 7, At2 RNAi 1, At2 RNAi 3, At2 RNAi 7, At3 RNAi, At3 RNAi 2 and At3 RNAi 5. To confirm genetic stability of the reduced transcript levels of *IPCS*, the transgenic lines were grown to obtain the T3 generation and the transcript levels of *AtIPCS1*, 2 and 3 quantified (Figure 4-7).

Quantification of *AtIPCS1* showed that the transgenic lines which had previously shown a decrease in transcripts of 57% and 77% for At1 RNAi 5 and At1 RNAi6 (Figure 4-9) had increased to 106% and 93% respectively. This indicated that RNAi of *AtIPCS1* transcripts was not genetically stable. In contrast, the reduction in *AtIPCS2* transcripts proved to be genetically stable with similar levels observed for the selected transgenic RNAi and At3 RNAi 5 had 26% and 16% of *AtIPCS3* wild-type transcript, compared to

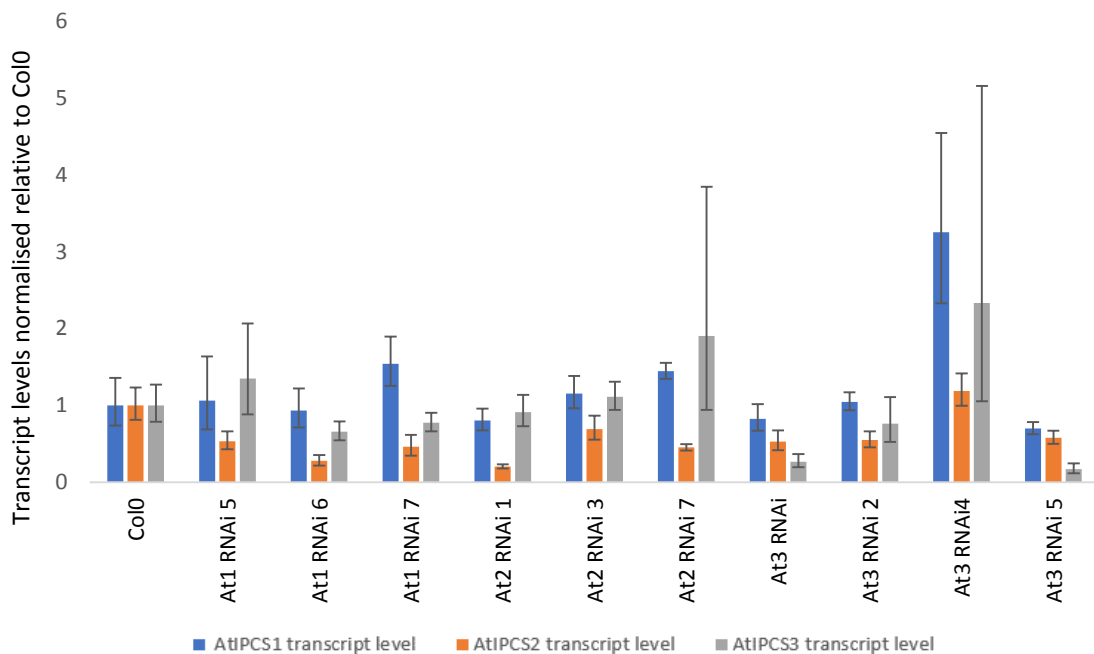


Figure 4-7: Relative transcript levels of *AtIPCS1*, 2 and 3 in the selected *Arabidopsis* transgenic lines from the T3 generation compared to wildtype. Nomenclature of transgenic line is as follows: the first number identifies the *IPCS* isoform and the second number refers to the clonal line. Total RNA was extracted from ten-day-old seedlings grown on MS agar and cDNA synthesised from 2 µg of total RNA. Transcript levels were analysed using SYBR green RT-PCR and are the average with standard error calculated for three technical replicates in each clonal line.

lines: At2 RNAi 1 (20%), At2 RNAi 3 (69%) and At2 RNAi 7 (45%). Similarly, At3 the earlier qPCR result which yielded 45% and 78%. Interestingly, all lines, except At3 RNAi 4, showed a reduction in *AtIPCS2* mRNA levels, 20-69% of wild-type.

It was observed that there was large variation between the biological duplicate results, in some part is owed to pipetting errors, but also to the fact that the analysed lines comprise of pools of different clonal lines consisting of hemizygous, homozygous and wildtype expression of the different isoforms of *AtIPCS*. To isolate a stable homozygous line, T3 transgenic clonal lines were collected separately and a progeny test carried out. The progeny test is based on the knowledge that the transformed T0 generation are heterozygous for the introduced transgene and are kanamycin resistance⁹.

Following self-pollination of the T1 generation, a mixture of homozygous (kanamycin resistant), hemizygous (kanamycin resistant) and wildtype (kanamycin sensitive) T2 generation is formed with a segregation ratio of 3:1 for transgenic lines harbouring the transgene. The same pattern of antibiotic selection also occurs in the T3 generation but with a segregation ratio of 5:3. If the parent of the T3 generation is homozygous, all seeds grown on kanamycin plates will survive as all of the progeny of a homozygous parent would be homozygous and therefore kanamycin sensitive. In contrast parent lines that are hemizygous will have some progeny that are kanamycin sensitive and not viable.

Of the 60 independent transgenic lines tested, 6 transgenic lines tested positive as homozygous lines with all seeds grown on kanamycin medium surviving. transgenic lines had reduced expression for at least one isoform of *AtIPCS* (Figure 4-8). However, RNAi effectively reduced transcript levels when targeted against *AtIPCS2* and 3; *AtIPCS1* RNAi did not lead to a decrease in *AtIPCS1* mRNA (Figure 4-8) which suggests that it has an essential role linked to the early developmental stages in plants.

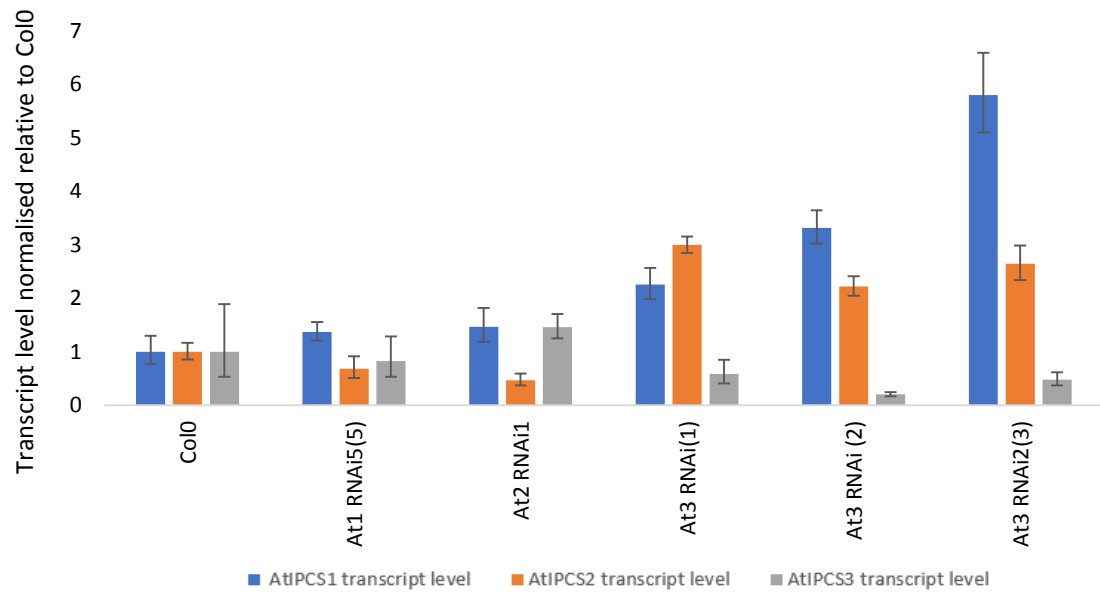


Figure 4-8: Relative transcript level of *AtIPCS1*, 2 and 3 in T3 homozygous *Arabidopsis* transgenic lines compared to wildtype using specific primers designed to detect the 3' UTR in each isoform. Nomenclature of transgenic line is as follows: the first number identifies the IPCS isoform and the second refers to the clonal line. Total RNA was extracted from ten-day-old seedlings grown on MS agar and cDNA synthesised from 2 µg of total RNA. Transcript levels were analysed using SYBR green RT-PCR and are the average with standard error calculated for three technical replicates in each clonal line.

4.2.3 *AtIPCS1-3* overexpression transgenic lines

Quantitation of transgenic lines over expressing *AtIPCS1-3* was carried out in the same manner. It was found that over-expression of *AtIPCS1* and *AtIPCS3* was highly efficient with very high levels of transcript detected, hundreds of folds higher than Col0. In contrast, over-expressing *AtIPCS2* had transcript levels only 5-7-fold higher than Col0 (Figure 4-9A-C).

The lower relative over-expression of *AtIPCS2* in the transgenic lines, was despite the fact that this isoform is the most highly expressed in *Arabidopsis*. These data suggested that the level of *AtIPCS2* expression is tightly regulated, which could indicate that this is because *AtIPCS2* functions as a negative regulator of PCD⁵, so it is vital for plant survival that the expression levels are tightly regulated. Therefore, transgenic lines with higher levels of *AtIPCS2* transcripts may not be viable, resulting in a selection pressure for lines

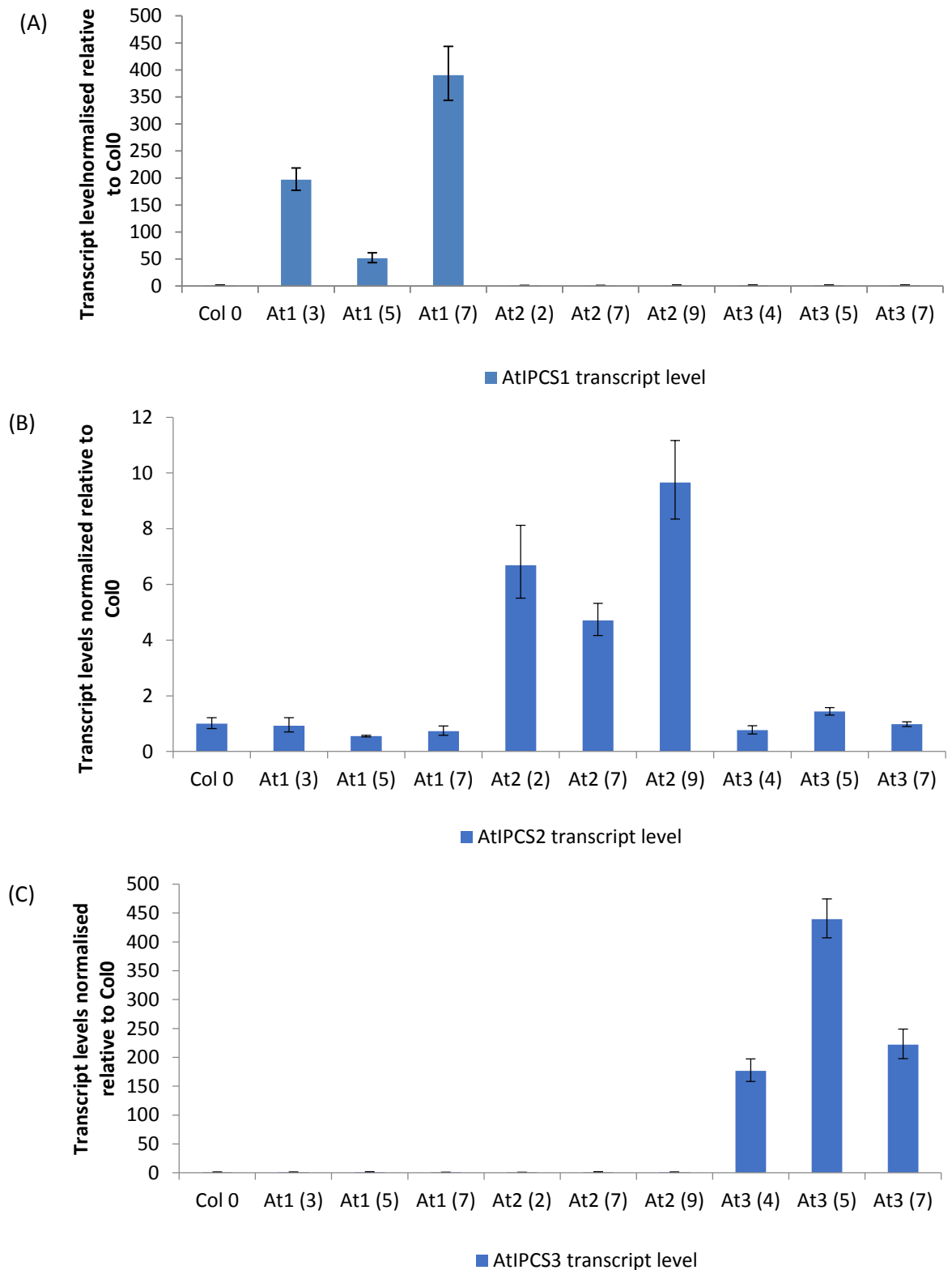


Figure 4-9: Quantification of *AtIPCS1*, 2 and 3 transcripts in respective overexpression transgenic lines *Arabidopsis* compared to wildtype using specific primers designed to detect each *IPCS* isoform (A) *AtIPCS1* (B) *AtIPCS2* (C) *AtIPCS3*. Nomenclature of transgenic line is as follows: the first number identifies the *IPCS* isoform and the second number in brackets refers to the clonal line. Total RNA was extracted from ten-day-old seedlings grown on MS agar and cDNA synthesised from 2 μ g of total RNA. Transcript levels were analysed using SYBR green RT-PCR and are the average with standard error calculated for three technical replicates in each clonal line.

with lower over-expression levels of *AtIPCS2*. These data also showed that the over-expression is target specific, with the transcript levels of the other isoforms found to be relatively unaffected (Figure 4-9A-C).

4.3 Phenotypic analysis of transgenic lines

Phenotypic analyses of the transgenic lines revealed no obvious phenotype at 10 days, however one transgenic line At3 RNAi 4 had seedlings displaying chlorotic leaves (Figure 4-10). When these were transferred to soil there was no growth.

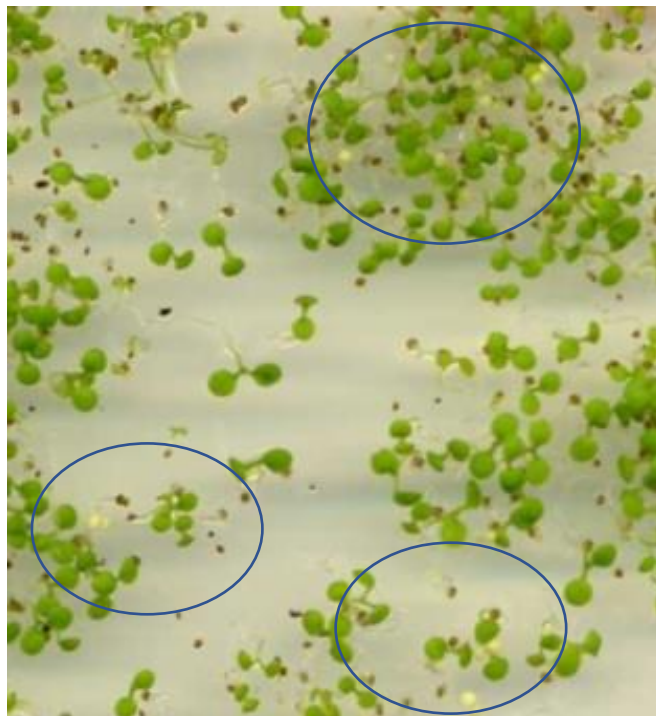


Figure 4-10: Phenotypic analysis of 13 days old at At3 RNAi 4 transgenic lines grown on MS agar; some seedlings have chlorotic leaves which are highlighted in blue circles.

Work carried out by Boyes et al. outlined the principle growth stages of Col0 from seed imbibition to senescence including the average period in days for each stage for plate-based and soil-based phenotypic analysis¹⁰. Phenotypic analysis of the transgenic lines compared to Col0 showed that RNAi transgenic lines had earlier inflorescence emergence

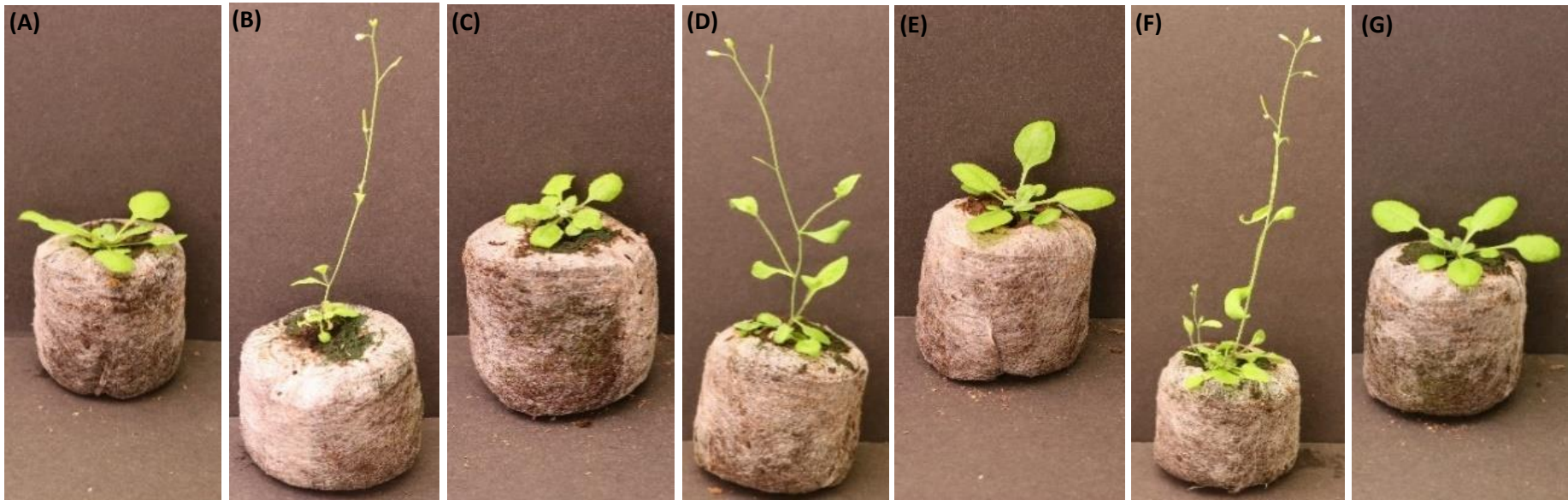


Figure 4-11: Phenotypic analyses of transgenic lines at 31 days (A) Col0 (B) At1 RNAi 7 (4) (C) At1 over exp 7 (D) At2 RNAi 1(3) (E) At2 over exp 9 (F) At3 RNAi (2) (G) At3 over exp 5.

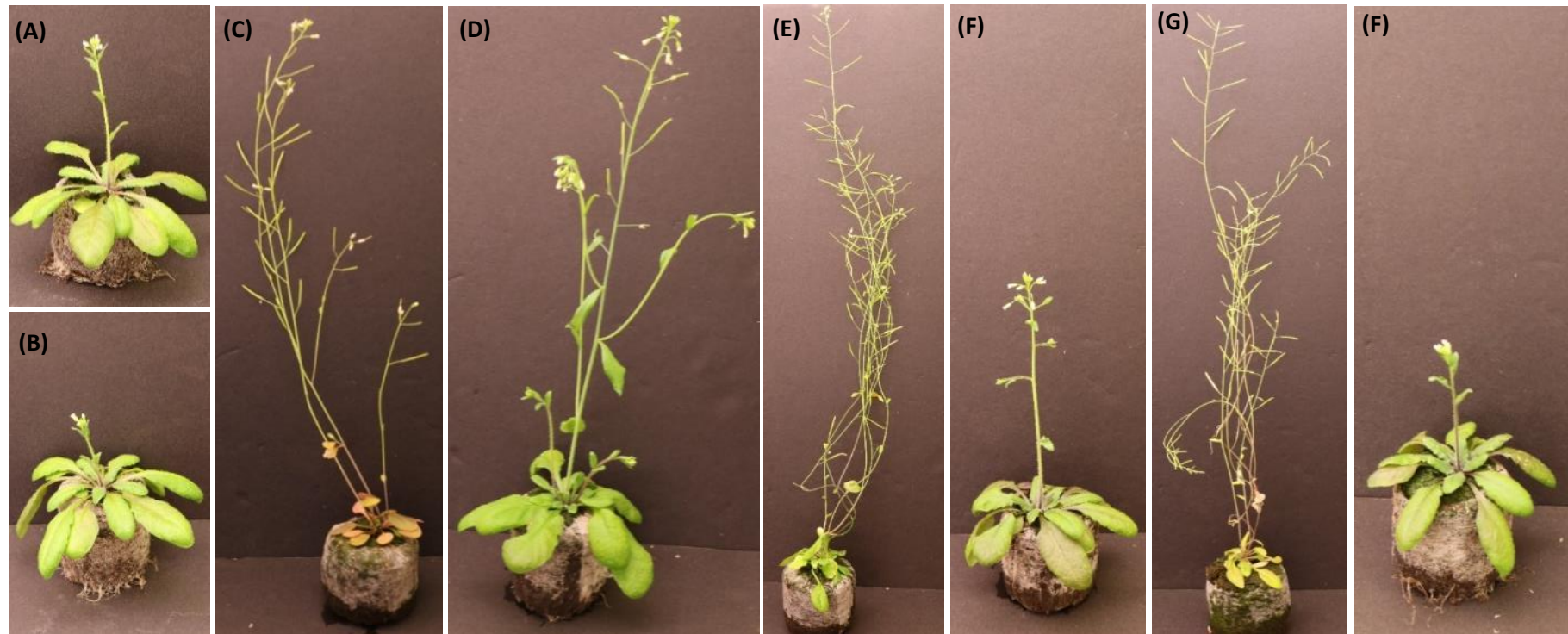


Figure 4-12: Phenotypic analyses of transgenic lines at 44 days (A and B) Col0 which had flowered with varying degrees in height (C) At1 RNAi 7 (4) (D) At1 over exp 7 (E) At2 RNAi 1(3) (F) At2 over exp 9 (G) At3 RNAi (2) (H) At3 over exp 5.

and flowering compared to over expressing transgenic lines and Col0. RNAi transgenic lines flowering was first captured at 31 days (Figure 4-11) whilst Col0 and the over-expressors at the same time point still showed no signs of flowering. IPCS overexpressing lines flowered (40 days) slightly earlier than Col0 (44 days) and were found to growing quicker (Figure 4-12).

This phenotype of early flowering has been shown to be a phenotype of plants grown in cold condition for extended periods of time and is triggered by a decrease in Flowering Locus C (FLC) transcript levels¹¹. This process termed vernalisation, is not an inheritable trait and requires low temperatures exposure in successive generations to promote flowering¹². In addition there is an FLC independent vernalisation pathway that promotes early flowering¹³, as does the autonomous pathway, which also controls flowering time in plants and requires the repression of FLC expression for early flowering¹⁴. The early flowering observed in RNAi transgenic lines are inheritable, implicating IPCS has a role to play in accelerating plant flowering time in *Arabidopsis*.

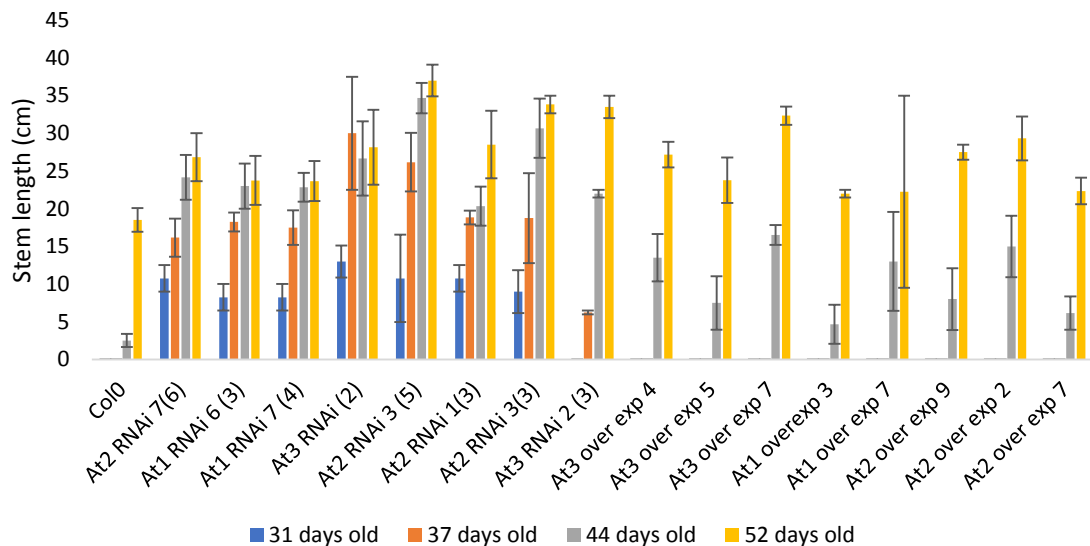


Figure 4-13: Tracking the growth of transgenic lines over 52 days; RNAi lines stem emergence (31 days) was earlier than overexpression transgenic lines and Col0 (44 days).

Another phenotype observed in the RNAi lines were much smaller rosette leaves and weak stems which needed support to stand upright, compared to Col0 and over-

expressing lines which had bigger rosette leaves and strong stems that were upright (Figure 4-12).

In addition, some RNAi transgenic lines showed early onset of senescence at 44 days in comparison to over expressing transgenic lines and Col0 (Figure 4-12). A comparison of plant growth showed that the RNAi lines exhibited initial acceleration in flowering relative to the overexpressing lines and Col0 (Figure 4-13). However, after late inflorescence emergence the overexpressing lines grew at a quicker rate and surpassed the RNAi lines in height.

4.4 Transcript quantification of *AtIPCS* homozygous lines from European seed bank

In an alternative approach, homozygous tDNA mutants of *AtIPCS1-3* were sought from the European seed bank, but only mutants for *AtIPCS2* (N696590) and *AtIPCS3* (N674600) were available. Transcript levels of *AtIPCS1* and *AtIPCS2* were quantified in the homozygous t-DNA mutant lines (Figure 4-14).

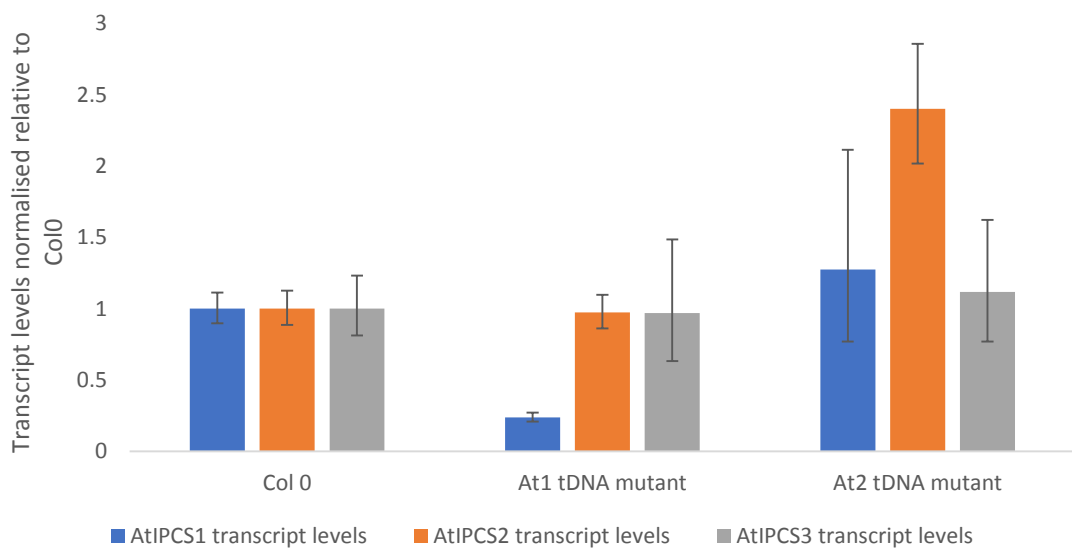


Figure 4-14: Transcript level of *AtIPCS1*, 2 and 3 in homozygous t-DNA mutants *AtIPCS1* and *AtIPCS2*.

The *AtIPCS1* t-DNA mutant showed a significant reduction in *AtIPCS1* transcripts, however, the *AtIPCS2* t-DNA mutant exhibited high levels of *AtIPCS2* mRNA. In *AtIPCS2* t-DNA mutants, the insertion was found to be located upstream of the *AtIPCS2* promoter, so disruption of expression would be far from certain. Indeed, studies of multiple t-DNA mutants have shown that insertion in the intron or exon regions are more effective than insertions occurring in the promoter or regions upstream of the promoter¹⁵. In comparison, the t-DNA insert for *AtIPCS1* was located in the intron sequence. Although this would have been spliced out, the significant reduction in *AtIPCS1* transcript levels suggested that transcripts were produced with decreased efficiency.

4.5 Conclusion

AtIPCS1-3 RNAi and over expressing transgenic lines were created and verified by qPCR. Homozygous lines were verified by progeny testing for RNAi lines. Phenotypic analyses of the transgenic lines yielded some interesting results, implicating IPCS in plant development particularly with regards to the robustness of the stem. In addition, IPCS was found to be implicated in vernalisation as indicated by the early flowering phenotype in RNAi *AtIPCS1-3* knock down transgenic lines, which is genetically stable and hereditary. The overexpressing lines also exhibited an early flowering phenotype compared to Col0 but was much later compared to the RNAi transgenic lines, indicating that reduction in *AtIPCS* transcripts has a more pronounced effect on the flowering time of plants. In generally the RNAi lines were unhealthy, with earlier onset of senescence and withering brown leaves when compared to over-expressers and Col0. These observations show that IPCS is necessary in plants development. For a deeper understanding of which biological pathways are affected by a change in IPCS expression, transcriptomic analyses were carried out, which will be discussed in the next chapter.

4.6 References

1. Earley, K. W.; Haag, J. R.; Pontes, O.; Opper, K.; Juehne, T.; Song, K.; Pikaard, C. S., Gateway- compatible vectors for plant functional genomics and proteomics. *The Plant Journal* **2006**, *45* (4), 616-629.
2. Hamilton, A. J.; Baulcombe, D. C., A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **1999**, *286* (5441), 950-952.
3. Hammond, S. M.; Bernstein, E.; Beach, D.; Hannon, G. J., An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *nature* **2000**, *404* (6775), 293.
4. Karimi, M.; Inzé, D.; Depicker, A., GATEWAY™ vectors for Agrobacterium-mediated plant transformation. *Trends in plant science* **2002**, *7* (5), 193-195.
5. Wang, W.; Yang, X.; Tangchaiburana, S.; Ndeh, R.; Markham, J. E.; Tsegaye, Y.; Dunn, T. M.; Wang, G.-L.; Bellizzi, M.; Parsons, J. F., An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*. *The Plant Cell* **2008**, *20* (11), 3163-3179.
6. Wee, E. G.-T.; Sherrier, D. J.; Prime, T. A.; Dupree, P., Targeting of active sialyltransferase to the plant Golgi apparatus. *The Plant Cell Online* **1998**, *10* (10), 1759-1768.
7. Livak, K. J.; Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ ΔΔCT method. *methods* **2001**, *25* (4), 402-408.
8. Zolman, B. K.; Monroe-Augustus, M.; Silva, I. D.; Bartel, B., Identification and functional characterization of *Arabidopsis* PEROXIN4 and the interacting protein PEROXIN22. *The Plant Cell* **2005**, *17* (12), 3422-3435.
9. Passricha, N.; Saifi, S.; Khatodia, S.; Tuteja, N., Assessing zygosity in progeny of transgenic plants: current methods and perspectives. *Journal of Biological Methods* **2016**, *3* (3), e46.
10. Boyes, D. C.; Zayed, A. M.; Ascenzi, R.; McCaskill, A. J.; Hoffman, N. E.; Davis, K. R.; Görlach, J., Growth stage-based phenotypic analysis of *Arabidopsis* a model for high throughput functional genomics in plants. *The Plant Cell* **2001**, *13* (7), 1499-1510.
11. Simpson, G. G.; Dean, C., *Arabidopsis*, the Rosetta stone of flowering time? *Science* **2002**, *296* (5566), 285-289.
12. Sheldon, C. C.; Rouse, D. T.; Finnegan, E. J.; Peacock, W. J.; Dennis, E. S., The molecular basis of vernalization: the central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences* **2000**, *97* (7), 3753-3758.
13. Michaels, S. D.; Amasino, R. M., Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. *The Plant Cell* **2001**, *13* (4), 935-941.
14. Simpson, G. G., The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of *Arabidopsis* flowering time. *Current opinion in plant biology* **2004**, *7* (5), 570-574.
15. Wang, Y. H., How effective is T-DNA insertional mutagenesis in *Arabidopsis*? *Journal of Biochemical Technology* **2008**, *1* (1), 11-20.

CHAPTER 5

RNASEQ ANALYSES OF TRANSENIC LINES

5.1 Chapter synopsis

In this Chapter, RNASeq was carried out on transgenic lines overexpressing each isoform of IPCS in *Arabidopsis thaliana*. Analyses of the changes in the transcriptome revealed the role of *AtIPCS* in plant defence response to biotic and abiotic stress. In addition, RNASeq analyses were also carried out on an *AtIPCS1* t-DNA insertion mutant to compare changes in the transcriptome with the over-expressor lines, thereby uncovering the effects of the dose dependent expression of *AtIPCS* and the global function of IPCS in plants.

5.2 Analyses of the transcriptome

Following the verification of the transgenic lines (Chapter 4) RNASeq was carried out on 10-day old over-expressor lines and 10 day old *AtIPCS1* t-DNA mutant line at principal growth stage 1.02; the RNAi lines were not utilized for RNASeq due to the limited time available. Principle component analysis (PCA) was carried out to quantify the variance between data sets (levels of gene transcription in each plant cell) in different samples (technical and biological replicates).

A PCA plot was created that captured the variance in the transcription patterns in each sample, accounting for 73% of the variance identified. From this plot, it was clear to see that all technical replicates of each biological sample, apart from one *AtIPCS2* over-expressor, At2-over-exp-2, clustered closely together, demonstrating that the transcriptomic data from the technical replicates, are similar, as expected (Figure 5-1). The variability in the variance between technical replicates for At2 over-exp-9 cannot be explained. However, a sample-to-sample heat map showing the similarities and dissimilarities between samples, reveals that all technical replicates of a given biological

sample, including At2-over-exp-9 cluster together (Figure 5-2). This showed that the technical replicates of At2-over-exp-9 are actually quite similar, despite the differences in variance revealed in the PCA plot.

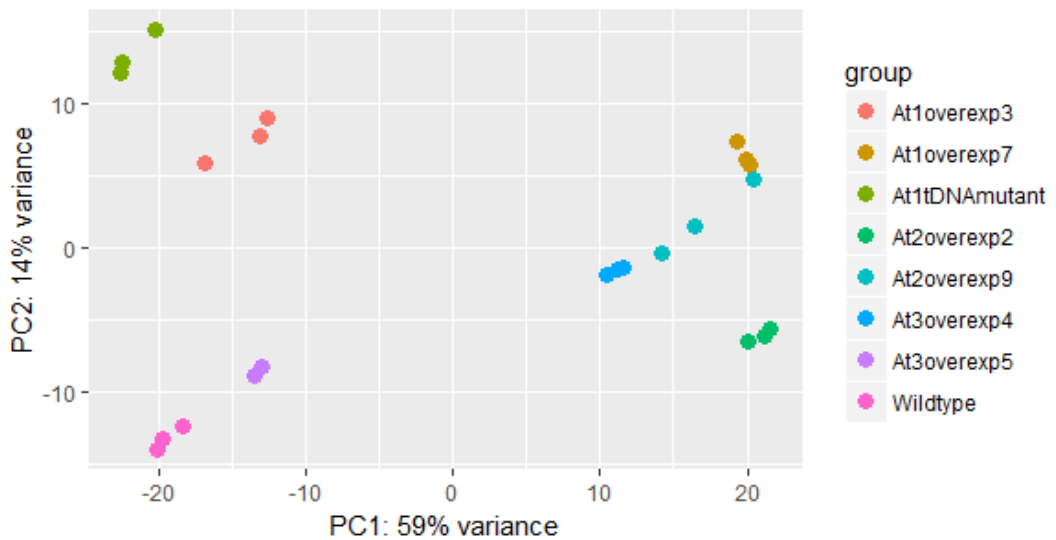


Figure 5-1: PCA plot of variance between transcriptome of technical and biological replicates for *AtIPCS1-3* over-expressor and *AtIPCS1* t-DNA mutant transgenic lines.

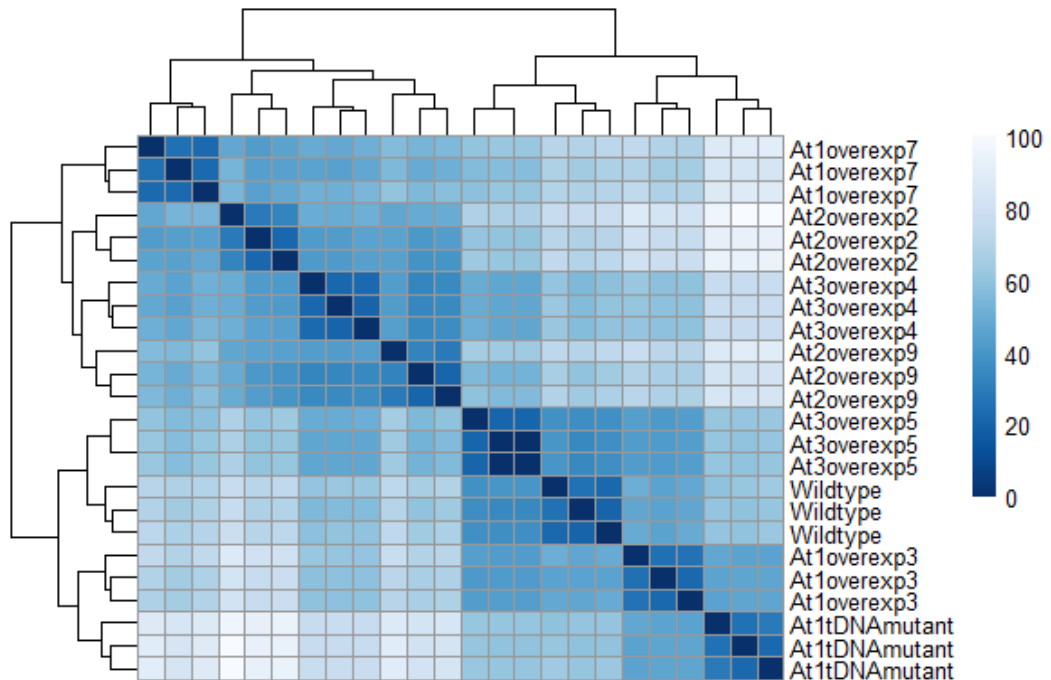


Figure 5-2: Heat map showing sample to sample distances for technical and biological replicates.

Functional characterisation of *OsIPCS1-3* has been carried out, and has revealed that the

expression of each IPCS isoform is modulated in response to abiotic stress (drought, cold and salt) to different degrees, with each isoform showing an organ specific expression pattern¹. *OsIPCS1* and *OsIPCS2* were found to be upregulated in response to cold and drought in the roots and stem, but specifically down-regulated in the leaves in response to cold. *OsIPCS3* expression was found to be upregulated in all tissues, in response to drought, cold and salt stress.

To date, there has been limited functional characterisation of the three IPCS isoforms in *A. thaliana*. Work done by Wang et al, has characterised *AtIPCS2* as a negative regulator of PCD² in plants, but little is known about *AtIPCS1* and *AtIPCS3*. These two homologues are likely redundant in function, and exhibit tissue-specific differential expression, with *AtIPCS2* found to be highly expressed in all tissues, whilst *AtIPCS1* expression is \log_2 fold ≥ 2 lower in expression in all tissues and *AtIPCS3* is highly expressed in flowers, and stems comparable to *AtIPCS2* expression levels³. *AtIPCS3* shows a \log_2 fold ≥ 3 lower expression levels than *AtIPCS2* in roots, rosette leaves, cauline leaves, and siliques and is lower in expression in comparison to *AtIPCS1* in all tissues apart from in siliques, flowers and stems³. The differential expression of the three isoforms, could be an indication of as yet unidentified, tissue specific regulatory roles and modulation of plant response to stimuli, based on the specific expression pattern of each isoform.

RNASeq of two biological replicates for each IPCS isoform over-expressor lines was carried out, with Col0 as control. Genes that were identified as upregulated with a \log_2 fold difference in expression in both biological replicates, compared to Col0, were carried forward for further analyses. It was found that 275 genes were upregulated in the *AtIPCS2* over-expressors, 70 in *AtIPCS1* and 19 genes in the *AtIPCS3* over-expressors; of these 15 were found to be upregulated in response to the overexpression of all three isoforms (Figure 5-3A). Regarding the down regulated genes, again *AtIPCS2* over expressors

transgenic lines had the highest number showing a \log_2 fold change ≥ 2 in expression, with 135 compared to 54 and 59 genes for *AtIPCS1* and *AtIPCS3* respectively (Figure 5-3B). Of these, 26 genes were downregulated in all three IPCS isoforms over-expressors.

The acquisition of high-throughput data from genomic and proteomic experiments, results in labour-intensive analyses to identify the involvement of a set of genes in a specific pathway. In order to achieve this, pathway analysis (also called enrichment analysis) is undertaken to identify the underlying biological function of these set of genes; this is referred to as the gene ontology⁴. The set of genes enriched under a specific GO are then compared to a set of genes associated with a specific biological pathway, and p-values generated by comparing the observed frequency of a GO term with the frequency expected by chance; GO annotation with a p-value ≤ 0.05 are considered to be enriched⁵. Singular enrichment analysis was carried out to categorise the genes identified, into the relevant biological pathways within which they function.

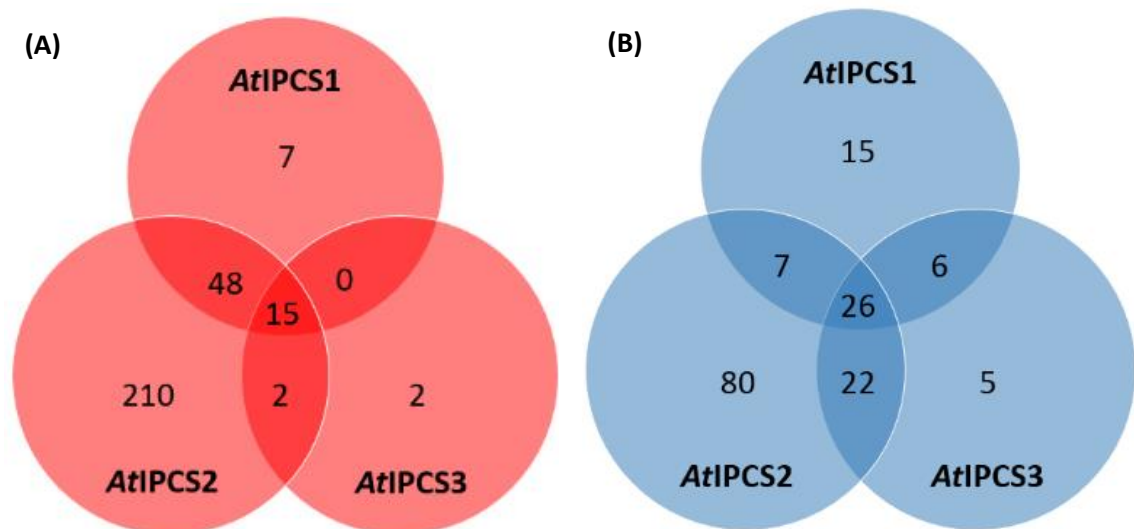


Figure 5-3: Venn diagrams showing the number of genes that were (A) upregulated (B) down regulated in the overexpression lines when compared to Col0.

5.3 Genes downregulated in response to the overexpression of *AtIPCS1*, 2 and 3

Analyses of genes that were downregulated and upregulated in response to the overexpression of *AtIPCS1*, 2 and 3 was carried out using agriGO (<http://bioinfo.cau.edu.cn/agriGO/analysis.php>). Genes downregulated in both *AtIPCS1* over expressors (At1-over-exp-3 and At1-over-exp-7) revealed a significant enrichment ($p = 1.87e-12$) for genes annotated with the GO term GO:0006950 response to stress, with 43.4% (23/53) of the genes associated with this term compared to 6.14% in the *Arabidopsis* transcriptome (Table 5-1). Other GO terms with significant enrichment include response to biotic stimulus (13/53) and defence response (13/53), with response to salicylic acid (7/53) and jasmonic acid stimulus (5/53) also showing enrichment. Of the 23 genes enriched under response to stress, 13 were associated with defence response and 9 were found to be downregulated by a \log_2 fold ≥ 2 in at least one biological replicate (highlighted in bold in Table 5-2).

Of particular interest are genes that showed a dose dependent decrease associated with higher transcript levels of *AtIPCS1* in the At1 over-exp-7 transgenic line; these genes included *plant defensin 1.2B*, the salicylic acid inducible pathogenesis-related genes, *PR1* and *PR2*, and an *ethylene/jasmonate responsive element plant defensin*, a well characterized component of the defence network against pathogens⁶ (Figure 5-2). *PR1* and *PR2*, and the *ethylene/jasmonate responsive element plant defensin*, showed a 2-fold decrease in transcript levels in At1 over-exp-7 transgenic lines, compared to At1 over-exp-3 which over expresses *AtIPCS1* at a lower level.

This implied that there is a link between the levels of *AtIPCS1* transcript and the expression of these genes. The roles of *PR* genes are well established in the plant response to biotic and abiotic stress; *PR2* and *PR5* expression level are elevated in response to plant

Table 5-1: GO enrichment of genes down regulated in response to the constitutive overexpression of *AtIPCS1*

| GO term | Description | Number in input list | Number in Background | p-value |
|----------------|--|-----------------------------|-----------------------------|----------------|
| GO:0050896 | Response to stimulus | 32 | 4057 | 3.50E-18 |
| GO:0006950 | Response to stress | 23 | 2320 | 1.50E-14 |
| GO:0051707 | Response to other organism | 13 | 599 | 2.10E-12 |
| GO:0009607 | Response to biotic stimulus | 13 | 638 | 4.60E-12 |
| GO:0006952 | Defence response | 13 | 766 | 4.30E-11 |
| GO:0051704 | Multi-organism process | 13 | 776 | 5.00E-11 |
| GO:0009814 | Defence response, incompatible interaction | 7 | 143 | 1.80E-09 |
| GO:0009751 | Response to salicylic acid stimulus | 7 | 200 | 1.70E-08 |
| GO:0045087 | Innate immune response | 7 | 347 | 6.30E-07 |
| GO:0009628 | Response to abiotic stimulus | 12 | 1471 | 7.60E-07 |
| GO:0006955 | Immune response | 7 | 367 | 9.10E-07 |
| GO:0002376 | Immune system process | 7 | 368 | 9.30E-07 |
| GO:0009753 | Response to jasmonic acid stimulus | 5 | 215 | 1.50E-05 |
| GO:0010033 | Response to organic substance | 10 | 1342 | 1.60E-05 |
| GO:0042221 | Response to chemical stimulus | 12 | 2085 | 2.60E-05 |
| GO:0009605 | Response to external stimulus | 5 | 429 | 0.00035 |
| GO:0009266 | Response to temperature stimulus | 5 | 485 | 0.00062 |
| GO:0009416 | Response to light stimulus | 5 | 596 | 0.0015 |
| GO:0009314 | Response to radiation | 5 | 613 | 0.0017 |
| GO:0009719 | Response to endogenous stimulus | 6 | 1068 | 0.0038 |
| GO:0005618 | Cell wall | 6 | 403 | 2.30E-05 |
| GO:0030312 | External encapsulating structure | 6 | 407 | 2.40E-05 |

Table 5-2: Genes down regulated by the constitutive overexpression of *AtIPCS1*, highlighted in red are genes responsive to JA signalling, in blue are genes responsive to SA signalling, in purple are genes responsive to SA and JA and in bold are genes with log₂ fold change ≥ 2

| Gene ID | Gene annotation | <i>At1-over-exp-3</i> | | <i>At1-over-exp-7</i> | |
|-----------|---|------------------------------|------------------|------------------------------|------------------|
| | | Log ₂ fold change | p-value | Log ₂ fold change | p-value |
| AT1G31580 | ECS1 (CARBOXYPEPTIDASE) | -1.6 | 1.60E-73 | -1.1 | 3.71E-37 |
| AT2G24850 | TAT3 (TYROSINE AMINOTRANSFERASE 3) | -1.8 | 6.29E-17 | -4.0 | 1.33E-53 |
| AT2G26020 | PLANT DEFENSIN 1.2B | -1.9 | 8.15E-18 | -5.0 | 1.44E-66 |
| AT5G52310 | LTI78 (LOW-TEMPERATURE-INDUCED 78) | -1.1 | 6.25E-52 | -1.5 | 5.40E-102 |
| AT2G14610 | PR1 (PATHOGENESIS-RELATED GENE 1) | -3.4 | 1.83E-172 | -7.3 | 4.08E-238 |
| AT1G75040 | PATHOGENESIS-RELATED GENE 5 | -1.4 | 3.27E-22 | -1.7 | 2.13E-33 |
| AT1G06160 | OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 | -1.0 | 1.14E-10 | -1.4 | 9.92E-17 |
| AT1G06040 | STO (SALT TOLERANCE) | -1.1 | 2.13E-28 | -1.5 | 1.78E-49 |
| AT2G14560 | LURP1 (LATE UPREGULATED IN RESPONSE TO HYALOPERON OSPORA PARASITICA) | -2.7 | 5.62E-112 | -3.4 | 1.13E-162 |
| AT5G10140 | FLC (FLOWERING LOCUS C) | -1.2 | 2.18E-09 | -1.5 | 4.16E-14 |
| AT1G19670 | CLH1 (CORONATINE-INDUCED PROTEIN 1) | -1.7 | 4.25E-75 | -1.8 | 9.20E-91 |
| AT4G14400 | ACD6 (ACCELERATED CELL DEATH 6) | -1.7 | 3.11E-73 | -1.5 | 5.03E-60 |
| AT5G57380 | VIN3 (VERNALIZATION INSENSITIVE 3) | -1.1 | 0.002783851 | -1.1 | 0.001246826 |
| AT5G37260 | RVE2 (REVEILLE 2) | -1.4 | 2.65E-46 | -1.2 | 1.18E-38 |
| AT3G57260 | PATHOGENESIS-RELATED PROTEIN 2 | -1.9 | 1.17E-30 | -5.3 | 4.64E-102 |
| AT3G56400 | WRKY70 | -1.0 | 3.44E-33 | -1.8 | 3.96E-89 |
| AT3G45860 | RECEPTOR LIKE PROTEIN-KINASE | -1.5 | 6.64E-09 | -2.9 | 3.18E-24 |
| AT5G44420 | ETHYLENE AND JASMONATE RESPONSIVE PLANT DEFENSIN | -2.7 | 1.49E-54 | -6.1 | 2.23E-122 |
| AT4G12490 | AZI3, LPID TRANSFER PROTEIN (LTP) | -1.3 | 7.93E-05 | -4.6 | 4.17E-43 |
| AT4G24350 | PHOSPHORYLASE FAMILY PROTEIN | -1.2 | 8.48E-38 | -1.2 | 1.08E-41 |
| AT3G25760 | AOC1 (ALLENE OXIDE CYCLASE 1) | -1.2 | 6.06E-40 | -1.7 | 4.85E-72 |
| AT2G40750 | WRKY54 | -1.3 | 4.41E-14 | -1.2 | 1.08E-13 |
| AT1G09080 | ATP binding BIP3 | -1.1 | 7.65E-06 | -2.5 | 6.79E-21 |

infection with the pathogen, *Erwinia amylovora*⁷. PR1 expression is elevated in response to SA⁸, ethylene and methyl jasmonate⁹, and all three PR genes are upregulated in response to drought¹⁰. The down regulation of these PR genes (*PR1*, *PR2* and *PR5*) in response to the constitutive overexpression of *AtIPCS1* implies that *AtIPCS1* is part of the plant defence signaling network.

Interestingly, between the two *AtIPCS1* over-expressors, a 3-fold difference in transcript levels of tyrosine amino transferase 3 (*TAT3*) was observed; this protein has been shown to increase in expression in response to wounding, and was hypothesized to have increased activity following pathogen attack in order to meet the high demand for prenylquinones that neutralize reactive oxygen species (ROS)¹¹. Another gene that showed a three-fold decrease in transcript levels was the systematic acquired resistance (SAR) specific protein, *azelaic acid induced 1* (*AZ11*) which interacts with DIR1 (Defective in Induced Resistance), forming a complex that contributes to the biosynthesis and accumulation of glycerol-3-phosphate (G3P), a molecule necessary for the spreading of SAR signaling to distal tissues in plants^{12, 13}.

The results from the transcriptomic analyses of genes downregulated in response to the overexpression of *AtIPCS1*, suggested that it is a negative regulator of the plant defence response to pathogens. This affects both the SA and JA signaling pathways, which counters the well-established JA/ET and SA antagonistic relationship found in most plant defence response against pathogens^{14, 15}. Studies have shown that SA signaling is usually instigated in response to biotrophic and hemibiotrophic pathogens, whilst JA signaling is activated by necrotrophic pathogens, with both signaling pathways acting as antagonists¹⁶. However, work by Yoashioka *et al.* showed that *crp22* (constitutive expresser of PR genes) mutants demonstrate a synergistic interplay between JA and SA signaling, with higher levels of SA, and constitutive expression of the *PR* genes and the

ethylene responsive defensin gene, *PDF1.2*¹⁷. The downregulation of genes involved in SA and JA plant defence signaling in response to the constitutive overexpression of *AtIPCS1* indicates that IPCS in plant defence against pathogens (necrotrophs, bio and hemibiotrophs) by utilizing both signaling networks.

The WRKY transcriptional factors also have well characterized roles in plant defence¹⁸; *WRKY 54* and *70* were shown to be downregulated in response to *AtIPCS1* overexpression (Table 5-2), and at low levels of expression are coupled to a decrease in SA biosynthesis after pathogen response¹⁹. In a separate study *wrky54/wrky70* double mutants have been shown to result in enhanced tolerance to osmotic stress²⁰ and to function as negative regulators of leaf senescence²¹, thereby showing their roles in plant response to biotic and abiotic stress. This affirms the role of *AtIPCS1* as a modulator of plant response to stress.

A decrease in gene expression of the Flowering Locus C (*FLC*) and vernalisation insensitive 3 (*VIN3*) genes, was also observed on *AtIPCS1* overexpression. This could explain, in part, the phenotypic differences (early flowering) observed between the over-expressors and Col0. The over-expressors exhibited a slightly earlier flowering (4 days) relative to Col0. When plants are exposed to cold, *VIN3* expression is induced and forms a heterodimer with vernalization 5 (*VRN5*) which is necessary for the repression of *FLC*, and results in early flowering²²; it therefore follows that a decrease in *VIN3* expression, should result in a decrease of the repression of *FLC* (up regulation of *FLC*) leading to either normal or delayed flowering. This is not what was observed from the transcriptomic analysis. Instead, a decrease in the transcript for *VIN3* and *FLC* was seen, which would suggest that there are other components which down regulate *FLC*, independent of *VIN3*. The down regulation of *FLC* combined with the slightly early flowering phenotype obser-

Table 5-3: GO enrichment of genes down regulated in response to the constitutive overexpression of *AtIPCS2*

| GO term | Description | Number in input list | Number in background | p-value |
|------------|-------------------------------------|----------------------|----------------------|----------|
| GO:0050896 | Response to stimulus | 72 | 4057 | 2.80E-34 |
| GO:0006950 | Response to stress | 46 | 2320 | 2.20E-22 |
| GO:0042221 | Response to chemical stimulus | 39 | 2085 | 5.60E-18 |
| GO:0010033 | Response to organic substance | 32 | 1342 | 1.20E-17 |
| GO:0051707 | Response to other organisms | 21 | 599 | 6.90E-15 |
| GO:0009607 | Response to biotic stimulus | 21 | 638 | 2.30E-14 |
| GO:0051704 | Multi-organism process | 22 | 776 | 9.60E-14 |
| GO:0006952 | Defence response | 21 | 766 | 6.90E-13 |
| GO:0009628 | Response to abiotic stimulus | 27 | 1471 | 2.60E-12 |
| GO:0009719 | Response to endogenous stimulus | 21 | 1068 | 2.80E-10 |
| GO:0009753 | Response to jasmonic acid stimulus | 11 | 215 | 5.70E-10 |
| GO:0009416 | Response to light stimulus | 15 | 596 | 5.10E-09 |
| GO:0009314 | Response to radiation | 15 | 613 | 7.40E-09 |
| GO:0048511 | Rhythmic process | 7 | 75 | 1.80E-08 |
| GO:0007623 | Circadian rhythm | 7 | 75 | 1.80E-08 |
| GO:0009751 | Response to salicylic acid stimulus | 9 | 200 | 6.40E-08 |
| GO:0009605 | Response to external stimulus | 12 | 429 | 6.20E-08 |
| GO:0009723 | Response to ethylene stimulus | 9 | 199 | 6.10E-08 |
| GO:0045087 | Innate immune response | 11 | 347 | 6.70E-08 |
| GO:0010200 | Response to chitin | 8 | 151 | 1.10E-07 |
| GO:0002376 | Immune system process | 11 | 368 | 1.20E-07 |
| GO:0006955 | Immune response | 11 | 367 | 1.20E-07 |
| GO:0009743 | Response to carbohydrate stimulus | 9 | 240 | 2.80E-07 |
| GO:0051716 | Cellular response to stimulus | 15 | 840 | 4.00E-07 |
| GO:0009725 | Response to hormone stimulus | 16 | 982 | 5.30E-07 |
| GO:0009611 | Response to wounding | 8 | 197 | 7.40E-07 |
| GO:0009617 | Response to bacterium | 8 | 247 | 3.80E-06 |
| GO:0010035 | Response to inorganic substance | 8 | 279 | 9.00E-06 |

Table 5-4: Genes down regulated by the constitutive overexpression of *AtIPCS2*, highlighted in red are genes responsive to JA signalling, in blue are genes responsive to SA signalling, in purple are genes responsive to SA and JA and in bold are genes with log₂ fold change ≥ 2

| Gene ID | Gene annotation | <i>At2-over-exp-2</i> | | <i>At2-over-exp-9</i> | |
|-----------|--|------------------------------|------------------|------------------------------|------------------|
| | | Log ₂ fold change | p-value | Log ₂ fold-change | p-value |
| AT5G47220 | ERF2 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 2) | -1.7 | 4.58E-25 | -1.1 | 7.33E-15 |
| AT2G24850 | TAT3 (TYROSINE AMINOTRANSFERASE 3) | -2.6 | 3.03E-28 | -2.3 | 4.60E-26 |
| AT1G16420 | MC8 (METACASPASE 8) | -1.6 | 3.84E-06 | -1.2 | 0.000470364 |
| AT4G12480 | PEARLI 1; LIPID BINDING A PUTATIVE LIPID TRANSFER PROTEIN | -1.9 | 1.94E-31 | -5.1 | 7.83E-172 |
| AT1G78290 | SERINE/THREONINE PROTEIN KINASE | -1.8 | 8.25E-47 | -2.0 | 1.39E-54 |
| AT4G37990 | ELI3-2 (ELICITOR-ACTIVATED GENE 3-2) | -2.3 | 7.52E-12 | -2.3 | 2.18E-12 |
| AT3G45290 | MLO3 (MILDEW RESISTANCE LOCUS O 3) | -1.1 | 5.16E-09 | -1.3 | 7.16E-12 |
| AT2G26020 | PDF1.2B (PLANT DEFENSIN 1.2B) | -4.9 | 1.47E-60 | -4.9 | 3.31E-62 |
| AT2G32680 | RLP23 (RECEPTOR LIKE PROTEIN 23) | -2.2 | 1.35E-13 | -2.3 | 3.16E-15 |
| AT2G26560 | PLA2A (PHOSPHOLIPASE A 2A) | -1.4 | 1.57E-26 | -2.6 | 1.57E-78 |
| AT2G14610 | PR1 (PATHOGENESIS-RELATED GENE 1) | -7.3 | 1.05E-206 | -7.9 | 4.60E-217 |
| AT2G46830 | CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) | -2.0 | 3.12E-12 | -1.1 | 1.04E-05 |
| AT1G06160 | ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59) | -2.5 | 9.89E-37 | -1.8 | 1.67E-24 |
| AT3G49620 | DIN11 (DARK INDUCIBLE 11) | -3.2 | 7.04E-106 | -4.1 | 5.37E-147 |
| AT1G06040 | STO (SALT TOLERANCE) | -1.3 | 2.23E-38 | -1.1 | 8.10E-27 |
| AT1G48000 | MYB112 (MYB DOMAIN PROTEIN 112) | -2.0 | 1.97E-10 | -1.2 | 5.54E-05 |
| AT2G14560 | LURP1 (LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA) | -4.1 | 5.67E-173 | -3.3 | 1.62E-149 |
| AT1G22770 | GI (GIGANTEA) | -2.4 | 6.55E-94 | -2.3 | 8.70E-91 |
| AT5G10140 | FLC (FLOWERING LOCUS C) | -1.4 | 5.23E-11 | -1.3 | 4.55E-10 |
| AT3G1550 | ANAC055 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 55) | -1.3 | 2.83E-20 | -1.5 | 4.16E-28 |
| AT1G76930 | EXT4 (EXTENSIN 4) | -1.2 | 1.21E-28 | -3.1 | 3.77E-175 |
| AT5G44420 | AN ETHYLENE- AND JASMONATE-RESPONSIVE PLANT DEFENSIN | -6.0 | 3.77E-111 | -6.1 | 3.17E-118 |
| AT2G23680 | STRESS-RESPONSIVE PROTEIN | -1.1 | 3.58E-14 | -1.3 | 1.09E-18 |
| AT1G61120 | TPS04 (TERPENE SYNTHASE 04) | -2.6 | 2.43E-15 | -1.7 | 5.55E-08 |
| AT1G75040 | PR5 (PATHOGENESIS-RELATED GENE 5) | -1.5 | 1.00E-25 | -1.8 | 5.50E-34 |
| AT1G52890 | ANAC019 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 19) | -1.2 | 2.90E-05 | -1.5 | 5.04E-07 |
| AT3G57260 | PR2 (PATHOGENESIS-RELATED GENE 2) | -4.8 | 2.99E-86 | -4.4 | 4.08E-87 |
| AT1G18330 | EPR1 (EARLY-PHYTOCHROME-RESPONSIVE1) | -1.6 | 3.85E-29 | -1.9 | 4.62E-40 |
| AT1G78410 | VQ MOTIF-CONTAINING PROTEIN | -1.8 | 9.42E-08 | -1.6 | 1.24E-06 |
| AT5G19880 | PEROXIDASE | -1.4 | 0.000104883 | -1.0 | 0.004183815 |

Table 5-4 continued: Genes down regulated by the constitutive overexpression of *AtIPCS2*, highlighted in red are genes responsive to JA signalling, in blue are genes responsive to SA signalling, in purple are genes responsive to SA and JA and in bold are genes with log₂ fold-change ≥ 2

| Gene ID | Gene annotation | <i>At2-over-exp-2</i> | | <i>At2-over-exp 9</i> | |
|-----------|--|------------------------------|------------------|------------------------------|------------------|
| | | Log ₂ fold-change | p-value | Log ₂ fold-change | p-value |
| AT2G18050 | HIS1-3 (HISTONE H1-3) | -2.5 | 2.90E-96 | -1.7 | 3.90E-55 |
| AT3G04720 | PR4 (PATHOGENESIS-RELATED 4) | -1.6 | 7.55E-69 | -1.5 | 1.20E-55 |
| AT2G43510 | TI1; SERINE-TYPE ENDOPEPTIDASE INHIBITOR | -2.5 | 6.34E-132 | -3.9 | 2.98E-227 |
| AT3G45860 | RECEPTOR-LIKE PROTEIN KINASE | -2.9 | 3.03E-22 | -3.0 | 8.20E-25 |
| AT3G51660 | MACROPHAGE MIGRATION INHIBITORY FACTOR FAMILY PROTEIN | -1.2 | 1.40E-16 | -1.1 | 6.63E-15 |
| AT1G71030 | MYBL2 (ARABIDOPSIS MYB-LIKE 2) | -1.2 | 1.04E-29 | -1.7 | 4.26E-55 |
| AT1G57630 | DISEASE RESISTANCE PROTEIN (TIR CLASS) | -1.5 | 8.34E-09 | -2.5 | 4.73E-18 |
| AT5G59780 | MYB59 (MYB DOMAIN PROTEIN 59) | -1.2 | 6.92E-29 | -1.7 | 9.77E-56 |
| AT4G12490 | LIPID TRANSFER PROTEIN (LTP) | -2.8 | 8.38E-18 | -4.7 | 1.76E-45 |
| AT4G23210 | PROTEIN KINASE FAMILY PROTEIN ENCODES A CYSTEINE-RICH RECEPTOR-LIKE KINASE (CRK13) | -1.7 | 8.20E-24 | -3.0 | 1.31E-52 |
| AT1G09080 | ATP BINDING BIP3 | -1.6 | 4.53E-10 | -1.6 | 6.04E-11 |
| AT4G02520 | GSTF2 (GLUTATHIONE S-TRANSFERASE PHI 2) | -1.2 | 2.82E-06 | -2.9 | 4.44E-28 |
| AT2G25000 | WRKY60 | -1.2 | 7.64E-11 | -1.4 | 9.79E-15 |
| AT2G02990 | RNS1 (RIBONUCLEASE 1) | -1.1 | 1.29E-08 | -1.7 | 1.82E-18 |

ved in the over-expressers is in line with the current understanding of early flowering in plants which requires reduced *FLC* transcripts²³.

Analyses of genes downregulated in *AtIPCS2* overexpressing lines, demonstrated a significant enrichment for GO terms, GO:0050896 response to stimulus (72/134), GO:0006950 response to stress (46/134). That response 34.3% of the genes associated with response to stress, which make up 6.1% of the *Arabidopsis* transcriptome (Table 5-3). Other GO terms showing significant enrichment include, GO: 0042221 (response to chemical stimulus), GO: 0010033 (response to organic substances), and GO: 0051707 (response to other organisms). Of the 46 genes enriched under the GO term response to stress, 17 genes were associated with defence response. Interestingly, genes that showed a dose dependent decrease associated with higher transcript levels of *AtIPCS1* (*plant defensin 1.2B*, *PR1*, *PR2*, and *ethylene/jasmonate responsive element plant defensin*) did not show the same dose dependent decrease in relation to higher *AtIPCS2* transcripts (Table 5-4) despite the 1/3 difference in *AtIPCS2* transcripts between At2 over-exp-2 and At2 over-exp-9 (higher levels of *AtIPCS2* transcripts in At2 over-exp-9). However, it is immediately clear that the plant defence system is more sensitive to expression levels of *AtIPCS2* than *AtIPCS1*; despite relatively low levels of *AtIPCS2* transcript increase (6-10 fold) compared to *AtIPCS1* transcripts (200-390 fold). The relatively low increase in *AtIPCS2* transcript level compared to *AtIPCS1* in the overexpression lines reflects *AtIPCS2* being the most expressed IPCS in plants compared to *AtIPCS1* and *AtIPCS3* in Col0. Genes identified to be down regulated in response to *AtIPCS2* overexpression, are comparable to the fold-change in expression of genes also observed to be down regulated in the transgenic line with the highest level of *AtIPCS1* transcripts (At1 over exp 7).

Analyses of the genes down regulated in *AtIPCS2* over-expressors associated with defence response are higher in numbers, with some identified that were not seen in the

AtIPCS1 over-expressors analyses. These include, *ERF2* which induces SAR via the JA signalling pathway resulting in resistance to *F. oxysporum*²⁴; *MC8*, which promotes programmed cell death in response to stress (hydrogen peroxide and UV light), and *MLO3* which confers resistance to powdery mildew in barley²⁵, and is probably in part, responsible for the reduced susceptibility to powdery mildew of *erh1/Col-gl* mutants². Also downregulated was *pEARL1*, which is a *cis*-jasmonate-inducible gene found to be upregulated in response to the enhancement of plant defence against herbivorous insects²⁶; *RLP23* which is down regulated in plant SAR response²⁷, *PLA2A* which shows elevated expression in response to *Botrytis cinerea* and *Pseudomonas syringae* with increased susceptibility to these pathogens observed in *Arabidopsis*²⁸, *EXT4* which is upregulated in response to hydrogen peroxide and the production of superoxide anion upon treatment with digitonin²⁹. *TPS04* which is responsible for the synthesis of the terpenoid, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and has been found to be produced as a by-product of activated JA signaling³⁰. *ANAC019* which is a regulator of JA signalling defence response³¹ and lines overexpressing this gene were found to have increased drought tolerance³², *GSTF2* which exhibits elevated transcript levels when *Arabidopsis* is inoculated with *P. syringae* and is inducible by SA and ethylene³³, and *CRK13* which also plays a role in plant resistance to *P. syringae* by the accumulation of SA³⁴. Also, among this list of down regulated genes identified in *AtIPCS2* over-expressors but not *AtIPCS1* over-expressors are *peroxidase*, *TIR* class of disease resistance proteins and *PR4*; which all play a role in plant defence response. From these analyses, it is clear that *AtIPCS2* expression affects a wider network of genes involved in plant defence responses compared to *AtIPCS1* which might be a result of its higher expression pattern in all tissues of *Arabidopsis*³.

A dose dependent decrease was found in the transcription of genes identified in response to the increase in *AtIPCS2* transcript levels in the transgenic lines. Interestingly 6 genes,

CCA1, *ORA59*, *LURP1*, *MYB112*, *TPS04* and *HIS1-3*, show a log₂ fold decrease in expression with an inverse relationship to *AtIPCS2* expression. The rationale as to why this has occurred is unclear. A higher number of genes are also identified for *AtIPCS2* over-expressors implicated in plant response to cold stress (*Gigantea*)³⁵, drought stress (*serine/threonine protein kinase* and *HIS1-3*)^{36,37}, salt and osmotic stress (*WRKY60*)³⁸.

It was found that genes involved in the regulation of circadian control of light responsive genes were down regulated. These included *CCA1* which encodes a transcription factor involved in the induction of the light-harvesting chlorophyll *a/b* gene associated with photosystem II³⁹, and *EPR1* which is inducible by irradiation with red light and regulated by phytochrome A and B⁴⁰. Overexpression of both genes is associated with delayed flowering, and in the case of *EPR1*, also results in cotyledon opening⁴⁰ whilst *CCA1* overexpression results in plants with longer hypocotyls and the disruption of the circadian clock which affects several genes³⁹. These genes are necessary for plant development, and in response to *AtIPCS2* overexpression are down regulated and probably contributed to the slightly earlier flowering (4 days) phenotype of these lines compared to Col0. In addition, the cold response gene *GI* was down regulated. The gene product regulates circadian rhythm and photoperiodic flowering by upregulating the expression of *CONSTANS* and *FLOWERING LOCUS T (FT)* which results in early flowering⁴¹.

Also, down regulated was *FLOWERING LOCUS C (FLC)* which is a MADS box protein and the regulator of flowering in the autonomous pathway⁴² and in response to vernalisation⁴³; down regulation of *FLC* promotes early flowering in both cases. Floral transition occurs not only through vernalisation and the autonomous pathway via the repression of *FLC*, but also through the photoperiodic pathway, GA pathway and the *FLC* independent vernalisation pathway⁴⁴ through a host of different intermediary genes and transcriptional regulators (Figure 5-4). A decrease in *FLC* transcript results in an early fl-

Table 5-5: GO enrichment of genes down regulated in response to the constitutive overexpression of *AtIPCS3*

| GO term | Description | Number in input list | Number in Background | p-value |
|----------------|----------------------------------|-----------------------------|-----------------------------|----------------|
| GO:0050896 | Response to stimulus | 33 | 4057 | 2.70E-17 |
| GO:0006950 | Response to stress | 24 | 2320 | 2.40E-14 |
| GO:0009628 | Response to abiotic stimulus | 15 | 1471 | 6.00E-09 |
| GO:0006952 | Defence response | 11 | 766 | 2.90E-08 |
| GO:0051707 | Response to other organism | 10 | 599 | 3.40E-08 |
| GO:0009607 | Response to biotic stimulus | 10 | 638 | 6.00E-08 |
| GO:0051704 | Multi-organism process | 10 | 776 | 3.60E-07 |
| GO:0009416 | Response to light stimulus | 8 | 596 | 4.40E-06 |
| GO:0009314 | Response to radiation | 8 | 613 | 5.40E-06 |
| GO:0009639 | Response to red or far red light | 5 | 210 | 2.20E-05 |
| GO:0042221 | Response to chemical stimulus | 12 | 2085 | 8.00E-05 |
| GO:0010035 | Response to inorganic substance | 5 | 279 | 8.30E-05 |
| GO:0009791 | Post-embryonic development | 7 | 705 | 0.00012 |
| GO:0009266 | Response to temperature stimulus | 6 | 485 | 0.00012 |
| GO:0009409 | Response to cold | 5 | 328 | 0.00017 |
| GO:0010033 | Response to organic substance | 8 | 1342 | 0.0011 |
| GO:0030528 | Transcription regulator activity | 12 | 2417 | 0.00032 |
| GO:0003700 | Transcription factor activity | 10 | 2173 | 0.0019 |

Table 5-6: Genes down regulated by the constitutive overexpression of *AtIPCS3*, highlighted in red are genes responsive to JA signalling, in blue are genes responsive to SA signalling, in purple are genes responsive to SA and JA and in bold are genes with log₂ fold-change ≥ 2

| Gene ID | Gene annotation | <i>At3 over-exp-4</i> | | <i>At3 over-exp-5</i> | |
|-----------|--|------------------------------|------------------|------------------------------|------------------|
| | | Log ₂ fold-change | p-value | Log ₂ fold-change | p-value |
| AT2G24850 | TAT3 (TYROSINE AMINOTRANSFERASE 3) | -1.8 | 6.10E-18 | -2.3 | 1.65E-24 |
| AT1G16420 | MC8 (METACASPASE 8) | -1.0 | 0.002428624 | -1.2 | 0.000589589 |
| AT1G78290 | SERINE/THREONINE PROTEIN KINASE | -1.0 | 2.73E-20 | -1.3 | 1.51E-29 |
| AT2G26020 | PDF1.2B (PLANT DEFENSIN 1.2B) | -2.6 | 3.65E-30 | -3.4 | 3.34E-40 |
| AT5G65080 | MAF5 (MADS AFFECTING FLOWERING 5) | -1.5 | 2.11E-07 | -1.7 | 5.09E-08 |
| AT2G14610 | PR1 (PATHOGENESIS-RELATED GENE 1) | -4.6 | 1.09E-252 | -4.9 | 2.85E-235 |
| AT1G06040 | STO (SALT TOLERANCE) | -1.0 | 6.04E-26 | -1.5 | 2.41E-48 |
| AT4G12480 | PEARLI 1; LIPID BINDING A PUTATIVE LIPID TRANSFER PROTEIN | -1.2 | 8.99E-13 | -2.0 | 3.20E-32 |
| AT1G48000 | MYB112 (MYB DOMAIN PROTEIN 112) | -1.2 | 6.45E-05 | -1.7 | 3.23E-08 |
| AT2G14560 | LURP1 (LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA) | -3.2 | 9.88E-155 | -2.2 | 5.56E-82 |
| AT1G61120 | TPS04 (TERPENE SYNTHASE 04) | -1.6 | 4.61E-07 | -1.8 | 6.72E-09 |
| AT5G10140 | FLC (FLOWERING LOCUS C) | -1.4 | 8.80E-12 | -1.1 | 3.25E-08 |
| AT1G19670 | CLH1 (CORONATINE-INDUCED PROTEIN 1) | -1.1 | 1.14E-34 | -1.2 | 2.36E-40 |
| AT1G22770 | GI (GIGANTEA) | -1.3 | 4.07E-33 | -2.4 | 1.27E-94 |
| AT3G57260 | PR2 (PATHOGENESIS-RELATED GENE 2) | -2.0 | 2.97E-36 | -2.2 | 8.40E-38 |
| AT1G78410 | VQ MOTIF-CONTAINING PROTEIN | -1.1 | 0.00072986 | -1.2 | 0.000163912 |
| AT2G43510 | TI1; SERINE-TYPE ENDOPEPTIDASE INHIBITOR | -1.1 | 2.90E-37 | -1.8 | 9.93E-77 |
| AT3G45860 | RECEPTOR-LIKE PROTEIN KINASE | -1.5 | 4.12E-09 | -1.7 | 4.81E-10 |
| AT5G44420 | PDF1.2A (PLANT DEFENSIN 1.2A) | -2.7 | 1.34E-55 | -3.6 | 7.78E-78 |
| AT4G37990 | ELI3-2 (ELICITOR-ACTIVATED GENE 3-2) | -1.8 | 3.22E-08 | -1.6 | 6.77E-07 |
| AT4G12490 | LIPID TRANSFER PROTEIN (LTP) | -1.9 | 1.32E-08 | -2.6 | 1.46E-15 |
| AT2G40750 | WRKY54 | -1.1 | 5.31E-11 | -1.2 | 1.02E-11 |
| AT4G02520 | GSTF2 (GLUTATHIONE S-TRANSFERASE PHI 2) | -1.4 | 3.33E-08 | -1.6 | 3.75E-10 |
| AT1G18330 | EPR1 (EARLY-PHYTOCHROME-RESPONSIVE1) | -1.3 | 1.98E-23 | -1.6 | 6.68E-30 |

is inherited, which is contrary to the current understanding of early flowering in plants. This is based on reduced *FLC* expression, with the levels of *FLC* returning to normal levels in subsequent generations and is reduced in plants undergoing vernalisation by exposure to cold temperatures⁴³.

Down regulation of *GI* would result in delayed flowering compared to Col10 which was not observed. The down regulation of these genes points to a link between IPCS expression and circadian control of light responsive genes which may be a result of MYB transcription factor binding sites, which were identified upstream of *AtIPCS1*, 2 and 3 promoter in chapter 2; this class of transcription factors are known to control plant development and plant response to abiotic and biotic stress⁴⁷.

Genes down regulated in response to the overexpression of *AtIPCS3* were found to be have a significant enrichment of GO term GO: 0050896 (response to stimulus) with 56% (33/59) of genes associated with this term compared to 10.7% in the *Arabidopsis* transcriptome. Other GO terms showing significant enrichment include GO:0006950 (response to stress), GO:0009628 (response to abiotic stimulus) and GO:0006952 (defence response) (Table 5-5). A dose dependent down regulation of genes identified in response to the overexpression of *AtIPCS3* was observed for *TAT3*, *PDF 1.2B*, *pEARL1*, *LURP1*, *GI*, *PDF1.2A* and *LPT*. Most of the genes identified as modulated in *AtIPCS3* over-expressors, had also been identified as responsive to *AtIPCS1* and *AtIPCS2* overexpression. It is clear, that there is a clustering of specific GO terms for genes down-regulated in *AtIPCS1*, 2 and 3 transgenic lines; these are mainly connected with the response to biotic and abiotic stress.

LURP1, which is upregulated in response to plant infection with oomycete *Hyaloperonospora parasitica* and required for basal defence against this fungal

pathogen⁴⁸, shows an inverse relationship in terms of expression, in response to *AtIPCS2* and *AtIPCS3* transcript levels (Table 5-4 and Table 5-6). This is different from the positive relationship found between *LURP1* down regulation and *AtIPCS1* expression (Table 5-2), the reasons for this differential relationship is unclear. Analyses of genes down regulated in response to the expression of all three *AtIPCS* isoforms was also carried out (Figure 5-7) and revealed 26 genes, of which 14 came under the GO term: 0050896 (response to stimulus), 7 genes under GO term: 0006950 (defence response) and 6 genes under GO term: 0009607 (response to biotic stress) with all genes identified under the GO term response to stress already identified under the GO term response to stimulus. Likewise, genes identified under GO term defence response were found to be also enriched under response to biotic stimulus.

Most of the genes enriched under response to stress for over-expressors of the three IPCS isoforms were expected as they had been identified in the analysis of genes enriched under GO term response to stimulus (Table 5-8). However, some genes were identified that were not grouped under response to stimulus but had been identified in the analyses of all three IPCS isoform over-expressors. These includes the glutamate receptor, *GLR2.7*, which encodes a functional channel involved in the allocation of Ca²⁺ ions to different calcium sinks for adaptation to K⁺ and Na⁺ stress and for normal development in plants⁴⁹, and *BDA1* which functions downstream of the SNC2 (Suppressor of NPR1, Constitutive2) receptor and has been shown to be a regulator of plant immunity. Loss of function of *BDA1* results in constitutive activated cell death and enhanced susceptibility to *P.syringae*⁵⁰. *HYH*, a positive regulator of blue light specific induction of gene expression and plant development (shown to inhibit hypocotyl growth)⁵¹, and *AIG1*, a GTP binding protein identified in *Arabidopsis* and found to confer resistance to plants infected with *Pseudomonas syringae* pv. *Maculicola* strain carrying the AvrRpt2 effector⁵².

Table 5-7: GO enrichment of genes down regulated in response to the constitutive overexpression of *AtIPCS1, 2* and *3*

| GO term | Description | Number in input list | Number in Background | p-value |
|----------------|-------------------------------|-----------------------------|-----------------------------|----------------|
| GO:0050896 | Response to stimulus | 14 | 4057 | 7.60E-08 |
| GO:0006952 | Defence response | 7 | 766 | 6.80E-07 |
| GO:0006950 | Response to stress | 10 | 2320 | 1.70E-06 |
| GO:0051707 | Response to other organism | 6 | 599 | 2.90E-06 |
| GO:0009607 | Response to biotic stimulus | 6 | 638 | 4.10E-06 |
| GO:0051704 | Multi-organism process | 6 | 776 | 1.20E-05 |
| GO:0009628 | Response to abiotic stimulus | 5 | 1471 | 0.003 |
| GO:0042221 | Response to chemical stimulus | 5 | 2085 | 0.013 |

Table 5-8: Genes down regulated by the constitutive overexpression of *AtIPCS1*, 2 and 3 respectively

| <i>Genes down regulated in all overexpressing IPCS transgenic lines and enriched under GO term :0050896 (Response to stimulus)</i> | |
|--|---|
| Gene ID | Gene annotation |
| AT2G29120 | GLR2.7 (GLUTAMATE RECEPTOR) |
| AT2G14610 | PR1 (PATHOGENESIS-RELATED GENE 1) |
| AT5G54610 | BDA1, ANKYRIN PROTEIN |
| AT2G24850 | TAT3 (TYROSINE AMINOTRANSFERASE 3); |
| AT3G45860 | RECEPTOR-LIKE PROTEIN KINASE |
| AT5G44420 | ETHYLENE- AND JASMONATE-RESPONSIVE PLANT DEFENSIN |
| AT3G57260 | PR2 (PATHOGENESIS-RELATED GENE 2) |
| AT2G26020 | PDF1.2B (PLANT DEFENSIN 1.2B) |
| AT1G06040 | STO (SALT TOLERANCE) |
| AT2G14560 | LURP1 (LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA) |
| AT5G10140 | FLC (FLOWERING LOCUS C) |
| AT3G17609 | HYH (HY5-HOMOLOG) |
| AT4G12490 | LIPID TRANSFER PROTEIN (LTP) |
| AT1G33960 | AIG1 (AVRRPT2-INDUCED GENE 1) |
| <i>Genes down regulated in all overexpressing IPCS transgenic lines and enriched under GO term: 0006950 (Defence response)</i> | |
| AT5G52310 | LTI78 (LOW-TEMPERATURE-INDUCED 78) |
| AT5G57380 | VIN3 (VERNALIZATION INSENSITIVE 3) |
| AT5G37260 | RVE2 (REVEILLE 2) |
| AT4G24350 | PHOSPHORYLASE FAMILY PROTEIN |
| AT3G56400 | WRKY70 |
| AT3G25760 | AOC1 (ALLENE OXIDE CYCLASE 1) |
| AT4G14400 | ACD6 (ACCELERATED CELL DEATH 6) |

The transcription factor *WKRY 70*, which has been shown to be capable of integrating the SA and JA signalling pathways in plant defence against pathogens⁵³, was also identified as downregulated in response to the overexpression of all IPCS isoforms, as was the SA inducible gene *ACD6* which is a positive regulator of plant defence against pathogens⁵⁴. From these analyses, the role of *AtIPCS1-3* in plant defence against pathogens has been established. The expression of *AtIPCS1*, 2 and 3 affects both the SA and JA signalling defence response, and so it is possible to conclude that the overexpression of *AtIPCS* isoforms, would make plants more susceptible to a wide range of pathogens.

5.5 Genes upregulated in response to the overexpression of *AtIPCS1*, 2 and 3

Genes that were upregulated in response to *AtIPCS1* overexpression were also analysed to determine other pathways affected. Significant enrichment ($p = 9.10E-27$) for genes annotated with the GO term GO:0015979 (photosynthesis), with 26.1% (18/69) of the genes associated with this term compared to 0.43 % in the *Arabidopsis* transcriptome (Table 5-9). Other GO terms with significant enrichment include lipid localization (8/69) and post-embryonic development (12/69). Of the 18 genes enriched under photosynthesis, 7 were found to be downregulated by a \log_2 fold-change ≥ 2 (highlighted in bold in Table 5-10). Genes upregulated and enriched under GO: 0010876 (lipid localization) and GO:0009791 (post-embryonic development) all show a dose dependent increase in expression relative to an increase in *AtIPCS1* transcripts in the transgenic lines.

NdhH a subunit of NDH (NADH dehydrogenase-like complex) which is responsible for facilitating the movement of electrons from the stroma to plastoquinone was found to be upregulated. It has been shown that plant mutants which are unable to fold the NADH

Table 5-9: GO enrichment of genes up-regulated in response to the constitutive overexpression of *AtIPCS1*

| GO term | Description | Number in input list | Number in Background | p-value |
|------------|--|----------------------|----------------------|----------|
| GO:0015979 | Photosynthesis | 18 | 162 | 9.10E-27 |
| GO:0019684 | Photosynthesis, light reaction | 12 | 103 | 2.60E-18 |
| GO:0006091 | Generation of precursor metabolites and energy | 15 | 285 | 8.40E-18 |
| GO:0010876 | Lipid localization | 8 | 24 | 8.20E-16 |
| GO:0009791 | Post-embryonic development | 12 | 705 | 6.40E-09 |
| GO:0044237 | Cellular metabolic process | 37 | 8722 | 4.10E-08 |
| GO:0008152 | Metabolic process | 41 | 10614 | 5.70E-08 |
| GO:0009987 | Cellular process | 42 | 11684 | 2.90E-07 |
| GO:0009737 | Response to abscisic acid stimulus | 8 | 378 | 5.30E-07 |
| GO:0009845 | Seed germination | 5 | 83 | 6.10E-07 |
| GO:0048316 | Seed development | 9 | 530 | 6.00E-07 |
| GO:0010154 | Fruit development | 9 | 557 | 9.00E-07 |
| GO:0033036 | Macromolecule localization | 8 | 462 | 2.30E-06 |
| GO:0048608 | Reproductive structure development | 10 | 978 | 1.20E-05 |
| GO:0003006 | Reproductive developmental process | 10 | 978 | 1.20E-05 |
| GO:0006869 | Lipid transport | 5 | 163 | 1.50E-05 |
| GO:0007275 | Multicellular organismal development | 14 | 2020 | 1.50E-05 |
| GO:0032501 | Multicellular organismal process | 14 | 2094 | 2.30E-05 |
| GO:0009409 | Response to cold | 6 | 328 | 3.40E-05 |
| GO:0055114 | Oxidation reduction | 5 | 203 | 4.00E-05 |
| GO:0022414 | Reproductive process | 10 | 1161 | 5.00E-05 |
| GO:0000003 | Reproduction | 10 | 1186 | 6.00E-05 |
| GO:0032502 | Developmental process | 14 | 2304 | 6.40E-05 |
| GO:0006412 | Translation | 11 | 1445 | 6.20E-05 |
| GO:0009725 | Response to hormone stimulus | 9 | 982 | 7.80E-05 |
| GO:0009719 | Response to endogenous stimulus | 9 | 1068 | 0.00015 |
| GO:0009266 | Response to temperature stimulus | 6 | 485 | 0.00028 |
| GO:0050896 | Response to stimulus | 18 | 4057 | 0.00028 |
| GO:0044085 | Cellular component biogenesis | 6 | 571 | 0.00065 |
| GO:0051179 | Localization | 11 | 1922 | 0.00071 |
| GO:0010033 | Response to organic substance | 9 | 1342 | 0.00076 |
| GO:0048856 | Anatomical structure development | 10 | 1726 | 0.0012 |
| GO:0042221 | Response to chemical stimulus | 10 | 2085 | 0.0045 |
| GO:0044249 | Cellular biosynthetic process | 17 | 4925 | 0.0065 |
| GO:0045735 | Nutrient reservoir activity | 10 | 63 | 1.20E-16 |
| GO:0005198 | Structural molecule activity | 11 | 659 | 3.60E-08 |
| GO:0003735 | Structural constituent of ribosome | 10 | 494 | 2.70E-08 |
| GO:0016651 | Oxidoreductase activity, acting on NADH or NADPH | 5 | 103 | 1.70E-06 |
| GO:0046906 | Tetrapyrrole binding | 5 | 136 | 6.20E-06 |
| GO:0008289 | Lipid binding | 5 | 323 | 0.00034 |
| GO:0008324 | Cation transmembrane transporter activity | 5 | 503 | 0.0024 |

Table 5-10: Genes upregulated by the constitutive overexpression of *AtIPCS1* under GO term: 0015979 (photosynthesis)

| Gene ID | Gene annotation | <i>At1-over-exp 3</i> | | <i>At1-over-exp 7</i> | |
|-----------|--|------------------------------|--------------------|------------------------------|-----------------|
| | | Log ₂ fold change | p-value | Log ₂ fold-change | p-value |
| ATCG00730 | A CHLOROPLAST GENE ENCODING SUBUNIT IV OF THE CYTOCHROME B6/F COMPLEX PETD | 1.3 | 2.93E-05 | 1.6 | 1.02E-06 |
| ATCG01110 | ENCODES THE 49KDA PLASTID NAD(P)H DEHYDROGENASE SUBUNIT H PROTEIN (NDhH) | 2.8 | 3.73E-19 | 4.0 | 4.72E-37 |
| ATCG01100 | NADH DEHYDROGENASE ND1 NDHA | 1.6 | 2.92E-06 | 2.6 | 2.62E-15 |
| ATCG00520 | ENCODES A PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY | 1.3 | 0.000136246 | 2.0 | 6.06E-09 |
| ATCG01090 | ENCODES SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX NDHI | 1.5 | 1.14E-05 | 2.5 | 1.60E-13 |
| ATCG00270 | PSII D2 PROTEIN PSBD | 1.8 | 5.79E-08 | 1.9 | 4.30E-09 |
| ATCG00680 | ENCODES FOR CP47, SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER | 1.0 | 0.000857587 | 1.2 | 0.00014644 |
| ATCG00710 | ENCODES A 8 KD PHOSPHOPROTEIN THAT IS A COMPONENT OF THE PHOTOSYSTEM II | 1.1 | 0.000866598 | 1.5 | 1.80E-06 |
| ATCG00360 | ENCODES A PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY | 1.5 | 6.99E-06 | 2.3 | 5.20E-12 |
| ATCG00300 | ENCODES PSBZ, WHICH IS A SUBUNIT OF PHOTOSYSTEM II. | 1.1 | 0.002252376 | 1.6 | 6.17E-06 |
| ATCG00440 | ENCODES NADH DEHYDROGENASE D3 SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX NDHC | 1.3 | 0.000167954 | 2.0 | 3.59E-09 |
| ATCG00720 | ENCODES THE CYTOCHROME B(6) SUBUNIT OF THE CYTOCHROME B6F COMPLEX | 1.1 | 0.000543643 | 1.4 | 5.73E-06 |
| ATCG00340 | ENCODES THE D1 SUBUNIT OF PHOTOSYSTEM I AND II REACTION CENTERS | 1.2 | 0.000120455 | 1.6 | 1.29E-06 |
| ATCG00580 | PSII CYTOCHROME B559 | 1.1 | 0.000365459 | 1.3 | 8.33E-05 |
| AT5G01600 | ENCODES A FERRETIN PROTEIN THAT IS TARGETED TO THE CHLOROPLAST | 1.1 | 1.19E-63 | 1.5 | 4.61E-116 |
| ATCG01060 | ENCODES THE PSAC SUBUNIT OF PHOTOSYSTEM I. | 1.3 | 5.19E-05 | 2.2 | 3.40E-12 |
| ATCG00540 | ENCODES CYTOCHROME F APOPROTEIN | 1.1 | 0.000430677 | 1.9 | 3.70E-09 |
| ATCG00280 | ENCODING A CP43 SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER | 1.5 | 2.31E-06 | 1.6 | 1.08E-06 |

subunit due to a defective chloroplast chaperonin subunit (Cpn60 β 4), cannot produce NADHh for the assembly of the NDH complex assembly with no phenotype reported as associated with the defect⁵⁵. From the list of genes up regulated, all are subunits of photosynthesis system I and II, which should contribute to an increase in the plant's ability to efficiently convert light energy to chemical energy for plant development.

For genes enriched under GO term lipid localization (Table 5-11), all apart for the seed storage proteins, were found to be expressed specifically in immature seeds but it is also thought that these genes may be expressed in vegetative tissues during plant development⁵⁶. Suppression of *olesin 1* resulted in large oil bodies (4–10 μm^2) compared to Col0 (1 μm^2) in embryo cells and a delay in germination^{57, 58}. Double mutants, *ole1 ole3* and *ole1 ole2*, were found to have even larger oil bodies (>10 μm^2) and had lower levels of olesin resulting in delays in germination⁵⁷. The mutant phenotype of large oil bodies in *ole1* single mutant, was reversed by complementation with a functional OLESIN gene, and the delay in germination found to be reversible by cold treatment or by growing the seeds in the presence of sucrose⁵⁸. In addition, upon undergoing freezing treatment, the delay in germination for the single mutants (*ole1-4*) was found to increase with an inverse relationship to the amount of olesin produced in the oil bodies. This indicated that olesin levels affect seed freezing tolerance⁵⁷. Storage proteins (2S albumin 1-4) were also upregulated in response to *AtIPCS1* overexpression, and are known to serve as nitrogen and sulphur reserves for plant germination^{59, 60}.

In addition there is a clear dose dependent relationship between the increase in *AtIPCS1* expression and the increasing upregulation of the genes which are involved in the nutrient reservoir and germination onset in seeds pathways. From these findings, it appears that *AtIPCS1* is linked to the seed development pathway, with overexpression of this isoform

resulting in an increase in expression of seed specific proteins which could, theoretically, lead to higher rates of successful germination.

Further analyses of upregulated genes show 12 are enriched under GO: 0009791 (post embryonic development) (Table 5-12), with *OLESINI-3* already identified as involved in seed germination. The other 9 genes upregulated include, *LEA* a protein known to accumulate during the final maturation stage of seeds termed desiccation, and in vegetative tissues in response to stress such as cold and drought⁶¹; *CRU3*, a seed storage protein that is phosphorylated in response to plant abscisic acid (ABA) treatment and found to play a role in protecting proteins needed for germination from oxidation⁶², *SI* the calcium binding oil body surface protein that facilitates the degradation of eicosenoic acid found in storage triacylglycerol in oil bodies and affects seedling growth post germination⁶³, and *EPR1* which is expressed specifically in the endosperm of germinating seeds but with an as yet uncharacterised role in plants, and with no phenotype detected in insertional mutants⁶⁴. In *Arabidopsis*, *EM6* which is expressed in all tissues in the embryo, has been shown to be necessary for normal seed development, with a T-DNA insertional mutant exhibiting a phenotype of premature seed dehydration at the distal end 13 days after flowering⁶⁵. *PER1*, a peroxidase proven to have antioxidant activity by protection of *E.coli* from cumen hydroperoxide, with overexpression shown to result in a delay in germination in seeds under-going osmotic stress⁶⁶, indicating that the upregulation of the protein serves to inhibit germination in response to stress.

FER1, a ferritin protein which accumulates in response to iron, has been shown to be involved in pathways that affect the aging process in *Arabidopsis*; a knock out mutant, exhibits a phenotype of early senescence in leaves, a reduction in chlorophyll concentration and reduced photochemical efficiency in PSII⁶⁷. Conversely upregulation

of this gene should result in high photochemical efficiency of PSII and delayed aging; this was not observed in any of the *AtIPCS* over-expressors. The upregulated gene *M10*, is specifically expressed in seeds and repressed 2 hours after imbibition; it accumulates in response to cold stress but is repressed in response to salt/drought stress and treatment with abscisic acid (ABA)⁶⁸.

Next, genes up regulated in response to *AtIPCS2* overexpression were analysed, and a significant enrichment found for GO term: 0015979 (photosynthesis) with 13.3% (30/226) of the genes associated with this term compared to 6.14% to 0.004% in *Arabidopsis* transcriptome (Table 5-13). Other GO terms significantly enriched include lipid localization (17/226) and post embryonic development (12/226). For GO term photosynthesis 30 genes are identified from 162 involved in this pathway in *Arabidopsis*, and for the GO term lipid localization 17 genes are identified as upregulated from 24 genes known to be involved in that process, which strongly indicated the there is a link between proteins involved in lipid localization and IPCS expression. Closer inspection of genes up regulated in response to *AtIPCS2* overexpression reveals that most of those involved in photosynthesis (Table 5-14) that were upregulated in response to *AtIPCS1* overexpression were also upregulated in response to *AtIPCS2* expression. However, there were some of these genes that were only upregulated in *AtIPCS2* over- expressing lines, these include: the F subunit of *NDH* (*NDHF*) which has been shown to affect the expression of H subunit (*NDHH*) in mutants exhibiting low transcript levels of *NDHF*, resulting in a decrease in NDH activity quantified by a measure of chlorophyll fluorescence and with no obvious phenotype⁶⁹, *PSB28* in *Synechocystis* 6803 has been shown to be involved in the biogenesis of chlorophyll binding proteins (CP47 and PsaA/PsaB) by facilitating the conversion of Mg protoporphyrin monomethyl ester to chlorophyll⁷⁰. Insertional mutants of *PSB28* have shown an accumulation of Mg protop-

Table 5-11: Genes upregulated by the constitutive overexpression of *AtIPCS1* under GO term:0010876 (lipid localization)

| Gene ID | Gene annotation | <i>At1 over-exp-3</i> | | <i>At1 over-exp-7</i> | |
|-----------|---------------------------|------------------------------|----------|------------------------------|-----------|
| | | Log ₂ fold change | p-value | Log ₂ fold-change | p-value |
| AT5G54740 | 2S SEED STORAGE PROTEIN 5 | 5.1 | 3.42E-97 | 8.2 | 3.86E-259 |
| AT4G25140 | OLEO1 (OLEOSIN 1) | 3.5 | 4.38E-45 | 7.1 | 2.82E-192 |
| AT4G27140 | 2S SEED STORAGE PROTEIN 1 | 4.2 | 5.35E-65 | 8.0 | 1.03E-238 |
| AT4G27150 | 2S SEED STORAGE PROTEIN 2 | 3.8 | 2.16E-40 | 7.3 | 2.16E-147 |
| AT4G27160 | 2S SEED STORAGE PROTEIN 3 | 5.0 | 1.84E-81 | 8.5 | 2.51E-239 |
| AT5G40420 | OLEO2 (OLEOSIN 2) | 1.4 | 1.45E-07 | 4.6 | 3.63E-70 |
| AT4G27170 | 2S SEED STORAGE PROTEIN 4 | 2.7 | 1.03E-19 | 5.7 | 5.84E-88 |
| AT3G27660 | OLEO4 (OLEOSIN 4) | 2.1 | 1.35E-15 | 5.2 | 7.26E-100 |

Table 5-12: Genes upregulated by the constitutive overexpression of *AtIPCS1* under GO term: 0009791 (post embryonic development)

| Gene ID | Gene annotation | <i>At1 over-exp-3</i> | | <i>At1 over-exp-7</i> | |
|-----------|---|------------------------------|-----------|------------------------------|-----------|
| | | Log ₂ fold change | p-value | Log ₂ fold change | p-value |
| AT3G22500 | LATE EMBRYOGENESIS ABUNDANT (LEA) PROTEIN ECP31 | 1.9 | 1.70E-09 | 4.7 | 8.46E-63 |
| AT3G27660 | OLEO4 (OLEOSIN 4) | 2.1 | 1.35E-15 | 5.2 | 7.26E-100 |
| AT4G28520 | CRU3 (CRUCIFERIN 3) | 5.8 | 9.91E-131 | 9.6 | 0 |
| AT4G25140 | OLEO1 (OLEOSIN 1) | 3.5 | 4.38E-45 | 7.1 | 2.82E-192 |
| AT4G26740 | S1 (SEED GENE 1) | 1.7 | 1.44E-08 | 5.1 | 8.42E-81 |
| AT2G27380 | EPR1 | 2.9 | 3.08E-31 | 6.0 | 6.18E-135 |
| AT2G40170 | EM6 (LATE EMBRYOGENESIS ABUNDANT 6) | 1.3 | 1.18E-06 | 3.8 | 1.09E-51 |
| AT1G48130 | 1-CYSTEINE PEROXIREDOXIN (AtPER1) | 1.4 | 1.87E-11 | 4.1 | 1.33E-98 |
| AT5G01600 | FER1; FERRITIN PROTEIN | 1.1 | 1.19E-63 | 1.5 | 4.61E-116 |
| AT5G40420 | OLEO2 (OLEOSIN 2) | 1.4 | 1.45E-07 | 4.6 | 3.63E-70 |
| AT1G03880 | CRU2 (CRUCIFERIN 2) | 2.4 | 1.77E-17 | 6.9 | 2.47E-149 |
| AT2G41280 | M10 ENCODES A HYDROPHILIC PROTEIN SIMILAR TO LATE EMBRYOGENESIS ACTIVATED (LEA) | 1.3 | 7.58E-05 | 3.8 | 1.77E-35 |

orphyrin IX monomethyl ester, lower levels of CP47 and PsaA/PsaB combined with slower photoautotrophic growth; there is currently no functional study of PSB28 that has been performed in *Arabidopsis*⁷⁰.

The calvin cycle enzyme, *SBPASE* activity in tobacco plants overexpressing the *Arabidopsis* gene was found to have a positive effect on rates of photosynthesis. This was associated with an up to 50% increase in the accumulation of the carbohydrates, sucrose and starch, and an increase in total leaf surface area and shoot biomass of up to 30%⁷¹. The two *PQL* isoforms 1 and 2 were also upregulated; work done by Yabuta et al. show that an insertional mutant did not display the usual increase in chlorophyll fluorescence upon exposure to actinic light which is associated with the activity of the NDH complex⁷². The minor light harvesting complex, *LHCA6*, was also upregulated. A *lhca6* mutant showed a reduction in NDH activity in immature and mature leaves (~60% compared to wildtype *Arabidopsis*) with no obvious phenotype⁷³. Again, the level of upregulation of genes identified in response to *AtIPCS2* overexpression was found to be about the same or in some cases exceeded the levels for the same genes in *AtIPCS1* over-expressors. This is despite the fact that the fold change in *AtIPCS1* expression was much than *AtIPCS2*.

Under the GO term lipid localization 9 /17 genes encode for lipid transfer proteins (LPT) (Table 5-15); LPTs like the yeast transfer lipid Sec14, facilitate the movement of phospholipids between membranes and has been shown to complement sec14 mutants⁷⁴. Some LPTs have been shown to have a role in plant defence, for example, insertional mutant *dir-1-1* (defective in induced resistance) exhibited increase susceptibility to *P. syringae*. DIR1 is hypothesised to function as a chaperon of lipid signals during SAR⁷⁵. The LTP, At5g05960, has been shown to be upregulated in the t-DNA mutant, *nhx1* (vacuolar Na⁺/H⁺ antiporter) in response to salt stress⁷⁶. The LPT orthologues TaLPT 1

Table 5-13: GO enrichment of genes upregulated in response to the constitutive overexpression of *AtIPCS2*

| GO term | Description | Number in input list | Number in Background | p-value |
|------------|---|----------------------|----------------------|----------|
| GO:0015979 | Photosynthesis | 30 | 162 | 1.70E-31 |
| GO:0010876 | Lipid localization | 17 | 24 | 1.80E-26 |
| GO:0006091 | Generation of precursor metabolites and energy | 26 | 285 | 2.50E-20 |
| GO:0008152 | Metabolic process | 143 | 10614 | 2.00E-18 |
| GO:0019684 | Photosynthesis, light reaction | 17 | 103 | 1.40E-17 |
| GO:0019748 | Secondary metabolic process | 28 | 489 | 7.80E-17 |
| GO:0044237 | Cellular metabolic process | 122 | 8722 | 4.00E-16 |
| GO:0009987 | Cellular process | 143 | 11684 | 1.30E-14 |
| GO:0050896 | Response to stimulus | 70 | 4057 | 1.30E-12 |
| GO:0015977 | Carbon fixation | 8 | 16 | 3.40E-12 |
| GO:0009409 | Response to cold | 19 | 328 | 7.00E-12 |
| GO:0009628 | Response to abiotic stimulus | 38 | 1471 | 9.20E-12 |
| GO:0009266 | Response to temperature stimulus | 22 | 485 | 1.40E-11 |
| GO:0044262 | Cellular carbohydrate metabolic process | 20 | 417 | 4.90E-11 |
| GO:0019760 | Glucosinolate metabolic process | 10 | 62 | 8.70E-11 |
| GO:0016143 | S-glycoside metabolic process | 10 | 62 | 8.70E-11 |
| GO:0019757 | Glycosinolate metabolic process | 10 | 62 | 8.70E-11 |
| GO:0006869 | Lipid transport | 13 | 163 | 4.10E-10 |
| GO:0016137 | Glycoside metabolic process | 11 | 104 | 6.00E-10 |
| GO:0016144 | S-glycoside biosynthetic process | 8 | 41 | 1.80E-09 |
| GO:0019761 | Glucosinolate biosynthetic process | 8 | 41 | 1.80E-09 |
| GO:0019758 | Glycosinolate biosynthetic process | 8 | 41 | 1.80E-09 |
| GO:0006950 | Response to stress | 43 | 2320 | 8.60E-09 |
| GO:0016138 | Glycoside biosynthetic process | 9 | 79 | 1.20E-08 |
| GO:0006790 | Sulfur metabolic process | 13 | 220 | 1.20E-08 |
| GO:0009719 | Response to endogenous stimulus | 27 | 1068 | 1.80E-08 |
| GO:0044272 | Sulfur compound biosynthetic process | 10 | 115 | 2.10E-08 |
| GO:0009737 | Response to abscisic acid stimulus | 16 | 378 | 2.30E-08 |
| GO:0009058 | Biosynthetic process | 70 | 5118 | 2.90E-08 |
| GO:0055114 | Oxidation reduction | 12 | 203 | 4.50E-08 |
| GO:0033036 | Macromolecule localization | 17 | 462 | 6.00E-08 |
| GO:0009767 | Photosynthetic electron transport chain | 7 | 46 | 8.90E-08 |
| GO:0005975 | Carbohydrate metabolic process | 23 | 866 | 9.30E-08 |
| GO:0042221 | Response to chemical stimulus | 38 | 2085 | 1.10E-07 |
| GO:0010033 | Response to organic substance | 29 | 1342 | 1.40E-07 |
| GO:0016051 | Carbohydrate biosynthetic process | 13 | 277 | 1.60E-07 |
| GO:0009664 | Plant-type cell wall organization | 8 | 79 | 1.90E-07 |
| GO:0009725 | Response to hormone stimulus | 24 | 982 | 2.10E-07 |
| GO:0022900 | Electron transport chain | 8 | 88 | 4.00E-07 |
| GO:0034637 | Cellular carbohydrate biosynthetic process | 10 | 177 | 9.00E-07 |
| GO:0034641 | Cellular nitrogen compound metabolic process | 16 | 506 | 1.00E-06 |
| GO:0009813 | Flavonoid biosynthetic process | 7 | 69 | 1.10E-06 |
| GO:0009699 | Phenylpropanoid biosynthetic process | 9 | 141 | 1.20E-06 |
| GO:0042398 | Cellular amino acid derivative biosynthetic process | 11 | 233 | 1.40E-06 |
| GO:0044249 | Cellular biosynthetic process | 63 | 4925 | 1.80E-06 |

Table 5-14: Genes upregulated by the constitutive overexpression of *AtIPCS2* under GO term: 0015979 (photosynthesis)

| Gene ID | Gene annotation | <i>At2 over-exp-2</i> | | <i>At2 over-exp-9</i> | |
|-----------|---|------------------------------|-----------|------------------------------|----------|
| | | Log ₂ fold change | p-value | Log ₂ fold-change | p-value |
| ATCG00520 | ENCODES A PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY | 2.4 | 3.56E-12 | 3.6 | 7.23E-26 |
| ATCG01090 | ENCODES SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX NDHI | 2.7 | 1.01E-15 | 4.0 | 4.89E-33 |
| ATCG00540 | ENCODES CYTOCHROME F APOPROTEIN | 2.2 | 1.72E-12 | 3.4 | 7.67E-27 |
| ATCG01010 | CHLOROPLAST ENCODED NADH DEHYDROGENASE UNIT. NDHF | 2.2 | 2.06E-10 | 3.4 | 7.33E-22 |
| ATCG00280 | CHLOROPLAST GENE ENCODING A CP43 SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER | 1.9 | 2.22E-09 | 3.0 | 1.05E-20 |
| ATCG00680 | ENCODES FOR CP47, SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER | 1.5 | 8.93E-07 | 2.5 | 2.18E-15 |
| AT4G28660 | PSB28 (PHOTOSYSTEM II REACTION CENTER PSB28 PROTEIN) | 1.6 | 6.17E-59 | 1.2 | 8.53E-37 |
| ATCG01100 | NADH DEHYDROGENASE ND1 NDHA | 2.9 | 1.82E-18 | 4.0 | 3.49E-33 |
| AT3G04790 | RIBOSE 5-PHOSPHATE ISOMERASE-RELATED | 1.8 | 1.32E-101 | 1.3 | 6.95E-58 |
| ATCG00270 | PSII D2 PROTEIN PSBD | 2.4 | 7.28E-13 | 3.4 | 1.13E-25 |
| ATCG01060 | ENCODES THE PSAC SUBUNIT OF PHOTOSYSTEM I | 2.6 | 8.05E-17 | 3.6 | 2.28E-30 |
| AT3G55800 | SBPASE (SEDOHEPTULOSE-BISPHOSPHATASE); | 1.2 | 1.64E-100 | 1.0 | 4.49E-67 |
| AT4G09650 | ATPD (ATP SYNTHASE DELTA-SUBUNIT GENE); | 1.5 | 7.39E-119 | 1.2 | 3.91E-76 |
| ATCG00730 | A CHLOROPLAST GENE ENCODING SUBUNIT IV OF THE CYTOCHROME B6 | 1.9 | 4.38E-09 | 2.8 | 6.43E-19 |
| ATCG01080 | NADH DEHYDROGENASE ND6 NDHG | 1.9 | 9.92E-08 | 3.1 | 5.52E-19 |
| ATCG00710 | ENCODES A 8 KD PHOSPHOPROTEIN | 1.7 | 5.64E-08 | 2.8 | 1.16E-17 |
| AT2G01590 | CRR3 (CHLORORESPIRATORY REDUCTION 3) | 1.2 | 1.06E-40 | 1.0 | 1.79E-31 |
| ATCG00580 | PSII CYTOCHROME B559 | 1.8 | 8.75E-09 | 2.4 | 2.18E-13 |
| ATCG00720 | ENCODES THE CYTOCHROME B(6) SUBUNIT OF THE CYTOCHROME B6F COMPLEX | 1.7 | 7.29E-08 | 2.9 | 4.62E-20 |
| AT3G01440 | PSB-LIKE PROTEIN 2 (PQL2) | 1.8 | 7.06E-107 | 1.5 | 2.74E-70 |
| AT1G14150 | PSB-LIKE PROTEIN 1 (PQL1) | 1.5 | 3.82E-75 | 1.2 | 1.54E-44 |
| ATCG01110 | ENCODES THE 49KDA PLASTID NAD(P)H DEHYDROGENASE SUBUNIT H PROTEIN | 4.2 | 1.35E-40 | 4.7 | 5.23E-52 |
| AT1G19150 | LHCA6; CHLOROPHYLL BINDING PSI TYPE II CHLOROPHYLL A/B-BINDING PROTEIN | 2.0 | 2.50E-114 | 1.6 | 5.98E-73 |
| AT1G60950 | FED A; 2 IRON, 2 SULFUR CLUSTER BINDING | 1.6 | 1.03E-63 | 1.5 | 1.68E-54 |
| ATCG00300 | ENCODES PSBZ, WHICH IS A SUBUNIT OF PHOTOSYSTEM II | 1.7 | 8.98E-07 | 3.2 | 1.04E-19 |
| ATCG00440 | ENCODES NADH DEHYDROGENASE D3 SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX NDHC | 2.7 | 6.65E-16 | 3.4 | 4.14E-24 |
| ATCG00340 | ENCODES THE D1 SUBUNIT OF PHOTOSYSTEM I AND II REACTION CENTERS. PSAB | 1.8 | 1.35E-08 | 2.9 | 4.71E-19 |
| ATCG00360 | ENCODES A PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY | 2.7 | 1.25E-15 | 3.8 | 1.03E-29 |
| AT3G16250 | NDF4 (NDH-DEPENDENT CYCLIC ELECTRON FLOW 1); | 1.4 | 1.02E-79 | 1.1 | 5.91E-52 |
| AT3G62410 | PROTEIN BINDING CP12-2 ENCODES A SMALL PEPTIDE FOUND IN THE CHLOROPLAST STROMA | 1.8 | 5.62E-79 | 1.4 | 5.57E-49 |

Table 5-15: Genes upregulated by the constitutive overexpression of *AtIPCS2* under GO term: 0010876 (lipid localization)

| Gene ID | Gene annotation | <i>At2 over-exp-2</i> | | <i>At2 over-exp-9</i> | |
|-----------|---|------------------------------|------------------|------------------------------|--------------------|
| | | Log ₂ fold change | p-value | Log ₂ fold-change | p-value |
| AT5G59310 | LTP4 (LIPID TRANSFER PROTEIN 4) | 3.7 | 2.07E-28 | 1.0 | 0.003587715 |
| AT5G48490 | DIR1 LIPID TRANSFER PROTEIN (LTP) | 1.2 | 4.21E-16 | 1.3 | 4.78E-18 |
| AT3G08770 | LTP6; LIPID BINDING PREDICTED TO ENCODE A PR (PATHOGENESIS-RELATED) PROTEIN | 2.0 | 3.96E-65 | 1.3 | 4.44E-27 |
| AT5G40420 | OLEO2 (OLEOSIN 2) | 2.5 | 9.95E-21 | 2.8 | 5.74E-25 |
| AT4G25140 | OLEO1 (OLEOSIN 1) | 4.6 | 2.85E-80 | 5.0 | 1.20E-91 |
| AT4G27140 | 2S SEED STORAGE PROTEIN 1 | 4.3 | 1.28E-68 | 6.3 | 1.95E-148 |
| AT4G27150 | 2S SEED STORAGE PROTEIN 2 | 4.7 | 1.09E-61 | 5.4 | 1.89E-81 |
| AT4G27160 | 2S SEED STORAGE PROTEIN 3 | 4.2 | 4.18E-58 | 6.3 | 1.28E-129 |
| AT5G64080 | LIPID TRANSFER PROTEIN (LTP) | 1.6 | 1.28E-30 | 1.2 | 3.85E-18 |
| AT5G05960 | LIPID TRANSFER PROTEIN (LTP) | 1.7 | 4.62E-51 | 1.2 | 6.31E-26 |
| AT3G18280 | LIPID TRANSFER PROTEIN (LTP) | 1.3 | 7.33E-28 | 1.1 | 3.46E-19 |
| AT5G54740 | LIPID TRANSFER PROTEIN (LTP) | 5.8 | 1.57E-127 | 6.3 | 5.98E-153 |
| AT5G55410 | LIPID TRANSFER PROTEIN (LTP) | 2.2 | 4.37E-12 | 2.0 | 1.50E-09 |
| AT4G27170 | 2S SEED STORAGE PROTEIN 4 | 1.8 | 6.48E-09 | 3.9 | 2.71E-39 |
| AT3G27660 | OLEO4 (OLEOSIN 4) | 2.8 | 6.81E-27 | 2.6 | 5.28E-23 |
| AT2G37870 | LIPID TRANSFER PROTEIN (LTP) | 2.9 | 2.88E-27 | 1.5 | 4.73E-08 |
| AT3G01570 | OLEO5 (OLEOSIN 5) | 1.5 | 8.26E-13 | 1.3 | 1.92E-08 |

Table 5-16: Genes upregulated by the constitutive overexpression of *AtIPCS2* under GO term: 0009791 (post embryonic development)

| Gene ID | Gene annotation | <i>At2-over-exp-2</i> | | <i>At2-over-exp-9</i> | |
|-----------|--|------------------------------|------------------|------------------------------|------------------|
| | | Log ₂ fold change | p-value | Log ₂ fold change | p-value |
| AT3G22500 | ATECP31 (LATE EMBRYOGENESIS PROTEIN ECP31) | 2.7 | 5.10E-20 | 2.2 | 6.09E-13 |
| AT4G24960 | ATHVA22D (HVA22-LIKE PROTEIN) | 1.0 | 2.41E-28 | 1.5 | 1.92E-57 |
| AT3G27660 | OLEO4 (OLEOSIN 4) | 2.8 | 6.81E-27 | 2.6 | 5.28E-23 |
| AT5G56030 | HSP81-2 (HEAT SHOCK PROTEIN 81-2) | 1.3 | 6.51E-80 | 1.0 | 3.92E-53 |
| AT4G28520 | CRU3 (CRUCIFERIN 3) | 6.0 | 5.56E-142 | 7.3 | 9.09E-207 |
| AT4G25140 | OLEO1 (OLEOSIN 1) | 4.6 | 2.85E-80 | 5.0 | 1.20E-91 |
| AT4G26740 | ATS1 (ARABIDOPSIS THALIANA SEED GENE 1) | 2.5 | 3.28E-18 | 2.9 | 4.39E-23 |
| AT2G27380 | ATEPR1 (EXTENSIN PROLINE-RICH 1) | 2.9 | 4.27E-33 | 5.1 | 6.03E-100 |
| AT2G40170 | GEA6 (LATE EMBRYOGENESIS ABUNDANT 6) | 2.5 | 3.49E-22 | 1.2 | 2.59E-05 |
| AT1G48130 | ATPER1 (THIOREDOXIN PEROXIDASE) | 2.0 | 7.06E-23 | 2.3 | 2.32E-29 |
| AT3G20210 | DELTA-VPE (CYSTEIN-TYPE ENDOPEPTIDASE) | 1.2 | 0.000532902 | 1.4 | 8.21E-05 |
| AT5G40420 | OLEO2 (OLEOSIN 2) | 2.5 | 9.95E-21 | 2.8 | 5.74E-25 |
| AT1G03880 | CRU2 (CRUCIFERIN 2) | 2.8 | 1.65E-22 | 4.4 | 2.05E-59 |
| AT3G58190 | LBD29 (LATERAL ORGAN BOUNDARIES-DOMAIN 29) | 1.5 | 2.69E-06 | 1.7 | 1.20E-07 |

and 2 were found to be upregulated in response to salt and drought stress in *Tritium aestivum*⁷⁷, showing that the plant LTPs expression are induced in response to biotic and abiotic stress. The function of most LTP are currently uncharacterized so it is not possible to hypothesize what the effects of the up regulation of the genes identified in response to *AtIPCS2* overexpression, will have on plant development. Genes upregulated under GO term post embryonic development with a \log_2 fold ≥ 2 in response to *AtIPCS2* transcript increase include *CRU2*, *CRU3*, *EPR1*, *S1* and *ECP31* (Figure 5-16).

For the *AtIPCS3* over-expressor there was a significant enrichment of upregulated genes associated with the GO term, GO: 0010876 (lipid localization) with 36.8% (7/19) of the genes associated with this term compared to 0.06% in the *Arabidopsis* transcriptome (Table 5-17). Other GO terms with significant enrichment include macromolecule localisation (7/19), lipid transport (5/7) and post embryonic development (5/19). Genes enriched under GO terms macromolecule localisation and lipid transport were also identified under the lipid localization GO term. Genes enriched under lipid localization and post-embryonic development that were upregulated in response to *AtIPCS3* overexpression, had already been identified in the responses to *AtIPCS1* and 2 overexpression. For the sake of completion the fold change in expression of these genes is shown in Table 5-18 and Table 5-19, with genes up-regulated by \log_2 fold-change ≥ 2 highlighted in bold. All genes identified showed a dose dependent increase in expression associated with higher transcript levels of *AtIPCS3* in At1 over-exp-7 compared to At1 over-exp-3.

Analyses of genes upregulated in response to increases in *AtIPCS1*, 2 and 3 expressions showed an expected significant enrichment for lipid localization as with 46.7% (7/15) of the genes associated with this term compared to 6.14% in the *Arabidopsis* transcriptome

(Table 5-20). These genes include *2S seed storage protein 1-4*, *OLESIN 1* and *OLESIN 4*. This showed that genes enriched under lipid localization, and found to be involved in the early stage plant development (embryonic), are conserved with respect to the response to *AtIPCS1*, 2 and 3 overexpression indicating that modulation of *AtIPCS* expression is likely to be vital for survival, with a decrease in the genes identified likely to lead to non-viable seeds.

Following the analysis of the overexpressing transgenic lines, the transcriptome of the At1 t-DNA mutant was also analysed to find out to what extent the decrease in *AtIPCS1* expression relative to Col0, would have on the pathways already identified as affected by *AtIPCS* expression.

5.6 Genes downregulated in response to *AtIPCS1* homozygous knock down

Genes down regulated in response to the decrease in *AtIPCS1* expression were found to be significantly enriched for genes annotated with the GO term GO: 0050896 (response to stimulus), with 40.3% (52/129) of the genes associated with this term compared to 6.14% in the *Arabidopsis* transcriptome (Table 5-21). Other GO terms with significant enrichment include secondary metabolic process (21/129) and response to stress (34/129). For comparison with At1 over-expressors, genes annotated under the GO term response to stress were analysed. Of the 34 genes, 17 were associated with defence response and 8 down regulated by a \log_2 fold change ≥ 2 (highlighted in bold in Table 5-22). Some genes that were downregulated in response to *AtIPCS1* overexpression were also found to be down regulated in response to knockdown of *AtIPCS1* expression. These included, *MC8*, *WRKY 54*, *PR5*, *WRKY 70*, *VQ containing motif protein 10*, and ATP binding *BIP3*.

Table 5-17: GO enrichment of genes upregulated in response to the constitutive overexpression of *AtIPCS3*

| GO term | Description | Number in input list | Number in Background | p-value |
|------------|--------------------------------------|----------------------|----------------------|----------|
| GO:0010876 | Lipid localization | 7 | 24 | 6.00E-18 |
| GO:0033036 | Macromolecule localization | 7 | 462 | 1.90E-09 |
| GO:0006869 | Lipid transport | 5 | 163 | 1.80E-08 |
| GO:0051179 | Localization | 7 | 1922 | 2.60E-05 |
| GO:0009791 | Post-embryonic development | 5 | 705 | 2.20E-05 |
| GO:0032502 | Developmental process | 7 | 2304 | 8.30E-05 |
| GO:0007275 | Multicellular organismal development | 6 | 2020 | 0.00035 |
| GO:0032501 | Multicellular organismal process | 6 | 2094 | 0.00042 |
| GO:0006810 | Transport | 5 | 1846 | 0.0018 |
| GO:0051234 | Establishment of localization | 5 | 1851 | 0.0019 |
| GO:0050896 | Response to stimulus | 7 | 4057 | 0.0026 |
| GO:0045735 | Nutrient reservoir activity | 9 | 63 | 1.80E-20 |
| GO:0008289 | Lipid binding | 5 | 323 | 5.00E-07 |

Table 5-18: Genes upregulated by the constitutive overexpression of *AtIPCS3* under GO term: 0010876 (lipid localization)

| Gene ID | Gene annotation | <i>At3 over-exp-4</i> | | <i>At3 over-exp-5</i> | |
|-----------|--|------------------------------|----------|------------------------------|-----------|
| | | Log ₂ fold change | p-value | Log ₂ fold-change | p-value |
| AT5G54740 | PROTEASE INHIBITOR/SEED STORAGE/LIPID TRANSFER PROTEIN (LTP) | 3.9 | 4.20E-57 | 7.7 | 9.99E-230 |
| AT4G25140 | ENCODES OLEOSIN1 | 2.5 | 1.01E-21 | 6.1 | 3.11E-141 |
| AT4G27140 | 2S SEED STORAGE PROTEIN 1 | 3.0 | 4.86E-32 | 7.2 | 6.10E-193 |
| AT4G27150 | 2S SEED STORAGE PROTEIN 2 | 2.3 | 8.24E-16 | 6.4 | 7.03E-115 |
| AT4G27160 | 2S SEED STORAGE PROTEIN 3 | 3.6 | 4.03E-43 | 7.3 | 2.59E-176 |
| AT4G27170 | 2S SEED STORAGE PROTEIN 4 | 1.8 | 6.80E-09 | 4.9 | 9.93E-64 |
| AT3G27660 | OLEO4 (OLEOSIN 4) | 1.1 | 8.65E-05 | 4.0 | 2.28E-58 |

Table 5-19: Genes upregulated by the constitutive overexpression of *AtIPCS3* under GO term: 0009791 (post embryonic development)

| Gene ID | Gene annotation | <i>At3 over-exp-4</i> | | <i>At3 over-exp-5</i> | |
|-----------|---------------------|------------------------------|----------|------------------------------|-----------|
| | | Log ₂ fold change | p-value | Log ₂ fold-change | p-value |
| AT4G28520 | CRU3 (CRUCIFERIN 3) | 4.2 | 6.51E-67 | 8.3 | 2.55E-271 |
| AT4G25140 | OLEO1 (OLEOSIN 1) | 2.5 | 1.01E-21 | 6.1 | 3.11E-141 |
| AT2G27380 | ATEPR1 | 1.5 | 4.85E-09 | 5.6 | 8.36E-120 |
| AT3G27660 | OLEO4 (OLEOSIN 4) | 1.1 | 8.65E-05 | 4.0 | 2.28E-58 |
| AT1G03880 | CRU2 (CRUCIFERIN 2) | 1.9 | 1.19E-10 | 5.5 | 6.22E-96 |

Table 5-20: GO enrichment of genes upregulated in response to the constitutive overexpression of *AtIPCS1, 2 and 3*

| GO term | Description | Number in input list | Number in Background | p-value |
|----------------|--------------------------------------|-----------------------------|-----------------------------|----------------|
| GO:0010876 | Lipid localization | 7 | 24 | 7.70E-19 |
| GO:0033036 | Macromolecule localization | 7 | 462 | 2.60E-10 |
| GO:0006869 | Lipid transport | 5 | 163 | 4.70E-09 |
| GO:0051179 | Localization | 7 | 1922 | 4.00E-06 |
| GO:0009791 | Post-embryonic development | 5 | 705 | 5.90E-06 |
| GO:0007275 | Multicellular organismal development | 6 | 2020 | 7.80E-05 |
| GO:0032501 | Multicellular organismal process | 6 | 2094 | 9.50E-05 |
| GO:0032502 | Developmental process | 6 | 2304 | 0.00016 |
| GO:0051234 | Establishment of localization | 5 | 1851 | 0.00056 |
| GO:0050896 | Response to stimulus | 7 | 4057 | 0.00049 |
| GO:0006810 | Transport | 5 | 1846 | 0.00056 |
| GO:0045735 | Nutrient reservoir activity | 9 | 63 | 9.80E-22 |
| GO:0008289 | Lipid binding | 5 | 323 | 1.30E-07 |

The down regulation of these genes seems to be a basal response to any alteration in the expression of *AtIPCS1* as the level of down regulation of these genes is never > 2 in the knock-down and the over-expressor lines. Other genes which had already been identified as down regulated in response to overexpression of either *AtIPCS2* or *AtIPCS3* included, *MC8*, *RNS1*, *TPS04* and *VQ motif containing protein 10*. Again these genes had a \log_2 fold-change > 2 (apart from *TPS04* down regulation in *At2* over exp 2 which had a \log_2 fold-change of 2.6) indicating a basal response to an alteration in *AtIPCS* expression. It is worth noting that none of the genes identified as enriched under the GO term response to stress were linked to cold, drought and salt stress. These had been previously identified as down regulated in response to *AtIPCS* overexpression.

NIMIN1, a defence related gene, found to be down regulated by a \log_2 fold-change of 3.2 in *At1* t-DNA mutant line, encodes a protein that interacts with NPR1, a regulator of SARs in plants. Treatment of plants with SAR inducing compounds, SA and 2,6-dichloroisonicotinic acid, resulted in transient expression of NIMIN, suggesting that is a sensitive and selective inducible target for specific SAR signals⁷⁸. It has also been suggested that the transient expression and accumulation of NIMIN1 indicates it is a regulator of late-SAR response by controlling the expression of PR1⁷⁹. The down regulation of this gene in response to the decreased *AtIPCS1* which was not identified in any of the *IPCS* over-expressors, indicated that the plant was even more sensitive to decreases *AtIPCS1* than it was to increased expression. Any further induction of SAR is effectively switched off, thereby further supporting role of *AtIPCS* as a negative regulator of plant defence response. Another gene down regulated by a \log_2 fold-change > 2 , and unidentified as responding to overexpression of *AtIPCS*, was *LOX2*. This gene has been shown to be necessary for JA accumulation in the plant response to wounding⁸⁰; its down

regulation indicated that the t-DNA mutant lacks the ability to induce the accumulation of JA and the associated stress response.

The camalexin biosynthesis enzyme, (*CYP71A13*) and flavin dependent monooxygenase (*FMO1*) were also down regulated in response to *AtIPCS1* knock-down. Previous studies have shown them both to be upregulated in plants infected with *Golovinomyces orontii*⁸¹. FMO1 is also a well characterized positive regulator of the programmed cell death effectors EDS1 (Enhanced disease susceptibility 1) and PADA4 (phytoalexin deficient 4) which regulate the accumulation of SA and are necessary for basal resistance to pathogens⁸². *PADA4* is also identified as down regulated (-1.1 fold) in this study, as are three TIR class of R (resistance) proteins which recognize a diverse set of pathogen effectors and are associated with the second line of plant defence against pathogens termed effector triggered immunity (ETI)⁸³. Also downregulated are MYB28 and MYB 29 encoding positive regulators of methionine-derived glucosinolates⁸⁴, which are upregulated in response to herbivore attack. Glucosinolates are broken down by thioglucosidases, releasing toxic compounds such as nitriles and isothiocyanates which are toxic to nematodes⁸⁵. In addition, *VSP2* a well characterized storage protein which has acid phosphatase activity, was also down regulated. *VSP2* expression has been shown to be enhanced in response to plant defence against the herbivory insect, *Sponoptera littoralis*⁸⁶.

The DIR genes which are responsible for the biosynthesis of lignin, have been shown in *Physcomitrella patens* to be induced upon inoculation with *B. cinerea* in order to reinforce the cell wall as a pathogen defence⁸⁷. The DIR proteins *DIR7* and *DIR 21*, which have been identified in this study as down regulated in response to *AtIPCS1* knock-down, have not been characterized in relation to plant response to stress in *Arabidopsis*. However, based on the expression of the DIR proteins identified in *P. patens*, it is probable that the

Table 5-21: GO enrichment of genes upregulated in response to the knock-down in expression of *AtIPCSI* in the t-DNA insertion mutant

| GO term | Description | Number in input list | Number in Background | p-value |
|------------|---|----------------------|----------------------|----------|
| GO:0050896 | Response to stimulus | 52 | 4057 | 4.10E-18 |
| GO:0019748 | Secondary metabolic process | 21 | 489 | 6.50E-17 |
| GO:0009987 | Cellular process | 82 | 11684 | 2.80E-14 |
| GO:0006950 | Response to stress | 34 | 2320 | 3.60E-13 |
| GO:0065007 | Biological regulation | 44 | 4188 | 4.20E-12 |
| GO:0009607 | Response to biotic stimulus | 17 | 638 | 1.10E-10 |
| GO:0050794 | Regulation of cellular process | 37 | 3375 | 1.20E-10 |
| GO:0016137 | Glycoside metabolic process | 9 | 104 | 2.00E-10 |
| GO:0009753 | Response to jasmonic acid stimulus | 11 | 215 | 3.80E-10 |
| GO:0051707 | Response to other organism | 16 | 599 | 3.70E-10 |
| GO:0016138 | Glycoside biosynthetic process | 8 | 79 | 6.80E-10 |
| GO:0050789 | Regulation of biological process | 37 | 3697 | 1.50E-09 |
| GO:0006952 | Defence response | 17 | 766 | 1.60E-09 |
| GO:0051704 | Multi-organism process | 17 | 776 | 1.90E-09 |
| GO:0044249 | Cellular biosynthetic process | 42 | 4925 | 9.50E-09 |
| GO:0009058 | Biosynthetic process | 43 | 5118 | 9.10E-09 |
| GO:0034637 | Cellular carbohydrate biosynthetic process | 9 | 177 | 1.70E-08 |
| GO:0042221 | Response to chemical stimulus | 25 | 2085 | 4.30E-08 |
| GO:0009751 | Response to salicylic acid stimulus | 9 | 200 | 4.60E-08 |
| GO:0016051 | Carbohydrate biosynthetic process | 10 | 277 | 5.90E-08 |
| GO:0019760 | Glucosinolate metabolic process | 6 | 62 | 1.30E-07 |
| GO:0016143 | S-glycoside metabolic process | 6 | 62 | 1.30E-07 |
| GO:0019757 | Glycosinolate metabolic process | 6 | 62 | 1.30E-07 |
| GO:0042398 | Cellular amino acid derivative biosynthetic process | 9 | 233 | 1.60E-07 |
| GO:0031347 | Regulation of defence response | 6 | 66 | 1.80E-07 |
| GO:0006575 | Cellular amino acid derivative metabolic process | 10 | 315 | 1.90E-07 |
| GO:0019438 | Aromatic compound biosynthetic process | 9 | 237 | 1.80E-07 |
| GO:0044272 | Sulfur compound biosynthetic process | 7 | 115 | 2.20E-07 |
| GO:0009698 | Phenylpropanoid metabolic process | 8 | 175 | 2.30E-07 |
| GO:0044262 | Cellular carbohydrate metabolic process | 11 | 417 | 2.70E-07 |
| GO:0051726 | Regulation of cell cycle | 7 | 127 | 4.10E-07 |
| GO:0009828 | Plant-type cell wall loosening | 5 | 40 | 4.60E-07 |
| GO:0016144 | S-glycoside biosynthetic process | 5 | 41 | 5.20E-07 |
| GO:0019761 | Glucosinolate biosynthetic process | 5 | 41 | 5.20E-07 |
| GO:0019758 | Glycosinolate biosynthetic process | 5 | 41 | 5.20E-07 |
| GO:0042440 | Pigment metabolic process | 7 | 134 | 5.80E-07 |
| GO:0080134 | Regulation of response to stress | 6 | 83 | 6.40E-07 |
| GO:0009699 | Phenylpropanoid biosynthetic process | 7 | 141 | 8.10E-07 |
| GO:0010033 | Response to organic substance | 18 | 1342 | 8.90E-07 |

Table 5-22: Genes upregulated by the decrease in *AtIPCS1* expression under GO term: 0006950 (response to stress)

| Gene ID | Gene annotation | <i>At1 tDNA mutant</i> | |
|-----------|--|------------------------------|--------------------|
| | | Log ₂ fold-change | p-value |
| AT1G17600 | DISEASE RESISTANCE PROTEIN (TIR-NBS-LRR CLASS) | -1.2 | 4.95E-06 |
| AT1G16420 | MC8 (METACASPASE 8) | -1.6 | 3.10E-06 |
| AT2G33380 | RD20 (RESPONSIVE TO DESSICATION 20) | -1.2 | 1.77E-05 |
| AT3G13650 | DIR7 | -1.1 | 1.69E-06 |
| AT2G30770 | CYP71A13 | -2.1 | 3.61E-10 |
| AT2G40750 | WRKY54 | -2.2 | 8.75E-31 |
| AT3G52430 | PAD4 (PHYTOALEXIN DEFICIENT 4) | -1.1 | 6.69E-16 |
| AT1G65870 | DIR21 | -1.4 | 9.42E-05 |
| AT1G75040 | PR5 (PATHOGENESIS-RELATED GENE 5) | -2.4 | 1.80E-47 |
| AT1G72920 | DISEASE RESISTANCE PROTEIN (TIR-NBS CLASS), | -1.1 | 1.30E-05 |
| AT2G02990 | RNS1 (RIBONUCLEASE 1) | -1.1 | 2.41E-09 |
| AT1G61120 | TPS04 (TERPENE SYNTHASE 04) | -2.9 | 3.77E-19 |
| AT1G19670 | ATCLH1 (ARABIDOPSIS THALIANA CORONATINE-INDUCED PROTEIN 1) | -1.7 | 1.14E-78 |
| AT2G41240 | BHLH100 (BASIC HELIX-LOOP-HELIX PROTEIN 100) | -3.3 | 1.22E-44 |
| AT5G07690 | ATMYB29 (ARABIDOPSIS THALIANA MYB DOMAIN PROTEIN 29) | -2.1 | 1.56E-13 |
| AT1G47890 | ATRLP7 (RECEPTOR LIKE PROTEIN 7) | -1.1 | 2.42E-05 |
| AT2G35380 | PEROXIDASE 20 (PER20) | -1.2 | 3.51E-18 |
| AT5G24770 | VSP2 (VEGETATIVE STORAGE PROTEIN 2) | -1.3 | 2.86E-13 |
| AT1G65060 | 4-COUMARATE:COA LIGASE (4CL) | -1.1 | 3.64E-06 |
| AT3G45140 | LOX2 (LIPOXYGENASE 2) | -1.7 | 5.15E-51 |
| AT4G17090 | CT-BMY (CHLOROPLAST BETA-AMYLASE) | -2.6 | 1.23E-243 |
| AT1G78410 | VQ MOTIF-CONTAINING PROTEIN 10 | -1.3 | 4.87E-05 |
| AT3G56400 | WRKY70 | -1.6 | 6.27E-68 |
| AT1G02450 | NIMIN1 (NIM1-INTERACTING 1) | -3.2 | 1.019475205 |
| AT1G18710 | ATMYB47 (MYB DOMAIN PROTEIN 47) | -1.7 | 1.07E-19 |
| AT1G19250 | FMO1 (FLAVIN-DEPENDENT MONOOXYGENASE 1) | -1.6 | 3.19E-10 |
| AT2G41480 | PEROXIDASE 25 | -1.7 | 1.39E-20 |
| AT3G22840 | ELIP1 (EARLY LIGHT-INDUCIBLE PROTEIN) | -1.0 | 0.002586795 |
| AT1G57630 | DISEASE RESISTANCE PROTEIN (TIR CLASS) | -1.1 | 1.44E-05 |
| AT1G68740 | PHO1 | -1.2 | 2.25E-09 |
| AT1G55210 | DISEASE RESISTANCE RESPONSE | -1.8 | 3.05E-45 |
| AT5G61420 | MYB28 (MYB DOMAIN PROTEIN 28) | -2.1 | 2.52E-64 |

DIR proteins are involved in the plant defence response to pathogens in *Arabidopsis* and *AtIPCS* functions as a negative regulator. These data showed that diverse components of the plant defence machinery, regulating the response to pathogens, are down regulated in response to changes in *AtIPCS* expression.

In relation to abiotic stress, two genes were notably down regulated in response to *AtIPCS1* knock-down: *CT-BMY* and *BHLH10*. *CT-BMY* encodes a beta amylase found in the chloroplast and induced in response to cold stress. In *Arabidopsis*, knock-down of *CT-BMY* was found to lead to reduced photosynthetic efficiency, suggesting that its accumulation might serve to increase plant freezing tolerance by the breakdown of glucans resulting in the accumulation of maltose and other oligosaccharides which act as protective solutes in response to stress⁸⁸. These sugars act as ‘compatible solutes’ for the continued function of macromolecules such as proteins and enzymes when plants are exposed to freezing temperatures by aiding water retention in cells⁸⁹, thereby reducing the risk of cell lysis when the osmotic flow favours the movement of water molecules into the cell. *BHLH100* was downregulated by log₂ fold-change of 3.3 in the insertion mutant; overexpression of the basic-helix-helix-loop has been shown to increase plant tolerance to high zinc (Zn) and nickel (Ni)⁹⁰. This suggested that *AtIPCS1* expression levels may affect plant metal homeostasis by acting as a negative regulator of response to metal stress.

5.7 Genes upregulated in response to a decrease in *AtIPCS1* expression

Analyses of genes upregulated in response to the decrease in *AtIPCS1* expression found them to be significantly enriched under the GO terms photosynthesis and lipid localisation (Table 5-23, 5-24 and 5-25). Further examination of genes upregulated under the GO term

Table 5-23: GO enrichment of genes upregulated in response to the knock down in expression of *AtIPCSI* tDNA insert mutant

| GO term | Description | Number in input list | Number in Background | p-value |
|------------|---|----------------------|----------------------|----------|
| GO:0050896 | Response to stimulus | 95 | 4057 | 1.60E-36 |
| GO:0010033 | Response to organic substance | 59 | 1342 | 3.60E-35 |
| GO:0042221 | Response to chemical stimulus | 70 | 2085 | 5.40E-35 |
| GO:0009719 | Response to endogenous stimulus | 47 | 1068 | 7.90E-28 |
| GO:0009725 | Response to hormone stimulus | 44 | 982 | 2.50E-26 |
| GO:0015979 | Photosynthesis | 20 | 162 | 2.90E-20 |
| GO:0009628 | Response to abiotic stimulus | 38 | 1471 | 4.70E-15 |
| GO:0006950 | Response to stress | 45 | 2320 | 2.30E-13 |
| GO:0019684 | Photosynthesis, light reaction | 12 | 103 | 2.20E-12 |
| GO:0070887 | Cellular response to chemical stimulus | 20 | 452 | 2.70E-12 |
| GO:0010876 | Lipid localization | 8 | 24 | 7.20E-12 |
| GO:0009743 | Response to carbohydrate stimulus | 15 | 240 | 1.60E-11 |
| GO:0009873 | Ethylene mediated signaling pathway | 10 | 74 | 4.20E-11 |
| GO:0009266 | Response to temperature stimulus | 19 | 485 | 7.10E-11 |
| GO:0009755 | Hormone-mediated signaling pathway | 16 | 321 | 8.30E-11 |
| GO:0032870 | Cellular response to hormone stimulus | 16 | 321 | 8.30E-11 |
| GO:0006091 | Generation of precursor metabolites and energy | 15 | 285 | 1.60E-10 |
| GO:0000160 | Two-component signal transduction system (phosphor-relay) | 10 | 92 | 2.90E-10 |
| GO:0009987 | Cellular process | 107 | 11684 | 1.40E-09 |
| GO:0010200 | Response to chitin | 11 | 151 | 2.00E-09 |
| GO:0009733 | Response to auxin stimulus | 15 | 360 | 3.40E-09 |
| GO:0009642 | Response to light intensity | 9 | 90 | 4.60E-09 |
| GO:0044237 | Cellular metabolic process | 86 | 8722 | 7.40E-09 |
| GO:0007242 | Intracellular signaling cascade | 19 | 659 | 9.30E-09 |
| GO:0051716 | Cellular response to stimulus | 21 | 840 | 1.70E-08 |
| GO:0009723 | Response to ethylene stimulus | 11 | 199 | 2.90E-08 |
| GO:0009605 | Response to external stimulus | 15 | 429 | 3.20E-08 |
| GO:0009416 | Response to light stimulus | 17 | 596 | 6.70E-08 |
| GO:0009646 | Response to absence of light | 5 | 16 | 9.30E-08 |
| GO:0009314 | Response to radiation | 17 | 613 | 9.90E-08 |
| GO:0032774 | RNA biosynthetic process | 22 | 1040 | 1.30E-07 |
| GO:0006351 | Transcription, DNA-dependent | 22 | 1039 | 1.30E-07 |
| GO:0009611 | Response to wounding | 10 | 197 | 2.70E-07 |
| GO:0009968 | Negative regulation of signal transduction | 6 | 43 | 3.10E-07 |
| GO:0009737 | Response to abscisic acid stimulus | 13 | 378 | 3.30E-07 |
| GO:0009409 | Response to cold | 12 | 328 | 5.00E-07 |

Table 5-24: Genes upregulated by the decrease in expression of *AtIPCS1* under GO term: 0015979 (photosynthesis)

| Gene ID | Gene annotation | Log ₂ fold-change | <i>At1 tDNA mutant</i> p-value |
|-----------|--|------------------------------|-----------------------------------|
| ATCG00730 | SUBUNIT IV OF THE CYTOCHROME B6/F COMPLEX PETD | 1.4 | 1.23E-05 |
| AT1G29920 | CAB2 (CHLOROPHYLL A/B-BINDING PROTEIN 2) | 1.2 | 3.18E-12 |
| ATCG00580 | PSII CYTOCHROME B559 | 1.3 | 8.64E-05 |
| ATCG01100 | NADH DEHYDROGENASE ND1 NDHA | 1.2 | 0.000267999 |
| ATCG00520 | PHOTOSYSTEM I ASSEMBLY PROTEIN YCF4 | 1.1 | 0.001040999 |
| ATCG01090 | SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX NDHI | 1.1 | 0.001370433 |
| ATCG00270 | PSII D2 PROTEIN PSBD; | 1.5 | 2.44E-06 |
| ATCG00680 | CP47, SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER | 1.2 | |
| ATCG00710 | PHOTOSYSTEM II REACTION CENTER PROTEIN H | 1.2 | 0.000212549 |
| ATCG00360 | PHOTOSYSTEM I ASSEMBLY PROTEIN YCF3 | 1.1 | 0.001002877 |
| ATCG00300 | PSBZ, WHICH IS A SUBUNIT OF PHOTOSYSTEM II | 1.1 | 0.002217157 |
| ATCG00440 | NAD(P)H-QUINONE OXIDOREDUCTASE SUBUNIT 3 | 1.0 | 0.002233798 |
| ATCG00540 | CYTOCHROME F APOPROTEIN | 1.1 | 0.000424669 |
| ATCG00340 | ENCODES THE D1 SUBUNIT OF PHOTOSYSTEM I AND II REACTION CENTERS. PSAB | 1.4 | 1.44E-05 |
| ATCG01110 | NAD(P)H-QUINONE OXIDOREDUCTASE SUBUNIT H | 1.9 | 6.32E-10 |
| AT5G01600 | ATFER1 | 1.1 | 1.09E-67 |
| ATCG01060 | PSAC SUBUNIT OF PHOTOSYSTEM I | 1.4 | 1.56E-05 |
| ATCG00720 | CYTOCHROME B(6) SUBUNIT OF THE CYTOCHROME B6F COMPLEX | 1.2 | 9.76E-05 |
| ATCG00280 | CP43 SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER | 1.4 | 8.36E-06 |
| AT1G27730 | STZ (SALT TOLERANCE ZINC FINGER) | 1.2 | 4.52E-17 |

Table 5-25: Genes upregulated by the decrease in expression of *AtIPCS1* under GO term: 0010876 (lipid localization)

At1 tDNA mutant

| Gene ID | Gene annotation | Log ₂ fold-change | p-value |
|-----------|-----------------------------------|------------------------------|-----------------|
| AT5G40420 | OLEO2 (OLEOSIN 2) | 1.3 | 3.97E-06 |
| AT4G25140 | OLEO1 (OLEOSIN 1) | 2.1 | 4.96E-15 |
| AT4G27150 | 2S SEED STORAGE PROTEIN 2 | 1.6 | 1.52E-07 |
| AT3G01570 | OLEO5 (OLEOSIN 5) | 1.3 | 4.17E-09 |
| AT5G54740 | 2S SEED STORAGE PROTEIN 5 | 3.6 | 6.33E-48 |
| AT4G24230 | ACBP3 (ACYL-COA-BINDING DOMAIN 3) | 1.3 | 6.87E-101 |
| AT3G27660 | OLEO4 (OLEOSIN 4) | 1.3 | 5.59E-06 |
| AT4G27160 | 2S SEED STORAGE PROTEIN 3 | 1.1 | 0.000254456 |

photosynthesis found the same genes observed as up-regulated in response to the overexpression of *AtIPCS1*, 2 and 3. Only one gene, *STZ*, was identified that had not been found in the overexpressing transgenic lines. *STZ* has a function in plant response to abiotic stress (Table 5-24) and have been shown to enhance tolerance to heat, salinity and osmotic stress in transgenic over-expressors and RNAi knock-downs⁹¹. Therefore, the downregulation of *STZ* in response to *AtIPCS1* indicates that *AtIPCS* affects the plant response to abiotic stress in a way that is advantageous for plant survival.

Genes up regulated under the GO enrichment term, lipid localization were found to encode the lipid transfer proteins and lipid storage proteins already identified as upregulated in response to *AtIPCS* overexpression. The only genes upregulated in response to overexpression of *AtIPCS1*, 2 and 3, but not upregulated on *AtIPCS1* knock-down were, *2S seed storage protein 1* and *4*. These data indicated that *AtIPCS* acts as a positive and negative regulator of lipid content in plants.

5.8 Conclusion

In this Chapter, the role of *AtIPCS* as a negative regulator of plant defence response against pathogens was indicated. The overexpression of each isoform resulted in the down regulation of genes involved in both JA and SA plant defence signalling, and these responded in a dose dependent manner to changes in the expression of each isoform. The PR genes are of particular interest because they are well characterized plant defence signals. In this study, it was found that overexpression of *AtIPCS1* and *AtIPCS3* down regulated these genes in a dose dependent manner. *AtIPCS2* did not show the same response; what was observed instead, was a heightened response resulting in an increase in the down regulation of these genes compared to over-expressors of *AtIPCS1* and *AtIPCS3*. The down regulation of these genes was also found to have reached a peak; the

same level of down regulation was observed for both *At2* over-expressor transgenic lines, despite the differences in *AtIPCS2* transcript levels.

In addition, the overexpression of *AtIPCS2* levels affected more genes in the defence response pathway than the other isoforms. From these data, it was clear that *AtIPCS2* is one of the principle regulators of plant defence response against pathogens, and that the other two isoforms have similar roles but perhaps as rheostats. The knock-down in *AtIPCS1* expression was also found to affect the plant defence pathway, resulting in the down regulation of the disease resistance TIR class of proteins, DIR proteins, *WRKY 70* and *PADA*. Interestingly, only *PR5* (out of all the other PR genes: *PR1*, *PR2* and *PR4*) was down regulated in response to *AtIPCS1* transcript decrease. In contrast, *PR1* and *PR2* in particular were found to show a heightened level of response to the overexpression of *AtIPCS*, especially *AtIPCS2*. This indicated whilst *PR1* and *PR2* are specifically induced in response to an increase in *AtIPCS* expression, *PR5* is induced as a basal response to any change in expression.

AtIPCS has a prominent role as a regulator of plant defence signalling, affecting not only pathways involved in response to fungal and bacterial pathogens, but also pathways involved in defence against parasites and herbivores. This showed that *AtIPCS* is part of a network of plant defence genes that transcends different defence pathways. This is probably because any change in the levels of phytoceramide, acts as an indiscriminate pro-apoptotic signal that mobilizes all defence networks in the push for plant survival.

AtIPCS levels also affect the plant response to abiotic stress; overexpression of *AtIPCS1*, 2 and 3, and knockdown of *AtIPCS1* resulted in the down regulation of genes involved in the response to salt, drought and cold stress. This is in alignment with the identification of transcription factor binding sites for genes involved in response to abiotic stress

upstream of the *AtIPCS1*, 2 and 3 promoters (Chapter 2). The down regulation of these genes could result in plants that are tolerant to cold, salt and osmotic stress.

Similarly, in response to the overexpression of *AtIPCS1*, 2 and 3, and knock-down of *AtIPCS1*, genes involved in photosynthesis and lipid localisation were upregulated. The link between IPCS expression and photosynthesis is unclear and could be an indirect effect. In relation to lipid localisation, the genes upregulated are those that have been identified as expressed specifically in seeds. The level of upregulation for these genes was similar in response to the overexpression of each isoform of *AtIPCS*. This indicated that the three isoforms have the same level of regulation of genes involved in lipid localization in seeds, and function to increase lipid content in seeds to ensure they reach maturity and germinate.

In conclusion, the RNASeq analyses have shown that changes in *AtIPCS* expression is a focal point for the induction of the plant defence response to biotic and abiotic stress, with other effects on plant photosynthesis efficiency and lipid content.

5.9 References

1. Liao, P.; Huang, J.; Tong, P.; Nie, W.; Yan, X.; Feng, Y.; Peng, H.; Peng, X.; Li, S., Characterization and expression analysis of inositolphosphorylceramide synthase family genes in rice (*Oryza sativa* L.). *Genes & Genomics* **2017**, *39* (5), 485-492.
2. Wang, W.; Yang, X.; Tangchaiburana, S.; Ndeh, R.; Markham, J. E.; Tsegaye, Y.; Dunn, T. M.; Wang, G.-L.; Bellizzi, M.; Parsons, J. F., An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*. *The Plant Cell* **2008**, *20* (11), 3163-3179.
3. Mina, J.; Okada, Y.; Wansadhipathi-Kannangara, N.; Pratt, S.; Shams-Eldin, H.; Schwarz, R.; Steel, P.; Fawcett, T.; Denny, P., Functional analyses of differentially expressed isoforms of the *Arabidopsis* inositol phosphorylceramide synthase. *Plant molecular biology* **2010**, *73* (4-5), 399-407.
4. Ashburner, M.; Ball, C. A.; Blake, J. A.; Botstein, D.; Butler, H.; Cherry, J. M.; Davis, A. P.; Dolinski, K.; Dwight, S. S.; Eppig, J. T., Gene Ontology: tool for the unification of biology. *Nature genetics* **2000**, *25* (1), 25.
5. Tipney, H.; Hunter, L., An introduction to effective use of enrichment analysis software. *Human genomics* **2010**, *4* (3), 202.

6. Penninckx, I.; Eggermont, K.; Terras, F.; Thomma, B.; De Samblanx, G. W.; Buchala, A.; Metraux, J.-P.; Manners, J. M.; Broekaert, W. F., Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *The Plant Cell* **1996**, *8* (12), 2309-2323.
7. Bonasera, J. M.; Kim, J. F.; Beer, S. V., PR genes of apple: identification and expression in response to elicitors and inoculation with *Erwinia amylovora*. *BMC Plant Biology* **2006**, *6* (1), 23.
8. Iglesias, M. J.; Terrile, M. C.; Casalengué, C. A., Auxin and salicylic acid signalings counteract the regulation of adaptive responses to stress. *Plant signaling & behavior* **2011**, *6* (3), 452-454.
9. Zhang, J.; Du, X.; Wang, Q.; Chen, X.; Lv, D.; Xu, K.; Qu, S.; Zhang, Z., Expression of pathogenesis related genes in response to salicylic acid, methyl jasmonate and 1-aminocyclopropane-1-carboxylic acid in *Malus hupehensis* (Pamp.) Rehd. *BMC research notes* **2010**, *3* (1), 208.
10. Liu, W.-X.; Zhang, F.-C.; Zhang, W.-Z.; Song, L.-F.; Wu, W.-H.; Chen, Y.-F., Arabidopsis Di19 functions as a transcription factor and modulates PR1, PR2, and PR5 expression in response to drought stress. *Molecular plant* **2013**, *6* (5), 1487-1502.
11. Lopukhina, A.; Dettenberg, M.; Weiler, E. W.; Holländer-Czytko, H., Cloning and characterization of a coronatine-regulated tyrosine aminotransferase from Arabidopsis. *Plant physiology* **2001**, *126* (4), 1678-1687.
12. Yu, K.; Soares, J. M.; Mandal, M. K.; Wang, C.; Chanda, B.; Gifford, A. N.; Fowler, J. S.; Navarre, D.; Kachroo, A.; Kachroo, P., A feedback regulatory loop between G3P and lipid transfer proteins DIR1 and AZI1 mediates azelaic-acid-induced systemic immunity. *Cell Reports* **2013**, *3* (4), 1266-1278.
13. Chanda, B.; Xia, Y.; Mandal, M. K.; Yu, K.; Sekine, K. T.; Gao, Q.-m.; Selote, D.; Hu, Y.; Stromberg, A.; Navarre, D., Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nature genetics* **2011**, *43* (5), 421-427.
14. Gupta, V.; Willits, M. G.; Glazebrook, J., Arabidopsis thaliana EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. *Molecular Plant-Microbe Interactions* **2000**, *13* (5), 503-511.
15. Petersen, M.; Brodersen, P.; Naested, H.; Andreasson, E.; Lindhart, U.; Johansen, B.; Nielsen, H. B.; Lacy, M.; Austin, M. J.; Parker, J. E., Arabidopsis MAP kinase 4 negatively regulates systemic acquired resistance. *Cell* **2000**, *103* (7), 1111-1120.
16. Glazebrook, J., Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **2005**, *43*, 205-227.
17. Yoshioka, K.; Kachroo, P.; Tsui, F.; Sharma, S. B.; Shah, J.; Klessig, D. F., Environmentally sensitive, SA- dependent defense responses in the cpr22 mutant of Arabidopsis. *The Plant Journal* **2001**, *26* (4), 447-459.
18. Pandey, S. P.; Somssich, I. E., The role of WRKY transcription factors in plant immunity. *Plant physiology* **2009**, *150* (4), 1648-1655.
19. Wang, D.; Amornsiripanitch, N.; Dong, X., A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS pathogens* **2006**, *2* (11), e123.
20. Li, J.; Besseau, S.; Törönen, P.; Sipari, N.; Kollist, H.; Holm, L.; Palva, E. T., Defense- related transcription factors WRKY70 and WRKY54 modulate osmotic stress tolerance by regulating stomatal aperture in Arabidopsis. *New Phytologist* **2013**, *200* (2), 457-472.
21. Besseau, S.; Li, J.; Palva, E. T., WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence in Arabidopsis thaliana. *Journal of experimental botany* **2012**, *63* (7), 2667-2679.

22. Greb, T.; Mylne, J. S.; Crevillen, P.; Geraldo, N.; An, H.; Gendall, A. R.; Dean, C., The PHD finger protein VRN5 functions in the epigenetic silencing of Arabidopsis FLC. *Current biology* **2007**, *17* (1), 73-78.
23. Henderson, I. R.; Dean, C., Control of Arabidopsis flowering: the chill before the bloom. *Development* **2004**, *131* (16), 3829-3838.
24. Berrocal-Lobo, M.; Molina, A., Arabidopsis defense response against Fusarium oxysporum. *Trends in plant science* **2008**, *13* (3), 145-150.
25. Wolter, M.; Hollricher, K.; Salamini, F.; Schulze-Lefert, P., The mlo resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. *Molecular and General Genetics MGG* **1993**, *239* (1), 122-128.
26. Matthes, M. C.; Bruce, T. J.; Ton, J.; Verrier, P. J.; Pickett, J. A.; Napier, J. A., The transcriptome of cis-jasmone-induced resistance in Arabidopsis thaliana and its role in indirect defence. *Planta* **2010**, *232* (5), 1163-1180.
27. Hung, C.-Y.; Aspesi Jr, P.; Hunter, M. R.; Lomax, A. W.; Perera, I. Y., Phosphoinositide-signaling is one component of a robust plant defense response. *Frontiers in plant science* **2014**, *5*.
28. La Camera, S.; Geoffroy, P.; Samaha, H.; Ndiaye, A.; Rahim, G.; Legrand, M.; Heitz, T., A pathogen- inducible patatin- like lipid acyl hydrolase facilitates fungal and bacterial host colonization in Arabidopsis. *The Plant Journal* **2005**, *44* (5), 810-825.
29. Wisniewski, J.-P.; Cornille, P.; Agnel, J.-P.; Montillet, J.-L., The extensin multigene family responds differentially to superoxide or hydrogen peroxide in tomato cell cultures. *FEBS letters* **1999**, *447* (2-3), 264-268.
30. Attaran, E.; Rostás, M.; Zeier, J., Pseudomonas syringae elicits emission of the terpenoid (E, E)-4, 8, 12-trimethyl-1, 3, 7, 11-tridecatetraene in Arabidopsis leaves via jasmonate signaling and expression of the terpene synthase TPS4. *Molecular plant-microbe interactions* **2008**, *21* (11), 1482-1497.
31. Bu, Q.; Jiang, H.; Li, C.-B.; Zhai, Q.; Zhang, J.; Wu, X.; Sun, J.; Xie, Q.; Li, C., Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. *Cell research* **2008**, *18* (7), 756.
32. Tran, L.-S. P.; Nakashima, K.; Sakuma, Y.; Simpson, S. D.; Fujita, Y.; Maruyama, K.; Fujita, M.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K., Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress I promoter. *The Plant Cell* **2004**, *16* (9), 2481-2498.
33. Lieberherr, D.; Wagner, U.; Dubuis, P.-H.; Métraux, J.-P.; Mauch, F., The rapid induction of glutathione S-transferases AtGSTF2 and AtGSTF6 by avirulent Pseudomonas syringae is the result of combined salicylic acid and ethylene signaling. *Plant and cell physiology* **2003**, *44* (7), 750-757.
34. Acharya, B. R.; Raina, S.; Maqbool, S. B.; Jagadeeswaran, G.; Mosher, S. L.; Appel, H. M.; Schultz, J. C.; Klessig, D. F.; Raina, R., Overexpression of CRK13, an Arabidopsis cysteine- rich receptor- like kinase, results in enhanced resistance to Pseudomonas syringae. *The Plant Journal* **2007**, *50* (3), 488-499.
35. Cao, S.; Ye, M.; Jiang, S., Involvement of GIGANTEA gene in the regulation of the cold stress response in Arabidopsis. *Plant cell reports* **2005**, *24* (11), 683-690.
36. Ascenzi, R.; Gantt, J. S., A drought-stress-inducible histone gene in Arabidopsis thaliana is a member of a distinct class of plant linker histone variants. *Plant molecular biology* **1997**, *34* (4), 629-641.
37. Mizoguchi, M.; Umezawa, T.; Nakashima, K.; Kidokoro, S.; Takasaki, H.; Fujita, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K., Two closely related subclass II SnRK2

protein kinases cooperatively regulate drought-inducible gene expression. *Plant and Cell Physiology* **2010**, *51* (5), 842-847.

38. Chen, H.; Lai, Z.; Shi, J.; Xiao, Y.; Chen, Z.; Xu, X., Roles of Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. *BMC plant biology* **2010**, *10* (1), 281.

39. Wang, Z.-Y.; Tobin, E. M., Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* **1998**, *93* (7), 1207-1217.

40. Kuno, N.; Møller, S. G.; Shinomura, T.; Xu, X.; Chua, N.-H.; Furuya, M., The novel MYB protein EARLY-PHYTOCHROME-RESPONSIVE1 is a component of a slave circadian oscillator in Arabidopsis. *The Plant Cell* **2003**, *15* (10), 2476-2488.

41. Mizoguchi, T.; Wright, L.; Fujiwara, S.; Cremer, F.; Lee, K.; Onouchi, H.; Mouradov, A.; Fowler, S.; Kamada, H.; Putterill, J., Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. *The Plant Cell* **2005**, *17* (8), 2255-2270.

42. Simpson, G. G., The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. *Current opinion in plant biology* **2004**, *7* (5), 570-574.

43. Sheldon, C. C.; Rouse, D. T.; Finnegan, E. J.; Peacock, W. J.; Dennis, E. S., The molecular basis of vernalization: the central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences* **2000**, *97* (7), 3753-3758.

44. Michaels, S. D.; Amasino, R. M., Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. *The Plant Cell* **2001**, *13* (4), 935-941.

45. Johanson, U.; West, J.; Lister, C.; Michaels, S.; Amasino, R.; Dean, C., Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. *Science* **2000**, *290* (5490), 344-347.

46. Michaels, S. D.; Amasino, R. M., FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *The Plant Cell* **1999**, *11* (5), 949-956.

47. Ambawat, S.; Sharma, P.; Yadav, N. R.; Yadav, R. C., MYB transcription factor genes as regulators for plant responses: an overview. *Physiology and Molecular Biology of Plants* **2013**, *19* (3), 307-321.

48. Knoth, C.; Eulgem, T., The oomycete response gene LURP1 is required for defense against *Hyaloperonospora parasitica* in Arabidopsis thaliana. *The Plant Journal* **2008**, *55* (1), 53-64.

49. Kim, S. A.; Kwak, J.; Jae, S.-K.; Wang, M.-H.; Nam, H., Overexpression of the AtGluR2 Gene Encoding an Arabidopsis Homolog of Mammalian Glutamate Receptors Impairs Calcium Utilization and Sensitivity to Ionic Stress in Transgenic Plants. *Plant and Cell Physiology* **2001**, *42* (1), 74-84.

50. Yang, Y.; Zhang, Y.; Ding, P.; Johnson, K.; Li, X.; Zhang, Y., The ankyrin-repeat transmembrane protein BDA1 functions downstream of the receptor-like protein SNC2 to regulate plant immunity. *Plant physiology* **2012**, *159* (4), 1857-1865.

51. Holm, M.; Ma, L.-G.; Qu, L.-J.; Deng, X.-W., Two interacting bZIP proteins are direct targets of COPI-mediated control of light-dependent gene expression in Arabidopsis. *Genes & Development* **2002**, *16* (10), 1247-1259.

52. Liu, C.; Wang, T.; Zhang, W.; Li, X., Computational identification and analysis of immune-associated nucleotide gene family in Arabidopsis thaliana. *Journal of plant physiology* **2008**, *165* (7), 777-787.

53. Li, J.; Brader, G.; Kariola, T.; Tapio Palva, E., WRKY70 modulates the selection of signaling pathways in plant defense. *The Plant Journal* **2006**, *46* (3), 477-491.

-
54. Lu, H.; Rate, D. N.; Song, J. T.; Greenberg, J. T., ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the Arabidopsis defense response. *The Plant Cell* **2003**, *15* (10), 2408-2420.
55. Peng, L.; Fukao, Y.; Myouga, F.; Motohashi, R.; Shinozaki, K.; Shikanai, T., A chaperonin subunit with unique structures is essential for folding of a specific substrate. *PLoS biology* **2011**, *9* (4), e1001040.
56. Becerra, C.; Puigdomenech, P.; Vicient, C. M., Computational and experimental analysis identifies Arabidopsis genes specifically expressed during early seed development. *BMC genomics* **2006**, *7* (1), 38.
57. Shimada, T. L.; Shimada, T.; Takahashi, H.; Fukao, Y.; Hara-Nishimura, I., A novel role for oleosins in freezing tolerance of oilseeds in Arabidopsis thaliana. *The Plant Journal* **2008**, *55* (5), 798-809.
58. Siloto, R. M.; Findlay, K.; Lopez-Villalobos, A.; Yeung, E. C.; Nykiforuk, C. L.; Moloney, M. M., The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. *The Plant Cell* **2006**, *18* (8), 1961-1974.
59. Youle, R. J.; Huang, A. H., Occurrence of low molecular weight and high cysteine containing albumin storage proteins in oilseeds of diverse species. *American Journal of Botany* **1981**, 44-48.
60. Krebbers, E.; Herdies, L.; De Clercq, A.; Seurinck, J.; Leemans, J.; Van Damme, J.; Segura, M.; Gheysen, G.; Van Montagu, M.; Vandekerckhove, J., Determination of the processing sites of an Arabidopsis 2S albumin and characterization of the complete gene family. *Plant Physiology* **1988**, *87* (4), 859-866.
61. Hundertmark, M.; Hinch, D. K., LEA (late embryogenesis abundant) proteins and their encoding genes in Arabidopsis thaliana. *BMC genomics* **2008**, *9* (1), 118.
62. Nguyen, T.-P.; Cuff, G.; Hegedus, D. D.; Rajjou, L.; Bentsink, L., A role for seed storage proteins in Arabidopsis seed longevity. *Journal of experimental botany* **2015**, *66* (20), 6399-6413.
63. Poxleitner, M.; Rogers, S. W.; Lacey Samuels, A.; Browse, J.; Rogers, J. C., A role for caleosin in degradation of oil- body storage lipid during seed germination. *The Plant Journal* **2006**, *47* (6), 917-933.
64. Dubreucq, B.; Berger, N.; Vincent, E.; Boisson, M.; Pelletier, G.; Caboche, M.; Lepiniec, L., The Arabidopsis AtEPR1 extensin- like gene is specifically expressed in endosperm during seed germination. *The Plant Journal* **2000**, *23* (5), 643-652.
65. Manfre, A. J.; Lanni, L. M.; Marcotte, W. R., The Arabidopsis group 1 LATE EMBRYOGENESIS ABUNDANT protein ATEM6 is required for normal seed development. *Plant physiology* **2006**, *140* (1), 140-149.
66. Haslekås, C.; Viken, M. K.; Grini, P. E.; Nygaard, V.; Nordgard, S. H.; Meza, T. J.; Aalen, R. B., Seed 1-cysteine peroxiredoxin antioxidants are not involved in dormancy, but contribute to inhibition of germination during stress. *Plant physiology* **2003**, *133* (3), 1148-1157.
67. Murgia, I.; Vazzola, V.; Tarantino, D.; Cellier, F.; Ravet, K.; Briat, J.-F.; Soave, C., Knock-out of ferritin AtFer1 causes earlier onset of age-dependent leaf senescence in Arabidopsis. *Plant Physiology and Biochemistry* **2007**, *45* (12), 898-907.
68. Raynal, M.; Guillemot, J.; Gueguen, C.; Cooke, R.; Delseny, M.; Gruber, V., Structure, organization and expression of two closely related novel Lea (late-embryogenesis-abundant) genes in Arabidopsis thaliana. *Plant molecular biology* **1999**, *40* (1), 153-165.
69. Favory, J.-J.; Kobayashi, M.; Tanaka, K.; Peltier, G.; Kreis, M.; Valay, J.-G.; Lerbs-Mache, S., Specific function of a plastid sigma factor for ndh F gene transcription. *Nucleic acids research* **2005**, *33* (18), 5991-5999.

-
70. Dobáková, M.; Sobotka, R.; Tichý, M.; Komenda, J., Psb28 protein is involved in the biogenesis of the photosystem II inner antenna CP47 (PsbB) in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant physiology* **2009**, *149* (2), 1076-1086.
71. Lefebvre, S.; Lawson, T.; Fryer, M.; Zakhleniuk, O. V.; Lloyd, J. C.; Raines, C. A., Increased sedoheptulose-1, 7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant Physiology* **2005**, *138* (1), 451-460.
72. Yabuta, S.; Ifuku, K.; Takabayashi, A.; Ishihara, S.; Ido, K.; Ishikawa, N.; Endo, T.; Sato, F., Three PsbQ-like proteins are required for the function of the chloroplast NAD (P) H dehydrogenase complex in *Arabidopsis*. *Plant and cell physiology* **2010**, *51* (6), 866-876.
73. Peng, L.; Fukao, Y.; Fujiwara, M.; Takami, T.; Shikanai, T., Efficient operation of NAD (P) H dehydrogenase requires supercomplex formation with photosystem I via minor LHCI in *Arabidopsis*. *The Plant Cell* **2009**, *21* (11), 3623-3640.
74. Jouannic, N.; Lepetit, M.; Vergnolle, C.; Cantrel, C.; Gardies, A. M.; Kader, J. C.; Arondel, V., Isolation of a cDNA from *Arabidopsis thaliana* that complements the sec14 mutant of yeast. *The FEBS Journal* **1998**, *258* (2), 402-410.
75. Maldonado, A. M.; Doerner, P.; Dixon, R. A.; Lamb, C. J.; Cameron, R. K., A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature* **2002**, *419* (6905), 399.
76. Sottosanto, J. B.; Saranga, Y.; Blumwald, E., Impact of AtNHX1, a vacuolar Na⁺/H⁺ antiporter, upon gene expression during short- and long-term salt stress in *Arabidopsis thaliana*. *BMC Plant Biology* **2007**, *7* (1), 18.
77. Jang, C. S.; Lee, H. J.; Chang, S. J.; Seo, Y. W., Expression and promoter analysis of the TaLTP1 gene induced by drought and salt stress in wheat (*Triticum aestivum* L.). *Plant Science* **2004**, *167* (5), 995-1001.
78. Weigel, R. R.; Bäuscher, C.; Pfitzner, A. J.; Pfitzner, U. M., NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from *Arabidopsis* that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. *Plant molecular biology* **2001**, *46* (2), 143-160.
79. Hermann, M.; Maier, F.; Masroor, A.; Hirth, S.; Pfitzner, A. J.; Pfitzner, U. M., The *Arabidopsis* NIMIN proteins affect NPR1 differentially. *Frontiers in plant science* **2013**, *4*.
80. Bell, E.; Creelman, R. A.; Mullet, J. E., A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **1995**, *92* (19), 8675-8679.
81. Pandey, S. P.; Roccaro, M.; Schön, M.; Logemann, E.; Somssich, I. E., Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of *Arabidopsis*. *The Plant Journal* **2010**, *64* (6), 912-923.
82. Bartsch, M.; Gobbato, E.; Bednarek, P.; Debey, S.; Schultze, J. L.; Bautor, J.; Parker, J. E., Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in *Arabidopsis* immunity and cell death is regulated by the monooxygenase FMO1 and the nudix hydrolase NUDT7. *The Plant Cell* **2006**, *18* (4), 1038-1051.
83. DeYoung, B. J.; Innes, R. W., Plant NBS-LRR proteins in pathogen sensing and host defense. *Nature immunology* **2006**, *7* (12), 1243.
84. Sønderby, I. E.; Burow, M.; Rowe, H. C.; Kliebenstein, D. J.; Halkier, B. A., A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in *Arabidopsis*. *Plant Physiology* **2010**, *153* (1), 348-363.
85. Lazzeri, L.; Curto, G.; Leoni, O.; Dallavalle, E., Effects of glucosinolates and their enzymatic hydrolysis products via myrosinase on the root-knot nematode *Meloidogyne*
-

-
- incognita (Kofoid et White) Chitw. *Journal of Agricultural and Food Chemistry* **2004**, 52 (22), 6703-6707.
86. Berger, S.; Mitchell- Olds, T.; Stotz, H. U., Local and differential control of vegetative storage protein expression in response to herbivore damage in *Arabidopsis thaliana*. *Physiologia Plantarum* **2002**, 114 (1), 85-91.
87. Ponce De Leon, I.; Schmelz, E. A.; Gaggero, C.; Castro, A.; Alvarez, A.; Montesano, M., *Physcomitrella patens* activates reinforcement of the cell wall, programmed cell death and accumulation of evolutionary conserved defence signals, such as salicylic acid and 12- oxo- phytodienoic acid, but not jasmonic acid, upon *Botrytis cinerea* infection. *Molecular plant pathology* **2012**, 13 (8), 960-974.
88. Kaplan, F.; Guy, C. L., RNA interference of *Arabidopsis* beta- amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. *The Plant Journal* **2005**, 44 (5), 730-743.
89. Yancey, P. H.; Clark, M. E.; Hand, S. C.; Bowlus, R. D.; Somero, G. N., Living with water stress: evolution of osmolyte systems. *Science* **1982**, 217 (4566), 1214-1222.
90. van de Mortel, J. E.; Schat, H.; Moerland, P. D.; van Themaat, E. V. L.; Van Der Ent, S.; Blankestijn, H.; Ghandilyan, A.; Tsiatsiani, S.; AARTS, M. G., Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd- hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **2008**, 31 (3), 301-324.
91. Mittler, R.; Kim, Y.; Song, L.; Coutu, J.; Coutu, A.; Ciftci-Yilmaz, S.; Lee, H.; Stevenson, B.; Zhu, J.-K., Gain- and loss- of- function mutations in *Zat10* enhance the tolerance of plants to abiotic stress. *FEBS letters* **2006**, 580 (28-29), 6537-6542.

CHAPTER 6

CONCLUSION AND FUTURE WORK

6.1 Conclusion

In this study two approaches have been utilised to understand the role of IPCSs in plants: a chemical approach involving the screen for inhibitors of *At*IPCS2 and *Os*IPCS2 combined with a genetic approach involving the analyses of transcriptome changes in response to the over expression and the knock down in expression of *At*IPCS.

Inhibitors of IPCS2 were identified which showed inhibitory activity at low μM , and exhibited herbicidal phenotypes associated with stress (purple patches) and PCD (chlorotic leaves) in an *in vivo* screen. The phenylamidine and triazinone classes of compounds have both been shown to be inhibitors of *At*IPCS2 and *Os*IPCS2, but showed differential selectivity for *At*IPCS2. Both were found to have little or no effect on AUR1, the yeast orthologue.

The identification of these two classes of compounds was interesting, in that the triazinone are known antifungals and the phenylamidines have herbicidal activity. These data also indicated that *At*IPCS2 could be both a herbicidal and antifungal target. The use of the phenylamidines as herbicides is quite promising in that they show obvious phenotypes of stress resulting from the application of the compounds on wildtype plants. The two phenylamidines tested show phenotypes associated with oxidative stress and PCD which result in the inhibition of plant growth. In the case of seedlings treated with compound 1, the PCD phenotype leads to the eventual death of the plant. Further studies will need to be conducted determine if the *in vivo* results can be replicated in mature plants, and whether constant contact between the compound and the plant is necessary to observe the phenotypes reported in this study.

The ability of *At*IPCS2 inhibitors to also have fungicidal effects can be explained by its role as a negative regulator of plant defence against pathogens as discovered from the

RNASeq transcriptomic analyses. *Arabidopsis* transgenic lines over-expressing *AtIPCS1*, 2 and 3, showed a marked decrease in the expression of genes involved in plant defence; this was heightened in response to *AtIPCS2* overexpression. The knock-down of *AtIPCS1* in a t-DNA insertion line mutant line also resulted in a decrease in genes associated with plant defence, however this did not include the PR genes. *PR1*, *PR2* and *PR5* were highly downregulated in *AtIPCS1*, 2 and 3 over-expressing plants and are well characterised as markers of SAR in plants. These results indicated the prominent role of *AtIPCS1*, 2 and 3 as negative regulators of plant defence. Perhaps these plant defence genes would be upregulated in a homozygous knockout of *AtIPCS2*, resulting in a less constrained response to pathogen attack and heightened resistance.

Another finding of this work was the observation of an early flowering phenotype in *AtIPCS* RNAi knock-down and over-expressers lines; this resembles the phenotype previously linked to vernalization in plants^{1, 2}. Combined with RNASeq data which identified the downregulation of several genes involved in abiotic stress in plants over expressing *AtIPCS*, there was a strong indication that the enzyme is also involved in abiotic stress response. As such manipulation of expression could increase plant stress tolerance, this demands further investigation.

In summary, this study has identified inhibitors of plant IPCS that have *in vivo* activity and may be tractable for further development towards herbicidal compounds. These compounds show differential selectivity for the dicot IPCS in *A. thaliana*, over the monocot IPCS in *O. sativa*; this indicated that inhibitors selective for a weed versus crop IPCS could be identified. In parallel, transcriptomic analyses of RNASeq analyses revealed that *AtIPCS1*, 2 and 3 are involved in the response to abiotic and biotic stress, with a prominent role as a negative regulator of the plant defence network. *AtIPCS1*, 2

and 3 also seems to have a role in enhancing plant stress tolerance, which could potentially be exploited in plant breeding strategies to create stress tolerant crops, increase yields and meet the challenge of increasing global agricultural output to sustain the projected 2-3 billion population increase by the year 2050.

6.2 Future work

Although these compounds do inhibit *AtIPCS2* still remains a chance that they also affect other targets. To test that these compounds are on target, a dose response assay should be carried out against *Arabidopsis AtIPCS2* over-expressers, knock-downs and wildtype. Theoretically, compounds targeting *AtIPCS2* should be less effective against the over-expressers, due to excess target, and more effective against knock-downs for the opposite reason. These identified inhibitors could also be tested against IPCS from crop and weed species to see if the observed differential selectivity is maintained.

RNASeq could also be used to analyses of wildtype plants and plants with *AtIPCS2* deleted or knocked-down, treated with the identified inhibitors. If the compounds are completely on target, an absence of *AtIPCS2* would preclude changes due to the inhibitor. The homozygous RNAi lines created in this study could also be used in transcriptomic analyses to see if the changes observed in the *AtIPCS1* t-DNA mutant also occur in response to the knock-down.

Work done by Owen et al. showed that the activity of triazinone possessing an R₂ haloalkyl group is enhanced against the ascomycetes, *Leptosphaeria nodorum*, *Magnaporthe grisea* and *Zymoseptoria tritici*³. Further activity enhancement was achieved by the replacement of the haloalkyl group with a benzyl or alkyl group. These triazinone derivatives are quite different from the triazinone identified as inhibitors of

AtIPCS2, in that they lack the thio linker and possess two benzyl groups that have halogens attached to them. Therefore, it is possible that these could be selective for plant over fungal IPCS. Different derivatives of triazinone may be able to protect plants from fungal infection by direct inhibition of pathogenic fungi IPCS, by inhibition of the plant IPCS, or by both. Inhibition of a negative regulator of PCD result in an enhanced plant defence response able to stop fungal growth. This latter effect, has been indicated by the previously observed, slightly lower susceptibility of *AtIPCS2* knockout plants to powdery mildew⁴.

To further test this hypothesis, IPCS from different ascomycetes species would need to be isolated, purified and assayed against the identified triazinone to see if the fungal IPCS is also inhibited if compounds selective for the *AtIPCS2* could be identified, a pathogen assay could also be designed to test the susceptibility of *Arabidopsis* to infection on triazinone treatment. The susceptibility of *AtIPCS2* knock-out or knock-down could be similarly analysed.

If *AtIPCS2* acts as a prophylactic to fungal infection, these experiments should show a reduction in fungal mass on chemical inhibition, genetic knock-out/knock-down of *AtIPCS2*. In addition, *AtIPCS2* over-expressor lines could also be assayed to see if the increase in expression has the opposite effect, making plants more susceptible to fungal infection. My results show an overall decrease in the expression of plant defense genes, on overexpression of *AtIPCS1*, 2 and 3, suggesting that this would be the case.

Similar to the proposal above, regarding the further analyses of plants treated with the identified compounds, RNASeq could be utilized to identify if genes associated with plant defense are upregulated to higher degree in *AtIPCS2* knockout/down or compound treated *Arabidopsis* during infection. Work done by Weeda *et al.* using this method identified the

role of melatonin in plant defense by treating plants with low (100 pM) and high (1 mM) concentrations and then comparing the genes upregulated in response to both treatments⁵. This experimental approach when applied to the study of *AtIPCS2* inhibition, would serve to further probe the hypothesized prophylactic effect of *AtIPCS2* inhibition in fungal infection by upregulation of plant defense genes.

One of the intriguing findings from this study was that an increase in the expression of *AtIPCS1*, 2 and 3 resulted in the upregulation of genes involved in the photosynthetic pathway. To test if this has an effect on the plants' ability to photosynthesize, the photosynthetic efficiency of PSII could be quantified by measuring the fluorescence of chlorophyll⁶. In addition, plant biomass could also be measured to see if there is an increase, correlated with *AtIPCS1*, 2 and 3 overexpression and upregulation of genes involved in the photosynthetic pathway.

The lipid storage and lipid transfer proteins were also upregulated in response to *AtIPCS1*-3 overexpression. To probe the effects of this on plant lipid content, lipidomic experiments could be performed; it would also be of interest to investigate how the sphingolipid and long chain base (LCB) content is altered in response to the overexpression and knock-down of *AtIPCS1*, 2 and 3. Finally, the over-expressor lines could also be subjected to abiotic stress such as cold, salinity and drought to find out if they have higher stress tolerance compared to Col-0 and if there is a link between higher levels of *AtIPCS1*, 2 and 3 expression, and an increase in stress tolerance. The experiments outlined could confirm the tractability of IPCS inhibition in crop protection, which would be advantageous to increase crop yields.

6.3 References

1. Henderson, I. R.; Dean, C., Control of Arabidopsis flowering: the chill before the bloom. *Development* **2004**, *131* (16), 3829-3838.
2. Sheldon, C. C.; Rouse, D. T.; Finnegan, E. J.; Peacock, W. J.; Dennis, E. S., The molecular basis of vernalization: the central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences* **2000**, *97* (7), 3753-3758.
3. Owen, W. J.; Sullenberger, M. T.; Loso, M. R.; Meyer, K. G.; Slanec, T. J., Synthesis and antifungal activity of 3- aryl- 1, 2, 4- triazin- 6- one derivatives. *Pest management science* **2015**, *71* (1), 83-90.
4. Wang, W.; Yang, X.; Tangchaiburana, S.; Ndeh, R.; Markham, J. E.; Tsegaye, Y.; Dunn, T. M.; Wang, G.-L.; Bellizzi, M.; Parsons, J. F., An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in Arabidopsis. *The Plant Cell* **2008**, *20* (11), 3163-3179.
5. Weeda, S.; Zhang, N.; Zhao, X.; Ndip, G.; Guo, Y.; Buck, G. A.; Fu, C.; Ren, S., Arabidopsis transcriptome analysis reveals key roles of melatonin in plant defense systems. *PloS one* **2014**, *9* (3), e93462.
6. Baker, N. R., Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu. Rev. Plant Biol.* **2008**, *59*, 89-113.

CHAPTER 7

MATERIALS AND METHODS

7.1 Experimental section

7.1.1 Materials

Forward and reverse primers for the cloning of *At*IPCS1-3 and *Os*IPCS1-3 into the pESC-LEU plasmid and the cloning of *At*IPCS1-3 into the pENTR/D-TOPO vector were purchased from Sigma; Quick-Load 1kb DNA ladder (NO4685, Dorset, UK) and Gel Loading Dye Blue (6X) (B70215, Massachusetts, USA) were purchased from BioLabs; alamarBlueR (DAL1025, Loughborough, UK) was purchased from Invitrogen; PCR high fidelity Master Mix kit (F531S, Loughborough, UK) was purchased from ThermoFisher; QIAprep DNA Miniprep kit was purchased from Qiagen (27104, Manchester, UK); High capacity cDNA reverse transcription kit were purchased from ThermoFisher (00539510, Loughborough, UK); Go-taq qPCR Master mix was purchased from promega (A600A, Southampton, UK); Reliaprep RNA tissue miniprep system was purchased from promega (Z6112, Southampton, UK); . In-Fusion® HD Cloning Kit with Cloning Enhancer was purchased from Clontech (638909, Saint-Germain-en-Laye, France); complete® EDTA-free Protease Inhibitor Cocktail Tablets were purchased from Roche Applied Science (05892791001, Sussex, UK). D (+) raffinose (RAF02, Norfolk, UK); Complete Supplement Mix (CSM) drop-out: -His -Leu -Trp -Ura (DCS1389, Norfolk, UK); yeast nitrogen base low fluorescence without amino acids, riboflavin (CYN6510, Norfolk, UK); D (+) galactose (GAL03, Norfolk, UK) and 5-FOA (5FOA01, Norfolk, UK) were purchased from Formedium; leucine (L8912), uracil (U0750), tryptophan (T0254), D (+) glucose (G8270), ammonium sulphate (A5132), tris base (tris hydroxymethyl amino methane) (252859), myriocin from *Mycelia sterilia* (M1177), L- α phosphatidylinositol ammonium salt from soybean (P0639), L- α phosphatidylinositol ammonium salt from bovine liver (P5766), cycloheximide (C7698), dimethyl sulfoxide (D8418), potassium formate (294454), potassium phosphate

monobasic (P9791), potassium phosphate dibasic (P3786), agar for plant cell culture (P9791), rifampicin (R3501) and ampicillin (A1593) were all purchased from Sigma Aldrich (Norfolk, UK); N-Hexanoyl NBD-phytoceramide (1628, Cambridge, UK) was purchased from Universal Biologicals; Aureobasidin A was purchased from AureoGen (Kalamazoo, USA); NBD-C6-ceramide (N1154, Dorset, UK) was purchased from Invitrogen; AG4-X4 resin (140-4341, Hertfordshire, UK) was purchased from BioRad; Molecular Biology Agarose Electrophoresis Grade (MB1200), kanamycin (K0126), spectinomycin (S0188) and timentin (T0190) were purchased from Melford (Suffolk, UK); Murashige and Skoog medium (M0222, Haarlem, Netherlands) was purchased from Duchefa Biochemie; bacto-Agar (214010, Surrey, UK) was purchased from Becton Dickinson and Company. Peat plugs were purchased from Jiffy Products (3825451, Kristiansand, Norway); ethidium bromide solution (46067, Buchs, Switzerland) was purchased from Fluka BioChemika; LoBind tubes for microsome work were purchased from Eppendorf (10708704, Loughborough, UK). The acid-washed glass beads 425-600 μm (G8772), glycerol (G5516), sucrose (S0389), polyethylene glycol 3350 (1546547), MgCl_2 (M2670), lithium acetate (517992), were all purchased from Sigma Aldrich (Dorset, UK). Yeast strains, lacking drug efflux pumps (*pdr1 Δ ::KanMX4* *pdr3 Δ ::KanMX4* *pdr16 Δ ::KanMX4* *pdr17 Δ ::KanMX4*) were provided by Prof. Stark, University of Dundee: MSY-23-3C (Parent Strain), MSY-23-3C-1A (pESC-Leu-*Lmj*IPCS), MSY-23-3C-3A (pESC-Leu-AUR1), and MSY-23-3C-6A (pESC-Leu-*Os*IPCS1).

7.1.2 Instruments and Equipment

Centrifugation steps were carried out using Beckman Coulter centrifuges or ultracentrifuges. Eppendorf tubes were spun down using a Sigma 1-14 microfuge. Eppendorf contents were dried using an Eppendorf Vacuum Concentrator 5301 from Brinkmann. Disruption of cells was performed using an IKA® Vortex Genius 3. For protein and cell quantification carried out using BOECO S-30 spectrophotometer. Quantitative PCR was carried out using the applied Biosystems 7300 machine. For the enzyme assay, the filtration step was carried out using multiscreen® solvent filter plates purchased from Millipore and OptiPlate-96 F black plates used for reading the fluorescence of NBD-IPCS from Perkin Elmer. V-shaped and flat bottom 96 well plates were purchased from Sigma Aldrich. HPTLC plates were from Merck and were analysed using a Fuji FLA-3000 plate reader and AIDA Image Analyser® software (version 3.52). The localisation experiments of 35s::GFP.*At*IPCS1-3 were performed with the Leica Stereo Fluor M165FC and Leica SP5 CLSM FLIM FCCS microscopes. Details of the buffers and solutions used are given in Table 7-1 and details of media composition is given in Table 7-2.

7.2 Table 7-1: Buffer composition

| Buffers | Final concentration | Volume/Weight | Stock solution |
|--|---------------------|---------------|---------------------------------------|
| Sodium chloride-Tris-EDTA (STE) buffer | 25 mM | 1.25 ml | Tris/HCl (1 M) |
| | 250 mM | 12.5 ml | Sucrose (1 M) |
| | 1 mM | 100 ul | EDTA (0.5 M) |
| | | 1 tab | Complete® Protease Inhibitor Cocktail |
| | | 50 ml | Water |
| Storage buffer | 50 mM | 2.5 ml | Tris/HCl (1 M) |
| | 10 w/v | 6.25 ml | Glycerol (80 % w/v) |
| | 5 mM | 0.25 ml | MgCl ₂ (1M) |
| | | 1 tab | Complete® Protease Inhibitor Cocktail |
| | | 50 ml | Water |
| Tris/EDTA/BSA buffer | 250 mM | 12.5 ml | Tris/HCl (1 M) |
| | 25 mM | 2.5 ml | EDTA (0.5 M) |
| | 15 mg/mL | 750 mg | Free fatty-acid BSA |
| | | 50 ml | Water |
| TE buffer | | 100 ml | 1 M Tris/HCl (pH7.5) |
| | | 20 ml | 500 Mm EDTA (pH8) |
| | | 880 ml | Ultrapure water |
| PEG 3350 (50%) | | 100 mg | PEG 3350 |
| | | 200 ml | Ultrapure water |
| TE/Li Acetate | | 5 ml | 1 M Li Acetate |
| | | 5 ml | 10x TE buffer |

| | | |
|----------------|-------|-----------------|
| | 50 ml | Ultrapure water |
| PEG/Li Acetate | 5 ml | 1 M Li Acetate |
| | 5 ml | 10x TE buffer |
| | 50 ml | 50% PEG 3350 |

7.3 Table 7-2: Media composition

| Growth media | Volume/Weight | Stock |
|--|---------------|---|
| Lysogeny broth | 25 g | Miller's LB broth |
| | 20 g | Bacto agar |
| | 1 ml | Ampicillin (100 mg/ml) |
| | 1 L | Distilled water |
| Murashige and Skoog (0.8% w/v) agar (pH 5.6) | 4.4 g | MS medium with vitamins |
| | 8 g | Plant tissue agar |
| | 1L | Distilled water |
| SD-H-L agar | 20 g | Glucose |
| | 1.93 g | Yeast Nitrogen base without amino acids and without ammonium sulphate |
| | 5 g | Ammonium Sulphate |
| | 15 g | Bacto agar |
| | 580 mg | Amino acid drop out supplement -His-Leu-Ura-Ttp |
| | 20 mg | Uracil |
| | 40 mg | Tryptophan |
| | 1 L | Distilled water |

| | | | |
|-------------------------------------|---------------|---|----------------------|
| SGR-H-L + FOA agar | 1 g | Galactose | |
| | 10 g | Raffinose penhydrate | |
| | 1.93 g | Yeast Nitrogen base without amino acids and without ammonium sulphate | |
| | 5 g | Ammonium Sulphate | |
| | 15 g | Bacto agar | |
| | 1 g | 5-Fluoroorthoic acid monohydrate | |
| | 580 mg | Amino acid drop out supplement - His-Leu-Ura-Ttp | |
| | 20 mg | Uracil | |
| | 40 mg | Tryptophan | |
| | 1 L | Distilled water | |
| | SGR-H-L media | 1 g | Galactose |
| | | 10 g | Raffinose penhydrate |
| 1.93 g | | Yeast Nitrogen base without amino acids and without ammonium sulphate | |
| 5 g | | Ammonium Sulphate | |
| 580 mg | | Amino acid drop out supplement -His-Leu-Ura-Tryp | |
| 20 mg | | Uracil | |
| 40 mg | | Tryptophan | |
| 1 L | | Distilled water | |
| Phosphate buffer (0.25 mM, pH 7) | 13.1g | KH ₂ PO ₄ | |
| | 26.8g | K ₂ HPO ₄ | |
| | 1L | Ultrapure water | |

7.4 Biological Procedures

All of the following biological procedures were carried out under sterile conditions unless stated otherwise. Plant were grown under 150–200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ lightening at 20°C and under a 16h photoperiod.

7.4.1 DNA extraction from bacteria

The QIAprep Spin Miniprep Kit was used to extract DNA from bacterial cultures by following manufacturer's instructions.

7.4.2 RNA extraction from plant

The ReliaPrep™ RNA tissue miniprep system kit was used to extract RNA from 1mg of plant material by following manufacturer's instructions.

7.4.3 cDNA synthesis

The RNA concentrations were quantified using nanodrop and all samples diluted to 2 μg in 10 μl total volume. To the RNA mixture was added 10 μl of Master mix of:

| Component | Volume per reaction (μl) |
|------------------------------------|---------------------------------------|
| 10x RT buffer | 2 |
| 25x dNTP Mix (100 mM) | 0.8 |
| 10x RT Random Primers | 2 |
| Nuclease-free water | 4.2 |
| Multiscribe™ Reverse transcriptase | 1 |

The mixture was mixed and spun down briefly, then placed in thermal cycler using the program below:

| | Step 1 | Step 2 | Step 3 | Step 4 |
|------------------|---------------|---------------|---------------|---------------|
| Temperature (°C) | 25 | 37 | 85 | 4 |
| Time (secs) | 160 | 7200 | 5 | ∞ |

7.4.4 Quantitative reverse-transcription real-time PCR

cDNA samples were diluted (1:50) and 5ul of the diluted sample was added to 10µl of the real-time PCR master mix, which consisted of:

| Component | Volume per reaction (µl) |
|----------------------|---------------------------------|
| 2x SYBR mix (ROX) | 7.5 |
| Forward primer (5µM) | 0.9 |
| Reverse primer (5µM) | 0.9 |
| Nuclease-free water | 0.7 |

Real-time PCR reaction was carried out using the following program:

| Temperature (°C) | Time (seconds) | Number of cycles |
|-------------------------|-----------------------|-------------------------|
| 50 | 120 | 1 |
| 95 | 600 | 1 |
| 95 | 15 | 40 |
| 60 | 60 | 1 |

7.4.5 Polymerase chain reaction protocol

The PCR mixture was prepared using 2x phusion HF master mix (10 μ L) and (1 μ L) of the forward and reverse primers with plasmid DNA (10 ng); final volume was adjusted to 20 μ L using distilled water. The PCR reaction was run on a PCR machine under the conditions described below. The DNA was stored at -20°C.

| Cycle step | Temperature (°C) | Time (seconds) | Number of cycles |
|----------------------|------------------|----------------|------------------|
| Initial denaturation | 98 | 30 | 1 |
| Denaturation | 98 | 10 | 30 |
| Annealing | 56 | 30 | |
| Extension | 72 | 100 | |
| Final extension | 72 | 600 | 1 |
| | 4 | hold | |

7.4.6 T4 ligase cloning

Agarose gel electrophoresis was used to quantify DNA concentration and for the DNA moles calculated. The molar ratio of insert to vector in the reaction was 1:3. T4 ligase cloning reaction was set up as follows:

| Component | Volume per reaction (μ l) |
|---------------------|-----------------------------------|
| T4 ligase enzyme | 0.5 |
| T4 ligase buffer | 1.5 |
| Vector | X μ l (~ 30ng) |
| Insert | Y μ l to give 1:3 molar ratio |
| Nuclease-free water | 20-X-Y |

The reaction mixture was incubated at 25°C for one hour; 5 µl of the reaction mixture was subsequently used for bacterial transformation. *AtIPCS1-3* were cloned into pENTR/D-TOPO vector using T4-ligase cloning method.

7.4.7 Gateway cloning

Creating multiple expression vector constructs for the target gene of interest (*AtIPCS1-3*) using traditional ligase-mediated cloning is time-consuming and laborious, hence the use of gateway cloning. Gateway cloning exploits the bacteriophage lambda recombination system, bypassing the use of ligase-mediated cloning¹. PCR amplification of the target sequence followed by T4 ligase cloning into the pENTR/D-TOPO ‘entry vector’ creates a plasmid that has the target DNA sequences flanked by *attL* recombination sequences. The *attL* sequence recombines with the *attR* sites using the LR clonase reaction mix which transfers the target sequence into a desired ‘destination vector’ (Figure 7-1). This destination vector usually carries the chloramphenicol resistance gene (*CmR*) and a *ccdB* gene which is lethal to most strains of *Escherichia coli*. the (*ccdB*) as a result it is possible to negatively select for the vector possessing the recombinant sites upon transformation of *E. coli* cells. This selection is combined with positive selection for an antibiotic resistance marker thereby making certain that only colonies harboring the ‘expression vector’ are selected.

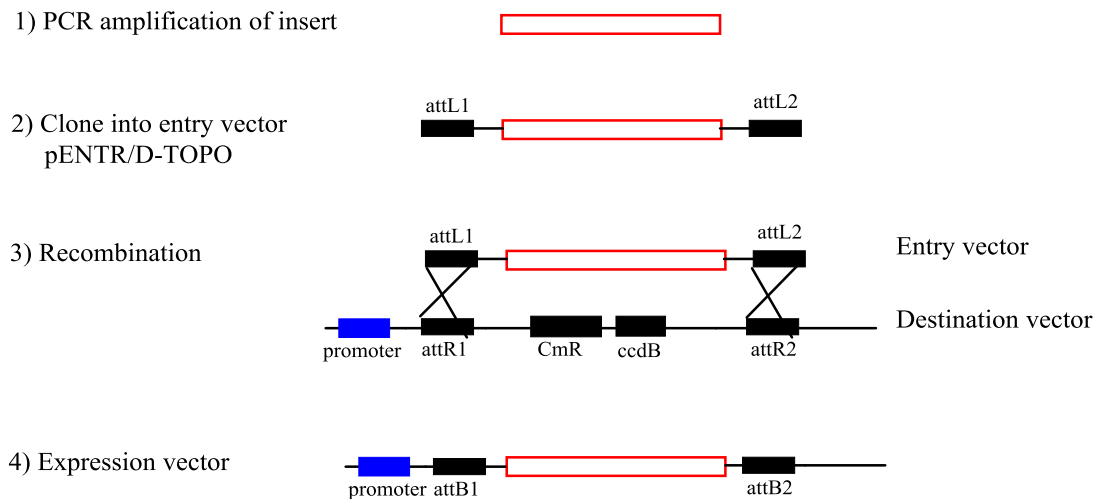


Figure 7-1: Overview of Gateway cloning (1) the target of interest is amplified by PCR (2) PCR products are mixed with the linearized pENTR/D-TOPO vector and ligation mediated by T4 ligase (3) Recombination between *attL* and *attR* sites is mediated by LR clonase with the target sequence recombined into a destination vector of choice (4) Resulting expression vector containing the target gene.

Cloning into the destination vector pK7WG2, carried out via Gateway LR clonase mediated cloning. The following components were added to a PCR tube:

| Component | Volume per reaction (µl) |
|--|--------------------------|
| Entry clone (50-150 ng/reaction) | 1-7 |
| Destination vector (150 ng/µl) | 1 |
| TE buffer, pH 8 | to a volume of 8 µl |
| LR Clonase TM II enzyme mix | 2 |

Reaction was incubated at 25°C for 1 hour and 4ul of mixture used for transformation.

7.4.8 In-Fusion cloning

Agarose gel electrophoresis was used to quantify the insert and plasmid DNA. Using the commercial InFusion cloning kit, the linearized vector was mixed with the insert DNA (1:3 ratios) and 5× In-Fusion HD enzyme premix (2 µl) added with the final volume

adjusted to 10 µl with distilled water. The mixture was then incubated at 50 °C for 15 mins and then used immediately for the transformation of *E. coli*.

7.5 Yeast culture

The *S. cerevisiae* strains lacking the drug efflux genes *pdr1Δ::KanMX4*, *pdr3Δ::KanMX4*, *pdr16Δ::KanMX4* and *pdr17Δ::KanMX4* which had been stored at -80°C in 80% were inoculated onto SGR-H-L agar and incubated at 30 °C for 3 days, then stored at 4°C. This was then used to inoculate SGR-H-L media (5 ml) which was incubated at 30 °C with 180 rpm shaking.

7.5.1 Frozen stocks

400 µl of 80% glycerol was mixed with 800 µl of yeast culture grown in SGR-H-L media and stored at -80°C.

7.5.2 Yeast transformation

Cells grown overnight in SGR-H-U media at 30°C were harvested by centrifugation (1,000 ×g for 5 minutes at room temperature). The supernatant was discarded, and the cells were resuspended in sterile 1x TE buffer (5 ml). The cells were spun down again (1,000 × g for 5 minutes at room temperature) and the supernatant discarded. The cell pellets were then resuspended in freshly prepared, sterile 1 × TE/LiAc buffer (1 ml) to form the competent yeast cells.

Purified plasmid (1 µg) and competent yeast cells ($OD_{600} = 0.6-0.8$, 0.1 ml) was added to Herring testes carrier DNA (10 µl) which had previously been boiled at 95 °C for 10 minutes; and mixed thoroughly. Freshly prepared, sterile PEG/LiAc (0.6 ml) was added and the mixture thoroughly mixed, followed by incubation with shaking at 30 °C for 30

minutes. After which DMSO (70 μ l) was added and mixed by gentle swirling. The competent yeast cells then underwent heat shocked at 42 °C for 15 minutes followed by chilling on ice for 2 minutes. The cells were spun down (14 000 rpm for 5 seconds at room temperature), the supernatant discarded. The cell pellets were resuspended in 1 \times TE buffer (0.2 ml) and the cells were plated onto SGR-H-L-U agar plates and incubated at 30 °C for 5 days.

7.5.3 Plasmid shuffle

SGR –H –L medium (0.1%:1%) was inoculated with yeast strains and incubated with shaking at 30 °C overnight and 200 μ l plated onto SGR –H –L +5FOA agar medium (0.1% galactose: 1% Raffinose) which was incubated for 4 days at 30 °C.

7.5.4 Yeast culture scale-up

Plasmid-shuffled yeast cultures were propagated in SGR –H –L medium (0.1% galactose: 1% Raffinose). Liquid medium (5 ml) was inoculated and incubated overnight at 30 °C until OD₆₀₀ had reached \geq 0.8. Fresh medium (250 ml) was added and the culture incubated at 30 °C until OD₆₀₀ had reached \geq 0.8. The culture was then diluted into fresh medium (1L) and incubated at 30 °C overnight until OD₆₀₀ =0.8-1.0.

7.5.5 Preparation of crude microsomal membranes

The yeast cells were harvested by centrifugation (4,000 \times g for 10 minutes at 4 °C) and resuspended in cold PBS (20 ml per litre). The cell suspension was then transferred into 50 mL falcon tubes (no more than 20 ml to each falcon tube) and the cells were pelleted by centrifugation (4000 rpm for 10 mins at 4°C). The centrifuge bottles were washed with 5 mL per litre of PBS and the washing transferred to falcon tubes and spun down (4 000

rpm for 10 mins at 4°C) supernatant from both centrifugation steps were discarded. The cell pellets were weighed and pre-chilled glass acid-washed glass beads (425-600 µm) to disrupt the cells were added (mass equal to 1.5 x mass of the pellet) and STE buffer was also added (volume in ml equal to the mass of the pellet in g). The cells were disrupted using pre-chilled, acid-washed glass beads (mass equal to 1.5x wet cell mass) for 30 cycles of 1 minute on the vortex machine, followed by 1 minute rest on ice. This mixture was then spun down (5 000 rpm for 15 minutes at 4 °C) and the supernatant was stored. STE buffer was added to cell pellets (volume in ml equal to 0.5x mass of wet cell mass) and the cell disruption cycle repeated for a further 20 cycles, followed by centrifugation (5500 rpm for 15 minutes at 4°C). Supernatant was transferred to ultracentrifuge tubes which were balanced to within 10 mg of each other using STE buffer and spun down (12500 rpm for 30 mins at 4°C); the supernatant was transferred to new ultracentrifuge tubes and spun down again (32 000 rpm for 90 mins at 4°C). Pellets were resuspended in the minimum amount of storage buffer and stored at -80°C. The protein content for each pellet and the supernatant was determined by Bradford assay².

7.5.6 Diffusion assay

Agarose was added to SGR –H –L medium (0.8%, 200 mL) and sterilised. To 15 mL of agarose SGR-H-L medium, OD₆₀₀ = 2 of liquid yeast culture were added, which was then mixed by gentle inversion. This was poured into a square plate and left to set. Aureobasidin A, myriocin and DMSO were each spotted onto the agarose surface in aliquots of 1 µl, 2 µl and 3 µl; the plate was then incubated at 30 °C for 3 days.

7.5.7 High performance thin layer chromatography (HPTLC) assay

Phosphatidyl inositol solution (50 mM) was dried in a rotavapor and a predetermined Unit/ μ l of active IPCS added. Tris/EDTA/BSA buffer (20 μ l) was added and the volume adjusted to 48 μ l with distilled water. This mixture was then incubated at 30 °C for 30 minutes, after which NBD-C6-ceramide (200 μ M) was added and then incubated at 30 °C for 60 minutes. The reaction was then quenched with 150 μ L of chloroform:methanol:water mixture (10:10:3). The mixture was spun down (14 000 rpm for 5 minutes at room temperature) and the aqueous layer was extracted. Upon the addition of chloroform (50 μ l), the aqueous layer was re-extracted and the organic extracts were combined and solvent dried in the rotavapor at 30 °C for 15 minutes. The pellets were resuspended in 20 μ l of chloroform:methanol:water mixture (10:10:3). On the HPTLC plate 5 μ L of the resuspended pellet was loaded and this was run using the solvent system chloroform: methanol: 0.25% (w/v) aqueous KCl mixture (55:45:10). NBD-IPC turnover was quantified and analysed on a TLC reader.

7.6 Experiments for high throughput screen

7.6.1 Frozen stocks for screening

Yeast strains were grown to an OD₆₀₀ of 0.5-0.7 and then spun down at 1000 rpm. The pellets were resuspended in a one to one mixture of SGR-H-L media and 20% glycerol, then frozen and stored at -80°C.

7.6.2 Primary screen, cellular assay

Cells were thawed on ice and diluted with SGR-H-L media (1:20) to obtain cells in the lag phase of growth with a starting OD₆₀₀ of 0.06 corresponding to 0.5×10^7 cell per ml.

The yeast cell culture was aliquoted (198 μ l) into 96-well plates using the Biomek FXP automated workstation with DMSO (2 μ l) and cycloheximide (2 μ l, 0.01 mM) dispensed into eight wells in the first column of the 96 well plates; the dispersion of DMSO and cycloheximide was alternated between a row of wells. Following this, these compound (10 μ M) were aliquoted into the remaining wells. Plates were sealed with a breathable membrane and incubated at 30°C for 24 hours. After 24 hours the OD₆₀₀ reading was obtained. Readings were carried out in duplicate for each strain and percentage inhibition calculated with this formula:

$$I (\%) = \frac{\Delta OD_{600nm}(NC - \text{Compound}) \times 100}{\Delta OD_{600nm} (NC - PC)}$$

Where NC is the average of the negative control (DMSO) and PC is the average of the positive control (cycloheximide)

For dose response, hit compounds were diluted (1:3) to obtain a range of concentrations from 68nM-50 μ M. Frozen yeast strains were thawed on ice and diluted with SGR-H-L media (1:20) and aliquoted into 96 well plates, then sealed with a breathable membrane and incubated for 24 hours at 30°C. Cell density was measured at OD₆₀₀. IC₅₀ values were determined using Graphpad Prism.

7.6.3 Secondary screen, enzyme assay

Filter plate wells are coated with AG4-X4 resin (10% w/v in ethanol) and prepared according to the protocol described by Mina et al³. For a 96 well plate assay set up, a mixture consisting of dried soy phosphoinositol solution (200 μ M), NBD phytoceramide (30 μ M) and phosphate buffer (pH7, 71 mM) was prepared. A second mixture made up of *At*IPCS2 microsomes (0.44 Unit/ μ l), *Os*IPCS2 microsomes (0.07 Unit/ μ l) and AUR1 microsomes (0.18 Unit/ μ l) respectively, were prepared with the addition of storage buffer

and phosphate buffer (pH7, 71 mM). The mixture consisting of PI and NBD-phytoceramide was combined with the microsome mix to get a final volume of 40 μ l aliquoted into a v-shaped 96-well plate. The hit compounds were assayed at 10 μ M in duplicate. Mixture was incubated for 60 mins at 30°C and quenched with 200 μ l of HPLC grade methanol. IPC was eluted from the resin using 4x 50 μ l potassium formate solution (1 M) in methanol and fluorescence quantified at λ_{Ex} 480 and λ_{Em} 540. Compounds showing an average of \geq 30% inhibition were taken on to the next stage for IC₅₀ determination. For IC₅₀ determination compounds were diluted (1:3) to obtain compound concentrations ranging from 68 nM-100 μ M, and screening carried out in triplicate for each hit. IC₅₀ was determined using GraphPad Prism.

7.7 Method for plant-based experiments

7.7.1 Agrobacterium transformation

Competent cells of agrobacterium strains GV3101 (for localisation experiments in tobacco) and C58C1 (for agrobacterium mediated plant transformation) were transformed with 1 μ l of expression vectors (pK7FWG2, pK7WG2 and pK7GWIWG2 (I) respectively) harbouring *AtIPCS1-3*. Cell were heat shocked for 5 mins at 37°C and 1 ml of lysogeny broth (LB) added and then incubated at 28°C for 3 hours at 140 rpm. Transformants were plated on LB (100 μ g/ μ l Rifampicin and 100 μ g/ μ l Spectinomycin) and incubated for 3 days at 28°C.

7.7.2 Agrobacterium mediated plant transformation

Following transformation of agrobacterium strain C58C1 with binary constructs, agrobacterium cells were cultured overnight in Lb (10 ml, 100 μ g/ μ l Rifampicin and 100 μ g/ μ l Spectinomycin) and then scaled up for growth in 200ml LB to use for plant

transformation. Wildtype *A. thaliana* plants were transformed using the floral dipping method⁴.

7.7.3 Plant growth conditions

Arabidopsis thaliana plants were grown on 0.8% Murashige and Skoog (MS) agar and transferred on to peat plugs after 10 days. Plants were grown at 20°C under 16h day/8 h night photoperiod.

7.7.4 Plant transformants selection

Transformed plants were grown to maturity, and the seeds collected. Selection of positive transformants was carried out on MS agar (50 µg/µl Timentin and 50 µg/µl Kanamycin) which were incubated for 6 days.

7.7.5 Tobacco infiltration

Agrobacterium GV1310 strain harbouring GFP::AtIPCS1-3 and ST RFP Golgi marker respectively were grown in Lb media (100 µg/µl Rifampicin and 100 µg/µl Spectinomycin). GV1310 agrobacterium strain harbouring a vector encoding a p35S cytosolic GFP marker was also grown in Lb media (100 µg/µl Rifampicin and 100 µg/µl Kanamycin) overnight at 28°C. cell density at OD₆₀₀ was quantified and diluted to an OD₆₀₀ = 0.1 for GFP::AtIPCS1-3 and ST RFP, OD₆₀₀=0.3 for p35S cytosolic GFP marker with a solution of MgCl₂ (10 mM). *Nicotiana benthamiana* was co-infiltrated with agrobacterium harbouring vectors encoding ST RFP and GFP::AtIPCS1-3 respectively. *N. benthamiana* was also infiltrated with agrobacterium encoding the p35S GFP cytosolic marker separately. After 48 hours, plants were viewed using the Leica Stereo Fluor M165FC and Leica SP5 CLSM FLIM FCCS microscopes.

7.7.6 *In-vivo* screening

A. thaliana seedling were grown for 10 days on 0.8% MS agar and then transferred to 1.2% MS agar containing compounds at a final concentration of 10 μ M and 40 μ M.

7.8 Bioinformatics analysis

Opening reading frames that encode for IPCS homologues in different plant species were identified by running BLAST search of *At*IPC1 nucleotide coding sequence against each species genome using the phytozome database (<https://phytozome.jgi.doe.gov/pz/portal.html>). Multiple amino acid sequence alignments of *At*IPC1-3, *Os*IPC1-3 and *Ench*IPC1 was carried out using ClustalW⁵ and shading done using Boxshade. Phylogenetic tree was constructed using MEGA 7.0⁶ based on the unweighted pair group method with arithmetic mean (UPGMA) and the evolutionary distances calculated using the maximum composite likelihood method⁷. Transmembrane topology was predicted using the transmembrane hidden Markov model (TMHMM) server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>)⁸. Conserved motifs in the promoter region, 1-1.5 kb upstream of the initiation codon were identified using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>)⁹ and PlantPAN 2.0 (<http://plantpan2.itps.ncku.edu.tw/>)¹⁰.

7.9 Materials preparation for RNASeq experiment

For RNASeq experiment, Col-0 and over-expressors of *At*IPC1, 2 and 3 were grown for 10 days and flash frozen in nitrogen. RNA extraction was carried out using the Plant RNeasy kit from Qiagen according to manufacturer's protocol, and the integrity of the RNA determined by running the samples on an Agilent 2100 Bioanalyzer to obtain a RIN

score. The RIN score was determined by measuring the ratio of 28S to 18S rRNA in the RNA sample, with a 1:2 ratio of 18S:28S being ideal for a RIN score of 10¹¹. Following DNase treatment, quality control of the samples were carried out using the Agilent 2100 Bioanalyzer before proceeding to sample preparation for RNASeq experiment.

7.9.1 RNASeq Experiment

Single end libraries for RNASeq were generated from DNase treated total RNA using Illumina Truseq stranded mRNA sample preparation kit and instruction manual. Briefly, mRNAs were fragmented and purified for use as template for the synthesis of double stranded cDNA. End repair of the double stranded cDNA was carried out and the 3' end adenylated. Sample specific indexing adapters were ligated to the ends of double stranded cDNA samples and amplified by PCR, then purified. Samples were normalized, pooled and then sequenced using Nextseq500 instrument to obtain 150 base pair single reads.

7.9.2 RNASeq analysis

The RNA sequence in Fastq format¹² were filtered and trimmed (sliding window 4:15 and 50 bp minimum) to remove low quality reads using Trimomatic¹³. Reads were aligned to the *Arabidopsis* genome (*Arabidopsis* Araport 2017) using STAR¹⁴. The sequence alignment files were sorted by name¹⁵ for HTSeq-count and indexed using SAMtools¹⁵. Files were then converted to BAM files and number of read mapped onto a genes calculated using HTSeq package¹⁶. Gene counts were normalized and compared sample by sample using DESeq2¹⁷ from Bioconductor¹⁸ in R¹⁹. Principle component analyses of the normalized counts was done in R using the DESeq package. Differential expression between over-expressors and Col-0 was determined using the DESeq package with a Log₂ fold-change output. GO term enrichment was performed for analysis of genes upregulated and downregulated in both biological replicates of each *AtIPCS* isoform over-expressor

using the agriGO analysis tool (<http://bioinfo.cau.edu.cn/agriGO/analysis.php>) with the default settings²⁰.

8.0 References

1. Earley, K. W.; Haag, J. R.; Pontes, O.; Opper, K.; Juehne, T.; Song, K.; Pikaard, C. S., Gateway- compatible vectors for plant functional genomics and proteomics. *The Plant Journal* **2006**, *45* (4), 616-629.
2. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **1976**, *72* (1-2), 248-254.
3. Mina, J., Thesis: Exploring IPC synthase: a potential anti-leishmanial drug target. **2010**.
4. Clough, S. J.; Bent, A. F., Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The plant journal* **1998**, *16* (6), 735-743.
5. McWilliam, H.; Li, W.; Uludag, M.; Squizzato, S.; Park, Y. M.; Buso, N.; Cowley, A. P.; Lopez, R., Analysis tool web services from the EMBL-EBI. *Nucleic acids research* **2013**, *41* (W1), W597-W600.
6. Kumar, S.; Stecher, G.; Tamura, K., MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution* **2016**, *33* (7), 1870-1874.
7. Tamura, K.; Nei, M.; Kumar, S., Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America* **2004**, *101* (30), 11030-11035.
8. Krogh, A.; Larsson, B.; Von Heijne, G.; Sonnhammer, E. L., Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology* **2001**, *305* (3), 567-580.
9. Lescot, M.; Déhais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van de Peer, Y.; Rouzé, P.; Rombauts, S., PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic acids research* **2002**, *30* (1), 325-327.
10. Chow, C.-N.; Zheng, H.-Q.; Wu, N.-Y.; Chien, C.-H.; Huang, H.-D.; Lee, T.-Y.; Chiang-Hsieh, Y.-F.; Hou, P.-F.; Yang, T.-Y.; Chang, W.-C., PlantPAN 2.0: an update of plant promoter analysis navigator for reconstructing transcriptional regulatory networks in plants. *Nucleic acids research* **2016**, *44* (D1), D1154-D1160.
11. Schroeder, A.; Mueller, O.; Stocker, S.; Salowsky, R.; Leiber, M.; Gassmann, M.; Lightfoot, S.; Menzel, W.; Granzow, M.; Ragg, T., The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC molecular biology* **2006**, *7* (1), 3.
12. Cock, P. J.; Fields, C. J.; Goto, N.; Heuer, M. L.; Rice, P. M., The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. *Nucleic acids research* **2009**, *38* (6), 1767-1771.
13. Bolger, A. M.; Lohse, M.; Usadel, B., Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30* (15), 2114-2120.
14. Dobin, A.; Gingeras, T. R., Mapping RNA- seq Reads with STAR. *Current protocols in bioinformatics* **2015**, 11.14. 1-11.14. 19.

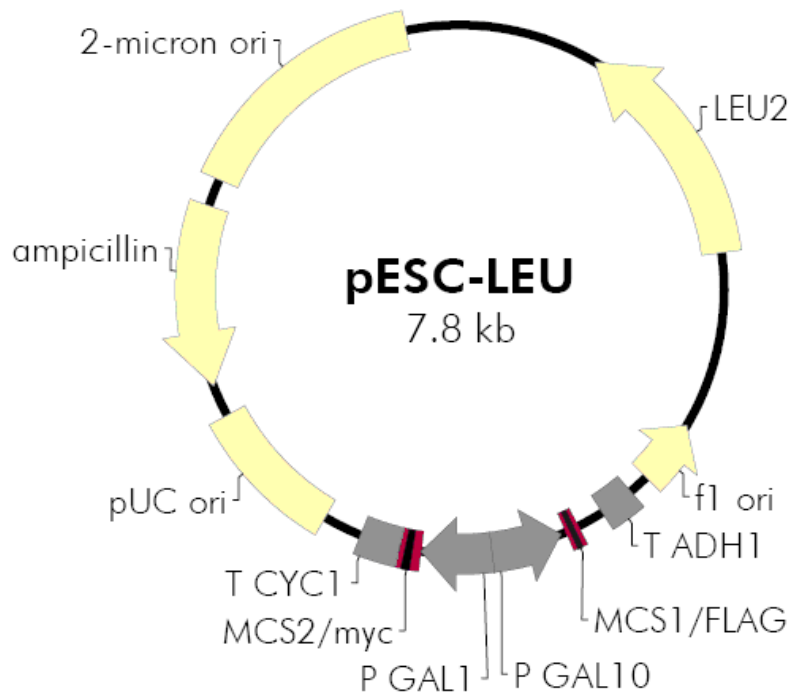
-
15. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R., The sequence alignment/map format and SAMtools. *Bioinformatics* **2009**, *25* (16), 2078-2079.
 16. Anders, S.; Pyl, P. T.; Huber, W., HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **2015**, *31* (2), 166-169.
 17. Love, M. I.; Huber, W.; Anders, S., Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* **2014**, *15* (12), 550.
 18. Huber, W.; Carey, V. J.; Gentleman, R.; Anders, S.; Carlson, M.; Carvalho, B. S.; Bravo, H. C.; Davis, S.; Gatto, L.; Girke, T., Orchestrating high-throughput genomic analysis with Bioconductor. *Nature methods* **2015**, *12* (2), 115-121.
 19. Ihaka, R.; Gentleman, R., R: a language for data analysis and graphics. *Journal of computational and graphical statistics* **1996**, *5* (3), 299-314.
 20. Du, Z.; Zhou, X.; Ling, Y.; Zhang, Z.; Su, Z., agriGO: a GO analysis toolkit for the agricultural community. *Nucleic acids research* **2010**, *38* (suppl_2), W64-W70.

APPENDICES

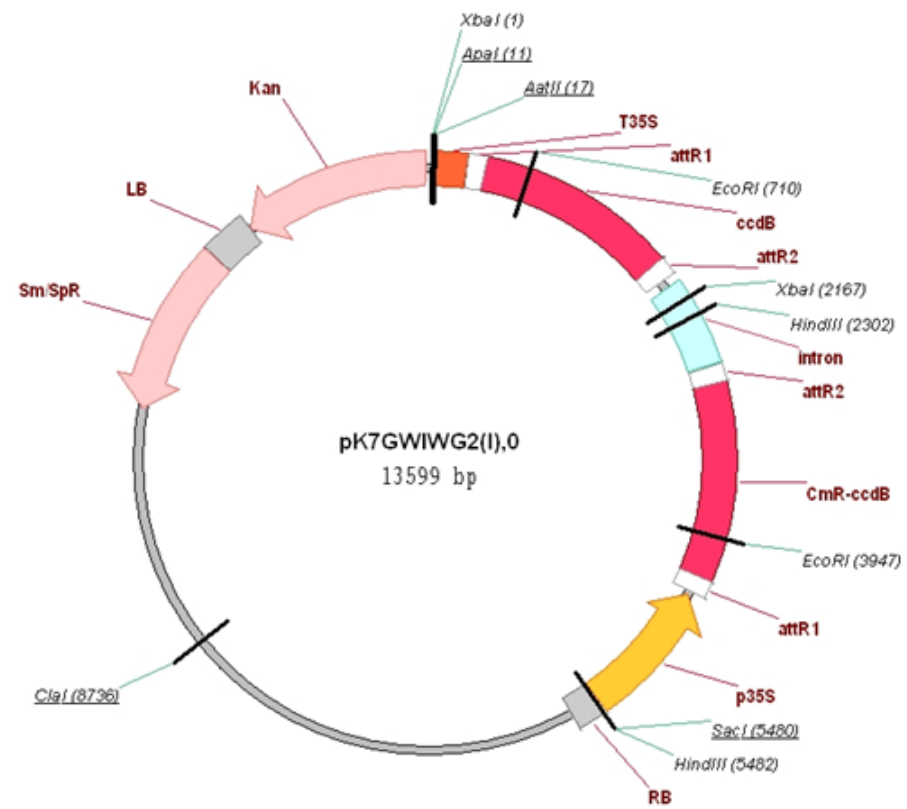
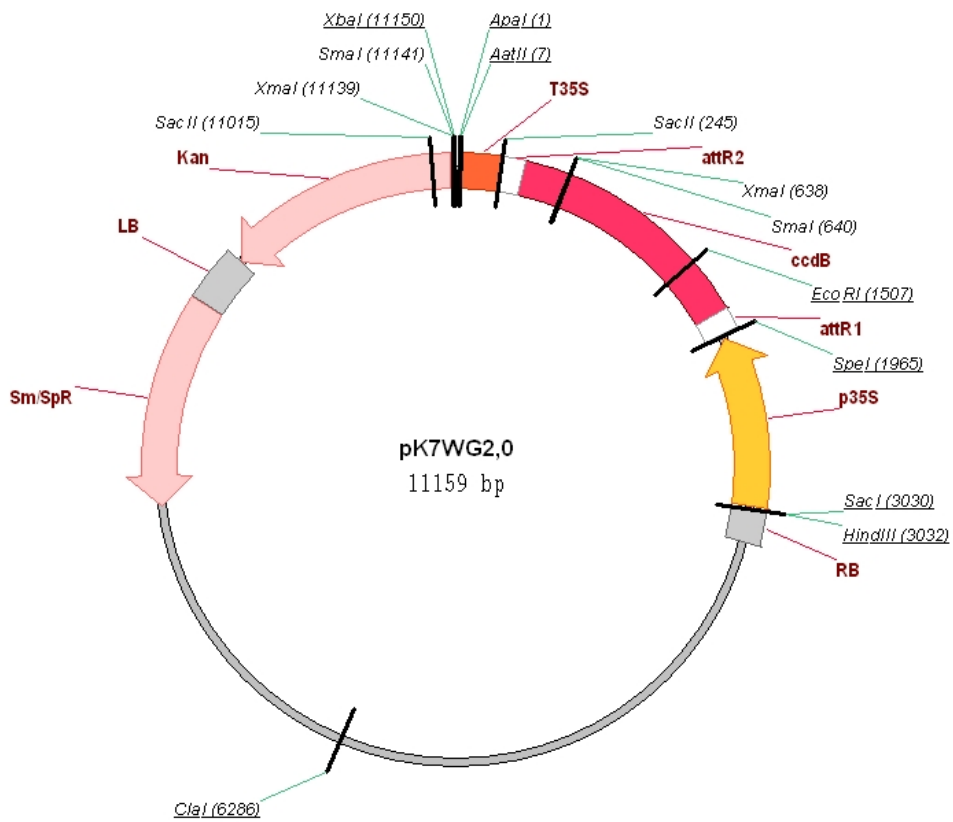
APPENDIX A – Transcription factor binding site motif sequence

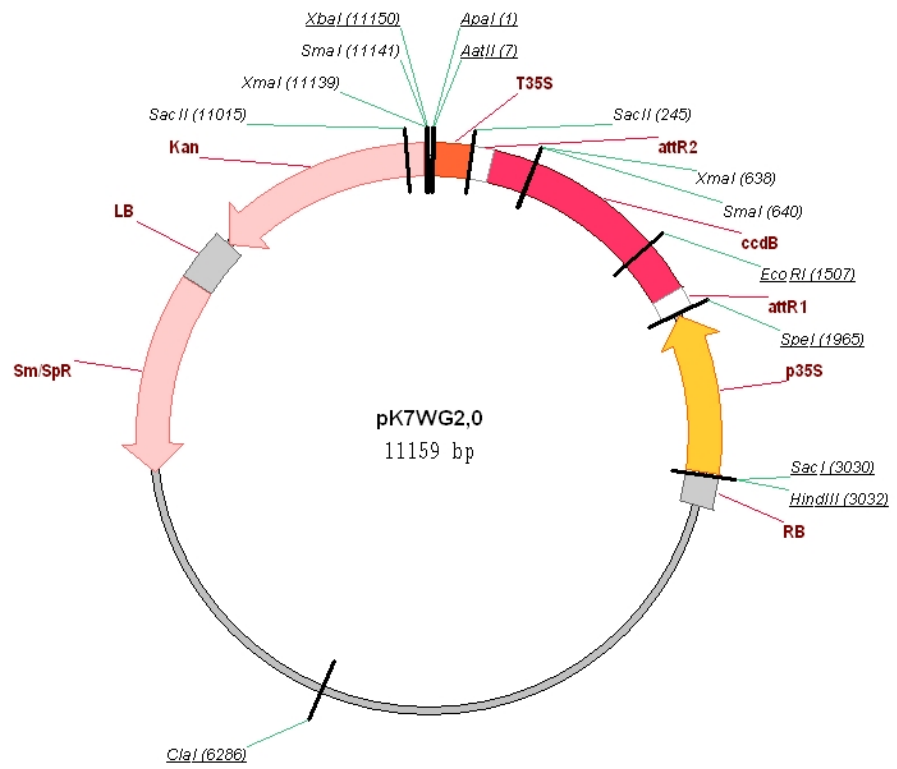
| Transcription factor | Motif sequence |
|-----------------------------|-----------------------|
| LS5ATPR1 | TCTACGTCAC |
| LS7ATPR1 | ACGTCATAGA |
| MYB1LEPR | GTTAGTT |
| WRKY 4 | TTGACCT |
| WRKY 18 | TTGACCT |
| WRKY 60 | TTGACCT |
| TGA 1 | TGACG |

APPENDIX B – pESC-LEU vector map

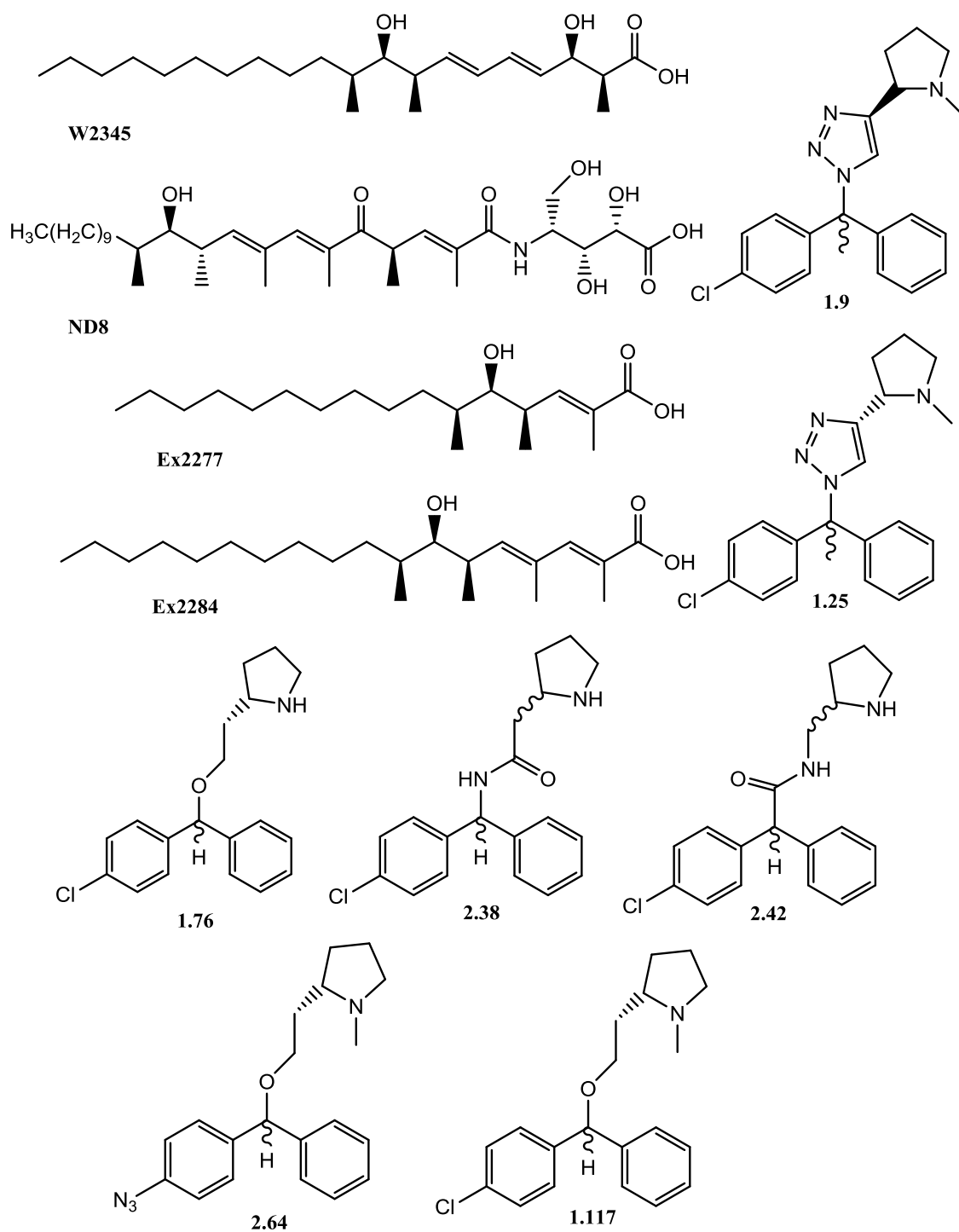


APPENDIX C – vector maps of expression plasmids used for plant genetic work





APPENDIX D – Structure of compounds tested against *At*IPCS1 complemented yeast strains



APPENDIX E – Primer sequences

Primers for infusion cloning of *At*IPCS 1,2, and 3 into pESC-LEU vector:

*At*IPCS1 FP: CTCACTATAGGGCCCCGGGATGACGCTTTATATTCGCCGC

*At*IPCS1 RP: TCCATGTGCGACGCCCGGGTCATGTGCCATTAGTAGCATT

*At*IPCS2 FP: CTCACTATAGGGCCCCGGGATGACACTTTATATTCGTCGT

*At*IPCS2 RP: TCCATGTGCGACGCCCGGGTCACGCGCCATTCATTGTGTT

*At*IPCS3 FP: CTCACTATAGGGCCCCATGCCGGTTTACGTTGATCGC

*At*IPCS3 RP: TCCATGTGCGACGCCCGGGTCAATGATCATCTGCTACATT

*Os*IPCS1 FP: CTCACTATAGGGCCCCGGGATGGCGGTTTACATCGCTCG

*Os*IPCS1 RP: TCCATGTGCGACGCCCGGGTCATGTGCCATTGGGAGTGGC

*Os*IPCS2 FP: CTCACTATAGGGCCCCGGGATGACGATTTACATAGCGCGG

*Os*IPCS2 RP: TCCATGTGCGACGCCCGGGTCATGTACCACCATTGACAGC

*Os*IPCS3 FP: CTCACTATAGGGCCCCGGGATGTCGGCCTTGTACCTTGCT

*Os*IPCS3 RP: TCCATGTGCGACGCCCGGGTCATGTTTTTGTGTTTCGGT

Internal primers for validation of IPCS expression in yeast mutants by colony PCR:

*At*IPCS1 FP: GGTCTGAGCTTGCCAGG

*At*IPCS1 RP: TGCCGTTGTTGATCAGG

*At*IPCS2 FP: CCTAACTATCACTGCCGT

*At*IPCS2 RP: GAGCAACACAGCAGTCCG

*At*IPCS3 FP: CGAGAAGGCTCCAAGCTC

*At*IPCS3 RP: GGTCCACTAGAACGTTCT

*Os*IPCS1 FP: TCTTGCTTGGCTGATGGC

*Os*IPCS1 RP: GTGGTTGATGTCCTCGCC

*Os*IPCS2 FP: GCTTCTTGCTTGGTTCAT

*Os*IPCS2 RP: GGCGTGATTGATGTCTTC

*Os*IPCS3 FP: GGTGCGGTGACTTGATAT

*Os*IPCS3 RP: CAATGTGCTTCCCATTCA

AUR1 FP: GGTCCACCAACTGTTTTGC

AUR1 RP: CTCAATTGAAGTGATGACC

Primers for *At*IPCS 1,2, and 3 cloning into pENTR/D-TOPO:

*At*IPCS1-NotI-F: GCGCGCGGCCGCCACAATGACGCTTTATATTCGCCGCG

*At*IPCS1-AscI-R: GCGCGGCGCGCCTCATGTGCCATTAGTAGCATTATCAGTGTG

*At*IPCS2-NotI-F: GCGCGCGGCCGCCACAATGACACTTTATATTCGTCGTGAATCTTCCAAG

*At*IPCS2-AscI-R: GCGCGGCGCGCCTCACGCGCCATTCATTGTGTTATC

*At*IPCS3-NotI-F: GCGCGCGGCCGCCACAATGCCGGTTTACGTTGATCGC

*At*IPCS3-AscI-R: GCGCGGCGCGCCTCAATGATCATCTGCTACATTGTTCTCGTTT

Primers to quantify *At*IPCS 1,2, and 3 transcripts in the overexpression transgenic lines using real-time PCR:

*At*IPCS1_F: TGCGTCCCGTAAACATTACA

*At*IPCS1_R: ACACCGTTCCCATTCAAGAG

*At*IPCS2_F: TACCAGATCGGACTGCTGTG

*At*IPCS2_R: GTGAACTCCGTTGCTGTCAA

*At*IPCS3_F: CTGGGCCGAATTATCATTGT

*At*IPCS3_R: CCTTCGTGTGCCGTATCTTT

Primers to quantify *At*IPCS 1,2, and 3 transcripts in the RNAi transgenic lines using real-time PCR:

*At*IPCS1_F: TGGTTGTCTTCTTCCTCGAC

*At*IPCS1_R: GAGCAGAGATCTCATGTGCC

*At*IPCS2_F: TGGTGGTGTCTGTCTAGAC

*At*IPCS2_R: GATCTTTTCACGCGCCATTC

*At*IPCS3_F: GCATGGTATACTGTGAACCT

*At*IPCS3_R: GGTGATCGGAGACATAAG

APPENDIX F – RNA quality control for RNASeq experiment

