Comparative Analyses of Primary Carbon Metabolism in Parasitic Plant Species

by
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Declaration

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Abstract

Most terrestrial plants make use of beneficial symbiotic associations to obtain nutrients (*eg.* nitrogen (N) and phosphorous (P)) from fungi in exchange for photoautotrophic carbon. However, plant parasitism (defined here as the ability of certain plants to parasitize other living material) has evolved in the plant kingdom and such plants obtain some, or all, of their nutritional needs from a host, which is severely negatively impacted by the parasite. While the physiological adaptations are well studied, the underlying molecular and biochemical mechanisms of plant parasitism remain largely unknown.

As a first approach, a biochemical blueprint of primary metabolites present within parasitic plant species was constructed. The metabolomes of nineteen parasitic plants, ranging from hemi- and holoparasitism to mycoheterotrophism, were profiled *via* gas chromatography mass spectrometry (GC MS) based technology and targeted spectrophotometric assays. Based on these analyses, three important observations were made. First, parasitic plants were severely carbon deprived, despite being successful in colonizing and exploiting their hosts. Second, the levels of organic acids participating in mitochondrial respiration decreased and certain amino acids and soluble protein content increased. This suggests that parasitic plants utilize alternative respiratory substrates to compensate for a limitation in carbon supply. Third, although characterized by reduced carbohydrate pools, minor sugars normally not associated with plant metabolism, dominated the soluble sugar pool. The presence and significance of one of these sugars, namely turanose (α-D-glucopyranosyl-(1→3)-α-D-fructofuranose), was further investigated.

Turanose biosynthetic reactions could be demonstrated in *Orobanche minor* extracts. Protein purification and mass spectrometry identification suggested that turanose biosynthesis occurred uniquely in parasitic plants. Future work will elucidate the functional significance of turanose metabolism in plant parasitism.

Taken together, this study significantly contributes to our understanding of plant parasitism through development of metabolic signatures associated with distinct parasitic classes. These biochemical profiles highlighted several important strategies and alternative metabolic pathways that are either expressed or constitutively activated during parasitism. This knowledge broadens the scope of using parasitic plants in several biotechnological applications or as a novel research tool to address fundamental questions in plant science.

Opsomming

Meeste landelike plante maak gebruik van voordelige simbiotiese assosiasies met swamme om voedinsgtowwe (bv. stikstof (N) en fosfor (P)) van hulle te verkry in ruil vir koolstof geproduseer deur die plant. Plant parasitisme (gedefinieer hier as die vermoë van sekere plante om ander lewende materiaal te parasiteer) het ontwikkel in die planteryk waar hulle sommige, of al hul voedings stowwe van 'n gasheer plant ontvang, wat erg negatief geraak word deur die parasiet. Terwyl die fisiologiese aanpassings goed gebestudeer is, is die onderliggende molekulêre en biochemiese meganismes van plant parasitisme steeds grootliks onbekend.

As 'n eerste benadering, was hierdie projek geïnisieer om 'n biochemiese bloudruk op te bou van primêre metaboliete teenwoordig in parasitiese plante. Die metabolome van negentien parasitiese spesies, wat wissel van hemi - en holoparasiete tot mikoheterotrofiese plante, is ondersoek deur gas chromatografie – massa spektrometrie (GC MS) gebaseerde tegnologie en geteikende spektrofotometriese toetse. Gebaseer op hierdie ontledings was drie belangrike waarnemings gemaak. Eerstens, parasitiese plante was erg koolstof arm, ten spyte daarvan dat hulle suksesvol is in die aanhegting en ontginning van voedingstowwe vanaf gasheer plante. Tweedens, die vlakke van organiese sure wat deelneem aan mitochondriale respirasie het afgeneem, terwyl sekere aminosure en oplosbare proteïen inhoude toegeneem het. Dit dui daarop dat parasitiese plante gebruik maak van alternatiewe respiratoriese substrate om te vergoed vir 'n beperking in koolstof aanbod. Derde, alhoewel parasitiese plante gekenmerk word deur verminderde koolhidraat inhoude, het skaarse suikers wat normaalweg nie verband hou met plant metabolisme nie, hulle oplosbare suiker inhoud oorheers. Die teenwoordigheid en betekenis van een van hierdie suikers, naamlik turanose (α -D -glucopyranosyl-(1→3)-α-D-fructofuranose), was verder ondersoek. Die sintese reaksie van turanose kan gedemonstreer word in *Orobanche hederae* uittreksels. Proteïen suiwering en massa spektrometrie identifikasie het voorgestel dat turanose biosintese uniek plaasvind in parasitiese plante. Toekomstige werk sal aandui wat die betekenis is van turanose metabolisme in plant parasitisme.

Saamgevat het hierdie studie aansienlik bygedra tot ons begrip van plant parasitisme deur ontwikkeling van metaboliese handtekeninge wat verband hou met onderskeie parasitiese klasse. Hierdie biochemiese profiele beklemtoon verskeie belangrike strategieë en alternatiewe metaboliese paaie wat óf uitgedruk of konstitutief geaktiveer word tydens parasitisme. Hierdie kennis verbreed die omvang van die gebruik van parasitiese plante in verskeie biotegnologiese toepassings of as 'n nuwe navorsings instrument om fundamentele vrae in plant wetenskap aan te spreek.

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''Hell - is sitting on a hot stone reading your own scientific publications''

Erik Ursin

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Publications arising from this thesis

Wiese AJ, Lloyd JR, Kossmann J, van der Merwe MJ. Metabolic requirements of plant parasitism is well-adapted for carbon starvation conditions through modulation of alternative respiratory substrate pools. To be submitted to New Phytologist

Other publications

Gouws L, Botes E, **Wiese AJ,** Trenkamp S, Torres-Jerez I, Tang Y, Hills PN, Usadel B, Lloyd JR, Fernie A, Kossmann J, Van Der Merwe MJ **(2012)** The plant growth promoting substance, lumichrome, mimics starch and ethylene-associated symbiotic responses in lotus and tomato roots. *Frontiers in Plant Science* 3: doi:10.3389/fpls.2012.00120.

Other outputs

Wiese AJ, Lloyd JR, Van Der Merwe MJ. The chemical nature of parasitic and myco-heterotrophic metabolism involves the reconfiguration of substrate usage in order to sustain the tricarboxylic acid cycle. Data from Chapter 3 presented at the 12th World Congress on Parasitic Plants (WCPP^{12}), Sheffield, United Kingdom, 15 – 20 July 2013.

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List of Abbreviations

N	nitrogen
P	phosphorous
%	percentage
°C	degrees Celcius
µg	micro-gram
µL	micro-litre
µm	micro-meter
µM	micro-molar
µmol	micro-mole
13C glucose	isotope-labelled carbon (glucose)
ABA	abscisic acid
abi	*ABA insensitive*
AGPase	ADP-glucose pyrophosphorylase
AKIN10	*Arabidopsis* SnRK
AM	arbuscular mycorrhizal
APGII	angiosperm phylogenetic group II
APX	ascorbate peroxidase
AtHXK1	*Arabidopsis* Hexokinase 1
bdr	below detection range
BSA	bovine serum albumin
CAB	chlorophyll a/b binding protein
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
DAG	days after germination
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e.g.	exempli gratia (for example)
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol tetraacetic acid
ein	*ethylene insensitive*
EMS	ethyl methanesulfonate
EST	expressed sequence tag
et al	et alli (and others)
flu	*Arabidopsis* conditional *fluorescent*
FW	fresh weight
g	gram
g	gravitational force
G6P	glucose 6-phosphate
GC MS	gas chromatography mass spectrometry
gin	*glucose-insensitive*
Glc	glucose

gun	*genome-uncoupled*
h	hour
H_2O_2	hydrogen peroxide
HXK	hexokinase
kDa	kilo-Dalton
kg	kilo-gram
KMC	K-means clustering
kV	kilo-Volt
L	litre
LC MS	liquid chromatography mass spectrometry
LHCB	light harvesting chlorophyll a/b
LUC	luciferase
M	molar
m y a	million years ago
m/z	mass to charge ratio
m^{-2} s^{-1}	metres per second
mg	milli-gram
$mg.ml^{-1}$	milli-grams per milli-litre (concentration)
$MgCl_2$	magnesium chloride
min	minutes
ml	milli-litres
mM	milli-molar
mRNA	messenger RNA
MS	Murashige and Skoog
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
NaCl	sodium chloride
nd	not detected
nl	nano-litres
nl/min	nano-litres per minute
NMR	nuclear magnetic resonance
O_2	oxygen
O_2^-	superoxide
otsA	*E. coli TPS*
otsB	*E. coli TPP*
PC	plastocyanin
PKIN1	potato SnRK
PQ	plastoquinone
PRL1	pleiotropic regulatory locus1
R	resolution
RBCL	large subunit of rubisco
RBCS	ribulose-1,5-biphosphate carboxylase small subunit
RNA	ribonucleic acid
RT	room temperature

RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
ROS	reactive oxygen species
SBE	starch branching enzyme
SDS	sodium dodecyl sulfate
SE	standard error
SLs	strigolactones
SNF1	sucrose non-fermenting 1
SnRKs	SNF1 related protein kinases
SRG	sugar regulated gene
Suc	sucrose
sun	*sucrose-uncoupled*
SuSy	sucrose synthase
SUTs	sucrose transporters
T6P	trehalose 6-phosphate
tin	*turanose-insensitive*
TPH	trehalose phosphate hydrolase
TPP	trehalose phosphate phosphatases
TPP	trehalose phosphate phosphatase
TPS	trehalose phosphate synthase
TPT	triose phosphate/phosphate translocator
Tre	trehalose
treC	*E. coli TPH*
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-HCl	Tris - hydrochloric acid
v	volume
V	volt
v/v	volume/volume
VvHT1	*Hexose transporter 1* from *Vitis vinifera*
w	weight
w/v	weight/volume
WT	wild type
α-Amy	α-Amylase

Chapter 1

General Introduction

Green plants use the process of photosynthesis to produce their own reduced carbon, which can be translocated to sink tissues (*e.g.* roots, developing organs, fruits) for utilization in biosynthetic processes. During plant evolution, certain genera of plants have lost their ability to photosynthesize and have become dependent upon other mechanisms to acquire carbon. This transition to heterotrophy was made possible through the relaxation of evolutionary pressure(s) usually associated with plastid genome maintenance (Bungard, 2004).

Parasitic plants rely on other living material for their supply of carbon (Hibberd et al., 1999). Photosynthetic capacities among these plants vary greatly, where some parasitic plants partly retain their ability to photosynthesize and are only partially dependent on their plant host (hemiparasitic plants), while others are unable to grow without host derived carbon (holoparasitic plants) (Hibberd and Jeschke, 2001). The above-mentioned parasitic plants all share a common feature by developing a haustorium, a root-like structure that function in host attachment, invasion and solute redirection (Fernandez-Aparico et al., 2008; Westwood et al., 2010) (see also section 2.3.3). However, parasitic plants can also obtain carbon and mineral nutrients indirectly from other plants in a process called mycoheterotrophism (Leake, 1994). During mycoheterotrophy, parasitic plants cheat their mychorrhizal fungal partner for essential carbon and nutrients (Smith and Read, 2008). Mycoheterotrophs do not develop haustoria but, similar to the other parasitic classes mentioned, can be distinguished between partial and fully parasitic depending on their photosynthetic ability.

To date, parasitic plants have received wide-spread attention due to their detrimental effects on crop yield and economic losses (Scholes and Press., 2008) and many research studies on plant parasitism addresses host response or resistance to parasitic invasion (Timko et al., 2012). However, studying plant parasitism may have several potential important consequences or provide novel insights into several interesting aspects of higher plant functions. Some of these important features to study range from the discovery of novel germination stimuli to the potential to uncover several important aspects of photosynthetic performance. Selected examples of these potential interesting research topics are listed below.

- Elucidating the mobile low molecular weight cues promoting *Striga* germination has led to the discovery of strigolactones (SLs) (Cook et al., 1966), a phytohormone exuded by host roots (Yoneyama et al., 2008) (see also section 2.3.1). SLs are implicated in a number

of important physiological roles in higher plants, including germination (Cook et al., 1966), root and shoot branching (Gomez-Roldan et al., 2008), arbuscular mycorrhizal colonization (Akiyama et al., 2005), as well as nutrient (Yoneyama et al., 2007) and abiotic stress responses (Bartoli et al., 2012).

- Holoparasitic plants only retain between 31 and 42 plastidial encoded genes (Wolfe et al., 1992b; Delannoy et al., 2011), compared to ~136 genes present in the plastid genome of the dicotyledonous model species, *Arabidopsis thaliana* (Sato et al., 1999). The genes that are retained in the parasitic lineages encode proteins involved in several steps of tetrapyrrole biosynthesis (Wickett et al., 2011), metabolite intermediates presumed to be involved in plastidial retrograde signaling (Strand et al., 2003; Terry and Smith, 2013). If parasitic plants still maintain plastidial retrograde signaling, studying them has the potential to uncouple the metabolic signals generated during plastidial dysfunction from photosynthetic performance and associated sugar metabolism.
- Parasitic plants can also be studied as useful models for tRNA import mechanisms as all the genes for the plastidial encoded machinery has been lost during evolution (Taylor et al., 1991; Wolfe et al., 1992a; Lohan and Wolfe., 1998).
- Increases in point mutation rates also decrease plastidial RNA editing sites needed for the correct expression of essential plastid localized genes in higher plants (Tillich and Krause, 2010). While this reduction could be due to the disappearance of their editing targets from the plastome (plastid genome), a more comprehensive investigation could serve as an important discovery tool for either alternative functions of these splicing and editing factors, or, alternatively, the level of coordination for optimizing plastidial function.
- Due to the achlorophyllous nature of holoparasitic plants, the energy-consuming process of photorespiration (*i.e.* the detoxification of the metabolite 3-phosphoglycerate produced during the oxygenation reaction of the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO)) are eliminated. The alterations and comparison between parasitic plant groups differing in their photosynthetic ability can provide useful clues to the plasticity observed for photorespiratory responses (Timm et al., 2012).
- Interestingly, plant parasitism has evolved in the same time scale (~20-40 m y a) (see also below) as C4 plants. C4 plants minimize photorespiration by the use of specialized leaf structures known as Kranz anatomy allowing for spatially separated biochemical pathways

that reduces oxygen in the vicinity of RuBisCO (Brown and Bouton., 1993; Nelson and Langdale., 1992). Plant parasitism has not evolved in monocotyledonous plants and further investigation into co-evolutionary mechanisms associated with a reduction in photorespiration and/or the absence of parasitic groups within (particularly) grass species would be of interest.

- Plant parasitism has arisen independently in dicotyledonous lineages (with only one known case in the gymnosperms) (Barkman et al., 2007) as a result of increased mutational rates (Bronham et al., 2013). However, at present it is not clear whether these independent evolutionary events have similar consequences or independent molecular and biochemical adaptations. Elucidating cohort changes, if any, will provide powerful clues to the origin of plant parasitism in plant evolution.
- Furthermore the consequences and modeling of plant evolution in a changing global climate can be enhanced by studying the alternative lifestyles of parasitic plant species. Indeed, modulation of parasitic metabolism upon elevated CO_2 concentrations suggests a high variability in growth responses in C3 host species (Matthies and Egli, 1999) while C4 hosts reduce the deleterious impacts of parasitic plants on growth performance (Watling and Press, 1997).
- Parasitic plants (with the exception of the mycoheterotrophy group) also develop specialized root-like structures called haustoria (De Candolle, 1813; Kuijit, 1969). This type of organogenesis is an interesting topic for the developmental genetic control of cell specialization and proposed neofunctionalization of gene products.
- Flowers from the parasitic plant *Rafflesia arnoldii* produces one of the largest single flower blooms recorded (diameter size may be over one meter and weighing 7 kg) (Davis et al., 2008) and macro evolutionary rate estimates suggest a bias in organ size selection within these species (Barkman et al., 2008), providing essential clues to natural selection during floral size evolution.
- Parasitic plants may also have several other useful applications. Extracts of certain parasitic plants are used in Eastern medicine and some exhibit anti-microbial activities (Xu et al., 2009; Cui et al., 2013; Meng et al., 2013), while fruits (Story, 1958), young shoots (Hiepko, 1979) and even flowers (Hooker, 1855; http://www.parasiticplants.siu.edu/ParasiteFood.html) are consumed in traditional

cultures. In addition, certain parasitic species serve ornamental and olfactory purposes in the household and perfume industries (http://waynesword.palomar.edu/ plnov99.htm).

- Moreover, parasitic plant species have been used as successful biological control agents for the control of invasive alien species (Shen et al., 2011; Li et al., 2012). It also contributes to species diversity (flora and fauna) in forests by acting as an important food and nutrient source during plant senescence (Watson and Herring, 2012; Fisher et al., 2013).
- In theory, parasitic plants can also be utilized as alternative bioreactors with many useful biotechnological application to harness or engineer useful products from these specific species; however, the biochemical potential within parasitic plants remain unknown.

With these attributes in mind and as a first and innovative approach, this study addresses the biochemical characterization of parasitic plants on a high-throughput, species-wide level.

Aims, Objectives and Layout of Thesis

The overall aim of this study was the detailed characterization of primary carbon metabolism in parasitic plants. More specifically, the identity, abundance and potential regulatory mechanisms of soluble sugar, amino acid and organic acid metabolism were investigated in a range of nineteen parasitic plant species (Aim1, Chapter 3). Furthermore, the biosynthetic and (putative) signaling pathways of a particular sugar, turanose, found to be an important biomarker in holoparasitic plant species, was further verified and elucidated in *Orobanche minor* extracts (Aim 2, Chapter 4&5).

The set objectives for each specific aim included

Aim1

a) Optimization and quantification of a range of soluble primary metabolites spanning the major plant families where plant parasitism occurs.
b) Evaluation of metabolite composition as a potential biomarker to discriminate between different parasitic plant lineages or lifestyles.
c) Identification of potential targets for pathway characterization or regulation to elucidate in future.

Aim 2 (Evaluation of a particular target identified in c) above)

d) Optimization of turanose biosynthetic assays for further characterization studies.
e) Verification of turanose metabolism in *O. minor*.
f) Elucidation of gene products responsible for turanose production in *O. minor*.
g) Evaluation of turanose metabolism in higher plant species.
h) Development of strategies to investigate turanose-specific signal transduction mechanisms.

The significance and implementation of these approaches greatly enhances our current understanding of plant parasitism and possible future directives are discussed in Chapter 6 (General Discussion) for broadening the scope of research questions based on these observations and analyses.

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Chapter 2

Evolutionary and Physiological Overview of Plant Parasitism

2.1 Introduction

Parasitic plants are a serious threat to agriculture, with *Striga* spp. being prevalent in semi-arid areas of Africa where they parasitize crops of cereals and cowpea, bringing about crop losses ranging between 30-90% (Scholes and Press., 2008; Kubo et al., 2009). *Orobanche* spp. predominate the Mediterranean region, where they infect crops of tomato, tobacco, faba bean and sunflower and are responsible for crop losses that can reach 33% in tobacco and 75% in tomato (Die et al., 2007; Kubo et al., 2009). Despite this, few molecular studies have been carried out on parasitic plants. The main exception to this is the genome sequencing efforts that led to the expansion and elucidation of phylogenetic classifications (see also section 2.2.2), as well as recent *de novo* sequencing efforts to characterize plastome and whole transcriptomes (http://ppgp.huck.psu.edu/plants.php). As mentioned in Chapter 2, parasitic plants could serve as potential biotechnological factories producing novel metabolites. Several interesting sugars have been profiled in plants (*e.g.* D-allose in *Oryza sativa*; Fukumoto et al., 2011) and we speculated that we would also profile rare/novel sugars in parasitic plants. In order to give a broad literature review on all topics covered in the next couple of chapters, we will focus on plant evolution with a subsequent section focusing on parasitic evolution and parasitic physiology as a consequence of that. Furthermore, this review will focus on sugar sensing and signaling to give a background on what roles sugars play in plants besides serving as metabolic intermediates. Lastly, a short review will be given on plant metabolomics as this was the major method utilized to characterize the metabolomes of parasitic plants.

2.2 Plant Evolution

2.2.1 Higher Plant Evolution

Prior to photosynthesis, the earth's atmosphere was largely anaerobic (Blankenship and Hartman., 1998). Oxygenic photosynthesis (where H_2O is split leading to the release of H^+ and O_2) is thought to have arisen around 2 billion years ago, as indicated by biogeochemical evidence. This created a sink for atmospheric CO_2, while subsequently increasing atmospheric O_2 concentrations (Blankenship, 2001; Blankenship, 2010; Nelson, 2011). The first photosynthetic organisms were probably anoxygenic, with cyanobacteria later developing two linked photosystems (Blankenship, 2001). When O_2 levels rose to a sufficient level, eukaryotic life started to evolve, acquiring molecular and biochemical functions that enhanced their cellular capacity in response to this atmospheric change. During the process of eukaryotic evolution cell number and

cellular complexity increased, due to the endosymbiotic acquisition of organelles such as mitochondria and plastids from free living α-proteobacteria and photosynthetic cyanobacterium, respectively (Reyes-Prieto et al., 2007).

Higher terrestrial plant life is thought to have evolved from a primitive multicellular aquatic green algal ancestor that migrated to moist terrestrial environments approximately 475 million years ago (m y a; Qui and Palmer., 1999; Waters, 2003). One of their earliest adaptive features was the acquisition of a waxy cuticle, stomata and gametangia (enclosed structures that protect gametes and embryos) in order to enhance desiccation tolerance. Fossil records indicate that terrestrial plants diverged from green algae around 460 m y a (Fig. 2.1; Bateman et al., 1998; Fiz-Palacios et al., 2011). To further ensure survival in the terrestrial environment, early land plants utilized symbiotic associations with fungi to improve nutrient acquisition from their abiotic environments (Bidartondo et al., 2011). Even though arbuscular mycorrhizal colonization is generally associated with roots, these associations are also possible for rootless plants, for example hornworts, where colonization is established in the thallus (Redecker et al., 2000). Fossil evidence indicates that mycorrhizal fungi were present during the same geological age as the earliest land plants (Wang et al., 2009). Taken together, this suggests that these early adaptive features and associations evolved early during autotrophic life and have been maintained since.

Approximately 420 m y a, plants evolved vasculature, which enabled them to transport water and organic carbon throughout the plant (Ligrone et al., 2012; Lucas et al., 2013). This decreased their dependence on living close to water to remain in a hydrated state. Vasculature also provided structural support to the plant, enabling upright growth. Around 375 m y a, vascular plants started producing structures that protected and nourished the developing embryo (Davis and Schaefer., 2011; Lucas et al., 2013). These first seed plants, referred to as the gymnosperms, were subsequently able to colonize drier regions, as the embryo was protected from desiccation within the enclosed seed. About 140 to 180 m y a, angiosperms evolved (Fig 2.1) which produce reproductive structures called flowers (Soltis et al., 2008). These carry seeds within ovaries, which later develop into a fruit, allowing seed dispersal. Seeds of the first angiosperms contained two cotyledons (dicotyledonous), which later fused leading to the evolution of monocotyledonous species.

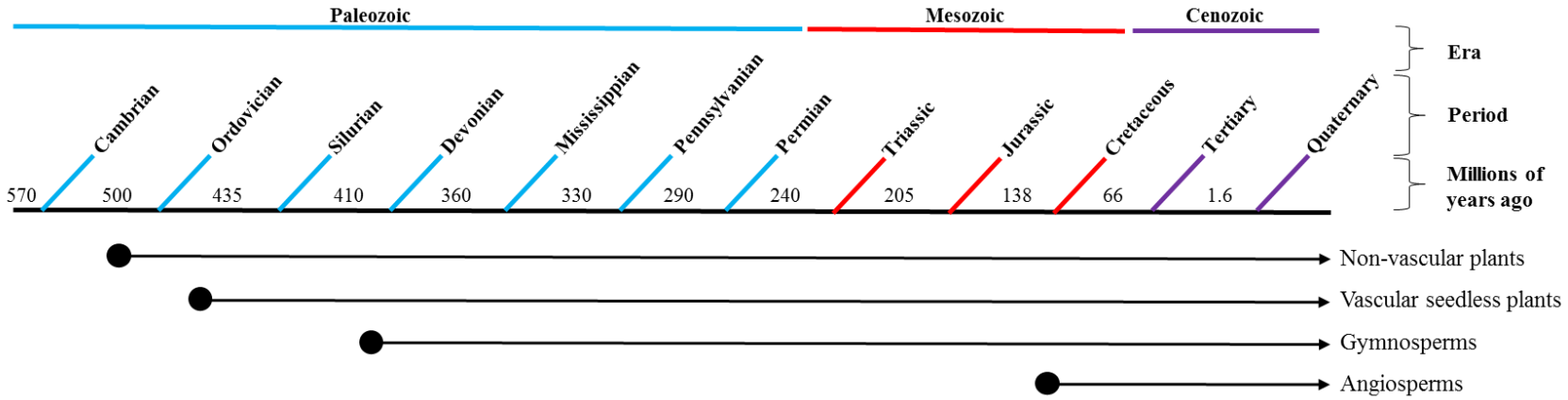

Figure 2.1. A geological time scale of the Phanerozoic Eon, encompassing the major events that took place in land plant evolution. Photosynthesis is thought to have occurred around 2 billion years ago. From there, aquatic plants migrated to land during the mid-Ordovician (~460 m y a). Vasculature evolved during the late Ordovician (~420 m y a) allowing plants to become less dependent on water. Seedplants (gymnosperms) appeared in the late Silurian – early Devonian Period (~375 m y a), allowing plants to spread to drier upland regions, with the evolution of flowering plants approximately 245 million years later in the Cretaceous Period (~130 m y a).

2.2.2 Parasitic Plant Evolution

Parasitic plants are only found in dicotyledonous angiosperms, where parasitism evolved independently 11-12 times (Barkman et al., 2007) and encompass several lineages (Fig 2.2). Approximately 4000 species of parasitic plants are found in 22 families and 265 genera, making up 1% of all angiosperms (Barkman et al., 2007). One can discriminate between parasitic plants based on host dependence and photosynthetic ability. With respect to host dependence parasitic plants can be classified into two groups, namely obligate parasites and facultative hemiparasites. The latter can complete their life cycles in the absence of a host, but will opportunistically parasitize host roots when coming into contact with them (Press, 1989). Obligate parasites on the other hand rely entirely on host contact for the completion of their life cycles. Within obligate parasites, one can discriminate between hemi- and holoparasitic plants based on photosynthetic ability. Hemiparasites are photosynthetically competent, although the degree of competency varies among species (Hibberd et al., 1998). Holoparasitic plants, on the other hand, are completely achlorophyllous and fully dependent on host plants for all their nutritional needs throughout their life cycle (de Pamphilis and Palmer, 1990).

Parasitic plants evolved from autotrophic plants to facultative hemiparasites by acquiring haustoria which allowed them to infect other plants (Westwood et al., 2010). Eventually this led to them developing a reliance on their hosts, accompanied by a narrowing of their host range both through a reliance on specific germination stimulants, as well as the loss of certain genes involved in photosynthesis (Revill et al., 2005). Holoparasitic plants appear to have lost the majority of plastidially localized genes involved in photosynthesis, for example the plastome size of *Epifagus virginiana* is reduced to 71 kilo base pairs, roughly 45% the size of the tobacco plastome (de Pamphillis and Palmer., 1990; Bungard, 2004). A unique parasitic genus within the various groups of obligate parasites is *Cuscuta*, which comprises both hemi- and holoparasitic species with varied photosynthetic abilities (Revill et al., 2005; Krause., 2008). A phylogenetic study using the plastome sequences of four *Cuscuta* species and *Epifagus virginiana*, showed that photosynthetic ability decreases with a loss in plastidially localized photosynthetic genes (Krause, 2008). Interestingly, most species within this genus retained the gene encoding the large subunit of RuBisCO (*rbcL*), the protein product of which, fixes CO_2 during photosynthesis (Bungard, 2004). Peptide sequences inferred from the *rbc*L gene from 15 *Cuscuta* spp show a conservation of the active site of the large subunit, however, photosynthesis among the most photosynthetically

competent species did not reach compensation point (Revill et al., 2005). The selection pressure to conserve *rbc*L might therefore be related to a function distinct from photosynthesis (Krause, 2008).

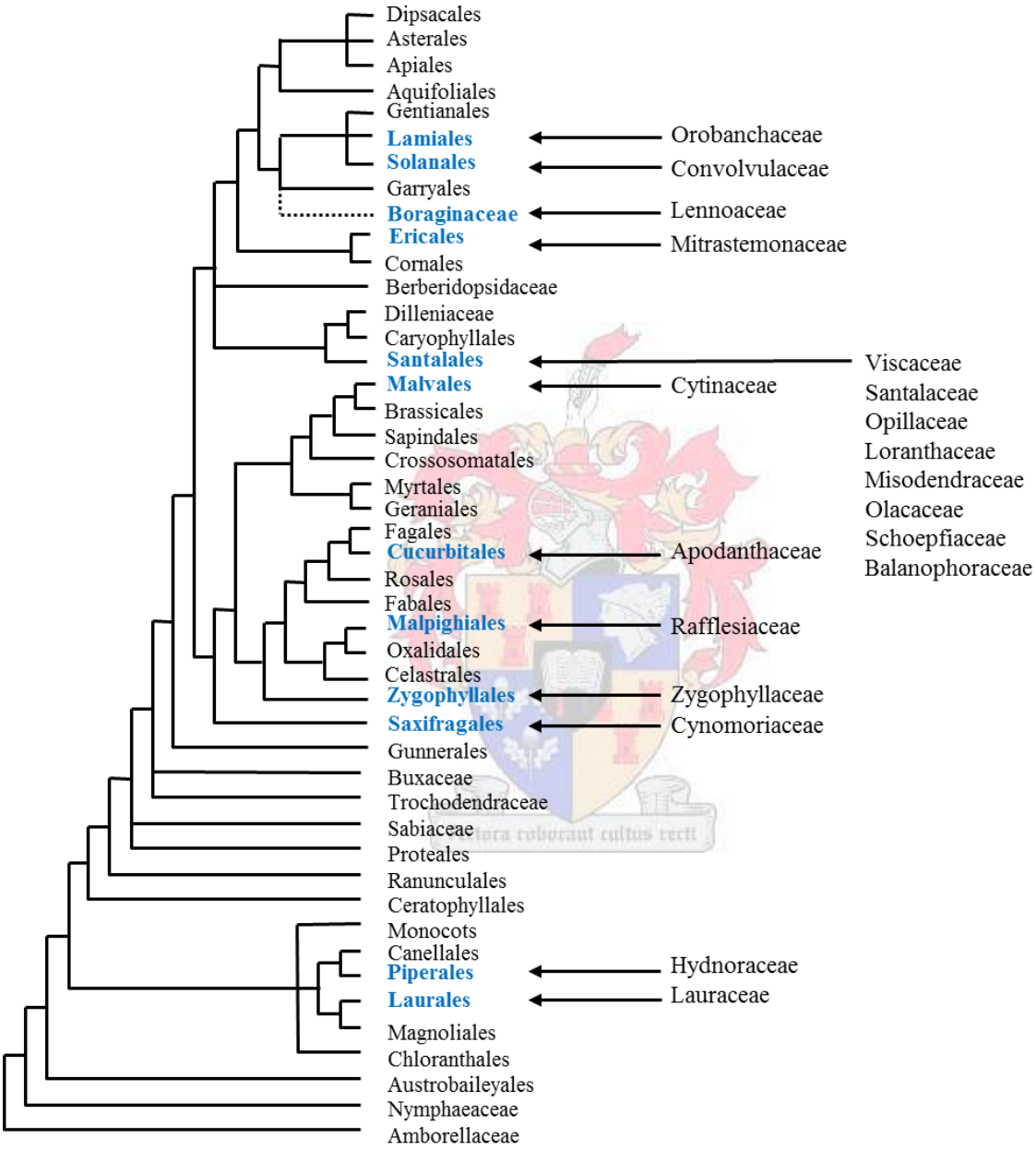

Figure 2.2. A phylogenetic tree constructed using the Angiosperm phylogenetic group II (APGII) system, indicating the lineages from which parasitic plants evolved. As can be seen from this figure, parasitic plants evolved 12 times from independent lineages (highlighted in blue). Indicated

in black arrows are the families containing parasitic species within these orders. (This tree was modified and reproduced with permission from Daniel Nickrent (www.parasiticplants.siu.edu).

Another form of plant parasitism exists whereby achlorophyllous plants parasitize mycorrhizal fungi that colonize the roots of autotrophic plants. The fungi acts, therefore, as a conduit for carbon flow from the autotrophic plant to the parasite This indirect form of parasitism is referred to as mycoheterotrophism a lifestyle that evolved from several lineages in all major plant groups (Watson, 2009). Their numbers are limited in the liverworts and seedless vascular plants, but widespread in vascular seed plants, especially the angiosperms where they occur in large numbers in both dicotyledonous and monocotyledonous plants (Merckx and Freudenstein, 2010). Similar to parasitic plants, one can discriminate between mycoheterotrophic plants based on fungal dependence. Full mycoheterotrophic plants are dependent on fungal supplied carbon and nutrients throughout their life cycles, whereas partially mycoheterotrophic plants utilize small amounts of fungal supplied solutes, even though they are autotrophic (Leake and Cameron., 2010). Lastly, initial mycoheterotrophic plants utilize fungal supplied solutes during seedling establishment, after which they depend on their own photosynthesis (Merckx and Freudenstein, 2010).

2.3 Parasitic Life Cycle and Emerging Molecular Adaptations

A consequence of the switch to a heterotrophic lifestyle was that it brought about major morphological changes in parasitic plants. Some of the changes include the need for germination requirements, haustoria development, transpiration and subsequent nutrient acquisition.

2.3.1 Germination

Parasitic plants germinate in response to chemical stimulants exuded by host roots (Cechin and Press., 1993; Runyon et al., 2009; Auger et al., 2012). Species within the family *Orobanchaceae* produce a large number of tiny seeds which can remain viable in the soil for many years, for example seeds of *Striga* species are on average 0.3 mm in diameter and can remain viable in the soil for over a decade (Cechin and Press., 1993; Vurro et al., 2005; Lambers et al., 2008). These tiny seeds contain scant reserves, necessitating germination close to a host root to

prevent seedling death (Lambers et al., 2008). Germination stimulants become less active the further away from the host plants they are, allowing parasitic plants to judge the availability and distance of a host (Albrecht et al., 1999) which helps to prevent the seedling from dying once germinated. Various germination stimulants have been isolated from both host and non-host species, however, the most effective ones utilized by most parasitic plants are strigolactones (SL; Kubo et al., 2009; Runyon et al., 2009; Auger et al., 2012). Autotrophic plants release SLs to enable arbuscular mycorrhizal (AM) fungi colonization of their roots, with their release being enhanced upon phosphate (Lopez-Raez et al., 2008) or nitrogen (Jamil et al., 2011) deficiency in soil. *Striga* germination negatively correlates with soil fertility, particularly nitrogen (N) availability, for example the germination of *S. asiatica* is reduced by 90% upon the application of N fertilizer on maize under field conditions (Cechin and Press., 1993). This use of host exuded germination stimulants is an elegant example of parasitic plants using their host's natural chemical pathways in order to coordinate their cues for germination.

2.3.2 Haustoria Development

Haustoria are masses of host and parasite tissue, consisting of both xylem and phloem that form a 'bridge' between the two plants (Ackroyd and Graves., 1997; Aly et al., 2009). Similar to germination, haustoria development will only start once a host exuded chemical is perceived by the parasite (Scholes and Press., 2008; Yoshida and Shirasu., 2012). These signals are distinct from germination stimulants and are referred to as haustorial inducing factors (HIFs; Lambers et al., 2008; Scholes and Press., 2008). A range of anthocyanins, flavonoids and simple phenolics exuded by *Zea mays* elicit haustorial differentiation in *Triphysaria versicolor*, marking the transition from vegetative to parasitic growth (Albrecht et al., 1999; Lambers et al., 2008). In order for the haustorium to penetrate the hosts root, parasitic plants produce cell wall degrading enzymes, for example pectin methylesterase (Yoshida and Shirasu, 2012). Following penetration, parasitic vascular cells come into contact with host plant vascular cells, subsequently diverting host sieve tubes and xylem conduits into the parasite (Lambers et al., 2008). Once a vascular connection is established a bridge forms which allows the translocation of water, nutrients and photosynthate from host to parasite (Aly et al., 2011).

2.3.3 Transpiration and Nutrient Acquisition

The haustorium is required for successful parasitism as it allows the withdrawal of solutes from the host (Kubo et al., 2009; Aly et al., 2011). In order to facilitate solute redirection into the parasite a water potential difference strong enough to redirect solute and water flow to them is created (Smith and Stewart., 1990; Ehleringer and Marshall., 1995; Ackroyd and Graves., 1997). This is achieved by ensuring their transpiration rates exceed that of the host plants (Lambers et al., 2008). It has been shown that the transpiration rates of hemiparasitic plants, such as *Striga* species, far exceed that of their host plants (Smith and Stewart., 1990). Factors that contribute to this low water potential in the parasite include an accumulation of solutes that maintain turgor pressure (*e.g.* mannitol in *Striga acuminatum*) and increased stomatal conductance (Ehleringer and Marshall., 1995; Lambers et al., 2008). The extraction of water from the host results in water stress, prompting the host plant to conserve water by decreasing transpiration (Ackroyd and Graves. 1997). As the leaf turgor pressure drops to zero abscisic acid (ABA) concentrations increase, subsequently causing guard cells to close (Smith and Stewart., 1990). The stomata of hemiparasitic plants are insensitive to ABA, resulting in them remaining open (Smith and Stewart., 1990; Ackroyd and Graves., 1997; Lambers et al., 2008).

2.4 Sugar Sensing and Signaling

Part of this study examines the role of a sucrose analog in sugar sensing and signaling in *Arabidopsis*. The rest of this section will survey what is currently known about this in higher plants. Apart from serving as substrates for several metabolic processes sugars also possess hormone-like functions, acting as signaling molecules which affect a range of physiological responses (Sinha et al., 2002). Plants rely on these signaling systems to provide them with information regarding internal (*e.g.* the status/concentrations of cellular metabolites) and external (*e.g.* light, temperature) conditions (Smeekens et al., 2010).

Sugar sensing and signaling was first demonstrated in *Saccharomyces cerevisiae*. This microorganism preferentially ferments sugars to ethanol in the presence of oxygen, giving it an advantage over other microorganisms that are less ethanol-tolerant. It developed sugar sensing and signaling mechanisms that would allow it to adjust its metabolism in order to make exclusive use of the carbon source (Rolland et al., 2006). When carbon availability changes, *S. cerevisiae* adapts by regulating its gene expression (Carlson, 1999). Several components have been implicated in

regulating gene expression *via* sugar signaling, including hexokinase 2 (HXK2), sucrose non-fermenting 1 (SNF1) protein kinase and the metabolic intermediate trehalose 6-phosphate (Rolland et al., 2006). Crosstalk between these pathways allows the yeast cell to adjust its response to carbon availability (Rolland et al., 2002). Findings in yeast prompted researchers to elucidate sugar sensing and signaling mechanisms in plants by complementing yeast mutants with plant orthologs and screening for mutants insensitive to sugars. From these efforts, three components that play a role in sugar sensing and/or signaling have been identified, namely HXK1, SNF1 related protein kinases (SnRKs) and trehalose 6-phosphate (T6P) (Fig 2.3). Only HXK has been verified to be a true sugar sensor in plants, with the roles of the other two components needing further verification.

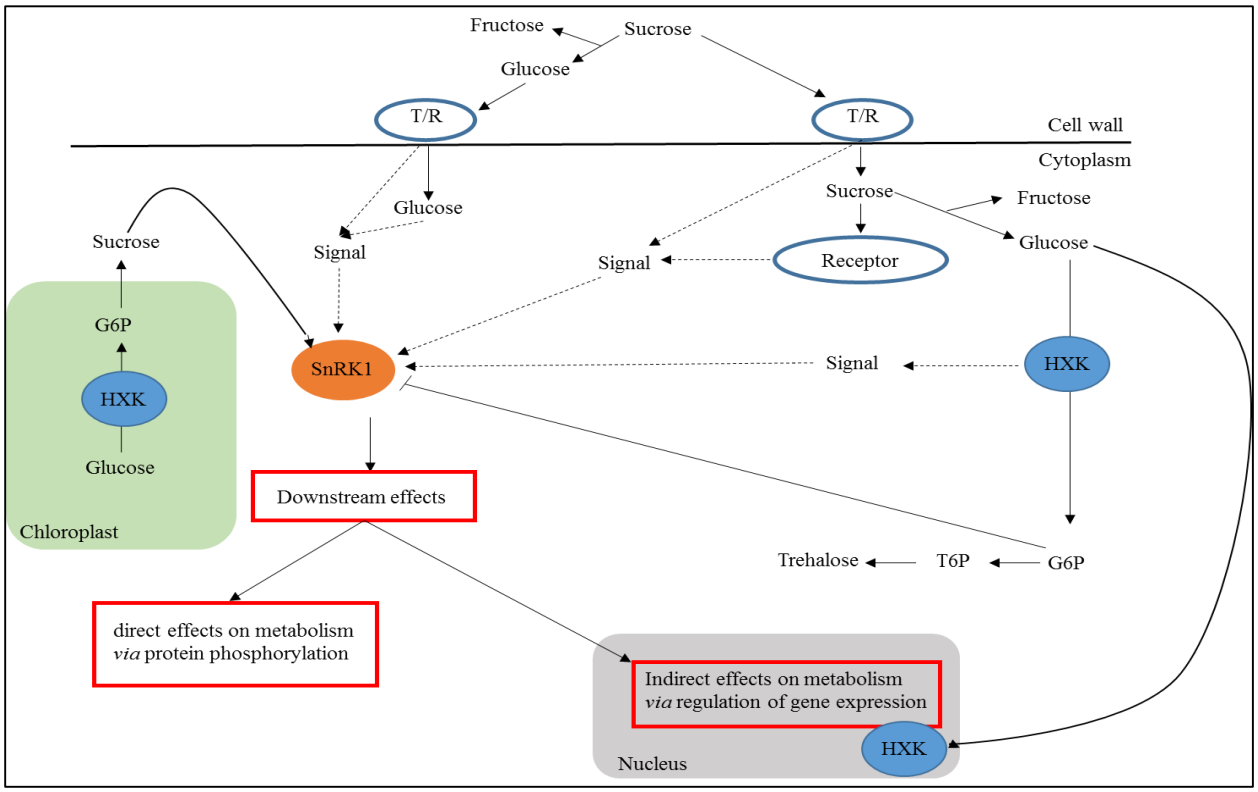

Figure 2.3. A schematic representation indicating sugar sensing and signaling mechanisms in plants (*adapted from Halford et al., 1999*). Dashed lines indicate a signal that is transduced. SnRK1= SNF1 related protein kinases, HXK=Hexokinase, G6P=glucose 6-phosphate, T6P=trehalose 6-phosphate, T/R=transporter and/or receptor.

2.4.1 Hexokinase as a sugar sensor and signaling component

The first evidence for HXK as a glucose sensor in plants came from complementation studies of yeast hexokinase mutants using an *Arabidopsis* expression library. Two *Arabidopsis* HXKs (AtHXK1 and AtHXK2) were identified, sequenced and shown to have high sequence similarity to the yeast and human HXKs (Jang et al., 1997). Plants expressing *HXK* in the sense orientation displayed a hypersensitive response when grown on 6% glucose, while antisense expressing plants were hyposensitive with normal development (Jang et al., 1997). The use of the non-metabolizable glucose analog, 2-deoxyglucose, demonstrated that this effect was not due to glucose metabolism, but rather due to its phosphorylation *via* HXK, making the latter a true glucose sensor (Jang et al., 1997; Sheen et al., 1999). To further establish a signaling function for AtHXK, the expression of three photosynthesis related genes (*chlorophyll a/b binding protein* (*CAB*), *ribulose-1,5-biphosphate carboxylase small subunit* (*RBCS*), *plastocyanin* (*PC*)) were monitored in WT and transgenic lines following exogenous application of glucose (Jang et al., 1997; Xiao et al., 2000). It was found that their expression was down-regulated WT plants, with sense-AtHXK plants showing a more severe repression (Xiao et al., 2000). Antisense-AtHXK plants however show no repression of the three genes, indicating that their regulation is mediated through a HXK-mediated pathway (Xiao et al., 2000). When *Arabidopsis* (*gin2*) hexokinase mutants were transformed with a gene encoding a catalytically inactive hexokinase, complementation of the sugar signaling functions was found to occur (Moore et al., 2003). This demonstrates that signaling is not a result of the activity of hexokinase *per se* (for example as a result of reduced ATP levels), but rather is intrinsic to the protein.

Since then a number of Arabidopsis mutants have been identified which are affected in sugar perception (Table 2.2). Interestingly a number of them are allelic to genes involved in hormone production and perception indicating the involvement of plant growth regulators in sugar signaling. For example, *gin6* is allelic to *abi4*, with the mutant plants able to germinate and develop normally on 7.5% glucose containing media and behaving like glucose-insensitive mutant plants (Arenas-Huerto et al., 2000). Furthermore, the phosphorylatable glucose analog, mannose, is able to inhibit the germination of the *sucrose uncoupled* 6 (*sun6*) mutant, which encodes the AP2 transcription factor that participates in ABA signaling (Huijser et al., 2000). Non-phosphorylatable glucose analogs are unable to do the same, indicating that *sun6* mutants participate in a HXK-mediated signaling pathway (Huijser et al., 2000). The *Arabidopsis gin2* mutant is insensitive to

auxin and hypersensitive to cytokinin, further providing evidence for AtHXK as a component in sugar signaling and indicating crosstalk between glucose and hormone signaling (Moore et al., 2003).

Table 2.1. Crosstalk between sugar and hormone signaling pathways (*adapted from Leon and Sheen., 2003*). *aba2,3/ABA2,3= ABA biosynthesis 2,3; hxk1/HXK1=hexokinase 1, ctr1/CTR1=constitutive triple response 1; abi4/ABI4= ABA insensitive 4.*

Mutant	Allelic to	Phenotype	Gene affected	Gene function	Reference
glucose insensitive (*gin*)					
gin1	*aba2*	growth retardation	*ABA2*	ABA biosynthesis	Zhou et al., 1998; Gibson et al., 2001; Cheng et al., 2002
gin2	*hxk1*	growth retardation; delayed leaf senescence	*HXK1*	HXK1 = glucose sensor and hexose phosphorylation	Rolland et al., 2002
gin4	*ctr1*	growth retardation	*CTR1*	negative regulator of ethylene signaling	Cheng et al., 2002
gin5	*aba3*	wilts	*ABA3*	ABA biosynthesis	Arenas-Huerto et al., 2000
gin6	*abi4*	osmotolerant; salt resistant	*ABI4*	AP2 transcription factor in ABA signaling	Arenas-Huerto et al., 2000
sugar insensitive (*sis*)					
sis1	*ctr1*	osmotolerant	*CTR1*	negative regulator of ethylene signaling	Gibson et al., 2001
sis4	*aba2*	osmotolerant; wilts	*ABA2*	ABA biosynthesis	Laby et al., 2000
sis5	*ctr1*	osmotolerant	*ABI4*	AP2 transcription factor in ABA signaling	Laby et al., 2000
sucrose-uncoupled (*sun*)					
sun6	*abi4*	sucrose, glucose & mannose insentive	*ABI4*	AP2 transcription factor in ABA signaling	Huijser et al., 2000

2.4.2 SNF1-related protein kinases

Hexokinases are not the only kinases involved in sugar sensing and signaling. Another protein kinase implicated is the Sucrose non-fermentable Related Kinase 1 (SnRK1), identified based on similarity to the yeast SNF1 (Alderson et al., 1991). *Arabidopsis* has three SnRK paralogs, with two, *AKIN10* and *AKIN11*, being expressed (Rolland et al., 2006). SnRK1 most likely inhibits biosynthetic and growth processes, while inducing degradation processes thus enabling the plant's survival under starvation conditions (Nunes et al., 2013a; Nunes et al., 2013b). AKIN10 regulates approximately 1000 genes involved in the starvation response (Nunes et al., 2013a). For example, it has been found that AKIN10 and AKIN11 interact with an N-terminal domain of the Pleiotropic Regulatory Locus1 (PRL1) protein, with the *prl1* mutant de-repressing glucose-responsive genes (Bhalerao et al., 1999).

In addition to regulating the plants response to stress conditions, SnRK also regulates genes involved in carbon metabolism (Slocombe et al., 2004). Transgenic potato plants expressing a potato SnRK (*PKIN1*) in antisense orientation driven by a tuber specific promoter show a decrease in SnRK activity (Purcell et al., 1998). Furthermore, *SuS* expression and sucrose synthase activity in tubers decreased (Purcell et al., 1998). When grown on 250 mM sucrose, excised leaves expressing antisense-*PKIN1* are unable to induce *SuS* expression. These data suggest a role for the potato SnRK in the regulation of carbon metabolism (Purcell et al., 1998). Antisense expression of a fragment of a wheat (*Triticum aestivum*) SnRK1 in cultured wheat embryos supplied with or without sugars, show that α-Amy1 and α-Amy2 expression is induced by sugar starvation (Laurie et al., 2003). When tubers discs from potato plants expressing antisense-*SnRK1* were supplied with 200 mM sucrose, redox activation of AGPase took place, suggesting a role for sucrose in the latter *via* a SnRK1 mediated pathway (Tiessen et al., 2003).

2.4.3 Trehalose

Trehalose is a disaccharide made up of two glucose units which serves as a storage reserve and protectant against cellular damage in invertebrates and fungi (Eastmond and Graham., 2003; Schluepmann et al., 2003; Nunes et al., 2013b). In plants, trehalose 6-phosphate (T6P) is formed from glucose 6-phosphate and UDP-glucose *via* trehalose phosphate synthase (TPS) (Goddijn and Dun.,1999; Nunes et al., 2013b). Subsequently, T6P gets dephosphorylated *via* trehalose phosphate phosphatase (TPP), yielding trehalose (Goddijn and Dun.,1999; Nunes et al., 2013b).

Roles for trehalose or intermediates of trehalose synthesis, in sugar signaling have been identified by looking at the expression of photosynthesis related genes upon treatment with these sugars. One of the genes identified to be regulated by trehalose is the SnRK *AtKIN11*, whose expression is induced fivefold upon trehalose feeding of WT seedlings (Schluepmann et al., 2004). Furthermore, trehalose is able to induce *APL3*, which encodes a subunit of the starch synthesising protein ADP-glucose pyrophosphorylase *Arabidopsis* (Wingler et al., 2000). The incubation of tuber discs from WT and antisense-SnRK1 expressing potato lines in 100 mM trehalose for 1h, results in an increase in redox activation of ADP-glucose pyrophosphorylase (AGPase) in WT plants, with near abolished activation in antisense lines (Kolbe et al., 2005). Several genes have been identified whose expression correlates with T6P levels, but which becomes suppressed when sucrose is supplied in the medium (Schluepmann et al., 2004). Further evidence for T6P as a component of sugar signaling, came from expression studies using *E. coli* orthologs of trehalose biosynthesis genes expressed in *Arabidopsis*. Transgenic *Arabidopsis* plants expressing the *E. coli otsA* (*TPS*) gene accumulate T6P, while plants overexpressing *otsB* or *treC* (*TPP* and *trehalose phosphate hydrolase* (*TPH*) respectively), contain low levels of this intermediate (Schluepmann et al., 2003). Seedlings that constitutively expressed *TPS* are dark green and accumulate anthocyanin along the rim of the cotyledons, while plants constitutively expressing *TPP* or *TPH* have cotyledons that expand a few days after the WT with bleached areas (Schluepmann et al., 2003). Transgenic seedlings over expressing *treC* develop normally on 100 mM trehalose media, whereas WT seedlings accumulate T6P and undergo growth arrest (Schluepmann et al., 2004). When metabolizable sugars are added to the latter medium, WT seedlings become rescued (Schluepmann et al., 2004). Furthermore, transgenic *Arabidopsis* lines over expressing *otsA* show a higher redox activation of AGPase than plants over expressing *otsB*, indicating that T6P, rather than trehalose, is involved in the redox activation of AGPase (Kolbe et al., 2005). The importance of this observation *in vivo* has recently been disputed with a larger effect of T6P being noted on starch degradation (Martins et al., 2013). Taken together, these studies point to a central role for T6P or trehalose in sugar signaling.

2.5 Plant Metabolomics

Genome sequencing, mRNA profiling, expressed sequence tag (EST) sequencing along with several other technologies, have allowed researchers to comprehensively analyze the genomes and transcriptomes of organisms (for example Kehoe et al., 1999; The *Arabidopsis*

initiative, 2000). However, recent advances in mass spectrometry have allowed metabolomes (the set of metabolites found within a single organism) to be studied. These can be considered as the biochemical phenotype of an organism, consisting of the end products of gene expression (Oliver et al., 2002).

Plants are thought to contain about 200 000 different metabolites (Bino et al., 2004) and plant metabolomics seeks to identify and quantify these using various analytical technologies such as liquid chromatography-mass spectrometry (LC MS), nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC MS) (Tweeddale et al., 1998 Roessner et al., 2000). Metabolites are subsequently identified by comparing spectra to authentic standards contained within spectral libraries (*e.g.* NIST, Wiley; Bino et al., 2004). Not all of the metabolites within a plant can be profiled due to variance in their chemical properties (Bino et al., 2004). For example, approximately 70% of peaks within a typical GC MS analysis of a plant extract remain unidentified (Bino et al., 2004). Gas chromatography mass spectrometry (GC MS) was first used to quantify metabolites found within potato tuber extracts (Roessner et al., 2000). Over 150 compounds were accurately quantified (Roessner et al., 2000), however, today, using more advanced techniques such as GC/time-of-flight MS technology one can quantify more than 1000 metabolites (Weckwerth et al., 2004).

Plant metabolomics has several applications in plant biology. These include the analysis of transgenic and mutant plants to study the effect of a genetic modification as well as the study of natural variation between plants in order to identify novel biochemical pathways. It has proven useful for the characterization of metabolomes of mutant plants that are phenotypically indistinguishable from the WT. Applying GC TOF MS to distinguish between the metabolomes of WT and sucrose synthase isoform II (SUS2)-antisense expressing potato plants, researchers were able to distinguish between the plants based on the metabolite compositions of their leaves and tubers (Weckwerth et al., 2004). In addition to distinguishing between mutant and WT plants, plant metabolomics can also be used to look at how plants respond to different treatments. Applying both GC TOF MS and LC MS to look at how the metabolome of *Arabidopsis* is affected by different sulfur treatments it was found that, under sulfur-deficient conditions, *Arabidopsis* seedlings catabolize glucosinolates as a sulfur source as their levels were significantly lower than seedlings grown under control conditions (Zhang et al., 2011a). Furthermore, metabolite profiling has also proven useful in distinguishing between different stages of fruit development (Zhang et

al., 2011b) or producing fruit chemotypes (Bhatia et al., 2013). Taken together, these examples together with various other applications in biology, making this technology essential in omics approaches to studying plants.

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Chapter 3
New insights gained from the carbon/respiratory requirements during plant parasitic evolution

New insights gained from the carbon/respiratory requirements during plant parasitic evolution

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3.1. Abstract

Metabolomics is a powerful tool to dissect complex phenotypic and metabolic traits. In parasitic plants, the alteration(s) in metabolic diversity can be potentially exploited in order to either effectively manage the detrimental effects these plants have on agricultural yield or, alternatively, be harnessed to be economically useful. Here we characterized a range of holo- and hemiparasitic (representing both root and shoot parasites) plants spanning six plant genera on a biochemical level through metabolite profiling. A major adaptation was the low levels and prevailing underrepresentation of tricarboxylic acid (TCA) cycle organic acids (notably citrate and malate) in the total organic acid pools. In addition, holoparasitic species were characterized by increased levels of total soluble protein and a 6- to 40-fold increase in the branched chain amino acids, isoleucine and leucine. In contrast to autotrophic plant metabolism, minor sugars (arabinose, turanose and palatinose) dominated the sugar pools, with the sucrose analogues, turanose and palatinose, representing between 20 and 95 % of the total. An underrepresentation of primary carbon intermediates (with the exception of starch), nitrogen (N) assimilating compounds (glutamine) and photorespiratory metabolites (glycine and serine) were hallmarks of the majority of the parasitic plant groups. Taken together, this suggests that the primary carbon metabolism of parasitic plants is uniquely adapted to sustain the resulting parasitic association despite low carbon and nitrogen inputs.

3.2. Introduction

Parasitic plants rely on other living material for their supply of carbon (Hibberd et al., 1999). The transition to this heterotrophic lifestyle was made possible through the relaxation of evolutionary pressure to conserve photosynthetic processes, which led to the subsequent (selective loss) of plastidial genes (Westwood et al., 2010). Photosynthetic capacities among these plants vary greatly, where some retain their ability to photosynthesize (hemiparasites) and others are completely achlorophyllous and unable to grow without host supplied photosynthate (holoparasites) (Hibberd and Jeschke, 2001). These plants develop invasive haustoria which they use to gain access to host plant vasculature (Kubo et al., 2009). Once the connection is established, they function as active sinks redirecting solutes away from autotrophic sink tissues, subsequently leading to a decrease in host biomass accumulation (Dale and Press, 1998). The latter consequence of parasitism leads to crop losses that affect approximately 300 million farmers worldwide, with

annual losses of more than $10 billion in Sub Saharan Africa (Parker, 2009). This has led to research into possible control methods in curbing parasitism of agricultural important crops. Autotrophic plants release strigolactones (SLs) into the rhizosphere to enable arbuscular mycorrhizal (AM) fungal colonization of their roots, which subsequently aids in nutrient acquisition from the soil (Rani et al., 2008). Root parasitic plants (*e.g. Striga, Orobanche* and *Phelipanche* spp) however, have evolved to make use of this system in order to locate host plants, only germinating when SLs are present within their vicinity (Kani et al., 2008; Cardoso et al., 2011). The exogenous application of methionine to the roots of *Lycopersicon esculentum* (tomato) seedlings inhibits the germination of *Orobanche ramosa* seeds, even after a treatment with GR-24, the synthetic strigolactone analog (Vurro et al., 2005). The latter control method proved very promising as the greatest amount of damage caused by parasitic plants takes place during early extraction, once the haustorium is established (Alakonya., 2012). Furthermore, the manipulation of mannitol metabolism in *Orobanche aegyptiaca* showed the potential of biotechnology as a possible and effective control method (Aly et al., 2009). Aside from their devastating effect on agriculture, parasitic plants may also have several other useful applications as discussed in Chapter 2. In order to develop more efficient control methods or harness useful products from them, a more detailed understanding of their metabolism is needed. Our current understanding of parasitic plants on a biochemical level is still very limited and uncovering further information regarding their metabolism could yield more clues on their possible management or potential biotechnological uses. In this study a range of parasitic plant species were profiled and here we reveal new insights into their carbon or respiratory requirements.

3.3. Materials and Methods

3.3.1. Plant material

The following species were investigated in their natural surroundings in the field. Hemiparasitic species included *Moquiniella rubra*[*], *Viscum capense*[*], *Thesidium fragile, Osyris compressa, Osyris speciosa, Thesium spicatum, Thesium aggregatum, Thesium commutatum, Thesium strictum* and *Thesium virgatum*. Holoparasitic species included *Cassytha ciliolate*[*], *Cuscuta campestris*[*], *Harveya squamosa, Orobanche hederae, Orobanche ramose* and *Hyobanche sanguinea*. Autotrophic host plant species included *Lycium ferocissimum, Othonna arborescens,*

Brachylaena nerfolia and *Trachyandra diverticulata*. Two *Phalaenopsis* hybrid orchids at different developmental stages (2 weeks old, 5 months old) were also included into this study. All parasitic plants harvested were flowering at the time of sampling, with replicates sampled from a single population in the field. Autotrophic species selected for this study were only sampled if infected by a parasitic plant. Replicates for autotrophic plants were taken from the same area in the field, with all replicates being parasitized by the same parasite. Samples were carefully excised, rapidly flash-frozen using liquid nitrogen, homogenized and kept at -80^{o}C until sample processing.
* Stem parasites. The rest of the parasitic group are all root parasites.

3.3.2. Starch extraction and measurement

Starch was extracted from plant material and measured spectrophotometrically as described by Müller-Roeber et al. (1992). Starch was visualized in *O. hederae* tubercules using Lugol staining according to Sehnke et al., (2000). Tubercules from *O. hederae* were harvested and destained in boiling 80% (v/v) ethanol, rinsed in distilled water (dH_2O) and stained with the Lugol solution (5% (w/v) iodine, 10% (w/v) potassium iodide). Following staining, the tubercules were briefly destained with dH_2O and placed on a light box for visualization.

3.3.3. Protein extraction and measurement

Protein content was extracted according to Gibon et al (2004). Approximately 20 mg homogenized tissue stored at -80°C was extracted with 500 µL of extraction buffer (10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 1 mM β-mercaptoethanol) and the sample vortexed for 10 min. Samples were left at room temperature for 15 min, after which a second vortexing step took place. These were subsequently centrifuged at 27369 *g* for 10 min and the supernatant transferred to fresh microcentrifuge tubes. This crude protein extract was subsequently used for protein measurement. Protein concentrations were determined based on the measured absorbance values and comparison with a linear authentic standard curve using bovine serum albumin (BSA) as a standard (Bradford, 1976).

3.3.4. Primary metabolite profiling *via* GC MS technology

To analyze the levels of primary metabolites a GC quadropole MS protocol for plant material (Roessner et al., 2001) was adapted and optimized for the metabolite profiling of parasitic tissue. Approximately 100 mg homogenized tissue extracted with 1400 µl 100% pre-chilled methanol and 39.4 µg [^{13}C] U-glucose (in HEPES buffer) added as internal standard. The mixture was incubated for 15 min at 70°C, centrifuged for 10 min at 32000 *g* and the supernatant transferred to clean microcentrifuge tubes. To the supernatant, 750 µl chloroform and 1500 µl deionized water (dH_2O) was added, the samples vortexed for 15 s and centrifuged at 2549 *g* for 15 min. From the polar phase, 150 µl was then dried down under vacuum and the dried residue subsequently re-dissolved and derivatized for 90min at 30ºC in 80 µl 30 mg.ml^{-1} methoxyamine hydrochloride (in pyridine). Subsequently samples were trimethylsilylated by a 30 min treatment at 37ºC containing 140 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). *n*-Alkane retention time standard mixture was also added prior to trimethylsilylation. Samples were then injected splitless into a GC quadropole MS (ThermoFinnigan, UK) and run according to specifications of Roessner *et al*. (2001).

The analysis of primary metabolites was performed on a gas chromatograph, Agilent technologies network GC system, model 6890N, coupled to a Agilent technologies inert XL EI/CI Mass Selective Detector (MSD), model 5975B (Agilent Technologies Inc., Palo Alto, CA). The GC MS system was coupled to a CTC Analytics PAL autosampler. Seperation of the metabolites was performed on a Restek 12723-127 column (30 m, xx mm ID, 0.25 µm film thickness), from Agilent Technologies. Analyses were carried out using helium as the carrier gas with a flow rate of 1.0 ml/min. The injector temperature was maintained at 250°C and the oven temperature programmed as follows: 150 °C for 1 min; and then ramped up to 310 °C at 7 °C/min and held for 2 min and finally ramped up to 320 °C at 15 °C/min and maintained for 7 minutes. The total running time was 33.52 min. The MSD was operated in full scan mode and the source and quad temperatures maintained at 240°C and 150°C, respectively. The transfer line temperature was maintained at 280°C. The mass spectrometer was operated under electron impact mode at ionization energy of 69.92 eV, scanning from 50 to 650 m/z.

Data pre-processing for baseline correction, scaling and alignment was conducted with MetAlign software, with parameters as specified in MetAlign v 200410 (www.metalign.wur.nl/UK/). For targeted metabolite analyses, authentic standards and calibration

curves was constructed and metabolite identities and annotations cross-checked with the Golm metabolome database (csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd).

3.3.5. Turanose enzyme activities

Crude protein extracts from *O. hederae*, was used to measure for turanose biosynthetic activity. Biosynthetic enzyme assays were carried out at room temperature in a 50 mM Tris-HCl buffer (pH 7.0) for 30min containing 2mM putative substrate(s) (sucrose, palatinose, melezitose, glucose and fructose 6-phosphate, fructose and glucose 6-phosphate, fructose and UDP-glucose, fructose and glucose 1-phosphate, fructose and ADP-glucose, fructose 6-phosphate and glucose 6-phosphate, fructose 6-phosphate and UDP-glucose). Reactions were stopped by a 1 min heating period at 95ºC. Samples were centrifuged at 2549 *g* for 15 min and the proteins separated from the sugars using exclusion columns (Amicon ® Ultra Centrifugal Filter Units), with a 3kDa cut off point. Turanose was detected *via* GC QUAD MS technology with running conditions as described previously (Roessner et al., 2001).

3.3.6. Statistical analyses

For statistical analysis of the data, a two-sided homoscedastic *Students t*-test was used (Excel software, Microsoft, Seattle). Analysis of variance (ANOVA) was performed using Statistica software (StatSoft Inc., 1997). Principal component and K-means clustering analyses of the metabolite datasets were performed by the algorithms embedded in http://metagenealyse.mpimp-golm.mpg.de/ using Bayesian PCA for missing value estimation.

3.4. Results

In order to gain a better understanding into the nature of parasitic metabolism, several aspects of haustorial and root primary carbon metabolism were investigated in seventeen parasitic plants (representative of holoparasitic, hemiparasitic and mycoheterotrophic metabolism) and four autotrophic host plants (representative of autotrophic metabolism), respectively (Table S1).

Total protein content assayed indicated that, on average, holoparasitic species contained higher protein levels compared to both hemiparasitic and autotrophic species, with one holoparasite (*Hyobanche sanguinea*) showing a 3.6 fold increase in protein content compared to

the autotrophic *Brachylaena nerifolia* (Table 3.1). Within the hemiparasitic group, *Moquiniella rubra* and *Viscum capense* showed increased protein content compared to the rest of the group. Four autotrophic host plants (*Hedera helix, Lycium ferocissimum, Othonna arborescens* and *Trachyandra diverticulata*) showed a decrease in protein content compared to their parasitic partners (*O. hederae, M. rubra, V. capense* and *Cuscuta campestris* respectively) (Table 3.1). The fourth host plant (*B. nerifolia*) however, showed an increase in protein content compared to its parasitic partner *Cassytha ciliolata*. Protein content assayed from the *Phalaenopsis* orchids showed that upon maturation, when the orchid becomes autotrophic (5 month old *Phalaenopsis*), the plant accumulates less protein (Table 3.1). Starch measurements indicated that holoparasitic (except for *C. ciliolata*) and autotrophic plants accumulate similar levels of starch, with hemiparasitic plants (except for *T. virgatum*) accumulating less (Table 3.1). Furthermore, the young *Phalaenopsis* orchid accumulated less starch compared to the maturing plant.

Next we applied GC MS technology to compare sugar, amino acid and organic acid levels within all four life styles. These measurements indicated that, on average, amino acid content in *Phalaenopsis* and hemiparasitic species were much lower than found in holoparasitic and autotrophic species. Two hemiparasitic plants (*V. capense*, *O. compressa*) however, showed a 3.6 fold increase in amino acid content compared to the rest of the hemiparasitic group. The average amino acid content in the holoparasitic group was similar to that of the autotrophic group, with *O. hederae* showing a significant decrease in amino acid content compared to its host *H. helix*.

Looking at the organic acid content within these plants, we saw high concentrations of organic acids in four hemiparasitic species (*V. capense*, *T. fragile*, *T. commutatum*, *T. strictum*) two holoparasitic species (*H. sanguinea*, *H. squamosal*), both *Phalaenopsis* orchids and the autotrophic plant *H. helix* (Table 3.1). Lesser amounts of organic acid content were observed in *T. spicatum*, *T. virgatum* and *L. ferocissimum*, with the latter having an organic acid concentration of 0.44 mM (a 56 fold reduction compared to its parasitic partner *M. rubra*) (Table 3.1).

Sugar content profiles indicated that *T. fragile*, *T. spicatum*, *H. sanguinea* and the maturing *Phalaenopsis* orchid had relatively high sugar levels (Table 3.1). The maturing *Phalaenopsis* orchid had a two fold increase in sugar content compared to the young *Phalaenopsis* orchid, while the autotrophic plant *H. helix* had a 10 fold increase in sugar content compared to its parasitic

partner *O. hederae*. *Thesium virgatum*, *C. ciliolate* and *O. ramose* showed a severe reduction in sugar content compared to the rest of the parasitic group. The remaining plants had similar sugar levels.

Table 3.1. Major metabolite pools in species investigated. Protein and starch levels were determined for holoparasitic, hemiparasitic and autotrophic plant material as described in the materials and methods section. Protein is expressed as $mg.g^{-1}$ FW and starch as µmol hexose equivalents.g^{-1} FW. Amino acid, organic acid and sugar levels were determined for holoparasitic, hemiparasitic and autotrophic plant material through GC MS, with values expressed as mM. Values are presented as mean ± SE of four individual plants and values determined to be significantly different ($P< 0.05$) from the respective host using *Students t*-test are in bold script. *bdr* – below detection range

	Protein (mg.g⁻¹ FW)	Starch (µmol hexose equivalents.g⁻¹ FW)	Σ Amino Acids (mM)	Σ Organic Acids (mM)	Σ Sugars (mM)
Hemiparasitic Species					
M. rubra	**354.0 ± 44.2**	121.8 ± 4.8	**15.8 ± 2.8**	**24.7 ± 2.8**	**85.6 ± 10.3**
V. capense	**2090.0 ± 254.0**	122.1 ± 11.6	342.0 ± 23.0	**60.6 ± 6.0**	47.9 ± 8.3
T. fragile	227.2 ± 42.1	163.3 ± 22.4	25.9 ± 3.0	56.4 ± 5.7	181.5 ± 10.2
O. compressa	551.1 ± 87.8	143.4 ± 22.8	342.0 ± 10.7	29.0 ± 3.5	32.6 ± 11.3
O. speciosa	312.7 ± 43.7	170.7 ± 8.4	13.0 ± 1.1	28.8 ± 2.0	38.3 ± 12.2
T. spicatum	282.7 ± 21.9	161.8 ± 28.6	128.6 ± 5.3	2.4 ± 0.5	296.0 ± 15.4
T. aggregatum	260.7 ± 16.0	76.1 ± 11.9	52.2 ± 17.2	22.8 ± 1.2	61.2 ± 16.5
T. carinatum	180.3 ± 12.4	157.4 ± 14.7	42.4 ± 13.9	18.9 ± 1.7	34.0 ± 10.8
T. commutatum	413.3 ± 44.6	108.4 ± 21.5	72.0 ± 1.3	63.5 ± 4.2	41.5 ± 2.0
T. strictum	489.0 ± 45.1	142.1 ± 23.0	15.2 ± 3.2	48.4 ± 17.2	68.4 ± 16.9
T. virgatum	313.5 ± 29.1	204.7 ± 30.3	10.1 ± 2.1	2.3 ± 0.7	12.9 ± 4.0
Holoparasitic Species					
C. cilliolata	**450.9 ± 88.9**	*bdr*	108.0 ± 10.7	44.7 ± 4.1	**10.4 ± 2.8**
C. campestris	**1230.0 ± 146.8**	195.8 ± 19.6	109.4 ± 7.7	24.6 ± 2.3	31.7 ± 2.7
H. squamosa	537.6 ± 71.6	181.1 ± 31.5	135.8 ± 9.6	80.8 ± 4.7	16.0 ± 1.7
H. sanguinea	2470.4 ± 218.0	216.6 ± 24.1	677.1 ± 3.9	127.1 ± 13.7	189.9 ± 10.4
O. hederae	**1487.9 ± 141.9**	**204.9 ± 29.9**	**421.8 ± 31.3**	**20.8 ± 2.7**	**47.9 ± 4.4**
O. ramosa	1490.9 ± 260.6	279.1 ± 43.3	342.0 ± 33.8	17.3 ± 4.5	14.5 ± 0.3
Mycoheterotrophic species					

2 week old *Phalaenopsis*	**17.5**	**±**	**4.78**	61.3	±	3.15	91.3	±	5.8	96.7	±	4.6	66.2	±	5.2
5 month old *Phalaenopsis*	**11.8**	**±**	**0.31**	83.0	±	3.9	94.9	±	7.3	69.5	±	4.8	160.4	±	5.9
Autotrophic Species															
L. ferocissimum	202.2	±	35.1	197.0	±	31.0	118.3	±	6.2	0.4	±	0.1	32.1	±	0.7
O. arborescens	555.5	±	71.5	273.9	±	22.4	65.9	±	4.5	11.8	±	0.4	43.3	±	1.8
B. nerifolia	812.7	±	108.4	256.8	±	27.3	118.3	±	10.8	42.8	±	6.2	40.6	±	5.8
T. diverticulata	497.2	±	97.6	217.4	±	16.7	111.0	±	11.8	25.1	±	2.2	29.2	±	4.0
H. helix	34.3	±	2.4	94.8	±	4.0	722.9	±	23.2	130.1	±	7.5	495.7	±	16.2

To look at how these plants cluster together based on metabolome composition, a principal component analysis (PCA) was carried out (Figure 3.1), revealing four major clusters. Subsequently, a K-means clustering analysis was carried out (Figure 3.2) to look at which metabolites are responsible for the clustering observed in the PCA analysis.

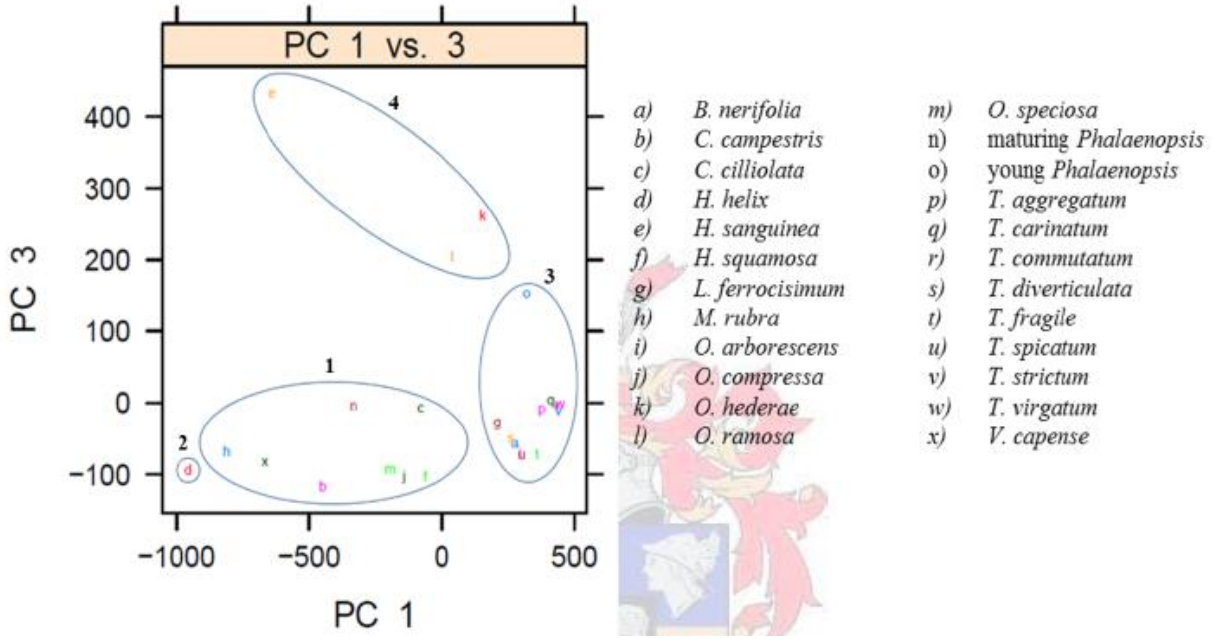


Figure 3.1. A Principal Component Analysis (PCA) of individual metabolites present within all plants. Results are displayed in a plot diagram with PC1 and PC3 plotted against each other. There are four clusters, with species indicated in letters a through x. Species corresponding to respective letters within clusters are indicated to the right of the plot diagram.

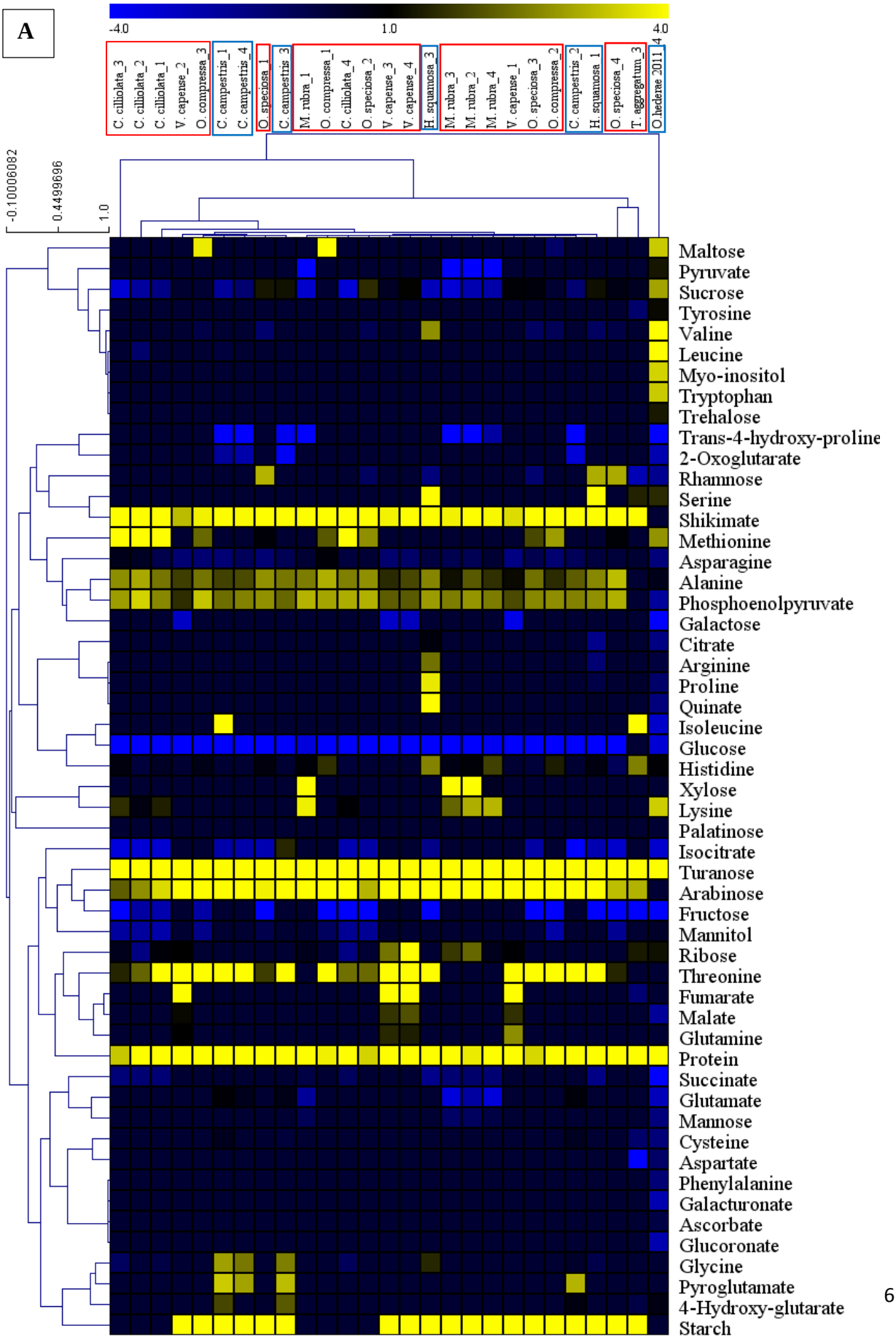
The first cluster (Figure 3.1.(1)) contained only parasitic plants (*C. ciliolate, V. capense, O. compressa, C. campestris, O. speciosa, M. rubra, H. squamosal, T. aggregatum, O. hederae*), with the clustering appearing to be due to increased levels of shikimate, turanose, arabinose, threonine, asparagine, PEP and overall protein content (Figure 3.2.A). Furthermore, a decrease in the levels of glucose, isocitrate, fructose, trans-4-hydroxy-proline and to some extent sucrose were also responsible for these plants clustering together (Figure 3.2.A).

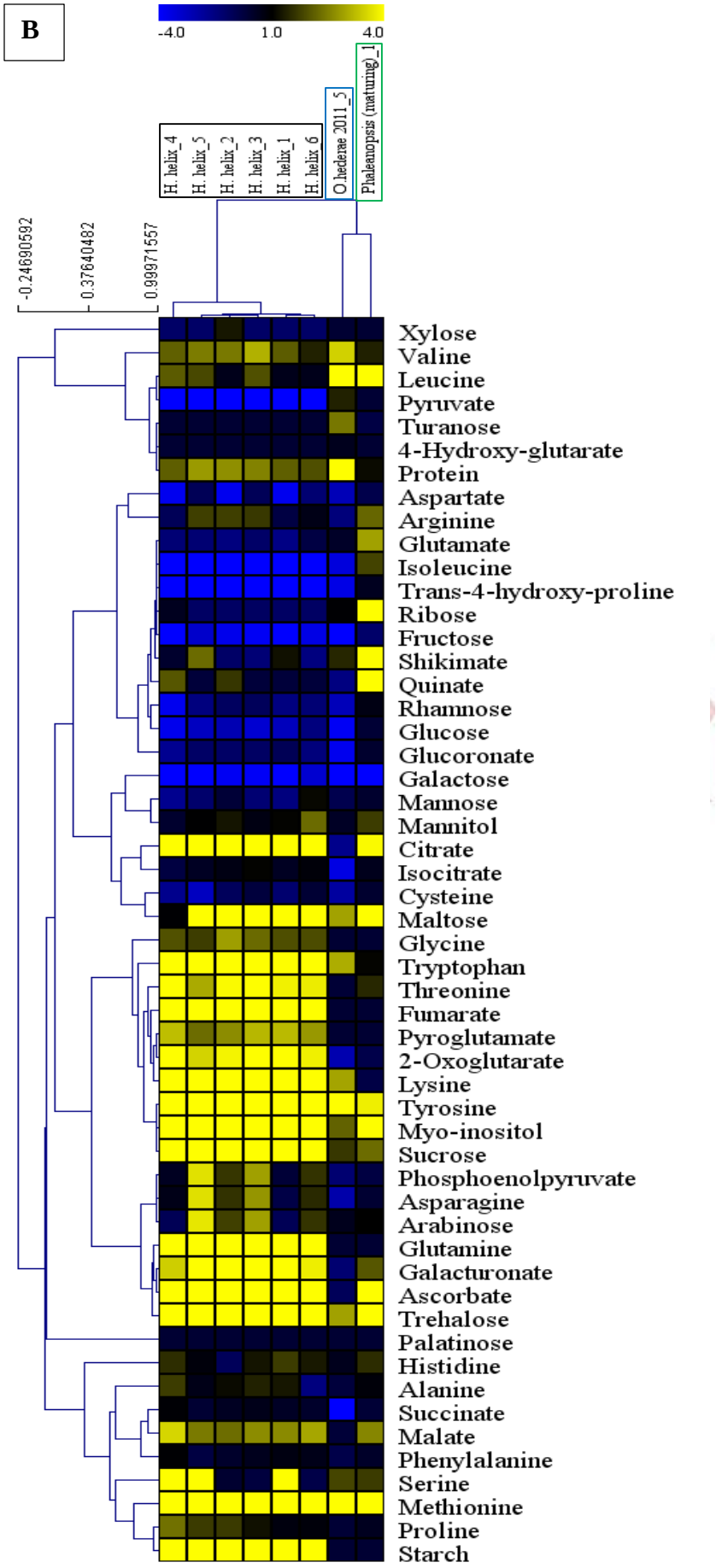
The second cluster (Figure 3.1 (2)) contained all replicates of *H. helix*. This clustering appears to be due to increased levels of maltose, citrate, tryptophan, threonine, fumarate, pyroglutamate, 2-oxoglutarate, lysine, tyrosine, myo-inositol, sucrose, glutamine, galacturonate,

ascorbate, trehalose, methionine and starch (Figure 3.2.B) and decreased levels of pyruvate, isoleucine, trans-4-hydroxy-proline, fructose, glucose and galactose (Figure 3.2.B).

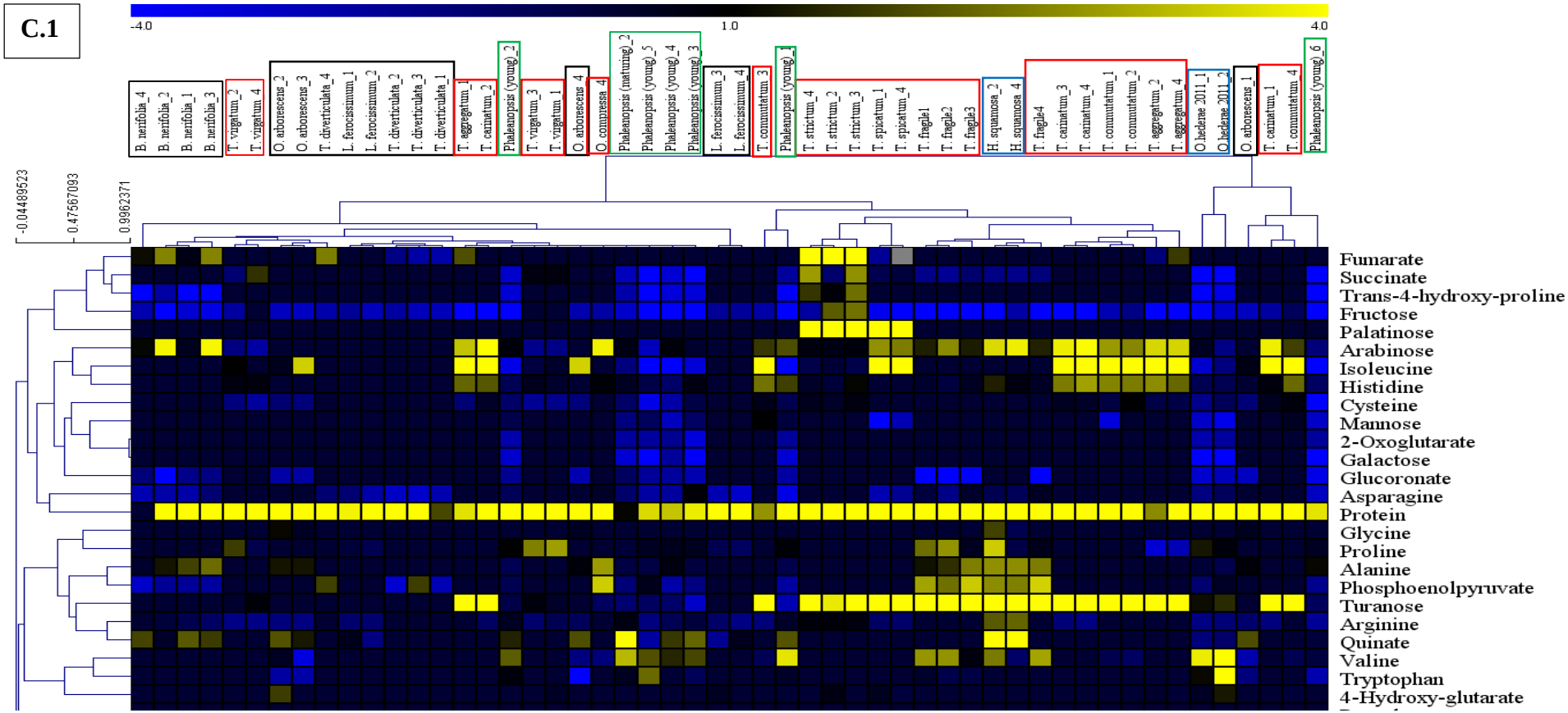
The third cluster (Figure 3.1 (3)) contained representatives of all four lifestyles, with most of the autotrophic species (excluding *H. helix*) found within this cluster. This cluster contained high levels of protein and starch for most plants. The autotrophic plants within this cluster showed increased levels of threonine, rhamnose, aspartate, glutamine, tyrosine, serine and sucrose, while the parasitic plants within the same sub-cluster, most notably the hemiparasitic species and *H. squamosa*, showed increased levels of turanose, shikimate, isoleucine, arabinose, histidine and serine (Figure 3.2.C). The *Phalenopsis* orchids within this cluster contained intermediate to high levels of protein, lysine, citrate, tyrosine, serine, ascorbate and phenylalanine, with very low levels of succinate, trans-4-hydroxy-proline, fructose, isoleucine, histidine, cysteine, mannose, 2-oxoglutarate, galactose and glucoronate (Figure 3.2.C).

The fourth cluster (Figure 3.1 (4)) contained most of the replicates for the two *Orobanche* species and *H. sanguinea*, along with replicates of two *Thesium* species. This clustering appears to be due to increased levels of leucine, isoleucine, palatinose, threonine, turanose, arabinose and shikimate, coupled to decreased levels of of glucose and sucrose (Figure 3.2.D). *Hyobanche sanguinea* showed high levels of ribose, serine, citrate, arginine, xylose and quinate, coupled to low levels of fructose, mannose, glutamate and histidine (Figure 3.2.D).

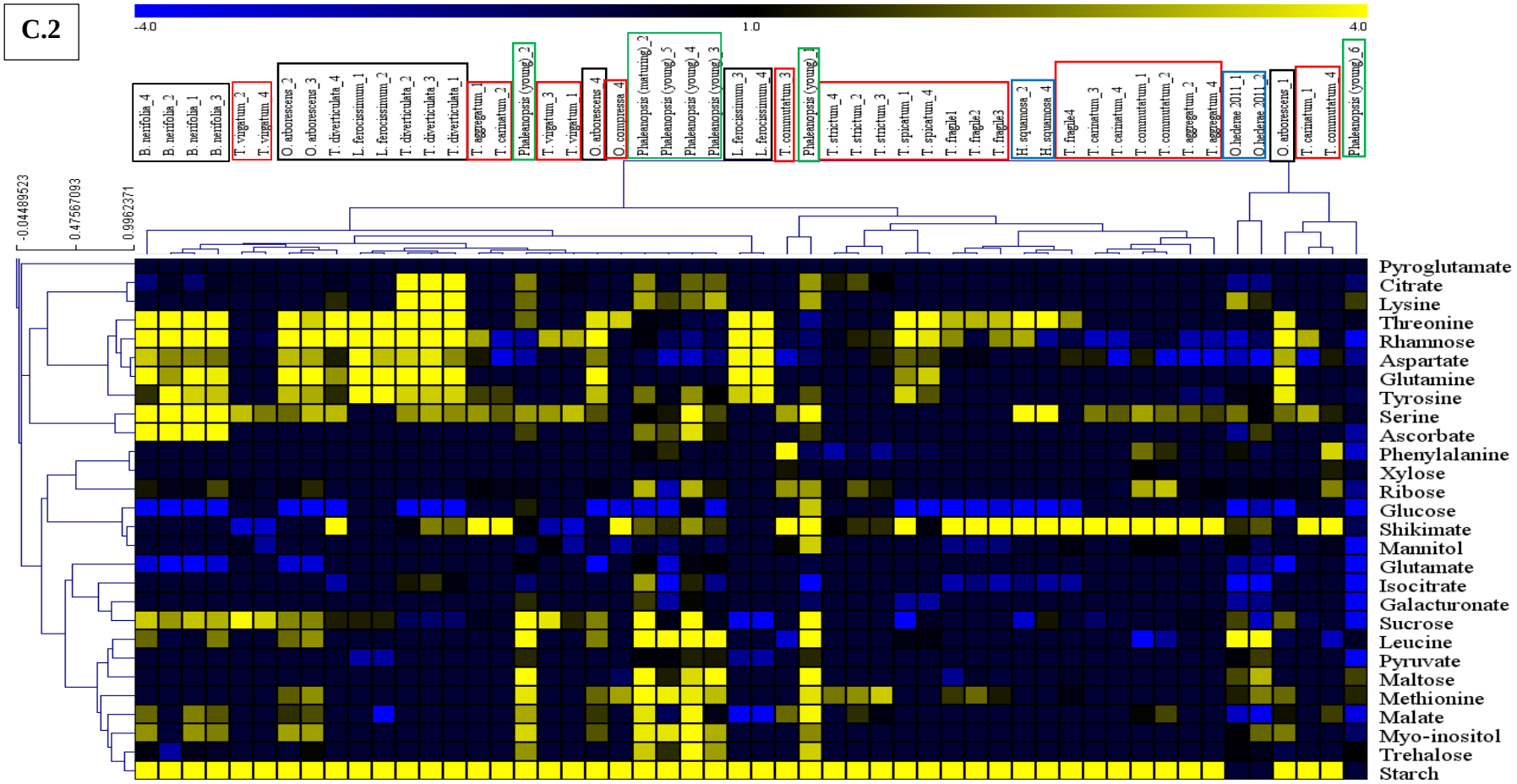




C.1



C.2



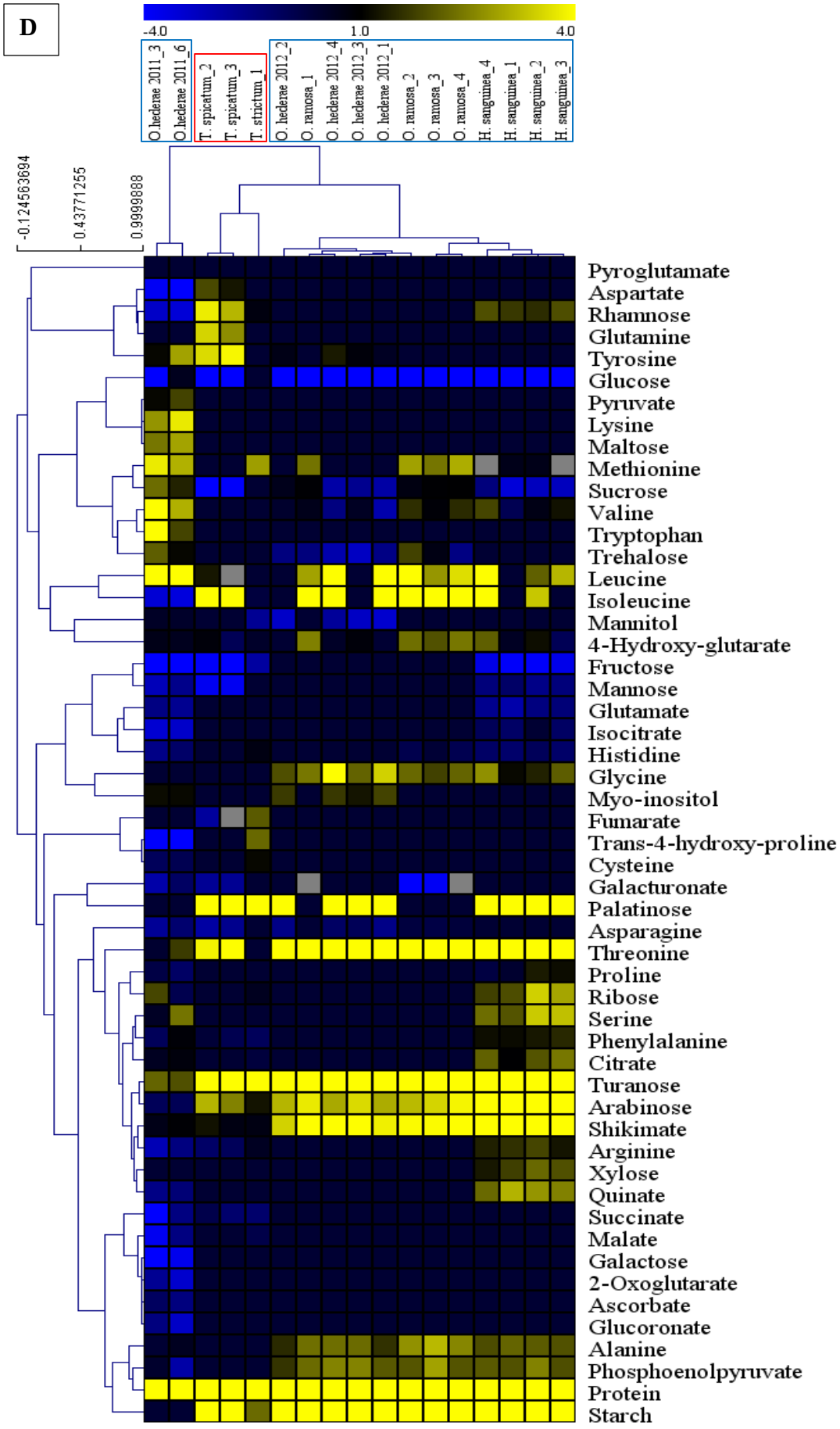
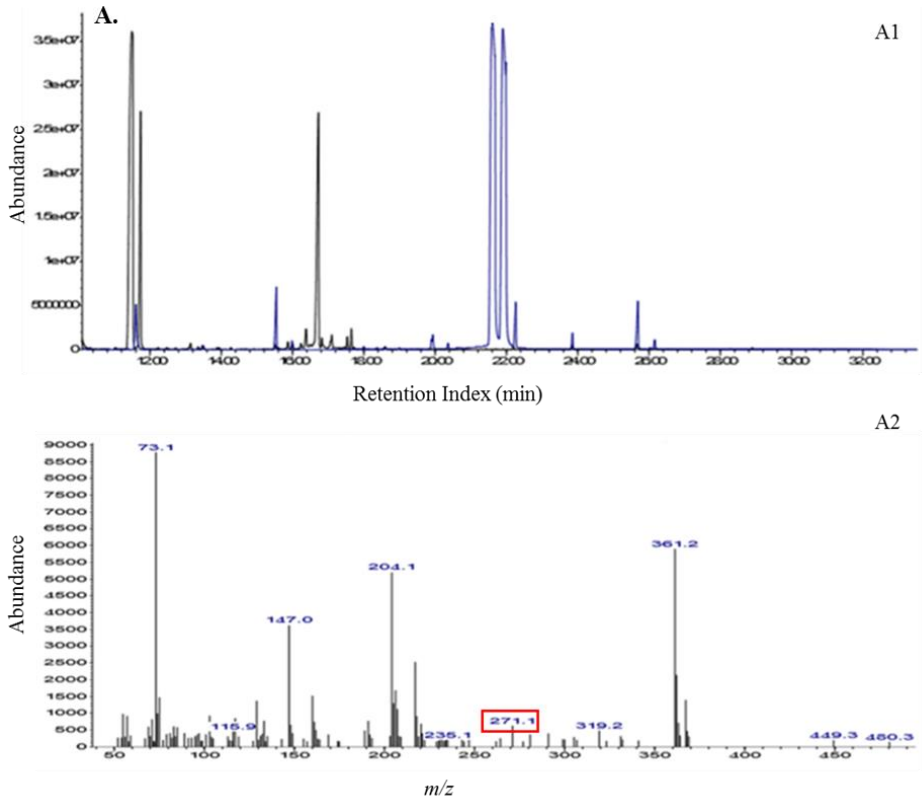


Figure 3.2. K-means clustering analysis of metabolites profiled within holoparasitic, hemiparasitic, mycoheterotrophic and autotrophic plants. Red boxes indicate hemiparasitic species, blue boxes holoparasitic species, green boxes mycoheterotrophic species and black boxes autotrophic species. The colour scale bar above specie names indicate individual metabolite levels, ranging from high (yellow) to very low/undetected (light blue). The scale bar represents numerical values based on the actual values on a log 2 scale.

To confirm the presence of the unusual sugar turanose within these plants, primary metabolites were again extracted from *O. hederae* and one sample spiked with turanose prior to derivitization and subsequent GC MS analysis (Figure 3.2.A). The peak representing turanose was found to be present within both the unspiked control and the spiked sample, as was shown when the chromatograms were overlaid (Figure 3.2.A.1). Furthermore, the mass spectra of these peaks contained the mass to charge ration (*m/z*) 271, unique to turanose (Figure 3.2.A.2). Subsequently, substrate specificity was determined for the 'turanose synthase' protein and it was found that turanose is produced from glucose 6-phosphate and fructose, indicating an enzyme with hexosyl transferase activity (Figure 3.2.B). To verify the high levels of starch measured in *O. hederae*, tubercules from this plant were destained and subsequently stained with Lugol solution (Figure 3.2.C), showing that *O. hederae* does indeed accumulate high levels of starch.



B.

Substrates tested (2mM)	Turanose detected
Sucrose	-
Palatinose	-
Melezitose	-
Glucose + Fructose 6-Phosphate	-
Fructose + Glucose 6-Phosphate	+
Fructose + UDP-Glucose	-
Fructose + Glucose 1-Phosphate	-
Fructose + ADP-Glucose	-
Fructose 6-Phosphate + ADP-Glucose	-
Fructose 6-Phosphate + UDP-Glucose	-

C.

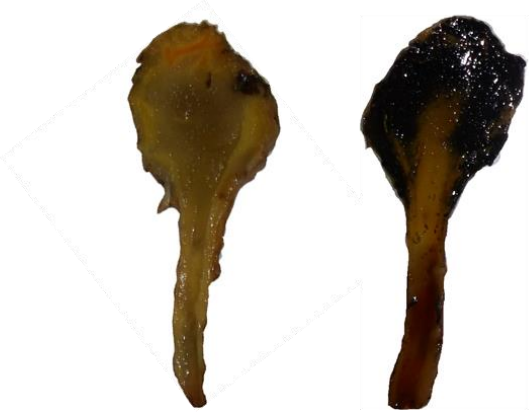


Figure 3.3. Validation of the presence of starch and turanose metabolite levels and turanose metabolic enzyme activity in *O. hederae*. (A) Gas chromatography-mass spectrometry (GC-MS) identification of turanose in plant extracts with and without spiking control. (B) Putative substrates tested for turanose biosynthetic activity. In each incubation, 2mM substrate was added to the assay mix, incubated for 30 min and the presence of turanose validated by GC-MS technology as described previously A negative sign (-) indicates no turanose detected while a positive sign (+) indicates the presence of turanose. (C) Lugol staining of *O. hederae* tubercules for the verification of the presence of starch.

3.5. Discussion

Plant parasitism is a highly successful life strategy that has evolved independently approximately eleven times (see Chapter 2). Due to its severe economic impacts on agronomical important crops and legumes (see Chapter 2), understanding the biochemical nature of this weedy species requires immediate attention. Research concerning the germination cue for *Striga* and *Orobanche* spp. has identified strigolactones (SLs), a new class of plant hormones, as a positive regulator of parasitic seed germination (Cook et al., 1966) and active research are pursuing the elucidation of the strigolactone biosynthetic and signaling pathways as potential molecular targets to reduce the spread and devastating effects of these parasitic weeds (Alder et al., 2012). Apart from the germination cue, far less is known about the exact metabolic nature of parasitism. Recent sequencing paradigms have started to elucidate the genomes and transcriptomes of this interesting set of land plants and have revealed that, in contrast to the achlorophyllous appearance of the holoparasites, a conservation of chlorophyll/heme biosynthesis and plastidial genes are maintained (Wickett et al., 2011). This suggests that other regulatory mechanisms, apart from photosynthate, contribute to the evolutionary development of parasitism. Furthermore, plastid genome sequencing of the mycoheterotrophic orchid *Neottia nidus-avis* have shown that it has lost most genes coding for photosynthetic subunits and RNA polymerases and retained all the genes associated with protein synthesis and translation (Logacheva et al., 2011). Parasitic plants are also known to be subjected to nuclear mitochondrial horizontal gene transfer mechanisms (Yoshida et al., 2010), yet the functional significance of this is far from elucidated. Understanding how parasitic plants function is an important step toward limiting their devastating economic losses.

In this study, a comparison of root and haustorial metabolism of 5 autotrophic, 17 parasitic and 2 mycoheterotrophic plants were made. Analyses of the primary metabolomes of these plants showed that they cluster into four groups based on metabolite composition as determined by a principal component analysis (PCA; Figure 3.1). Subsequently, a K-means clustering (KMC) analysis was carried out to determine which metabolites are responsible for this clustering (Figure 3.2) and it was found that each cluster is characterized by a unique mode of metabolism.

The first cluster (Figure 3.1 (1), 3.2.A) included only parasitic plants with the clustering appearing to be attributed to a decrease in metabolites associated with photosynthesis (sucrose, glucose and fructose with the exception of starch), a decrease in metabolites associated with the tricarboxylic acid (TCA) cycle (citrate, isocitrate, succinate), an increase in minor sugars (turanose, arabinose), protein and the organic acids shikimate and phosphoenol pyruvate (PEP). Arabinose is a major constituent of plant cell walls (Moore et al., 2013) and has been found in marama bean (*Tylosema esculentum*) seeds (Mosele et al., 2011) and certain resurrection plants (*e.g. Myrothamnus flabellifolia*; Moore et al., 2013). The other minor sugar, turanose, is an isomer of sucrose and is less common in higher plants. Turanose also dominates the sugar pool in the cluster containing *O. hederae* (Figure 3.2.D) and, to verify its presence, samples were spiked with turanose prior to GC MS analysis (Figure 3.3.A). Analysis of the data found that turanose was indeed present in *O. hederae*, indicating that this plant, together with the others containing turanose, have the capacity to manufacture this sugar.

The organic acids shikimate and PEP dominated the organic acid pool within this cluster. In plants, shikimate serves as a precursor to several aromatic amino acids (*e.g.* phenylalanine, tyrosine, tryptophan), which plants use as building blocks for protein biosynthesis and as precursors to secondary metabolites, some of which serve in defense responses (Herrmann, 1995; Weaver and Herrmann., 1997). In the shikimate pathway, chorismate is synthesized in multiple steps starting with PEP and erythrose-4-phosphate as substrates, from where it can be utilized in separate pathways that lead to the synthesis of the three aromatic amino acids (Herrmann, 1995). The high proportion of these organic acids profiled in this cluster suggests that these plants utilize them to synthesize aromatic amino acids. However, aromatic amino acid contents were not determined to be that high and so it could be that these plants rapidly convert the newly synthesized aromatic amino acids into secondary metabolites, possibly used in defense responses. Alternatively, these amino acids could have been utilized in protein biosynthesis, explaining the high protein levels quantified in this cluster. The low levels of TCA cycle intermediates, especially citrate and isocitrate, indicates that respiration might be fueled by alternative substrates, or that N metabolism is decreased in these plants, as these metabolites are implicated in both processes (Popova et al., 1998).

The second cluster included all replicates of *H. helix* (Figure 3.1 (2), 3.2.B), with the clustering appearing to be attributed to an increase in metabolites associated with photosynthesis

and subsequent phloem translocation (sucrose, starch). Both the levels of fumarate, one of the major translocated organic acids (Pracharoenwattana et al., 2010) and ascorbate, another organic acid that plays multifaceted roles in photosynthesis (Nunes-Nesi et al., 2011) and which is transported from the shoot to roots to maintain the root redox state (Herschbach et al., 2010), were significantly increased in the host root metabolism. This plant also accumulates high levels of glutamine, an amino acid involved in N assimilation (Corruzi and Zhou, 2001). Furthermore, these plants accumulate high levels of the sugar-alcohol *myo*-inositol, which serves as a transport and storage form of fixed carbon within several plants (Schneider et al., 2008). *Myo*-inositol accumulates under conditions of stress with mutants in the rate limiting step of its biosynthesis showing a hypersensitive response and localized programmed cell death induced by pathogen attacks (Meng et al., 2009; Donahue et al., 2010). Hence, *H. helix* might be accumulating this sugar alcohol to serve as a protectant against *O. hederae* to lessen the extent to which this parasite acquires nutrients from its vascular system.

The third cluster (Figure 3.1 (3), 3.2.C) contained all the *Thesium* species, both developmental stages of the *Phalaenopsis* orchids, all autotrophic host species (with the exception of *H. helix*), *Thesidium fragile* and 2 replicates of *Harveya squamosa*. This cluster formed various sub-clusters, with the mutual factors combining them within this larger cluster being the high levels of protein and starch. The sub-clustering of the autotrophic plants are attributed to an increase in metabolites associated with photosynthesis (sucrose), N assimilation (glutamine, aspartate) and the minor sugar, rhamnose. The second sub-cluster contained the majority of the hemi parasitic species and similar to the first cluster (Figure 3.2.A), this cluster contains high levels of turanose, arabinose and shikimate, coupled to a decrease in metabolites associated with photosynthesis (sucrose). Furthermore, an increase in the branched chain amino acid isoleucine and overall protein content was coupled to a decrease in organic acids associated with the tri-carboxylic acid (TCA) cycle (isocitrate, succinate). The two developmental stages of the *Phalaenopsis* orchid were also found in this cluster, however, even though they cluster together one can still discriminate between the two developmental stages based on metabolite composition. The young *Phalaenopsis* seedlings showed a severe reduction in major carbohydrate metabolism. Starch, maltose (the breakdown product of starch) and sucrose levels were significantly reduced. As the major form of phloem transported carbon, analysis of this metabolite could thus discriminate between the young and

maturing metabolism of *Phalaenopsis* orchids.

Similar to cluster one (Figure 3.2.A), the increased protein content and levels of branched chain amino acids and lysine coupled to a decrease in the levels of TCA cycle intermediates, further suggests that these plants (with regards to the parasitic plants and the young *Phalaenopsis* orchid) utilize alternative substrates to fuel their mitochondrial respiration. Recently Obata et al. (2011) demonstrated that, upon oxidative stress, an enhancement of protein breakdown and utilization of branched chain amino acids and lysine compensates for a reduced electron supply in the mitochondrial TCA cycle *via* an alternative functional electron transfer flavoprotein (ETF) complex (identified by Ishizaki et al., 2006). Once branched chain amino acids are released *via* protein breakdown, they undergo a process of transamination, decarboxylation and esterification to yield CoA esters (Taylor et al., 2004). The latter is subsequently oxidized to produce electrons which are fed into the TCA cycle *via* the ETF to ubiquinone (Taylor et al., 2004). One of the enzymes involved in this process is the branched chain keto acid dehydrogenase, and enzyme that is induced under conditions of sugar starvation (Fujiki et al., 2001). In general, it is now accepted that, upon carbon limitation, plants can utilize proteins, lipids and amino acids as alternative substrates to fuel respiration by synthesis of isovaleryl-CoA or hydroxyglutarate substrates that allow electron transport through the ETF: ubiquinone oxidoreductase or complex II electron transport systems (Araujo et al., 2011).

The occurrence and levels of these metabolites in parasitic metabolism lead us to believe that parasitic and mycoheterotrophic plants preferentially utilize proteins as a respiratory substrate. In further support of this, the recent plastid genome sequencing of the mycoheterotrophic orchid *Neottia nidus-avis* have shown that, while it has lost most genes coding for photosynthetic subunits and RNA polymerases, it retained all the genes associated with protein synthesis and translation (Logacheva et al., 2011). The evolutionary significance of this finding suggests that the presence/absence of organic acids could play an important and integral role in establishing or shifting the symbiotic and parasitic interdependence.

Even though we propose that these plants utilize proteins and branched chain amino acids as alternative respiratory substrates, it is however peculiar that the levels of branched chain amino acids accumulate together with protein levels. As mentioned in above, under conditions of oxidative stress, an enhancement of protein breakdown takes place releasing branched chain amino acids to be used for respiration (Araujo et al., 2011). Why these two accumulate together remains

to be elucidated. This increase in protein content could be due to TCA cycle organic acids being used for the synthesis of amino acids which are later used for protein biosynthesis. However, why these plants would sacrifice the fuelling substrates for amino acid synthesis, only to utilize the latter as alternative substrates would be very peculiar.

The last cluster contained mostly holoparasitic species (Figure 3.1 (4), 3.2.D). The holoparasitic plants contained very high levels of starch, the branched chain amino acids leucine and isoleucine, an array of minor sugars (palatinose, turanose, arabinose), the organic acid shikimate and intermediate levels of the sugar alcohol *myo*-inositol. Starch levels were verified in *O. hederae via* Lugol staining of tubercules (Figure 3.3.D). Furthermore, these plants showed a severe reduction in the photosynthetic metabolites sucrose and glucose. The low levels and prevailing under-representation of TCA cycle organic acids (notably citrate and malate) in the total organic acid pools, coupled to increased levels of total soluble protein and a 6- to 40-fold increase in the branched chain amino acids, isoleucine and leucine, indicated that this cluster also fuels its respiration with the latter substrates as was demonstrated for the first and third cluster.

Furthermore, the presence and predominance of turanose, *myo*-inositol, shikimate, tyrosine and tryptophan (Fig 3.2.A, 3.2.C, 3.2.D) in clusters 1,3 and 4, collectively, suggest that the involvement and maintenance of local auxin gradients might play a role in either the establishment or maintenance of host-symbiont relationships. A *turanose insensitive* (*tin*) activation tagged mutant is allelic to *WOX5* and has higher levels of the auxin, indole-3-acetic acid (IAA) and its conjugates (Gonzali et al., 2005). In addition, high levels of the sugar alcohol, *myo*-inositol, was also observed in the *H. helix* host (Fig 3.2.B). *Myo*-inositol plays an important role as a precursor to phosphorylated inositol secondary messengers, as well as one of the major sugars that form conjugated IAA, which further modulates auxin metabolism and plays an integral role in maintaining distinct auxin gradients (Chen and Xiong, 2010). Furthermore, the increased levels of aromatic amino acids (tyrosine and tryptophan) as well as shikimate, the precursor to flavonoids (an important group of compounds that interact with auxin distribution), was also observed, suggesting that local and systemic auxin gradients are modulated, possibly in order to alter sink relationships that would facilitate the successful invasion of the host metabolism. Taken together with the associated metabolite changes observed, it suggests that the respiratory requirement of

alternative substrates by parasitic plants, may modulate aspects of auxin and nitrogen metabolism, in order to maintain its unique metabolism.

3.6. Conclusion

In contrast to autotrophic plant metabolism, minor sugars dominated the sugar pools of parasitic plants, with the sucrose analogues, turanose and palatinose, representing between 20 and 95 % of the total sugar pool. A decrease in metabolites associated with primary carbon metabolism (with the exception of starch), N assimilation (glutamine) and photorespiration (glycine and serine) was hallmarks of the majority of parasitic plants. While obligate holoparasitic and initial mycoheterotrophic metabolism are expected to have many divergent adaptation and metabolic strategies, it was interesting to observe 1) a severe reduction in major carbohydrate pools (sucrose, starch and fructose) in the roots associated with 2) reduced TCA cycle intermediates and 3) a predominance of protein, valine, leucine and lysine levels or percentage to the total amino acid pool. Taken together, it suggested that alternative substrate usage to fuel mitochondrial respiration might be evident in both lifestyles.

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Supplemental Information

Supplemental Table S1 List of species investigated.

Investigated species	Lifestyle	Family	Corresponding parasitic member	Host organ parasitized
Moquiniella rubra	Hemiparasitic	Loranthaceae		Stem
Viscum capense		Santalaceae		Root
Thesidium fragile		Santalaceae		Root
Osyris compressa		Santalaceae		Root
Osyris speciosa		Santalaceae		Root
Thesium spicatum		Santalaceae		Root
Thesium aggregatum		Santalaceae		Root
Thesium carinatum		Santalaceae		Root
Thesium commutatum		Santalaceae		Root
Thesium strictum		Santalaceae		Root
Thesium virgatum		Santalaceae		Root
Cassytha ciliolata	Holoparasitic	Lauraceae		Stem
Cuscuta campestris		Convolvulaceae		Stem
Harveya squamosa		Orobanchaceae		Root
Hyobanche sanguinea		Orobanchaceae		Root
Orobanche hederae		Orobanchaceae		Root
Orobanche ramosa		Orobanchaceae		Root
Brachylaena neriifolia	Autotrophic	Asteraceae	*C. ciliolata*	Stem
Trachyandra diverticulata		Xanthorrhoeaceae	*C. campestris*	Stem
Othonna arborescens		Asteraceae	*V. capense*	Stem
Lycium ferocissimum		Solanaceae	*M. rubra*	Stem
Hedera helix		Araliaceae	*O. hederae*	Root
Phalaenopsis hybrid	Mycoheterotrophic	Orchidaceae		

Chapter 4
Biochemical and Molecular Characterization of Turanose Metabolism

Biochemical and molecular characterization of turanose metabolism

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Anna J. Wiese

- All experimental work
- GC MS data analysis
- Writing

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- Supervisor
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James R. Lloyd

- Supervisor
- Writing suggestions
- Funding

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- Supervisor
- Funding

4.1. Abstract

Photoautotrophic sugar sensing and signaling is an important mechanism to transduce and coordinate the plants biochemical and energy state for optimum performance. Some higher plants have, however, abandoned photosynthesis and accumulate a significant array of minor sugars, such as turanose, a sugar with several applications in the food industry. Following substrate elucidation for turanose biosynthesis (Chapter 3), we set out to purify the 'turanose synthase' protein from *Orobanche hederae*. Here we combined classical protein purification methods with high-throughput mass spectrometry peptide elucidation and this data is currently being used to identify the turanose synthase gene.

4.2. Introduction

In chapter 3 it was found that one of the major sugars dominant in *Orobanche hederae*'s metabolome is that of turanose (α-D-glucopyranosyl-(1→3)-α-D-fructofuranoside; Chapter 3). This is a non-reducing disaccharide isomer of sucrose (α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside), differing in the chemical link position between the glucose and fructose moieties (Thompson et al., 2001; Sinha et al., 2002; Gonzali et al., 2005). Turanose was first discovered through the weak acid hydrolysis of melezitose (Wang et al., 2012) and since then has mainly been studied in microorganisms. Bakers yeast (*Saccharomyces cereviseae)* can completely hydrolyze turanose *via* α-glucosidase to yield glucose and fructose, which can be further fermented (Yoon et al., 2003). In bacteria such as *Deinococcus geothermalis* and *Neisseria polysaccharea*, turanose can be synthesized by amylosucrase (Büttcher et al., 1997; Guerin et al., 2011), a glucosyltransferase that forms insoluble α-1,4-linked glucan chains accompanied by the release of fructose (De Montalk et al., 2000). When incubated in up to 2.5M sucrose as a substrate, amylosucrase from *Neisseria polysaccharea* yielded 14-56% turanose (Albenne et al., 2004; Wang et al. 2012).

Turanose was considered not to be present in higher plants and was also presumed not to be metabolized by them (Sinha et al., 2002; Gonzali et al., 2005). These general presumptions have recently been questioned as, in sugarcane, many non-metabolizable disaccharides (including turanose) have been shown to be catabolized by enzyme extracts assayed under neutral invertase conditions (Wu and Birch, 2011). Turanose is a major constituent in honey (Sporns et al., 1992; Da Costa Leite et al., 1999), with its composition depending on the nectar source (Kaskoniene et

al., 2010). More recently, it has been shown to occur in pollen (Szczêsna, 2007), strawberry fruit (Zhang et al., 2010) and garden cress leaves (Voitsekhovskaya et al., 2009).

Sucrose is the precursor to glucan, a polysaccharide that enables oral bacteria to adhere to tooth surfaces (Thompson et al., 2001). Furthermore, it can be fermented to lactic acid by *Streptococcus mutans*, initiating the demineralization of tooth enamel (Thompson et al., 2001). Because turanose is half as sweet as sucrose, but is non-metabolizable by the majority of oral bacteria (Shibuya et al., 2004), it could become a non-cariogenic alternative to sucrose in the food industry. Furthermore, turanose has been used in medicine to diagnose Pompe's diseasewhere glycogen accumulates in tissues lacking α-glucosidase activity (Salofsky and Nadler., 1971). It is also an inhibitor of the acid form of α-glucosidase, making it useful in the study of this disease (Fluharty et al., 1973; Salafsky and Nadler., 1973; Wang et al., 2012). The current retail price of turanose is approximately 20 000 USD/Kg, although this price may well decrease with the development of production systems using amylosucrase. An alternative system using plants would be an ideal way of producing turanose on a larger scale and to do this, a further understanding of turanose metabolism in plants is needed. To address this we evaluated turanose metabolism in *O. hederae*.

4.3. Materials and Methods

4.3.1 Plant material

For screening of turanose synthase activity, haustorial material was sampled from *Orobanche hederae*, obtained from the Botanical Gardens, University of Stellenbosch. Haustorial material was carefully excised, rapidly flash-frozen in liquid nitrogen, homogenized and kept at -80°C until sample processing.

4.3.2 Chemicals

Commercially available chemicals were purchased from Sigma Aldrich (Steinheim, Germany) and Roche (Mannheim, Germany), unless stated otherwise.

4.3.4 Turanose enzyme activities

Crude protein extracts from *O. hederae* was used to identify turanose biosynthetic activity. Assays were carried out at room temperature for 30 min in 50 mM Tris-HCl (pH 7.0) containing 2mM putative substrate(s) (fructose and glucose 6-phosphate). Reactions were stopped by heating at 95ºC for 1 min. Samples were centrifuged at 2549 *g* for 15 min and proteins in the supernatant were separated from the sugars using exclusion columns (Amicon ® Ultra Centrifugal Filter Units), with a 3kDa cut off. Turanose was detected *via* GC QUAD MS technology with running conditions as described previously (Roessner et al., 2001).

4.3.5 Turanose profiling *via* GC MS technology

The presence of turanose was detected with a GC QUAD MS protocol optimized for *O. hederae* metabolite profiling (Chapter 3). Synthesis incubations were briefly dried and the residue re-dissolved and derivatized for 90 min at 30ºC in 80 µl 30 $mg.ml^{-1}$ methoxyamine hydrochloride (in pyridine). Subsequently samples were trimethylsilylated by 30 min incubation with 140 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) at 37ºC. *n*-Alkane retention time standard mixtures was added prior to trimethylsilylation. Samples were injected splitless into a GC quadropole MS (ThermoFinnigan, UK) and separated according to specifications in Chapter 3. Data pre-processing for baseline correction, scaling and alignment was conducted with MetAlign software and the crmn package in R (Redestig et al, 2009). Turanose presence and concentrations was also verified by a linear calibration curve from an authentic standard.

4.3.6 Protein purification

25 g material was extracted with 603 mL extraction buffer. The crude protein extract was dialyzed overnight in Buffer A (50 mM Tris-HCl (pH 8.8)) before being passed through a 2 µm filter. Approximately 250 mg protein was subjected to anion exchange chromatography (HiTrap Q HP, 5 ml) (Amersham Biosciences). Proteins were loaded onto the column in Buffer A before the column was washed using 50 ml of the same buffer. Elution was performed with a linear gradient between Buffer A and Buffer B (50 mM Tris-HCl (pH 8.8), 1 M NaCl) where Buffer B completely replaced Buffer A after 50 ml. Seventy five 1ml fractions were collected containing eluted proteins. . These were subsequently stored on ice at 4ºC and tested for activity. Fractions positive for turanose synthase activity were pooled and dialyzed overnight in Buffer C (50 mM

acetic acid buffer (pH4.5)). These were loaded onto a 5 ml cation exchange (HiTrap SP HP) column (Amersham Biosciences) in Buffer C and washed with 50 ml of the same buffer. Proteins were eluted using a gradient where Buffer C was completely replaced with Buffer D (50 mM acetic acid (pH4.5), 1M NaCl) after 50ml. Fifteen 5 ml fractions were collected and tested for activity.

4.3.7 Liquid Chromatography Mass Spectrometry

Twenty µl of fractions positive for turanose synthase activity were added to 80 µl SDT lysis buffer (0.1 M DTT, 4% SDS, 100 mM Tris-HCl pH 7.6) and 100 µl UA buffer (100 mM Tris-HCl pH 8.5, 8M urea) and the mixture placed on a filter (Amicon ultra 0.5 centrifugal filter, 10 kDa, Millipore) and centrifuged for 40 min at 14 000 *g* (all subsequent centrifugation steps were carried out at this speed). After addition of200 µl UA buffer the samples were centrifuged for 40 min. Alkylation was acheived by addion of 100 µl 0.05 M iodoacetaminde in UA buffer, incubation at RT for 5 min and centrifugation for 30 min. After addition of 100 µl UB buffer (0.1 M Tris-HCl pH 8.0, 8M urea) the mixture was centrifuged for 30 min, after which theer was a further addition of 100 µl UB buffer and 30 minutes centrifugation. One hundred µl 50 mM NH_4HCO_3 was added and centrifuged for 30 min, with the latter step repeated. Forty µl trypsin was then added and the mixture incubated at 37 °C for 17 h in a high hunidity chamber. Following this, the filter was placed in a clean microcentrifuge tube and centrifuged for 40 min before 40 µl 0.5 M NaCl solution added. The sample was further centrifuged for 20 min and acidified by the addition of 4 µl CH_2O_2. The filtrate was desalted using C18 StageTips (Thermo Scientific, Bremen, Germany), dried down *in vacuo* and stored at -20^{0}C. Dried peptides were re-dissolved in 5% (w/v) acetonitrile in 0.1% (w/v) CH_2O_2 prior to nano-LC chromatography.

Liquid chromatography mass spectrometry of samples was performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. For liquid chromatography, separation was performed on an EASY-Column (2 cm, ID 100 µm, 5 µm, C18) pre-column followed by a XBridge BEH130 NanoEase column (15 cm, ID 75 µm, 3.5 µm, C18) with a flow rate of 300 nl/min. Elution from the column was achieved using the following gradient: 5-17 % B in 5 min, 17-25% B in 90 min, 25-60% B in 10 min, 60-80% B in 5 min and kept at 80% B for 10 min. Solvent A consisted of 100% water in 0.1 % CH_2O_2, while solvent B consisted of

100 % acetonitrile in 0.1% CH_2O_2.

The mass spectrometer was operated in a data-dependent mode, with data acquired using the Xcaliber software package. The precursor ion scan MS spectra (*m/z* 400 – 2000) was acquired in Orbitrap with resolution R = 60000 with the number of accumulated ions being 1 x 10^6. The 20 most intense ions were isolated and fragmented in linear ion trap (number of accumulated ions 1.5 x 10^4) using collision induced dissociation. The lock mass option (polydimethylcyclosiloxane; *m/z* 445.120025) enabled accurate mass measurement. In data-dependent LC-MS/MS experiments, dynamic exclusion was used with 60 s exclusion duration. Mass spectrometry conditions were 1.8 kV, capillary temperature of 250 °C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS and an activation Q-value of 0.25 and activation time of 10 ms were also applied for MS/MS.

Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) was used to identify proteins via automated database searching (Mascot, Matrix Science, London, UK) of all tandem mass spectra against the Swissprot database. Carbamidomethyl cysteine was set as fixed modification and oxidized methionine, N-acetylation and deamidation (NQ) was used as variable modifications. Proteins were considered positively identified when they were identified with at least 2 tryptic peptides per proteins, a Mascot score of more that $p<0.05$ as determined by Proteome Discoverer 1.3. Percolator was also used for validation of search results. In Percolator a decoy database was searched with a FDR (strict) of 0.02 and FDR (relaxed) of 0.05 with validation based on the q-value.

4.4. Results and Discussion

Most plants produce sucrose as a metabolizable simple sugar which can be transported from source to sink tissues. Some plants, such as sugarcane, store significant amounts of sucrose which can be extracted and sold to consumers. However sucrose leads to a number of medical problems including dental caries. One way to overcome this would be to convert sucrose to one of its isomers as these taste sweet, but cannot be metabolized by oral bacteria (Shibuya et al., 2004). One sucrose isomer is turanose and understanding its metabolism will aid in producing organisms that manufacture turanose which can be used by industry. Identifying a gene encoding a turanose synthase would allow the production of turanose either in micro-organisms or agricultural crops, such as sugarcane. This has already proven to be a successful approach for palatinose production.

Sugarcane lines expressing sucrose isomerase in the vacuole accumulate large amounts of palatinose, with no decrease in sucrose content (Wu and Birch., 2007). Furthermore, 47% of transgenic sugarcane lines evaluated over a period of three years, produce palatinose, with some accumulating up to 217 mM, 33% of the total sugar pool, in cane-juice (Basnayake et al., 2012).

Previously it was shown that Orobanche hederae crude protein extracts produced turanose from fructose 6-phosphate and glucose (Chapter 3), indicating an enzyme with hexosyl transferase activity. Following this substrate elucidation, purification of turanose synthase activity (Fig 4.1) from *O. hederae* crude extracts was undertaken in order to isolate the biosynthetic protein responsible for this conversion.

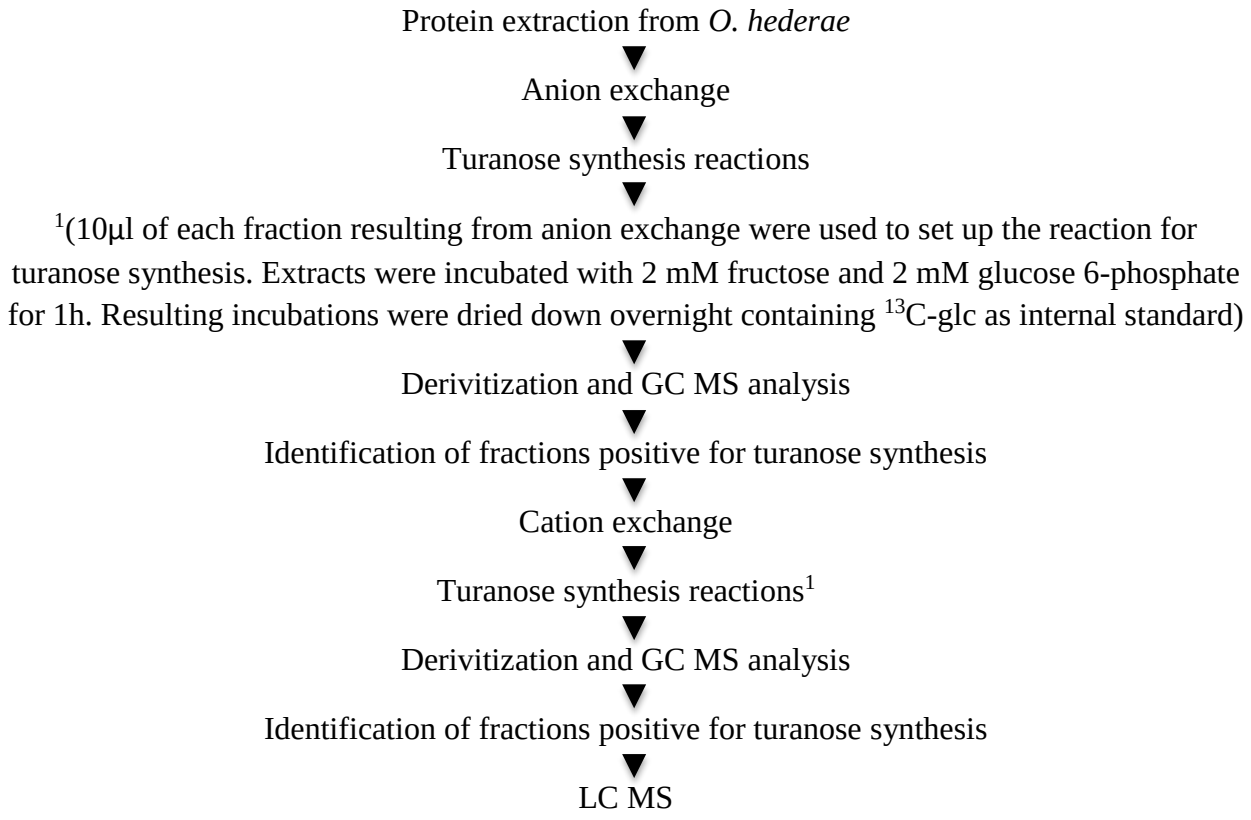

Figure 4.1. Purification strategy. Large scale protein extraction from *O. hederae* was followed by anion exchange and the resulting fractions used in turanose synthesis reactions. Incubations contained 10 µl from the respective fraction, 2 mM glucose and 2 mM fructose 6-phosphate as substrates in a 50 mM Tris-HCl buffer. Fractions positive for turanose formation, as determined *via* GC MS analysis, were pooled and the process repeated for the next chromatography step (cation exchange). Subsequent fractions positive for turanose formation were sent for LC MS analysis for protein identification.

Protein extracts were separated using anion exchange chromatography and fractions collected incubated with fructose and glucose 6-phosphate with the resulting products analyzed via GC MS. Several fractions (1 through 4 and 16) showed turanose synthase activity (Fig 4.2). These fractions were pooled and subjected to cation exchange chromatography. From this chromatography step, fractions 3 and 12 through 15 (Figure 4.3) were selected for protein identification *via* LC QQQ MS technology.

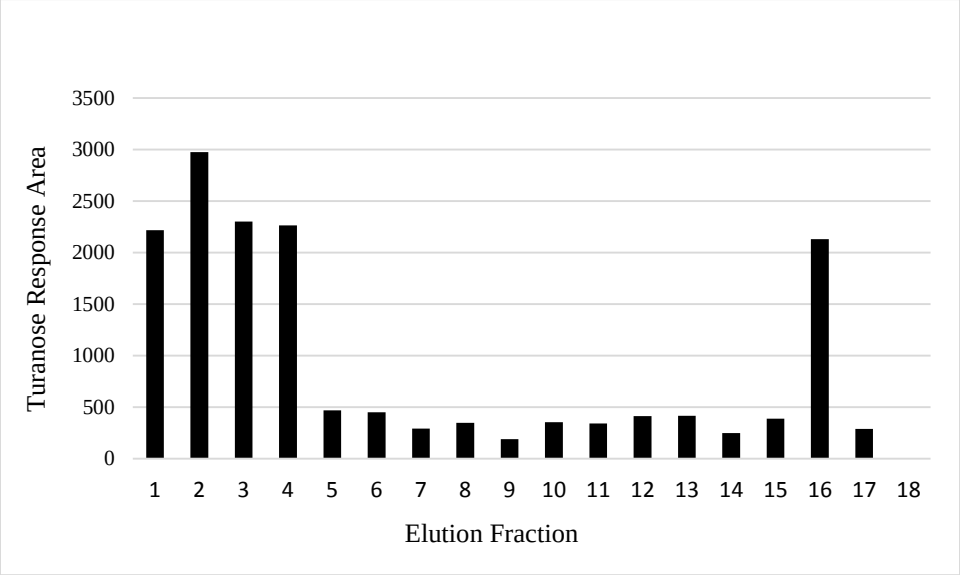

Figure 4.2. Anion exchange chromatography. Graphs represent eluted fractions and their respective synthesizing activities. Fractions 1 through 4 and 16 showed very high turanose response areas and were selected for the subsequent anion exchange chromatography.

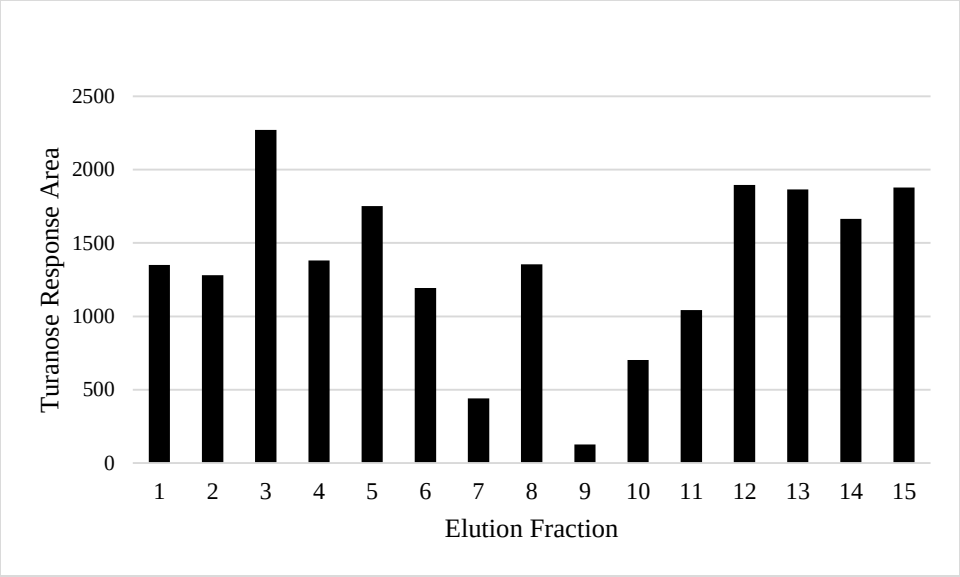

Figure 4.3. Cation exchange chromatography of fractions 1 through 4 and 16 from the cation exchange chromatography step synthesizing turanose *in vitro*. Graphs represent eluted fractions and their respective synthesizing activities. Fractions 3 and 12 through 15 showed very high turanose response areas and were selected for the LC MS peptide elucidation.

Peptide sequences resulting from LC QQQ MS are currently being searched against an assembled transcriptome of *Orobanche aegyptiaca*, a sister species of *O. hederae* to identify the gene encoding turanose synthase.

4.5. Conclusion

In this study, I partially purified the turanose synthase protein and am currently using it to identify the gene encoding it. Furthermore, by demonstrating that turanose is produced in crude and partially purified enzyme extracts from *O. hederae*, we have shown that it is not an artefact of the analytical technique, but that these plants have the ability to synthesize this compound, strengthening the observations from chapter 3.

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Chapter 5
Production of *Arabidopsis* Mutants Altered in Their Sensitivity to Growth on Turanose

Production of *Arabidopsis* mutants altered in their sensitivity to growth on turanose

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5.1. Abstract

Plants can sense the concentrations of sugars by a number of mechanisms, for example by utilizing SnRK proteins and trehalose 6-phosphate (Chapter 2.4). There is evidence that, although turanose has never been described in higher plants, they can also sense this sugar (Gonzali et al., 2005). This indicates a potential novel regulatory mechanism involving turanose. An EMS mutagenized seed population was screened on plates containing 90 μM turanose for seedlings showing a *turanose-insensitive* (*tin*) phenotype. Putative mutants that were identified underwent a second round of screening to confirm the phenotype. From these experiments we have identified six *Arabidopsis tin* mutants which are turanose insensitive. Mutant phenotypes include normal vegetative development, white necrotic lesions at leaf margin edges, reduced organ size, yellow pigmentation in mature leaves and accelerated flowering. We hypothesize that the genes mutated represent components of a novel sugar perception pathway in higher plants.

5.2. Introduction

As discussed in Chapter 2.3, sugars perform signaling roles in plants, regulating genes involved in photosynthesis, sink metabolism and defense responses (Sinha et al., 2002).

Plants that contain turanose (*e.g. Orobanche hederae*), should also contain sensing mechanisms that enables its perception to occur. Turanose has been shown to be transported by certain sucrose transporters (SUTs) that partake in phloem loading and unloading (Sivitz et al., 2007). Not all SUTs are substrate specific and it was found that the Arabidopsis AtSUC9 translocator transports a wide range of glucosides, including turanose (Sivitz et al., 2007). This suggests that plants already contain the machinery necessary for turanose transport. On a physiological level, turanose-treated *Arabidopsis* seedlings are characterized by a short hypocotyl and primary root, in contrast to the reverse phenotype when seedlings are treated with sucrose (Gonzali et al., 2005). A screen of activation tagged Arabidopsis lines identified one mutant which affected a homeobox gene, *Wox5* (Gonzali et al., 2005). They demonstrated that *Wox5* expression was upregulated by turanose and that the mutation affected a number of pathways including some involved in auxin homeostasis (Gonzali et al., 2005). In addition, these plants also had increased adventitious roots (Gonzali et al., 2005) and enhanced anthocyanin levels (Teng et al., 2005). While it has been proposed that turanose play a role in sugar signaling as a sucrose analogue, these distinct responses to turanose question this generalization. Furthermore, the induction of soluble

carbohydrates by gibberellins (GA) during barley germination has shown that turanose, similar to glucose and sucrose, represses α-amylase transcript abundance without destabilizing the mRNA (Loreti et al., 2000). Upon adding turanose to cassava storage roots, starch branching enzyme (SBE) expression was induced to almost the same extent as with sucrose application (Baguma et al., 2008). Furthermore, like sucrose, turanose is able to sustain the semidian oscillation of *SBE* transcript levels (Baguma et al., 2008).

The persistence of turanose in non-photosynthetic parasitic *O. minor* plants, the decrease in turanose levels upon development in orchids (*Phalaenopsis*) and an involvement in starch metabolism (the major transient carbohydrate store during photosynthesis (Loreti et al., 2000; Baguma et al., 2008), suggest that one plausible functional role of turanose could involve the modulation of photosynthetic activity. In order to identify additional *turanose-insensitive* mutants, we set about screening an EMS mutagenized seed population under similar conditions as Gonzali et al., (2005) and identified six mutants altered in growth on turanose containing media compared to WT seedlings.

5.3. Materials and Methods

5.3.1 Plant material and chemicals

An *Arabidopsis thaliana* ethyl methanesulfonate (EMS) mutagenized seed collection of Col::LUC (Ng et al., 2013) was used for screening purposes. Commercially available chemical compounds were purchased from Sigma Aldrich (Steinheim, Germany) and Roche Diagnostics (Mannheim, Germany), unless specified otherwise.

5.3.2 *tin* mutant screening

Seed collections were screened for mutants grown on vertical agar plates containing ½ MS, 1% (w/v) agar and 90 µM turanose, 90 µM sucrose or no added sugars (control). Seedlings were observed under a microscope 5 days after germination (DAG), to identify individuals showing normal hypocotyl and root development. Putative *turanose insensitive* (*tin*) mutants were collected and transferred to petri dishes containing ½ MS, 1% (w/v) agar and 90 µM sucrose in the light for 15 days (14/10-h photoperiod at 150 µmol photons m^{-2} s^{-1}) to allow safe transfer to the soil. Seeds collected from these were rescreened on turanose to confirm the phenotype.

5.3.3 Hypocotyl Elongation Assays

Software embedded in the ImageJ program was used to determine hypocotyl lengths of seedlings grown on turanose, sucrose and control media. These values were subsequently used to determine whether growth differences observed between WT and mutant lines were statistically significant.

5.3.4 Statistical Analyses

For statistical analysis of the data, a two-sided homoscedastic *Students t*-test was used (Excel, Microsoft, Seattle).

5.4. Results and Discussion

It has always been assumed that plants can sense the amounts of sugars present within their cells and react to alterations in these by affecting gene expression and enzyme activities. This would be advantageous for the plant in order to react to changing environmental conditions both over the short and long terms. Studies over the past 20 years have identified several mechanisms by which plants do this (Chapter 2.4; Rolland et al., 2006). *Arabidopsis* is sensitive to germination when grown on turanose (Gonzali et al., 2005), indicating that turanose might act as a signaling molecule in Arabidopsis. Here we produce a number of mutants that may be involved in this pathway.

5.4.1. Screening for putative *turanose insensitive* mutants

Screening of an EMS mutagenized seed collection revealed 49 putative mutants displaying a *turanose-insensitive* (*tin*) phenotype. These seedlings were grown on sucrose plates in the light for 15 days for safe transfer to soil (Fig 5.1). Seeds were subsequently collected and grown on turanose, sucrose and control media to confirm the phenotype (Fig 5.2).

Fig 5.1. Representative photograph of phenotypes associated with *turanose-insensitive* (*tin*) mutants. Representatives of some of the putative *tin* mutant lines are shown in the figure above, with *tin* phenotypes including altered growth and leaf yellowing.

5.4.2. Second round of screening for confirmation of phenotype

From these 49 putants six positive mutants (Table 5.1) were identified that were overcame the repression of hypocotyl elongation when placed on exogenous turanose media, but which responded when either no exogenous sugar or exogenous sucrose were present (Fig 5.2). Hypocotyl elongation assays indicated that the growth of control plants was significantly decreased compared with the mutants (Fig 5.3). Furthermore, the growth of the WT seedlings on turanose containing media was significantly lower than their growth on sucrose containing or control media containing no sugar (Figure 5.3).

Table 5.1. Growth of positive *tin* mutants compared to Col::LUC WT seedlings. (+) indicates improved growth over WT, (-) indicates similar growth to WT and (*) indicates low rates of germination (however seeds that were able to germinate, showed better growth compared to WT).

Mutant Line	90 µM turanose	90 µM sucrose	control
MST -A2-1-18	+	+	+
MST-A3-1-5	+	+	+*
MST-A4-1-10	+	-	+*
MST-A1-2-9	+	+	+
MST-A1-2-18	+	-	-
MSTA1-2-2	+*	+*	+*

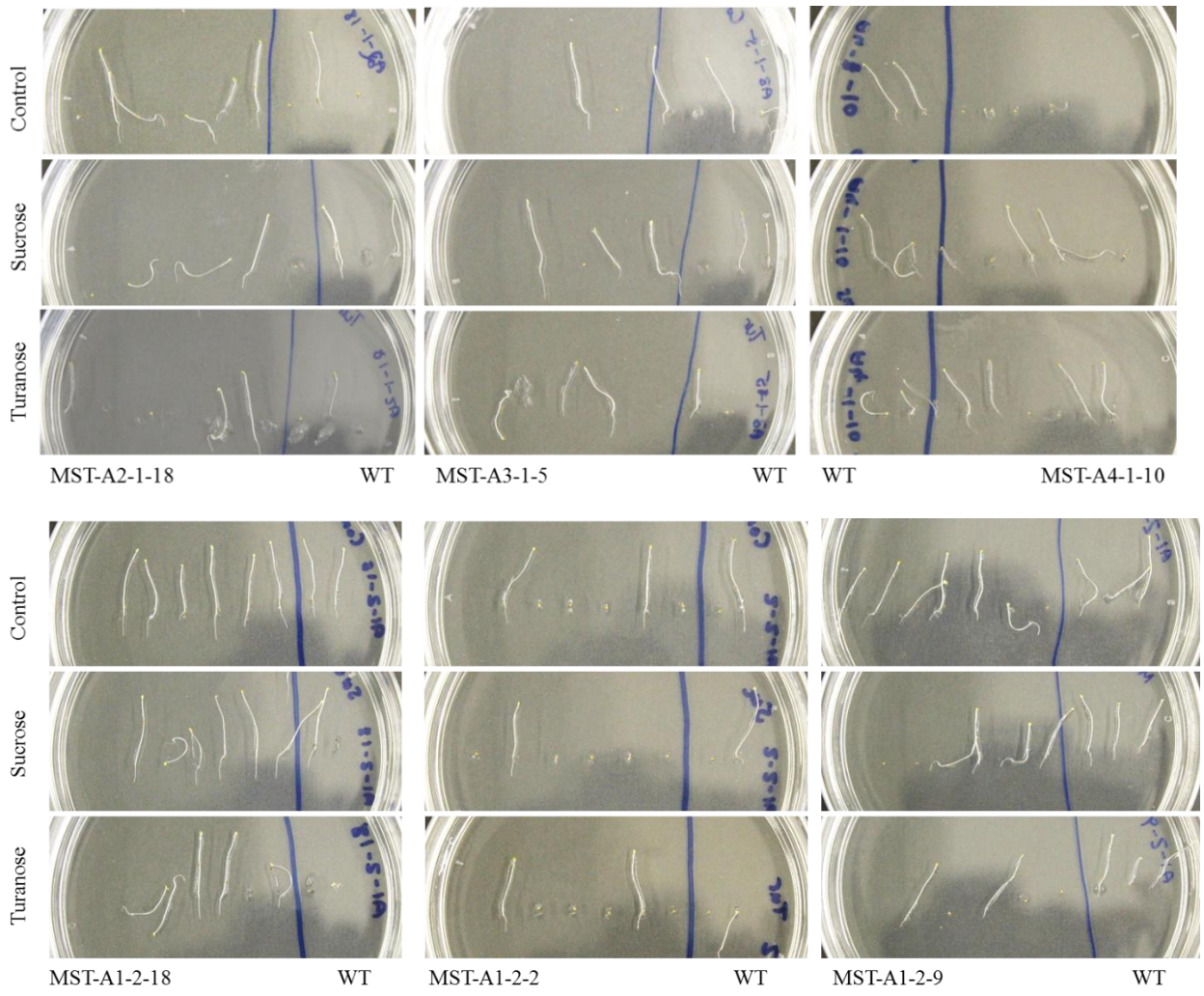

Figure 5.2. Confirmed *tin* mutants resulting from second screen on turanose, sucrose and control media. Confirmed *tin* mutants that show improved germination and growth on the respective media compared to Col::LUC WT seedlings.

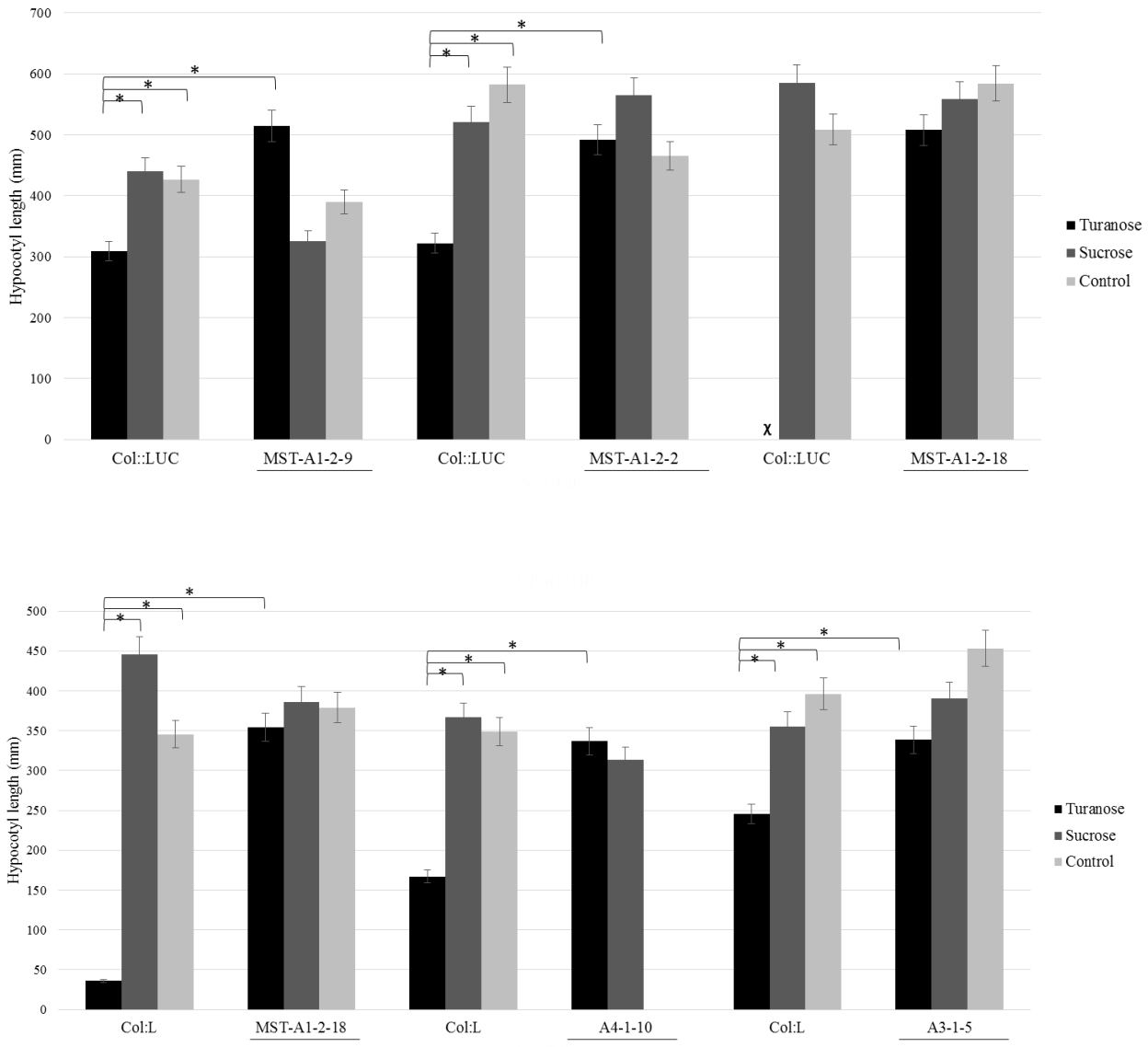

Figure 5.3. Hypocotyl elongation assays. Seeds were grown on 90 µM turanose, 90 µM sucrose or control media containing no sugars. Hypocotyl length was determined using the ImageJ program. Growth of WT seedlings on turanose were compared to WT growth on sucrose media, WT growth on control media and mutant lines grown on turanose media. (*) indicate values determined to be significantly different ($P < 0.05$; $n=3$) from the respective comparisons using Students t-test. (χ) indicates no germination.

Subsequently, seedlings were transferred to soil to observe the phenotype 2 weeks later (Fig 5.4).

Figure 5.4. Representative images of phenotypes observed for confirmed *tin* mutants. Six mutants confirmed from a secondary turanose insensitivity screen ranged from normal vegetative development (putative *tin 5* mutant) to mutants with accelerated cell death phenotypes observed by white necrotic lesions at leaf margin edges (red arrows, putative *tin 3* and *tin 7* mutants), reduced organ size (*tin 2* mutant), yellow pigmentation in mature leaves (*tin 4*) and accelerated flowering (*tin 3* and *tin 6*) compared to the control (Col::LUC).

Several of the mutants show interesting phenotypes in terms of cell death and alterations in flowering time, which indicates that some of them are altered in their developmental processes (Figure 5.4). Some of the *tin* mutants demonstrated normal vegetative development (although no physical phenotype is observed, metabolically, this mutant could differ from the WT), others contained organs of reduced size and yellow pigmentation in mature leaves (Figure 5.4). Although the number of complementation groups has not yet been determined, the diversity of phenotypes indicates that more than one gene has been mutated. We hypothesize that the genes mutated represent components of a novel sugar perception pathway in higher plants.

Positive mutants with confirmed *tin* phenotypes have been crossed to a mapping line (LerxA3-1-5) and the mutated genes are currently being identified by next generation mutation mapping (Li et al., 2008). This will allow the identification of novel components of a potential turanose perception pathway in *Arabidopsis*.

5.5. Conclusion

Results obtained from this project and future work will contribute to our understanding of how higher plant metabolism functions. Future work will allow us to examine if there is a new pathway of sugar signalling in higher plants. The identification of parts of the pathway through mutational analysis will allow us to build a model of how the control mechanism works which would also be of high scientific importance as it would allow us to manipulate the pathway. This will potentially provide us with the ability to engineer crop plants with increased yields.

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Chapter 6
General Discussion

Parasitic plants have an enigmatic role – featuring as "The Golden Bough" in ancient Greek mythology to being a central feature in the modern-day pagan ritual of kissing under mistletoe (*Santales*) during Christmas times. These plants may have several important roles (see Chapter 1), however, they have proved to be serious agricultural pests. Despite this, few molecular studies have been carried out in parasitic plants. The main aim of this project was to develop a metabolic blueprint of several parasitic plants. Furthermore, turanose biosynthesis and signaling was further elucidated in *O. hederae* and *A. thaliana* respectively, a sugar found to predominate parasitic plant metabolism.

6.1 Novel carbon metabolism in parasitic plants and future prospects

In Chapter 3, we utilized plant metabolomics to profile the metabolomes of 5 autotrophic, 17 parasitic and 2 mycoheterotrophic plants and found that parasitic metabolism is very different compared with the metabolism of the model plant *Arabidopsis* and the autotrophic plants profiled in this study. In addition to fuelling their mitochondrial respiration with different substrates compared to autotrophic plants, parasitic plants accumulate an array of interesting metabolites, not generally encountered in autotrophic metabolism. Several of these metabolites were absent in autotrophic species, making them opportune targets for developing herbicides that could curb parasitic growth on crop plants, as they would potentially kill the parasite, without affecting the host plant. In order to do this, the synthesis of these metabolites (e.g. turanose, arabinose, palatinose) first need to be characterized in order to identify the proteins that can be used in herbicide screens, or to develop silencing constructs for the genes that encode the proteins. This second approach has proven successful in curbing the growth of parasitic plants through silencing of mannitol metabolism (Aly et al., 2009).

Furthermore, data generated from Chapter 3, points to the power of metabolomics in profiling non-model species. This was clearly demonstrated for the two *Phalaenopsis* orchids, where one could discriminate between the two developmental stages (mycoheterotrophism and autotrophism) based on metabolome composition. In the future, one could profile metabolites in the non-polar phase, as the data in Chapter 3 only considered metabolites found in the polar phase. Looking at lipids and other non-polar metabolites might shed further light on the metabolism of parasitic plants. Data generated from Chapter 3 touched on the metabolism of parasitic plants, with further studies potentially contributing significantly towards our understanding regarding these plants.

The profiling of the metabolomes of parasitic plants revealed several interesting metabolites that could be exploited for their use in industry. The amino acid trans-4-hydroxyproline can be used for the synthesis of pharmaceuticals like carbapanem antibiotics (Shibata et al., 1986; Hara and Kino., 2009). Arabinose has several applications in the food industry where it serves as a low calorie alternative to sucrose (http://www.futaste.com/pharma_en/index.php?categoryid =26&m2_articleid=82). Another sugar with various applications in industry is turanose due to it being sweet and non-mtabolizable (Shibuya et al., 2004). Furthermore, turanose has been used in the medical field to diagnose Pompe's disease, pointing to another use for this sugar outside of consumption. Understanding turanose metabolism could potentially aid in producing plants that have higher productivity and produce turanose that can be used by industry. This sugar predominated the sugar pools in various parasitic species and hence the following aims of this project was to further elucidate turanose metabolism and possible turanose signaling mechanisms in higher plants.

6.3 Elucidation of turanose biosynthesis and future prospectsne

Autotrophic plants produce sucrose as a metabolizable simple sugar which it can translocate to sink tissues for use in other biosynthetic processes, however, as found in Chapter 3, parasitic plants accumulate very low levels of this sugar while accumulating especially high levels of minor sugars such as turanose. In Chapter 4 we partially purified the 'turanose synthase' protein and this in the future will contribute significantly to our understanding of turanose metabolism. Once the gene responsible for encoding 'turnose synthase' is identified, this gene can be expressed in *E. coli* to purify recombinant protein that will allow us to characterize the protein biochemically. Furthermore, this gene can be either expressed in *Arabidopsis* to explore potential regulatory roles of this sugar in a model plant or in sugarcane to try and produce commercially viable quantities of turanose.

6.4 Production of six positive *turanose-insensitive* (*tin*) mutants and future prospects

The persistence of turanose in parasitic plants suggest that these plants contain sensing mechanisms that enables its perception to occur. Plants use sugar sensing mechanisms to monitor the amounts of sugars present within their cells in order to react to changes in sugar levels by affecting gene expression and enzyme activities. *Arabidopsis* is sensitive to germination when grown on turanose (Gonzali et al., 2005). Using the latter finding and an EMS-mutagenized seed population, we screened for seedlings able to overcome developmental arrest when grown on

turanose. We have identified 6 positive *turanose-insensitive* (*tin*) mutants that are currently undergoing next generation mutation mapping to allow for the identification of mutant alleles that allow *Arabidopsis* seeds to germinate on turanose. Once the mutated genes have been identified, one can order more mutants from various *Arabidopsis* stock centres in order to confirm that a mutation in the identified gene does in fact confer turanose insensitivity. Following confirmation, one can determine why the mutation causes turanose insensitivity.

6.5 Conclusion

In this project we have demonstrated that parasitic plants are characterized by a unique mode of metabolism where they fuel their mitochondrial respiration with alternative substrates compared to autotrophic plants. These plants also accumulate an array of minor sugars, one of them, turanose. This sugar was further investigated in *O. hederae* to identify the protein responsible for its synthesis and in *A. thaliana* to isolate additional *tin* mutant plants. Future work into both the latter projects will shed further light on turanose metabolism and possible signaling within higher plants.

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