Early leaf development in rice (*Oryza sativa* L.): structure, physiology, and gene expression

A thesis submitted by

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Abstract

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Rice (*Oryza sativa* L.) is a key staple crop, but novel approaches are needed to address the growing gap between supply and demand. Increasing the efficiency of photosynthesis is currently a major target for rice improvement, however the onset of this process during rice leaf development is poorly characterised. In addition, the spatial and temporal coordination of photosynthesis with the development of structural elements important to photosynthetic performance is not well understood. In this thesis I describe a series of experiments designed to address this gap in our knowledge of rice leaf development. Firstly, I used chlorophyll fluorescence microscopy to investigate the physiology of developing rice leaf primordia. I also carried out a histological analysis to probe the patterning and differentiation of the vasculature at the same stages of early leaf development. In parallel, the acclimation of rice leaves to light was used to probe the developmental limits of plasticity of these traits. Using this combined study of structure and physiology, I identified the P3/P4 transition as a key stage in the onset of photosynthesis as well as the development of physiologically relevant structural parameters. Moreover, by performing an RNA-Seq analysis of the P3, P4 and P5 stages of rice leaf development, I uncovered a number of gene expression changes correlated with these specific developmental processes. These data were then compared to published gene expression data from maize leaf primordia, allowing the identification of genes putatively underpinning differences between leaf development in *C*₃ and *C*₄ grasses. My results identify the P3/P4 transition as a pivotal stage in rice leaf development where several processes for the initiation of photosynthetic competence are co-ordinated. As well as identifying gene targets for future manipulation of rice leaf structure/function, my data highlight a developmental window during which such manipulations are likely to be most effective.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CAM</td>
<td>Crassulacean acid metabolism</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeat/ Cas9 nuclease mediated genetic modification</td>
</tr>
<tr>
<td>Cₜ</td>
<td>threshold cycle at which PCR amplification was detected</td>
</tr>
<tr>
<td>das</td>
<td>days after sowing</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPOR</td>
<td>light independent (dark functional) protochlorophyllide oxidoreductase</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EdU</td>
<td>ethynyldeoxyuridine</td>
</tr>
<tr>
<td>ETR</td>
<td>electron transport rate</td>
</tr>
<tr>
<td>Fₘ₀</td>
<td>maximum fluorescence in the dark</td>
</tr>
<tr>
<td>Fₘ'</td>
<td>maximum fluorescence in the light</td>
</tr>
<tr>
<td>F₀</td>
<td>zero (background) fluorescence</td>
</tr>
<tr>
<td>FP</td>
<td>maize foliar leaf primordium</td>
</tr>
<tr>
<td>FPKM</td>
<td>fragments per kilobase of transcript per million mapped reads</td>
</tr>
<tr>
<td>Fₛ</td>
<td>steady state fluorescence</td>
</tr>
<tr>
<td>Fᵥ</td>
<td>variable fluorescence</td>
</tr>
<tr>
<td>Glut-tRNA</td>
<td>glutamine- transfer ribonucleic acid</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>HL</td>
<td>high light</td>
</tr>
<tr>
<td>HP</td>
<td>maize husk leaf primordium</td>
</tr>
<tr>
<td>IRRI</td>
<td>International Rice Research Institute</td>
</tr>
<tr>
<td>IVD</td>
<td>interveinal distance</td>
</tr>
<tr>
<td>LHC</td>
<td>light harvesting complex</td>
</tr>
<tr>
<td>LL</td>
<td>low light</td>
</tr>
<tr>
<td>LPL</td>
<td>LICOR programming language</td>
</tr>
<tr>
<td>LV</td>
<td>large vein</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Moloney-murine leukaemia virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mestome sheath</td>
</tr>
<tr>
<td>MX</td>
<td>metaxylem</td>
</tr>
<tr>
<td>NADP-ME</td>
<td>nicotinamide adenine dinucleotide phosphate- malic enzyme</td>
</tr>
<tr>
<td>NBT/BCIP</td>
<td>nitro-blue tetrazolium/ bromo-chloro-indolylphosphate</td>
</tr>
<tr>
<td>NPQ</td>
<td>non-photochemical quenching</td>
</tr>
</tbody>
</table>
P  plastochron
P1  P1 stage leaf
P2  P2 stage leaf
P3  P3 stage leaf
P4  P4 stage leaf
P5  P5 stage leaf
PAM  pulse amplitude modulated fluorometer
PAR  photosynthetically active radiation
PBS  phosphate buffered saline
PCA  principal component analysis
PCR  polymerase chain reaction
Ph  phloem
POR  light dependent protochlorophyllide reductase
PPR  pentatricopeptide repeat protein
PSI  photosystem I
PSII  photosystem II
QTL  quantitative trait locus
r value  correlation coefficient
r' value  coefficient of determination
rbcS  Rubisco small subunit
RIN  RNA integrity number
RNA  ribonucleic acid
RNA-Seq  messenger ribonucleic acid sequencing
rSAP  recombinant Shrimp Alkaline Phosphatase
RT-PCR  reverse transcription polymerase chain reaction
Rubisco  ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP  ribulose-1,5-bisphosphate
SAM  shoot apical meristem
SSC  saline sodium citrate buffer
SV  small vein
TAE  Tris/acetic acid/EDTA buffer
TALEN  transcription activator-like effector nuclease
TE  transposable element
TEM  transmission electron microscopy
TF  transcription factor
UHP  ultra-high purity
UTP  uridine triphosphate
v/v  volume/volume
VBS  vascular bundle sheath
w/v  weight/volume
Xy  xylem
ΦPSII  quantum efficiency of photosystem II
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Chapter 1

Introduction
1. Introduction

1.1. Food security and rice crop improvement

1.1.1. Rice and Food security: current situation and future prospects

Ensuring global food security is one of the most pressing issues of the 21st century. The global population is set to increase from approximately 7 billion currently to 9.7 billion people in 2050 (United Nations, 2015). In addition to requiring a greater amount of food, rising demand for meat and dairy mean that an 85% increase in primary foodstuffs is required between 2013 and 2050 (Ray et al., 2013). One of the world’s key crops is rice. Rice is the second most important crop plant after maize in terms of production (around 746 Mt per year, FAOSTAT 2013), and the single most important in terms of direct supply of calories through food rather than feed (IRRI Rice Almanac, 2003). In addition, it is the staple food for some of the world’s poorest and most rapidly growing populations, predominantly in Asia and Africa. Indeed, rice can form up to 70% of caloric uptake in some Asian countries, including Myanmar and Bangladesh (Dawe, 2000). However, in recent years rice has hit a yield plateau, as have many other major staple crops. Although there was a 36% yield increase of rice in 1970-1980, only a 7% increase was seen in 2000-2010 (Long, 2014; Long et al., 2015). Yield trends in China clearly show this recent plateau (Figure 1.1). Thus, novel approaches are needed to address the growing gap between rice supply and demand.

![Figure 1.1. Average annual yield of rice per unit land area in China. Each data point is the average of all harvested areas in China. The line is a third-order polynomial best fit to the trend of yield against time. Source: FAOSTAT 2007/Zhu et al., 2010.](image-url)
1.1.2. Improvement and stasis in rice yields

Rice yields have improved rapidly over the last century to keep pace with the growing world population. In particular, the Green Revolution was a phase of unprecedented yield increases of major food crops including rice. It started in the 1960s with the introduction of dwarf rice varieties such as IR8, which allowed increased fertilizer use without lodging (Leung et al., 2015). In the 1980s, the key Green Revolution variety IR64 was released. IR64 was adaptable to a relatively wide range of environments, had good disease resistance and eating quality, and good genetic yield potential (the maximal theoretical yield a variety can produce under optimal conditions). This combination of traits made it the most popular rice variety ever, and at its peak it was grown on an astonishing 9 million hectares (Leung et al., 2015). However, despite many improvements in biotic and abiotic stress tolerance since then, genetic yield potential has remained almost completely static (Long and Ort, 2010). There are several potential reasons for this stasis. Breeding for a good harvest index and for strong early vigour have pushed partitioning efficiency and the efficiency of light capture during the growing season to their biological limits (Evans, 2013; Evans, 1997; Long, 2014; Long et al., 2015; Long and Ort, 2010; Long et al., 2006). This leaves only the efficiency of radiation energy conversion to biomass as a target for improvements in genetic yield potential. Losses of energy occur at every stage of energy conversion from radiation energy to biomass (Figure 1.2). Photosynthetic conversion efficiency is currently around 0.02 for major cereal crops, which is a fifth of the theoretical efficiency of 0.1 for C₃ crops/ 0.13 for C₄ crops (Long and Ort, 2010). In fact, rice has particularly poor conversion efficiency (2.2 grams of biomass per mega joule light energy) compared to other crops, including the C₃ crop wheat (2.8 g/MJ) and the C₄ crops maize (3.5 g/MJ) and sorghum (2.8 g/MJ) (Kiniry et al., 1989; Smillie, 2011). However, the biochemical processes underlying conversion efficiency show very little variation between rice varieties and breeding markers are often lacking, making it near impossible to improve most of them by breeding (Long et al., 2015; Sinclair et al., 2004). The anatomical parameters underlying conversion efficiency show slightly more variation, but the links and trade-offs between them are poorly understood, potentially limiting their use in breeding programmes (Long et al., 2006). This is exemplified by the strong apparent links between vein density and leaf width in rice, and the strong conservation of the small lobed nature of the mesophyll cells in rice (Feldman et al., 2014; Smillie et al., 2012). Thus, although breeding for improved photosynthetic conversion efficiency has been suggested, innovations in other methods of crop improvement may ultimately prove more successful. This includes the use of genetic engineering. In addition, the conserved nature of the photosynthetic process may actually be favourable for genetically engineering improved crops, as success in one plant may be relatively easy to translate to another.
There are several reasons to believe improving photosynthetic conversion efficiency would improve yields. The first is the observation that C₄ plants, which have increased photosynthetic rates because they concentrate CO₂ around the key photosynthetic enzyme Rubisco, convert radiation energy to biomass more efficiently than C₃ plants do (Evans, 2013; Hibberd et al., 2008). The second observation is that enriching atmospheric CO₂, which increases photosynthetic rates, can lead to increased biomass accumulation and grain yield (Ainsworth and Long, 2005; Ainsworth et al., 2004; Mitchell et al., 1999). The third is that, compared to modelled optima, many juvenile spikelets of high yielding varieties are not converted to filled grains even under well fertilized conditions (Sheehy et al., 2001; Smillie, 2011). Thus, there is evidence to suggest yield is limited by the supply of photosynthate resource, rather than a lack of sink capacity (Sheehy et al., 2001; Smillie, 2011). Therefore, if high partitioning efficiency can be maintained, increasing photosynthesis should increase biomass and thus crop genetic yield potential.
1.2. Photosynthesis

1.2.1. Key reactions of photosynthesis

Rice plants undertake C₃ photosynthesis (Figure 1.3). In this process, light is captured by the pigment chlorophyll and used to fix carbon dioxide and produce sugars, thus converting light energy from the sun into the chemical energy of organic molecules (Raven et al., 2005). The key processes of photosynthesis take place in chloroplasts, which are present in all green parts of plants. In the chloroplast thylakoid membrane, chlorophyll molecules in antenna complexes trap light energy, which is transferred to the P₆₈₀ chlorophyll molecules of the photosystem II (PSII) reaction centre (Horton et al., 1996; Rochaix, 2014). This results in excitation of an electron in a reaction centre chlorophyll molecule, which is transferred to an electron acceptor. Low energy electrons from water molecules replace the electrons lost from P₆₈₀, and oxygen is produced as a by-product of this photolysis (Messinger and Renger, 2007; Renger, 1987). The protons released in this water splitting reaction contribute to the establishment of a proton gradient across the thylakoid membrane. The energized electrons from PSII move along an electron transport chain to photosystem I (PSI), losing energy at every transfer step (Allen, 2003). This transfer generates a proton gradient across the thylakoid membrane via the cytochrome b₆/f complex (Kurisu et al., 2003). The flow of protons from the thylakoid lumen back into the stroma through ATP synthase drives the synthesis of ATP. The energized electrons are accepted by the P₇₀₀ chlorophyll molecules of the PSI reaction centre, and ultimately passed on from here to the coenzyme NADP+, generating NADPH. Alternatively, cyclic electron flow around PSI occurs to generate only ATP (Munekaga et al., 2004).

Subsequently, this ATP and NADPH are used to fix CO₂ that has entered the leaf through stomata and airspaces, and to reduce it to organic carbon through the reactions of the Calvin-Benson cycle in the stroma of chloroplasts (Benson et al., 1950; Flexas et al., 2008; Michelet et al., 2013). The enzyme Rubisco carboxylates ribulose 1,5-bisphosphate to form 3-phosphoglycerate. Through a series of enzymatic reactions in which electrons are provided by NADPH and energy by ATP, glyceraldehyde 3-phosphate is generated, most of which is eventually converted to sucrose (for transport) or starch (for storage) (Goldschmidt and Huber, 1992; Shinano et al., 2006). Ribulose 1,5-bisphosphate is regenerated to be used in another turn of the Calvin-Benson cycle.
1.2.2. Options for improving photosynthesis

In the light of the need to increase crop yields, work is currently in progress to improve the efficiency of these photosynthetic processes in plants. A wide range of options for improving photosynthesis exists (Figure 1.4). Several of these are aimed at optimising light interception. These include improving canopy architecture, reducing antenna size in the upper leaves of the crop canopy, or altering the pigment distribution in the canopy (Ort et al., 2011; Reynolds et al., 2011; Zhu et al., 2010). Optimising light penetration to lower leaves in this way has been shown to be beneficial to crops grown in monocultures, and may be far from optimal due to
evolutionary selection pressure for shading out competing plants (Song et al., 2013). Incorporating algal pigments into plants to extend the range of wavelengths that can be used in photosynthesis has also been suggested, and could possibly be used in conjunction with the above options (Chen and Blankenship, 2011).

Figure 1.4. Options for improving photosynthesis. 1. Modifications to the crop canopy. Canopy architecture, pigment distribution and antenna length distribution in the canopy have been considered. 2. Modifications to leaf structure. Mesophyll and stomatal CO2 conductance as well as leaf thickness and vascular patterning have been considered. 3. Modifications to cell organization. Optimization of chloroplast distribution and movement as well as mitochondrial location within the cell may be targeted. 4. Modifications to the electron transport chain. Increasing the range of wavelengths that can be used in photosynthesis may be useful, as may an increase abundance of cytochrome f (accompanied by ATP synthase upregulation) and faster mechanisms of relaxation of photoprotection. 5. Modifications to carbon fixation. Upregulation of sedoheptulose bisphosphatase; an improved Rubisco; more efficient phosphoglycolate metabolism; or carbon concentrating mechanisms (including bicarbonate transporters, algal structures such as pyrenoids or carboxysomes, as well as C4 photosynthesis) may lead to increased efficiency of photosynthesis.
However, as discussed above, improving the efficiency of photosynthetic energy conversion may harbour more opportunity than targeting light interception. One of the processes that may be targeted is that of photosynthetic electron transport. For example, increasing cytochrome f content may increase electron transport capacity, although a concurrent increase in ATP synthase content may also be necessary (Chida et al., 2007; Peterhansel et al., 2008; von Caemmerer and Evans, 2010). Alternatively, faster relaxation of photoprotection through the use of algal systems or the modification of xanthophyll cycle dynamics may allow faster recovery of photosystem II efficiency after light stress (Murchie and Niyogi, 2011).

Other options for optimising photosynthesis are aimed at carbon fixation. Leaf conductance to CO$_2$ may not yet be optimal for CO$_2$ provision to the enzymes involved in its fixation. Optimising stomatal patterning, mesophyll airspace structure and chloroplast arrangement within cells have been considered, though trade-offs with drought, heat and light stress tolerance may exist (Araus et al., 2002; Bartoskova et al., 1999; Evans, 1997; Gounaris et al., 1984; Taylor et al., 2012). Another option is the upregulation of enzymes involved in the regeneration of RuBP, which share metabolic control of CO$_2$ fixation with Rubisco (Raven et al., 2005). In particular, upregulating sedoheptulose bisphosphatase has been pinpointed as an option by models, and increases grain yield under high CO$_2$ conditions (Raines, 2011; Rosenthal et al., 2011).

Several efforts aim to reduce losses through photorespiration. This process is an unavoidable consequence of the oxygenase activity of the key carbon fixation enzyme Rubisco (Farquhar et al., 1980). As Rubisco is also an oxygenase, phosphoglycolate can be produced as a result of its activity. The process of recycling this phosphoglycolate to the Calvin-Benson cycle intermediate phosphoglycerate is known as photorespiration and is considered wasteful, as energy is used and carbon is lost in these metabolic reactions (but see (Peterhansel et al., 2010). Rice has particularly high levels of photorespiration, since it is a C$_3$ plant and is largely grown under tropical, high temperature conditions which increase Rubisco oxygenase activity (Brooks and Farquhar, 1985). It is estimated that approximately 33% of the total level of Rubisco in a rice plant may be functioning as an oxygenase under current atmospheric CO$_2$ concentrations at 30°C (Evans and von Caemmerer, 2000). In general, photorespiration reduces the productivity of C$_3$ crops by over 30% (Ogren, 1984; Zhu et al., 2004).

Engineering a Rubisco with a higher rate of catalysis or higher CO$_2$ specificity may adapt this enzyme to our increasingly warm, relatively high oxygen world (compared to the conditions under which Rubisco evolved) (Parry et al., 2013; Spreitzer and Salvucci, 2002). The use of a less wasteful bacterial pathway of phosphoglycolate metabolism has already been shown to result in increased net photosynthetic efficiency in *Arabidopsis* (Kebeish et al., 2007). Alternatively, building
up an entirely new CO₂ fixation pathway in plants from bacterial components has been one of the more radical proposals (Bar-Even et al., 2010; Fuchs, 2011; Mattozzi et al., 2013). However, given the many roles of Calvin cycle intermediates in other aspects of plant metabolism, signalling and development, this would likely require extensive rewiring of a multitude of plant systems.

Recently, efforts have focused on saturating Rubisco with carbon dioxide in order to suppress its oxygenase activity and thus reduce photorespiration. Carbon dioxide may be concentrated around Rubisco through the use of cyanobacterial bicarbonate transporters (Price et al., 2008). Even greater gains may be possible if algal carbon concentrating mechanisms such as carboxysomes and pyrenoids can be put to use in higher plant cells (Meyer and Griffiths, 2013; Zarzycki et al., 2013). However, plants have evolved carbon concentrating mechanisms of their own, and these promise the largest gains. C₄ photosynthesis and crassulacean acid metabolism (CAM) are two complex traits plants have evolved to reduce losses through photorespiration (Hibberd et al., 2008; Meyer and Griffiths, 2013). Both are thought to have evolved on many separate occasions in response to past decreases in atmospheric CO₂, which are known to increase the oxygenase activity of Rubisco (Christin and Osborne, 2014; Sage et al., 2011). C₄ photosynthesis involves a spatial separation of photosynthetic processes, whereas CAM involves temporal separation of activities. Current efforts are focused on C₄ photosynthesis as a means of improving the yield of C₃ crops, particularly rice (extensively reviewed in (Evans and von Caemmerer, 2000; Hibberd et al., 2008; Kajala et al., 2011; Langdale, 2011; Leegood, 2013).

The predicted contribution of each of these suggested improvements to increased conversion efficiency ranges from 13-15% for the use of photorespiratory bypasses or optimised relaxation of photoprotection to 30-60% for the introduction of C₄ photosynthesis or algal carbon concentrating mechanisms, or the dramatic reduction of Rubisco oxygenase activity through engineering of the enzyme itself (Long et al., 2015; Long et al., 2006). The timescale to implementation varies from around 1-10 years for alterations which have already led to improved seed yield in model species, to an unknown number of years (10-30) for the most complex alterations (reviewed in (Long et al., 2015; Long et al., 2006)).

Several recent developments make this a good time to attempt these ambitious projects in crop improvement. Firstly, developments in computing power and crop modelling make in silico exploration of synergistic effects, trade-offs and their consequences for using combinations of traits possible at the level of cells, whole plants and canopies (Boote et al., 2013; Song et al., 2013; Zhu et al., 2013). In addition, developments in genetic engineering such as the use of zinc finger nucleases, TAL-effector nucleases (TALENs) and particularly CRISPR make it possible to place
exogenous sequences into particular places in the genome (Jiang et al., 2013b; Long et al., 2006). Such directed insertions make positional effects on the expression of transgenes less of an issue, enabling more accurate comparisons between transformation events. These enhanced tools may also make it easier to use combinations of gene alterations. As many of the traits (such as leaf thickness, airspace volume, chloroplast surface area and C₄ photosynthesis) affecting photosynthetic efficiency are controlled by complex networks, modifying multiple genes in the same plant is likely to be essential (Tholen et al., 2012). Understanding leaf development in rice and the genetic mechanisms underlying it are thus crucial to engineering a more efficient photosynthetic process in this crop.

1.3. Structure and development of leaves

1.3.1. Rice leaf structure and anatomy

Leaves are the primary site of photosynthesis in plants. As such, their anatomy has been extensively studied, and is a key target for efforts to improve photosynthetic efficiency. Leaf shape and structure vary greatly between species. In monocots, leaves often lack a petiole and consist of a sessile blade, a ligule and a sheath that surrounds the plant stem (Raven et al., 2005). In grasses such as rice, leaves are strap-shaped, and contain several different tissues (Figure 1.5). The epidermis is the outermost layer of the leaf, which in rice provides protection of internal tissues from drought through its cuticle and relatively thick cell walls, and from pests through trichomes and silica knobs (Luo et al., 2012). Bulliform cells arranged above veins in the rice epidermis provide another drought protection mechanism, facilitating leaf rolling (Price et al., 1997; Zhang et al., 2009). The rice epidermis is patterned with stomata on both the ad- and abaxial side (Luo et al., 2012). As is typical for monocots, the structures of the rice epidermis are arranged in parallel, in contrast to the random arrangement of structures on most dicot leaves (Luo et al., 2012; Raven et al., 2005).

Below the epidermis, the rice mesophyll or ‘middle tissue layer’ is specialized for photosynthesis. Rice mesophyll cells are small and heavily lobed (Sage and Sage, 2009). This increases the mesophyll cell surface area available for CO₂ diffusion. In addition, organelle distribution within cells is such that CO₂ recapture from photorespiration is maximised, with mitochondria in the middle of cells and chloroplasts around the outside and spaces between chloroplasts along the cell membrane filled with stroma-filled protrusions (stromules) (Sage and Sage, 2009). Thus, despite having a relatively thin layer of mesophyll, rice leaves still have a relatively high photosynthetic capacity compared to other cereal crops. For example, despite having only 50% of the mesophyll thickness of wheat, rice has 80% of its photosynthetic capacity (Tholen et al., 2012).
Veins run parallel through the rice mesophyll, with small (secondary) veins being defined as those that are more or less completely embedded in the mesophyll and large (primary) veins defined as larger veins that are associated with protruding ribs on the leaf surface (Raven et al., 2005). Much smaller tertiary veins connect minor and major veins, often running perpendicular to them. Veins contain xylem tissues, involved in the provision of water to the mesophyll, and phloem tissues, which export photosynthate from the mesophyll to sink tissues. These tissues are surrounded by a mestome sheath and bundle sheath, neither of which contain many chloroplasts in rice, in contrast with the large green bundle sheath cells seen in C₄ Kranz anatomy (Dengler et al., 1994; Leegood, 2008).

1.3.2. Development of leaves

Considering the development of leaves may provide insight into how they can be manipulated to enable more efficient photosynthesis. In leaf development, as in other aspects of plant development, cells communicate with each other continuously, and position plays a major role (Braybrook and Kuhlemeier, 2010). Thus, a myriad of mobile signals have been implicated in leaf
development, including hormones, small RNA gradients and mechanical forces (Barkoulas et al., 2007; Benkovics and Timmermans, 2014; Besnard et al., 2011; Boudaoud, 2010; Dumais, 2007; Hay et al., 2004). This network results in a complex but robust growth process involving both cell division and cell expansion (Figure 1.6).

Initial recruitment of cells from which leaves are to be formed occurs from the shoot apical meristem (SAM), a dynamic layered and zoned body of cells. The transcription factor (TF) WUSCHEL (WUS) interacts with the ligand CLAVATA3 and its heterodimeric receptor encoded by CLAVATA1 and CLAVATA2 to maintain a pool of stem cells (Clark, 2001). From these cells, the site of formation of a leaf primordium is selected by the presence of an auxin signalling maximum. A family of dynamic auxin efflux carriers, the PIN-proteins, plays an important role in this process (Kuhlemeier, 2007). The growing leaf then acts as an auxin sink, an auxin depletion zone is formed, and the next leaf is initiated at the point furthest away from this leaf, giving rise to spiral phyllotaxis in many plants including the model plant *Arabidopsis thaliana* L. (Deb et al., 2015; Kuhlemeier, 2007). However, modelling has shown that this process is also capable of giving rise to other phyllotactic patterns (Smith et al., 2006). Rice, for example, displays distichous phyllotaxis (Itoh et al., 1998; Itoh et al., 2000). In addition to auxin, cytokinin signalling has also been implicated in this process (Itoh et al., 2012). As leaf primordia are formed expansins are upregulated, loosening the cell wall and promoting leaf outgrowth (Fleming et al., 1997; Pien et al., 2001; Reinhardt et al., 1998). Thus, it is clear that leaf formation requires changes in mechanical forces as well as phytohormone concentrations and transcription factor expression.

![Figure 1.6: Initiation of leaf formation and leaf axis formation by hormones, transcription factors and small RNAs. ‘ad’, adaxial side of leaf, ‘ab’, abaxial side of leaf, KAN, KANADI TF, YAB, YABBY TF, GA, Gibberellin, SAM, shoot apical meristem. Modified from (Barkoulas et al., 2007).](image)
The early stages of leaf formation are characterised by the transition from cell proliferation, indeterminacy and inhibition of differentiation to cell differentiation and determinacy. A range of transcription factors are involved in this process (Veit, 2004). Key amongst these are the KNOX genes, which are implicated in inhibition of gibberellin synthesis, are repressed in simple leaves by ASYMMETRIC LEAVES1/ROUGHSHOEATH2/PHANTASTICA (ARP) MYB-transcription factors (Hay and Tsiantis, 2009) (Figure 1.6). This leads to a switch from inhibition of differentiation to determinacy and leaf formation.

Axis formation is another key process determining leaf shape. The interaction between abaxializing transcription factors (such as KANADI and YABBY-family TFs) and adaxializing transcription factors (such as REVOLUTA, PHABULOSA and PHAVOLUTA-class III HD-ZIP TFs) results in leaf blade outgrowth (Emery et al., 2003; Eshed et al., 2004; Prigge et al., 2005) (see Figure 1.6). In turn, the polarised expression of these transcription factors is regulated by several small RNAs (e.g. miR165), which are themselves regulated by AGO10 (Liu et al., 2009) (Figure 1.6).

Later steps in leaf primordium development involve the differentiation of various leaf structures. This includes the development of veins, in which auxin canalization plays an important role (Berleth et al., 2000; Nelson and Dengler, 1997). In many dicots, the mesophyll differentiates into palisade mesophyll on the adaxial side of the leaf, whereas spongy mesophyll is the fate of abaxial cells (Fleming, 2005). Epidermal cell fate is determined by numerous well studied transcription factors. These include the positive (GL1, TTG and GL3) and mobile negative (TRY and CPC) agents of the reaction-diffusion process that regulates trichome development, and various regulators of stomatal guard cell specification (MUTE and FAMA) and patterning (TMM) (MacAlister et al., 2007; Nadeau and Sack, 2002; Schellmann et al., 2002; Yang and Sack, 1995).

### 1.3.3. Monocot leaf development

Although most of the developmental processes described above have been best studied in the model dicotyledonous plant *A. thaliana*, monocots including maize (*Zea mays* L.) and rice have also been studied (Itoh et al., 2005; Ohtsu et al., 2007). One major difference between monocots and dicots is the location of the SAM within the plant. In monocots, the SAM is found near the base of the plant, whereas the dicot SAM is found at the apex. In addition, a monocot leaf primordium is formed from a ring of cells around the SAM, rather than from a clump of cells on one side (Freeling, 1992). However, the underlying principles of monocot and dicot leaf development are largely conserved. For instance, the ARP/KNOX module that controls determinacy and the interaction between abaxializing YABBY/KANADI TFs and adaxializing class III HD-ZIP TFs are both also found in maize (Juarez et al., 2004; Timmermans et al., 1999).
However, monocot development also has several unique features. One example is the CLAVATA/WUSCHEL module (Nardmann and Werr, 2006). Although this is conserved in monocots, the expression patterns of maize and rice orthologues of these genes suggest they are not directly involved in meristem maintenance. This seems to be carried out by an entirely different mechanism, possibly involving FLORAL ORGAN NUMBER1 (FON1) and FON2 in roles played by CLAVATA1 and CLAVATA3 (respectively) in dicots (Suzaki et al., 2006).

Maize is the best studied monocot in terms of leaf development. The maize KNOX-family TF KNOTTED1 maintains indeterminacy in the SAM (Jackson et al., 1994). In certain domains of the SAM it is repressed by the ARP TF ROUGH SHEATH2, resulting in recruitment of the cells here into leaf primordia (Schneeberger et al., 1998). In addition, the NARROW SHEATH1 and NARROW SHEATH2 TFs recruit cells from two domains to either side of the ROUGH SHEATH2 domain (Nardmann et al., 2004; Scanlon et al., 1996), giving rise to the leaf margins of a ring shaped leaf primordium. The location of the blade/sheath boundary is later established by LIGULELESS2, whereas LIGULELESS1 plays a more specific role in formation of the ligule tissue at this boundary (Walsh et al., 1998).

1.3.4. Leaf development in rice

Rice leaf development is less well studied than that of maize. However, the rice genome has been sequenced and several large scale studies of gene expression during rice development are available (Goff et al., 2002; Jiao et al., 2005; Wang et al., 2010). Itoh et al. (2005) describe six stages of leaf development, of which the first four show the most morphological change. Initial recruitment of leaf founder cells is coordinated by repression of OSH1 (a KNOX-gene) and OsPNH1 upregulation (Itoh et al., 2005). The additional upregulation of OsSCR and the YABBY-family TF DROOPING LEAF (DL) results in a cascade of events (Itoh et al., 2005). The leaf primordium protrudes from the SAM and takes on a hood-like shape as the leaf margin elongates around the SAM. Subsequently, differentiation of the vascular bundle and formation of unique monocot structures such as the ligule and the leaf blade/sheath boundary takes place. Finally, leaf cells differentiate into sclerenchymatous, bulliform, silica, cork and epidermal cells as the blade elongates. Following downregulation of OsPNH1, OsSCR and DL, the sheath elongates rapidly, leading to the emergence of the leaf blade from the sheath of the previous leaf and eventual bending of the leaf at the blade/sheath boundary, such that the adaxial surface of the leaf blade, the main site of photosynthesis, receives more sunlight.

Rice improvement has until recently mainly taken place through breeding. Thus, many rice genes that affect leaf developmental traits important to yield have been identified through QTL analysis. Many of these loci are auxin-related, which is consistent with the known importance of
auxin in many aspects of *Arabidopsis* development (Baylis et al., 2013; Hay and Tsiantis, 2009; Hobbie et al., 2000; Sabatini et al., 1999). For instance, the rice NARROWLEAF1 (NAL1) QTL is known to be involved in the regulation of leaf thickness and width, and is a classical rice dwarfing locus (Qi et al., 2008; Takai et al., 2013). The NAL1 protein was found to encode a plant specific protein possibly involved in polar auxin transport (Qi et al., 2008). More recently, it has been found that NAL1 modulates mesophyll cell divisions between vascular bundles to change vascular patterning, and certain naturally occurring protein variants increase leaf thickness, which pleiotropically increases leaf Rubisco and chlorophyll content and thus photosynthetic rate per unit leaf area (Taguchi-Shiobara et al., 2015; Takai et al., 2013).

The rice NARROWLEAF2 and NARROWLEAF3 loci were also initially identified through QTL analysis. In rice, mutations in WUSCHEL-RELATED HOMEOBX 3A (OsWOX3A) underlie the defects in leaf polarity, margin development and vasculature seen in both *nal2* and *nal3* mutants (Cho et al., 2013; Ishiwata et al., 2013). As with NAL1, auxin transport plays a role, with OsWOX3A thought to interact with PIN protein expression to affect lateral cell proliferation (Cho et al., 2008). A third narrow leaf mutant (*nal7*) is allelic to the constitutively wilted *cow1* mutant, with both having narrow leaves due to a defective flavin-containing monooxygenase of the YUCCA family, which is known to be involved in auxin biosynthesis in *Arabidopsis thaliana* (Fujino et al., 2008; Woo et al., 2007). Phytohormones other than auxin have been implicated in other aspects of rice plant development, including through studies of the DWARF1 (gibberellin signal transduction), DWARF61 (brassinolide receptor kinase) and DWARF53 (strigolactone signalling repressor) genes (Ashikari et al., 1999; Jiang et al., 2013a; Yamamuro et al., 2000; Zhou et al., 2013). However, mutants in these genes often display dwarfing and pleiotropic developmental defects rather than those specific to leaf shape.

### 1.3.5. Links between photosynthetic development and leaf morphogenesis

Leaves are the primary site of photosynthesis, but the links between the development of leaf morphology and the development of photosynthesis are poorly understood. Few studies have been done in *A. thaliana*, rice or any other species to study the onset of photosynthesis in leaf primordia. Thus, it is unknown when a leaf first becomes photosynthetically active, and particularly how photosynthetic development and leaf development are linked (Jarvis and Lopez-Juez, 2013).

Photosynthesis takes place in chloroplasts, and as such their biogenesis and differentiation is crucial to the development of photosynthetic activity in leaves. Plastids replicate through binary fission, betraying their prokaryotic endosymbiont origins (Pyke, 2013; Yoshida et al., 2012). Meristematic cells have fewer than ten proplastids, whereas leaf mesophyll cells can have up to
100, and the number of plastid DNA copies increases from around ten to around 50 per plastid concurrently (Jarvis and Lopez-Juez, 2013; Lopez-Juez and Pyke, 2005). The chloroplast compartment size per cell is tightly regulated and cell type specific, and the proteins regulating chloroplast compartment size are starting to be understood (Jarvis and Lopez-Juez, 2013; Larkin et al., 2016). There is some evidence that plastid DNA copy number may act as a checkpoint for plastid development. Leaves with defects in plastid DNA proliferation have abnormal plastid division and show growth defects, which may also point to a link between plastid proliferation and leaf cell differentiation (Garton et al., 2007).

Indeed, several other mutants also point to links between leaf development and plastid biogenesis. The white sections of variegated leaves often have defective palisade mesophyll development (Aluru et al., 2001; Lopez-Juez and Pyke, 2005; Yu et al., 2007). In addition, the plastid division mutant crumpled leaf has cells lacking chloroplasts accompanied by abnormal leaf lamina expansion (Chen et al., 2009). However, given the many roles of chloroplasts in central metabolism, it has been suggested that these defects are simply due to a lack of synthesis of essential building blocks such as lipids needed by growing cells, or possibly a perturbation of lipid hormone biosynthesis in cells lacking plastids (Babiychuk et al., 2011; Kode et al., 2006).

1.3.6. Development of photosynthesis: recent advances

In recent studies, the development of photosynthesis in very early leaf primordia has received more attention. For instance, a maize microarray study on laser capture micro dissected shoot apices showed that there is no significant difference in the number of photosynthesis-related genes upregulated between the SAM proper and leaves at the P0 and P1 stages (Brooks et al., 2009). Two photosynthesis related genes were found to be upregulated in the SAM proper compared to the P0 and P1 stage leaves, namely a magnesium chelatase subunit H family protein and a ferredoxin-dependent glutamate synthase, and two other photosynthesis related genes were upregulated in the P1 and P0 stage leaves compared to the SAM, namely protoporphyrinogen oxidase and a chloroplast channel forming outer membrane protein (Brooks et al., 2009). However, only a limited number of photosynthesis-related genes could be detected by the microarray chip used in this study, as it was enriched in SAM-derived cDNAs.

A second useful recent study characterized the entire transcriptome of developing maize foliar and husk leaf primordia using RNA-Seq in the context of international efforts to optimise photosynthesis in rice by engineering C₄ photosynthesis (Wang et al., 2013). Substantial changes in photosynthetic gene expression were seen even in very young maize leaf primordia, with maize foliar leaves showing a greater complexity of photosynthetic gene expression during development compared to maize husk leaves (Wang et al., 2013). Other studies of maize leaf photosynthetic
development have been carried out on leaf gradients of older developing leaves (Li et al., 2010; Majeran et al., 2010). These have revealed much about the relationship between the sink-source transition and chloroplast differentiation, and identified roles for known light-associated transcription factors and novel cell-type specific proteases involved in shaping chloroplast protein complements (Li et al., 2010; Majeran et al., 2010). More recently, the rice leaf developmental gradient has also been similarly transcriptomically profiled and compared to that of maize (Wang et al., 2014).

However, to date, no study has been carried out of photosynthetic development in leaf primordia of rice. Many of the leaf developmental processes described above, including the shift from indeterminate to determinate growth, the establishment of polarity, and the most fundamental aspects of tissue patterning occur in developing leaf primordia rather than the older developing leaves profiled in gradient studies. In addition, the functional physiology of photosynthesis during leaf development has been given little attention, and is likely to change rapidly as primordia develop. Thus, although there is likely to be a link between this photosynthetic development and fundamental aspects of leaf morphological differentiation, the developmental stages at which an interaction between these developmental processes is likely to occur are relatively poorly studied. This is particularly true for monocots such as rice, in which a better understanding of these developmental processes could open up opportunities for engineering a more efficient photosynthetic process.
1.4. Thesis aims
- To characterise morphological changes during rice leaf development
- To identify the point at which developing rice leaves gain the ability to carry out electron transport
- To identify the gene expression changes underlying specific events in rice leaf morphological and physiological development
- To identify conserved and diverged gene expression patterns during leaf development in the C₃ grass rice and the C₄ grass maize

1.5. Thesis objectives
- To perform histological analyses of rice leaves at different stages of development
- To perform light environment transfer experiments to identify the developmental window within which rice leaf morphology displays developmental plasticity
- To image the chlorophyll fluorescence dynamics of developing rice leaves
- To carry out a targeted RNA-Seq analysis on developing rice leaves
- To compare RNA-Seq data on developing rice leaf gene expression to equivalent data from maize

1.6. Thesis hypotheses
- The development of structures such as vasculature and stomata occurs at early developmental stages
- Structural parameters affecting photosynthetic performance acclimate to the external environment, but only within a defined developmental window
- The development of photosynthesis occurs later in development than the laying down of morphological parameters
- Biochemical aspects of photosynthesis acclimate within a different developmental window to structural aspects
Chapter 2

Methods
2. Methods

2.1. General chemicals
Analytical and molecular grade chemicals were ordered from Sigma-Aldrich, Fluka, BDH, Fisher Scientific or Melford. Enzymes and reagents were supplied by Roche, Bioline, Promega, Invitrogen or New England BioLabs. All primers were synthesized by Sigma Life Science; plasmid miniprep kits, PCR purification kits and gel extraction kits were from Qiagen and RNA extraction kits were from Sigma. Water used for preparing reagents was deionised ultra-high purity (UHP) water from an ELGA ion exchange system. Nuclease free water was from Ambion; RNAse free water for use in in situ hybridisation was generated using diethylpyrocarbonate treatment (Sigma).

2.2. Plant material and growth conditions
Rice seeds (Oryza sativa var. IR64) were sown on moist tissue paper in petri dishes and incubated at 28 °C at an irradiance of 700µmol m$^{-2}$ s$^{-1}$ for 7 days before being transferred to growth in hydroponics (Makino et al., 1997; Narawatthana 2013 (Thesis)). The hydroponic system, which was maintained in a CONVIRON BDR16 growth chamber (Controlled Environments Ltd., Manitoba, Canada), consisted of 1.5ml microcentrifuge tubes with an opening in the bottom floating on hydroponic growth medium in a polystyrene rack, which was covered with black plastic to prevent algal growth (Figure 2.1). The hydroponic growth medium contained 1.4 mM NH$_4$NO$_3$, 0.6 mM NaH$_2$PO$_4$·2H$_2$O, 0.5 mM K$_2$SO$_4$, 0.8 mM MgSO$_4$, 0.009 mM MnCl$_2$·4H$_2$O, 0.001 mM (NH$_4$)$_6$Mo$_7$O$_24$·4H$_2$O, 0.037 mM H$_3$BO$_3$, 0.003 mM CuSO$_4$·5H$_2$O, 0.00075 mM ZnSO$_4$·7H$_2$O, 0.2 mM CaCl$_2$·2H$_2$O, 0.07 mM Fe-EDTA, and the pH was adjusted to 5.5 using KOH (Murchie et al., 2005). Plants were grown at an irradiance of 700 µmol m$^{-2}$ s$^{-1}$ (except for light acclimation experiments, where low light grown plants were grown at an irradiance of 250 µmol m$^{-2}$ s$^{-1}$) with a 12h/12h light/dark cycle. Light was supplied from a combination of metal halide and tungsten halogen bulbs. Humidity was 50%, CO$_2$ was ambient and temperature 28 °C. For experiments in which plants were transferred between light conditions at different developmental stages of leaf five, the protruding length of the third leaf was used as a proxy for the developmental stage of the younger leaf five inside it (van Campen et al., 2016). For bulking of rice seeds, seedlings germinated in petri dishes as described above were transferred at 7 days after sowing to 900ml square pots containing a 4:1 (v/v) mixture of Levington M3 compost and vermiculite. These rice plants were grown under the same irradiance and atmospheric conditions as seedlings grown in hydroponics.
2.3. Histology

2.3.1. Leaf thickness measurements and vascular development series
1 cm segments were cut from mature 5th leaf blades (grown as described before) and incubated in Carnoy’s solution (4:1 (v/v) ethanol (Fisher Scientific): acetic acid (Sigma)) for 24 hours, dehydrated in 100% ethanol for 24 hours, pre-infiltrated with 1:1 (v/v) Technovit Liquid 1 (TAAB): ethanol for 24 hours, and twice with 100% Technovit 1 for 24 hours. Leaves were vacuum infiltrated with each new solution for 20 minutes. Samples were then embedded in Technovit 7100, dried at 37 °C overnight and sectioned (2µm thickness) using a microtome (Leica RM2145). Images were taken at a 10x magnification using a light microscope (Olympus DP71). Thickness was measured at the bulliform cells using Adobe Photoshop version 12.0. The average of three measurements was taken per leaf. All measurements were taken in the same region along the width of the leaf (Figures 2.2, 2.3 and 2.4), in between the first and second major veins away from the midvein, avoiding bulliform cells directly adjacent to major veins.

Figure 2.2. Diagram of region of leaf section used for thickness measurements (hatched area). Blue: midrib; light green: tissue in between major veins; dark green: major vein. Not to scale.
2.3.2. **TEM**

For transmission electron microscopy, primordia at different developmental stages were dissected into primary fixative (3% (v/v) glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4)). Further fixation and processing were carried out by Chris Hill (Sheffield Electron Microscopy Unit). Samples were fixed for 2-3 days in primary fixative before two fifteen minute washes with 0.1M phosphate buffer at 4°C. Samples were then incubated in secondary fixative (2% (w/v) aqueous osmium tetroxide) at room temperature for one hour before another two fifteen minute washes with 0.1M phosphate buffer (pH7.4) at 4°C. Samples were dehydrated using a graded ethanol series (15 minutes each in 75%, 95%, 100%, 100% ethanol, 1 x 100% ethanol dried over anhydrous copper sulphate). Following this, samples were transferred to glass vials to which 2-3 ml propylene oxide was added and incubated for 15 minutes at room temperature. This step was
then repeated. Samples were then infiltrated with 50:50 Araldite resin/propylene oxide overnight, followed by transfer to 100% Araldite, in which samples were incubated for 6-8 hours at room temperature. Samples were then transferred to moulds containing fresh 100% araldite resin supplemented with BDMA accelerator and baked in an oven at 60°C for 48-72 hours. A diamond knife on a Leica UC 6 microtome was used to cut 85-90 nm sections, which were mounted on copper TEM grids and stained with uranyl acetate for 5-10 minutes in the dark and then with Reynold’s lead citrate for a further five minutes, before being imaged using a FEI Tecnai G2 Spirit TEM (Hillsboro, OR, USA).

2.3.3. Confocal imaging
P3 and P4 stage leaf primordia were dissected as above, mounted in water in a 2mm-depth coverwell (Grace Biolabs), and imaged using an inverted Zeiss LSM510 Meta confocal microscope under a Plan-Neofluar 10x objective with a numerical aperture of 0.3. Excitation was with a 488nm argon laser (50.5% transmission). Emitted light was detected at 650-710nm as a result of a 650-710nm bandpass filter; the pinhole diameter was 96 µm, and a line average of four images was taken. Noise in the image background only was removed using Adobe Photoshop (version 12).

2.4. Gas exchange analysis
Light response curves were recorded on mature 5th leaves. Leaves were deemed mature as soon as blade elongation was no longer occurring (measured on individual leaves; this usually occurred around 20 days after sowing in high light grown leaves and slightly later in leaves grown in or transferred to low light). The probed leaf area of around 0.75cm² was positioned 1/3 of the total length of the leaf blade away from the leaf tip. Absorbance of this area was recorded using an Imaging PAM (Heinz Walz GmbH). Plants were dark adapted for 5 minutes prior to an initial F₀/Fₘ and respiration rate measurement in the dark. Subsequently, plants were allowed to acclimate to the initial irradiance of 200µmol m⁻² s⁻¹ for ten minutes. Gas exchange and simultaneous fluorescence measurements were then recorded using a LICOR LI-6400 portable photosynthesis system (LICOR GmbH) at irradiances of 50, 100, 150, 200, 250, 300, 400, 500, 600, 800, 1000, 1250, 1500 and 2000µmol m⁻² s⁻¹ using a constant flow rate of 200µmol m⁻² s⁻¹, a sample CO₂ concentration of 400µmol m⁻² s⁻¹, and a block temperature of 28 °C with a relative humidity of at least 50% at all times. Plants were allowed to acclimate to each subsequent irradiance level for 3 minutes. The average of four fluorescence measurements was then recorded at 30 second intervals and the average of up to 54 photosynthetic assimilation rate measurements recorded at 5 second intervals were taken at each irradiance level (see Appendix 1 for details of data handling and of the LICOR autoprogram written). The LICOR IRGAs were matched
immediately prior to each measurement. All measurements were performed within the growth chamber plants were grown in.

2.5. Chlorophyll fluorescence imaging

Mature fifth leaves were imaged whilst attached to the plant and surrounded by ambient CO₂ and 100% relative humidity. P3 and P4-1 stage rice leaf primordia were dissected under a stereomicroscope (Leica MZ12) and mounted on cooled set 5% (w/v) agarose (Sigma) in a 35mm (2 mL volume) imaging dish (Ibidi). Imaging was carried out using a custom built chlorophyll fluorescence imaging system using a modified Olympus BX50WI microscope (Rolfe & Scholes, 2002). After exposing samples to an initial dark period of five minutes, the zero level of fluorescence was recorded (F₀) and a saturating pulse was applied (3000μmol m⁻² s⁻¹) to measure the initial maximum fluorescence (Fₘ). Subsequently, an actinic light was switched on (50μmol m⁻² s⁻¹ for primordia, 200μmol m⁻² s⁻¹ for P5 stage and mature leaves). These optimal induction irradiances to avoid photodamage of 50 μmol m⁻² s⁻¹ for P3 and P4 stage leaves and 200 μmol m⁻² s⁻¹ for P5 stage and mature leaves were determined through pilot experiments. During induction, Fₛ (steady state fluorescence) and Fₘ' (fluorescence during a saturating pulse of 3000μmol m⁻² s⁻¹) were recorded after 2s and 10s, then every 30s (in the first two minutes) and every 60s (in the remaining induction period) over a total period of 10min12s (primordia) or 15min12s (mature leaves). For light response curves, samples were then exposed to irradiances of 30, 50, 100, 150, 200, 300, 400 and 600 (and 800, 1500 for mature leaves) μmol m⁻² s⁻¹. After acclimation to each subsequent irradiance level (four minutes for leaf primordia, five minutes for mature leaves), four Fₛ/Fₘ' measurements were taken at 30 second intervals. Captured F₀, Fₘ, Fₛ and Fₘ' images were processed to calculate photosynthetic parameters using custom-built Image J-based software (Rolfe & Scholes, 2002). Absorbance was imaged by measuring the relative reflection of red and near-infrared light as described in Rolfe and Scholes (2010).

2.6. RNA-Seq

2.6.1. RNA extraction and Sequencing

For P3 stage leaves, 240 primordia were used per RNA sample; for P4 stage leaves, five primordia per sample (P4 stage leaves around 1 cm in length were used); and for P5 stage leaves, three leaves per sample (blade tissue down to the collar only). Samples were all harvested between three to five hours into the photoperiod to minimise the potential influence of circadian factors and the analysis was performed with three biological replicates. Samples were snap frozen in liquid nitrogen upon dissection/ harvesting, before being ground with an ice cold micropestle in a 1.5 mL Eppendorf tube. RNA was extracted using 500μl TriZol (Invitrogen) and cleaned up and DNAsé1 treated using the second column of the Sigma Plant Total RNA kit (Sigma Aldrich).
For P3 stage primordia, a batch of 20-40 primordia dissected during the same session were harvested into a single 1.5 mL tube kept cold in liquid nitrogen, and these batches were combined at the TriZol addition step. Samples were stored for up to three months at -80°C between harvesting and extraction. The quality of the resulting RNA was assessed using the Agilent 2100 BioAnalyzer (www.genomics.agilent.com), and all RIN values were found to be above 8. RNA-Seq was carried out at the Liverpool Centre for Genomic Research (www.liv.ac.uk/genomic-research) using RiboZero treated RNA with library construction following the Illumina TruSeq stranded mRNA protocol (www.illumina.com). 1 µg of input RNA was submitted for sequencing per sample. Sequencing (Illumina HiSeq 2000) produced paired end reads with a read length of 100 basepairs.

2.6.2. Transcript quantification and differential gene expression analysis

Bioinformatics analysis was carried out by Steve Kelly (University of Oxford). Paired end reads were subject to quality trimming and adaptor filtering using Trimmomatic (Bolger et al., 2014) using the settings “LEADING:10 TRAILING:10 SLIDINGWINDOW:5:15 MINLEN:50”. The quality filtered paired-end reads were then mapped to the complete set of CDS from version 7.0 of the *Oryza sativa* L. var. japonica MSU Release 7 using bowtie2 (Langmead and Salzberg, 2012) and transcript abundances were estimated using RSEM (Li and Dewey, 2011). All pairwise comparisons between developmental stages were made using DESeq (Anders and Huber, 2010), using the default normalization method and identifying differentially expressed genes as those with a Benjamini-Hochberg corrected p-value ≤ 0.05 (Benjamini and Hochberg, 1995). A PCA plot of all count data by replicate was generated using Simca-P+, version 12 and found to show clear clustering by developmental stage.

2.6.3. Validation of RNA-Seq data by quantitative RT-PCR

After RNA analysis by Illumina sequencing both in silico quality control and RT-PCR validation of the expression patterns of selected genes were used to assess the quality of the data. Validation of RNA-Seq data was carried out using RNA retained from the original samples submitted for RNA-Seq. M-MLV reverse transcriptase (Invitrogen, www.lifetechnologies.com) was used for cDNA synthesis following the manufacturer’s protocol, with 1µg of input RNA used and 500ng oligo (dT)$_{18}$ primer (Sigma).

Subsequently, RT-PCR amplification was carried out in an ABI StepOne Plus RT-PCR system using SYBR Green Master Mix (Invitrogen). Each 20 µL RT-PCR reaction contained 10 µL SYBR Green Master Mix, 0.4 µL 10 µM forward primer (Sigma), 0.4 µL 10 µM reverse primer (Sigma), 1 µL cDNA and 8.2 µL nuclease free water (Ambion). RT-PCR was performed in 96-well plates sealed with optical adhesive covers (Applied Biosystems, USA). Primers for RT-PCR
were designed using QuantPrime (Arvidsson et al., 2008) (details in Table 2.1). After an initial 20 second denaturation at 95 °C, RT-PCR reactions were run for 40 cycles, which consisted of 15 seconds of denaturation at 95 °C followed by 60 seconds of annealing/extension at 60 °C, with all ramp speeds standard. This was followed by melt curve analysis, which consisted of 15 seconds at 95 °C followed by 1 minute at 60 °C, with ramping from 60 °C to 95 °C occurring in 0.3 °C increments. Data were collected at the end of each amplification cycle and during melt curve analysis.

In order to ensure primers were suitable for highly accurate RT-PCR, a standard curve using four two-fold serial cDNA dilutions was plotted for each primer pair, and the primer pair efficiency and standard curve $r^2$ value were calculated (Table 2.1). In addition, melt curves for each primer pair were examined and found not to show multiple peaks. For standard curves, three technical replicates were used at each cDNA dilution; P5 stage derived cDNA was used in all cases except for testing MONOPTEROS primers, for which P3 stage derived cDNA was used, as MONOPTEROS had insufficient expression at P5 stage.

Having ensured primers had high efficiency and produced a linear standard curve, a comparative $C_T$ experiment was carried out. In this experiment, cDNA from each of three biological replicates for each developmental stage was used, except for P3 stage, where two biological replicates were used. Two technical replicates from two separate cDNA synthesis reactions were averaged for each biological replicate, and relative fold changes in expression between developmental stages were calculated using the $\Delta\Delta C_T$ method using StepOne software, version 2.2 (Applied Biosystems, U.S.A.) (Schmittgen and Livak, 2008), with P4 designated the control stage, and PROFILIN used as an internal control gene. Alternatively, DnaJ, which also shows a neutral expression pattern, gave similar results when used as an internal control gene.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer pair efficiency</th>
<th>$r^2$ value</th>
<th>F primer (5'-3')</th>
<th>R primer (5'-3')</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MONOPTEROS (LOC_Os04g56850)</td>
<td>101.24%</td>
<td>0.971</td>
<td>TGTGTCCCTTCG</td>
<td>TCCATCCGCCG</td>
<td>75</td>
</tr>
<tr>
<td>DROOPING LEAF (LOC_Os03g11600)</td>
<td>110.99%</td>
<td>0.982</td>
<td>AAGAGCACCCTTC</td>
<td>TGGCTTTGCAGC</td>
<td>106</td>
</tr>
<tr>
<td>THYLAKOID FORMATION 1 (LOC_Os07g37250)</td>
<td>84.95%</td>
<td>0.958</td>
<td>CAGAGCCAACCAT</td>
<td>TCGAGATCCCTG</td>
<td>73</td>
</tr>
<tr>
<td>IMMUNOPHILIN (LOC_Os07g30800)</td>
<td>98.91%</td>
<td>0.988</td>
<td>CGGGACATGAAA</td>
<td>AAGATGGTAGC</td>
<td>79</td>
</tr>
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<td>NARA5 (LOC_Os10g42240)</td>
<td>112.17%</td>
<td>0.921</td>
<td>GCCAGGCAATGG</td>
<td>TGCCCAATCTGT</td>
<td>60</td>
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<td>DNAJ (LOC_Os03g57340)</td>
<td>93.49%</td>
<td>0.986</td>
<td>TTCTACGAGCAGCA</td>
<td>CGTTGACAGCCT</td>
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<td>PROFILIN (LOC_Os06g05880)</td>
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<td>0.982</td>
<td>GGTGTCATCCG</td>
<td>AGCACAGGCCA</td>
<td>62</td>
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</table>

Table 2.1. RT-PCR primer details. Primer pair efficiency and $R^2$ value are derived from standard curves calculated for each primer pair using three technical replicates at four input cDNA dilutions.

2.7. In situ hybridisation

2.7.1. Cloning techniques

cDNA synthesized as described above from RNA extracted from the stage at which the gene was highest expressed was used as a template for PCR. The PCR amplifications were carried out using Q5 polymerase (NEB) in 50µL containing 10µL 5x Q5 reaction buffer (NEB), 1µL 10mM dNTPs (NEB), 2.5µL 10µM forward primer (Sigma), 2.5µL 10µM reverse primer (Sigma), 2µL cDNA and 0.5µL Q5 polymerase (NEB). Primers with Kpn1 and Sac1 restriction sites added were designed using NCBI PrimerBlast and are listed in table 2.2. PCR conditions were as follows: 30 seconds of initial denaturation at 98°C followed by 30 cycles of denaturation for 10 seconds at 98°C, annealing for 20 seconds at the appropriate annealing temperature and extension for 20 seconds per kb at 72°C, with a final extension at 72°C for two minutes. PCR
products were then double-digested with Sac1 and Kpn1 (both NEB) and purified using a QIAGEN PCR purification kit. The pBluescript SK- plasmid (Figure 2.5) was also double-digested with Sac1 and Kpn1 and mixed with 6x loading dye (containing 0.2% w/v bromophenol blue and 50% v/v glycerol; both Sigma) before being run on a 1% (w/v) agarose gel in 1x TAE buffer (1L 50x TAE buffer contained 242g Tris base (Sigma), 57.1mL glacial acetic acid (Sigma), 100mL of 0.5M EDTA (pH8; Sigma) and 750mL deionized water) alongside 5µL Hyperladder I (Bioline) for 30 minutes at 70V before gel purification using a QIAGEN Gel purification kit. Treatment with recombinant Shrimp Alkaline Phosphatase (rSAP; NEB) was carried out following the manufacturer’s protocol, including heat inactivation. The purified PCR products were ligated to the digested plasmid (2:1 ratio of insert: vector) using T4 DNA ligase (NEB) for two hours at room temperature.

Figure 2.5. Vector map of pBluescript SK-, showing Multiple Cloning Site and T7 and T3 promoters.
5µL of this ligation product was used to transform α-Select Bronze competent cells (Bioline) following the manufacturers protocol. Colonies formed after overnight incubation at 37°C of transformants on LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 10% (w/v) NaCl and 1% (w/v) agar; all Sigma) containing 100mg/mL ampicillin (Sigma) were analysed using colony PCR. The colony PCR amplifications were carried out using Standard Taq polymerase (NEB) in 25µL containing 2.5µL 10x Standard Taq reaction buffer (NEB), 0.5µL 10mM dNTPs (NEB), 0.5µL 10µM forward primer (Sigma), 0.5µL 10µM reverse primer (Sigma), and 0.125µL Standard Taq polymerase (NEB). Colony PCR conditions were as follows: denaturation for 5 minutes at 95°C, followed by 30 cycles of denaturation for 30 seconds at 95°C, 30 seconds of annealing at the appropriate annealing temperature (see Table 2.2) and extension for 1 minute per kb at 68°C, with a final extension at 68°C for five minutes. 10µL PCR product mixed with 2µL 6x loading dye were run on a 1% agarose gel in 1x TAE buffer for 30 minutes at 70V alongside 5µL Hyperladder I to check for the correct sized insert. Selected transformants were used to inoculate 5ml LB liquid medium cultures (1% tryptone, 0.5% yeast extract, 10% NaCl) containing 100mg/mL ampicillin, which were incubated at 37°C overnight with shaking at 200rpm (Orbisafe, SANYO, Japan). A QIAprep Spin Miniprep kit was used to extract the recombinant plasmid and the insert sequence was verified by DNA sequencing performed by the Core Genomic Facility at the University of Sheffield (http://genetics.group.shef.ac.uk/).
<table>
<thead>
<tr>
<th>Probe</th>
<th>Cloned insert length (bp)</th>
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<th>R primer</th>
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<td>GCTTAAGGAGAGGA</td>
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<td>(LOC_Os06g06510)</td>
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<td>GTACCAGAAAGGCA</td>
<td>GGTGTCCTCGAA</td>
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</tbody>
</table>

Table 2.2. Details of primers used for cloning of in situ hybridisation riboprobes. Note that all probes over 300bp were hydrolysed.

2.7.2. Riboprobe synthesis

5µg of the miniprep kit product described above was digested (using Kpn1 for antisense probes and Sac1 for sense probes) and cleaned up using QIAGEN PCR purification kits. 1µg of these linearised template plasmids were then used in 20µl in vitro transcription reactions also containing 2µL DIG RNA labelling mix (containing Digoxigenin-labelled UTP; Roche), 1µL RNase inhibitor, 2µL T3 (for Kpn1 cut plasmids to generate antisense probes) or T7 (for Sac1 cut
plasmids to generate sense probes) RNA polymerase (both 20 units/µL; Roche), and nuclease free water (Ambion). Reactions were incubated at 37°C for two hours in a thermocycler before addition of 1µL RNase-free DNase I (Roche) and incubation at 37°C for a further 15 minutes. To precipitate riboprobes, 2µL 0.2M EDTA, 2.5µL 4M LiCl and 75µL 100% ethanol (all Sigma) were added sequentially before overnight incubation at -20°C. Centrifugation at 12000g for 25 minutes at 4°C resulted in the formation of a pellet, which was washed with cold 70% ethanol and air dried before resuspension in 100µL nuclease free water (if hydrolysis was required) or 50µL 50% (v/v) formamide (Sigma) (if no hydrolysis was required). If necessary, 100µL probes in nuclease free water were hydrolysed by incubation at 60°C with 100µL 2x carbonate buffer (80mM NaHCO₃ (Sigma) and 120mM Na₂CO₃ (Fluka)) for the appropriate length of time to generate 200-300bp fragments using the following approximation: Time (t) = (Lᵢ - Lᵢ)/kLᵢLᵢ, where Lᵢ = initial probe (cloned insert) length, Lᵢ = desired final length, k = 0.11kb/min. After neutralising with 10µL 10% acetic acid (Sigma), probes were precipitated with 1/10th volume 3M NaAc pH 5.2 (Sigma) and 2 volumes ethanol at -20°C for 2 hours. Samples were spun down for 20 minutes at 4°C and 13000RPM, washed with 750µL 70% ethanol, and spun down again for 10 minutes at 4°C and 13000RPM. Pellets were then air dried and resuspended in 50µL 50% formamide (Sigma). 2-4µL of these riboprobes were run on a 1% agarose gel for 15 minutes at 60V in 1X TAE buffer made up using DEPC (diethylpyrocarbonate; Sigma) treated water in a clean gel tank. Probes were then stored at -20°C until use.

2.7.3. Tissue fixation

For all in situ hybridisation processes, the highest grade chemicals available were used. IR64 rice seedlings grown as described above were harvested at 13 days old. The base 5mm of the plant with the outermost leaf removed was cut from the plant using a clean scalpel and immediately immersed in fixative in 10ml vials (50% ethanol, 5% glacial acetic acid, 4% formaldehyde (Sigma) in DEPC treated water). After vacuum infiltration with two washes of cold fixative samples were incubated at 4°C overnight. The next day, samples were incubated at 4°C for 30 minutes in 50, 60, 70, 80, 90, and 100% ethanol sequentially. The 100% ethanol step was then repeated for 3x1 hour before samples were incubated at room temperature for 1 hour in 25% histoclear (National Diagnostics) /75% ethanol, 50% histoclear/50% ethanol, 75% histoclear/25% ethanol, and 100% histoclear (final step repeated twice). 10-15 chips per 10ml vial of Paraplast Xtra (Sigma) were added and samples were incubated overnight at room temperature. The vial was then incubated at 40°C for five hours with 10-15 chips of Paraplast Xtra added after every 2 hours. The vial was then moved to 58°C and incubated for one hour before the histoclear/Paraplast Xtra mix was replaced with pure molten Paraplast Xtra and incubated overnight at 58°C. A further two changes/day of liquid Paraplast Xtra were carried out over three days. After the final
change, samples were poured into pre-heated aluminium cake moulds at 58°C to give a Paraplast Xtra depth of 5-8mm. The position of samples within the blocks was adjusted using a heated needle before samples were left to set at room temperature for at least two hours before mounting onto sample holders. Samples were stored at 4°C before being sectioned on a microtome (Leica RM2145) at 8µm and mounted on 750µL of degassed DEPC-treated water on Polysine slides (VWR) on an RNAseZAP (Invitrogen) wiped hotplate (Leica) at 42°C and dried overnight. Slides were stored at 4°C.

2.7.4. Tissue pre-treatment

Selected slides were mounted in a sterile (baked) glass holder and brought through the following series of solutions at room temperature (200mL per sterile glass dish): histoclear (10 minutes), histoclear (10 minutes), 100, 90, 80, 60, 30% ethanol (1 minute each), DEPC-H$_2$O (5 minutes), 2xSSC (20 minutes; 1xSSC contains 150mM NaCl and 15mM NaHCO$_3$, pH 7.0). The slide rack was then placed in a pre-warmed dish at 37°C containing 100mM Tris pH 7.5, 50mM EDTA and 1µg/mL proteinase K (Sigma) for 30 minutes before being brought through the following solutions: 1xPBS (Sigma) containing 2mg/mL glycine (Sigma) (2 minutes); 1xPBS (2x2 minutes); 1xPBS containing 4% formaldehyde (10 minutes); 1xPBS (2x5 minutes); 0.1M triethanolamine (Sigma), pH8 (with stirring for 10 minutes, then another 10 minutes with the addition of 1mL acetic anhydride (Fluka)); 1xPBS (2x5 minutes); 30, 60, 80, 90, 100, and 100% ethanol (1 minute each). Slides in their slide rack were then incubated at 4°C for 120 minutes in a glass dish containing a small amount of 100% ethanol at the bottom.

2.7.5. Hybridisation and visualization

Probes were denatured at 80°C for two minutes before being cooled on ice and diluted appropriately in 50% formamide before mixing 30µL diluted probes with 120µL hybridisation buffer (for 18 slides, hybridisation buffer was made up of 300µL 10x salts (100mM Tris pH7.5, 10mM EDTA, 3M NaCl), 1200µL formamide, 600µL 50% dextran sulphate (Sigma), 30µL 100x Denhardt’s solution (ThermoFisher Scientific), 30µL RNAse free tRNA (Roche) and 240µL nuclease free water). 150µL of the hybridisation buffer/probe mix was added to each slide and a cover slip put on. Slides were incubated in a sealed slide box above DEPC treated water-soaked tissue paper in a hybridisation oven (Hybrigene) at 50°C overnight. The next day, slides were transferred back to a slide rack and taken through the following solutions: 0.2x SSC (30 minutes at 55°C; coverslips should fall off), 0.2x SSC (2x 30 minutes at 55°C), 1xNTE (0.5M NaCl, 10mM Tris pH 7.5, 1mM EDTA; 2x 5 minutes at 37°C), 1xNTE containing 20µg/mL RNAse A (30 minutes at 37°C), 1xNTE (2x5 minutes at 37°C), 0.2x SSC (2x 1 hour at 55°C), 0.2x SSC (5 minutes at room temperature), and 1xPBS (5 minutes at room temperature). Slides were then incubated in Roche Blocking buffer (for 18 slides, 200mL contained 20mL Roche blocking
reagent (Roche), 20mL 1M Tris pH 7.5, 12mL 2.5M NaCl and 148mL DEPC-treated water) followed by BSA blocking buffer (1L for 18 slides contained 10g BSA (Sigma), 100mL 1M Tris pH7.5, 60mL 2.5M NaCl, 3mL 100% Triton X (Sigma), 837mL DEPC treated water), both in a slide box at room temperature on a rocking table for 40 minutes. Slides were then drained on tissue paper, a PAP (hydrophobic) pen (Sigma) was used to draw around sections and antibody solution (for 18 slides, 2850µL antibody solution was made up of BSA blocking buffer containing 2.28µL Anti-DIG antibody (Roche)) was applied to each slide, before incubation in a sealed slide box above soaked tissue paper at room temperature for 90 minutes (without coverslips). Slides were then placed in fresh BSA blocking buffer (4x15 minutes at room temperature in a slide box on a rocking table as before). Slides were drained on tissue paper before incubation in a slide rack in a dish containing 200mL developing buffer (made up of 20mL 1M Tris pH7.5, 8mL 2.5M NaCl, 10mL 1M MgCl₂ (Sigma) and 162mL DEPC treated water; 2x 10 minutes at room temperature). Slides were again drained on tissue paper before being placed above soaked tissue paper in a slide box as before. A PAP (hydrophobic) pen was used to draw around slides again where necessary, and 150µL developing reagent (made using a NBT/BCIP tablet; Sigma) was pipetted to each slide. Slides were incubated in the dark at room temperature at least overnight, with colour development judged at regular intervals over the next three days using a stereomicroscope (Leica). The reaction was stopped by dipping in stopping buffer (200mL contained 2mL 1M Tris pH7, 0.4mL 0.5M EDTA and 197.6mL DEPC treated water) for 2x 10 minutes at room temperature in a slide rack. Slides were mounted using DPX mountant (Sigma) and allowed to harden for at least 2 hours before viewing.
2.8 Appendix 1

2.8.1 LPL script ‘Flexible LightCurve (Julia)’

/* AutoProgram
   Generated Thr Feb 28 2013 18:39:12
   by AutoProg Builder 1.0b
   Sequence = "GaaGbaGcaBdaCcDbFkAbdaCcDaFkCcFnCcFnCeFnCeFn* */

:FLOAT
ctlVal1 200
ctlVal2 400
ctlVal3 28
wait1 5
logEvery1 5
wait2 10
logEvery2 5
wait3 .5
logEvery3 5
wait4 .5
logEvery4 5
wait5 .5
logEvery5 5
wait6 3
logEvery6 5
wait7 .5
logEvery7 5
wait8 .5
logEvery8 5
wait9 .5
logEvery9 5
wait: 60
values1[50] { 0 }
values2[50] { 200 }
values3[50] { 50 100 150 200 250 300 400 500 600 800 1000 1250 1500 2000 }
values4[50] { 0 }

:PTR user[]
|
| :PTR { ctlVal1 "Flow rate (æmol/s):" } 

:PTR { ctlVal2 "Ref CO2 value (æmol/mol):" } 

:PTR { ctlVal3 "Block temp (C):" }
user LPPrompts IF RETURN THEN
LPRep
cntlVal 2 FlowSetNewTarget
cntlVal 2 MixerSetNewTarget
cntlVal 3 CoolSetNewTarget
1 :INT i1
values1 READY LPRegLoop NILOOP LPLoopStat
values1 i1 PICK VAL LampSetNewTarget
logEvery1 wait1 WaitWithLog lpAbort BREAKIF
LPMatch
DoFoFm
&i1 1 + DROP
ENDLOOP LPDeregLoop
1 :INT i2
values2 READY LPRegLoop NLOOP LPLoopStat
values2 i2 PICK VAL. 2 LampSetNewTarget
logEvery2 wait2 WaitWithLog lpAbort BREAKIF
LPMatch
DoFsFmp
logEvery3 wait3 WaitWithLog lpAbort BREAKIF
DoFsFmp
logEvery4 wait4 WaitWithLog lpAbort BREAKIF
DoFsFmp
logEvery5 wait5 WaitWithLog lpAbort BREAKIF
DoFsFmp
&i2 1 + DROP
ENDLOOP LPDeregLoop
1 :INT i3
values3 READY LPRegLoop NLOOP LPLoopStat
values3 i3 PICK VAL. 2 LampSetNewTarget
logEvery6 wait6 WaitWithLog lpAbort BREAKIF
DoFsFmp
logEvery7 wait7 WaitWithLog lpAbort BREAKIF
DoFsFmp
logEvery8 wait8 WaitWithLog lpAbort BREAKIF
DoFsFmp
logEvery9 wait9 WaitWithLog lpAbort BREAKIF
DoFsFmp
&i3 1 + DROP
ENDLOOP LPDeregLoop
1 :INT i4
values4 READY LPRegLoop NLOOP LPLoopStat
values4 i4 PICK VAL. 2 LampSetNewTarget
wait: 60 * LPMeasure lpAbort BREAKIF
&i4 1 + DROP
ENDLOOP LPDeregLoop
LPCleanup
}
:FCT WaitWithLog
{
60 * :FLOAT totalSecs
:FLOAT logEvery
GETMS totalSecs 1000 * + :LONG stopTimeMs
LOOP

stopTimeMs GETMS - :LONG remainingMs

remainingMs 1000 <= BREAKIF

remainingMs 1000 / logEvery MIN LPMeasure lpAbort BREAKIF

LPLog

ENDLOOP

}
2.8.2. Description of data handling for light response curves

Raw data collected using the 'Flexible LightCurve (Julia)' autoprogram was imported into Excel. The average of four fluorescence measurements recorded at 30 second intervals and the average of up to 54 photosynthetic assimilation rate measurements recorded at 5 second intervals were taken at each irradiance level. Since photosynthetic assimilation rate was logged every 5 seconds, the phase of initial acclimation to a new irradiance level could be identified and any data points collected during this phase were excluded. In addition, since the reference and sample CO$_2$ concentrations were also recorded every 5 seconds, any photosynthetic assimilation rate measurements taken at a time when input CO$_2$ was found to have been unstable could also be excluded. The photosynthetic assimilation rate at the end of acclimation at 200µmol m$^{-2}$ s$^{-1}$ was not found to be significantly lower to that reached at the same irradiance during the light response curve in any samples, indicating that the acclimation period was long enough. All samples analysed had an initial F$_{v}$/F$_{m}$ over 0.75. A representative analysis is illustrated in Figure S1.

![Figure S1](image_url)

Figure S1: A. Representative raw assimilation rate data collected during a light response curve at an irradiance of 100µmol m$^{-2}$ s$^{-1}$. B. Processed data with five data points removed; three were removed during the initial acclimation to the new irradiance and two were removed during a period of unstable input CO$_2$. 

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Chapter 3

Early leaf development in rice
3. Early leaf development in rice

3.1. Introduction

Rice is a C₃ grass whose seeds are the single most important staple food, providing a fifth of the world’s dietary energy supply (Elert, 2014). The source of all carbon in these seeds is photosynthesis and there is significant interest in improving this fundamental process through alteration of several morphological, biochemical and physiological traits in leaves (Hibberd et al., 2008; Long et al., 2006). However, there are still large gaps in our knowledge of leaf development in rice.

Historically, more work has been carried out to understand the morphological development of the fellow monocot maize (Zea mays). This includes work characterizing vascular development, the development of leaf margins and the ligule, and work identifying the genes regulating the transition between indeterminate and determinate growth (Freeling, 1992; Jackson et al., 1994; Jankovsky et al., 2001; Scanlon et al., 1996; Schneeberger et al., 1998; Walsh et al., 1998). Recently, maize leaf development has been studied in more detail to elucidate the developmental signals required for Kranz anatomy which enables the specific compartmentalisation of metabolic processes required for C₄ photosynthesis (Li et al., 2010; Majeran et al., 2010; Wang et al., 2013). International efforts are currently in progress to use this knowledge to improve crops that currently use C₃ photosynthesis by engineering their anatomy and biochemistry to allow the more water- and energy-efficient C₄ photosynthesis (Hibberd et al., 2008; Kajala et al., 2011). One of these C₃ crops is rice. However, basic knowledge of rice leaf development is limited. This is an issue that needs to be addressed if successful modifications of rice leaf anatomy for improved yield are to occur.

Kusumi et al. (2010) and Itoh et al. (2005) broadly characterise the different stages observed during rice leaf development. Rice leaves are initiated by a co-ordinated process of cell growth and division which leads to the formation of a protrusion on the shoot apical meristem (Itoh et al., 2005). This leaf primordium (which is enclosed within the encircling sheaths of older leaves) then undergoes further development to form a mature leaf which consists of a flattened blade and a sheath encompassing the younger leaves, which develop sequentially from the shoot apical meristem. The young plant thus consists of a series of concentric leaves all of which originate from the meristem and are produced in a series separated by a time period, termed the plastochron (P). The definition of leaves by their sequential order of formation (L1, L2, L3…) and plastochron age (P1, P2, P3…) allows the comparison of leaves from different plants at equivalent developmental stages.
P1 stage is characterised by a small protrusion forming on the flank of the shoot apical meristem. This protrusion then forms a hood shaped structure around the shoot apical meristem and taking on an elongated conical shape (P3 stage) (Itoh et al., 2005). The subsequent P4 stage is characterised by a phase of rapid elongation of the leaf blade and visible greening of the tissue. Due to the degree of elongation that occurs during P4, the stage can be sub-divided into stages (1-12) defined by particular blade lengths (Kusumi et al., 2010). At P5 stage the distal tip of the leaf starts to protrude from the sheaths of older leaves, being pushed up by rapid elongation of the more proximal sheath, before growth stops as the leaf reaches maturity (P6 stage).

Despite the fact that the gross morphology of rice leaf developmental stages has been previously studied, there is a gap in our knowledge when it comes to a precise analysis of the development of specific structures. The development of stomata and vasculature in particular are not as well studied in rice as they have been in maize, let alone Arabidopsis thaliana. The vasculature shows distinct modifications in C₄ plants compared to C₃ plants, and it has been argued that an in-depth understanding of C₃ vascular anatomy and C₄ ‘Kranz anatomy’ is crucial for the successful engineering of C₄ photosynthesis into C₃ crops (Hibberd et al., 2008; Kajala et al., 2011). Stomatal development in rice is also of key importance, particularly with regard to improving heat and drought tolerance in the light of global climate change, but this has recently been given more attention by others (Luo et al., 2012) (van Campen et al., 2016; Yaapar 2016, Thesis). Thus, I have focused here on the precise characterization of vascular development in early rice leaf primordia (P2 stage to mature leaves).

The development of vasculature, stomata and other morphological traits in plants is broadly governed by patterns of gene expression (Barkoulas et al., 2007; Braybrook and Kuhlemeier, 2010; Fleming, 2005). Indeed, the fact that many traits are found to be highly repeatable (have high heritability) within plants of the same genetic background under the same growth conditions has long been used in breeding programs (Dudley and Moll, 1969; Hallauer, 2007; Reeves and Cassaday, 2002). However, even within plants of a similar genetic background, phenotypic plasticity can be observed when plants are exposed to different environments (Sultan, 2000). In rice, a crop plant, many studies of the yield performance of a single variety in different environments demonstrate this (Braun et al., 2010; Muralidharan et al., 2002). However, the precise morphological and developmental aspects of this phenotypic plasticity are not often studied in crop plants. Thus, in addition to characterizing morphological
changes during leaf development, I aim to define the developmental window within which phenotypic plasticity is possible in rice.

Plants show developmental plasticity in response to a variety of environmental stimuli. In particular, a significant body of work has shown that many plants have the ability to acclimate their leaves to the light environment, leading to the formation of sun or shade leaves (Kim et al., 2005; Kubinova, 1991; Murchie and Horton, 1997; Oguchi et al., 2003; Terashima et al., 2006). The ambient environment is sensed by mature leaves, leading to the formation of an as yet uncharacterised signal that influences the morphogenesis of developing leaves at a distance from the mature leaves and facilitates their morphological and physiological acclimation. Although it is well established that leaves in many species (including rice) can undergo this acclimation (Hikosaka and Terashima, 1995; Murchie and Horton, 1997; Oguchi et al., 2003) and it is known that the cellular processes underpinning this acclimation occur during relatively early stages of leaf development (Jurik et al., 1979; Murchie et al., 2005; Sims and Pearcy, 1992), the point when developmental plasticity is lost has not been defined precisely. Thus, I have used transfers of plants at different developmental stages between different light environments to induce developmental changes. Since the transfer of plants from high irradiance (700 µmol m$^{-2}$ s$^{-1}$) to low irradiance (200 µmol m$^{-2}$ s$^{-1}$) showed a similar but stronger response than vice versa (both in thickness and in photosynthetic capacity) in previous experiments performed in the Fleming group, this experimental setup was chosen (Narawatthana 2013, Thesis).

3.2. Aims

- To characterize the early stages of rice leaf development
- To characterize vascular development in rice leaves
- To define the developmental window within which morphological and physiological acclimation to light can take place.
3.3. Brief methodology

General methods are described in Chapter 2; details are shown below of the specific methods used in this chapter.

3.3.1. Plant material and histology

IR64 rice was grown as described in chapter 2. For experiments in which plants were transferred between light conditions at different developmental stages of leaf five, the protruding length of the third leaf was used as a proxy for the developmental stage of the younger leaf five inside it (van Campen et al., 2016). For plants grown in high light (at an irradiance of 700µmol m$^{-2}$ s$^{-1}$) a clear filter (cellophane) was positioned approx. 10cm above the canopy; for plants grown in low light (at an irradiance of 250µmol m$^{-2}$ s$^{-1}$), a neutral density filter was positioned approx. 10cm above the canopy. For leaf thickness measurements and studies of vascular development, 1cm segments were cut from mature 5th leaf blades and embedded in Technovit 7100 as described in chapter 2. Thickness was measured at the bulliform cells using Adobe Photoshop as described in chapter 2.

3.3.2. Physiology- light response curves

Light response curves were recorded on mature 5$^{th}$ leaves. Leaves were deemed mature as soon as blade elongation was no longer occurring (measured on individual leaves; this usually occurred around 20 days after sowing in high light grown leaves and slightly later in leaves grown in or transferred to low light). The probed leaf area of around 0.75cm$^2$ was positioned 1/3 of the total length of the leaf blade away from the leaf tip. Absorbance of this area was recorded using an Imaging PAM (Heinz Walz GmbH). Plants were dark adapted for 5 minutes prior to an initial $F_0/F_m$ and respiration rate measurement in the dark. Subsequently, plants were allowed to acclimate to the initial irradiance of 200µmol m$^{-2}$ s$^{-1}$ for ten minutes. Gas exchange and simultaneous fluorescence measurements were then recorded using a LICOR LI-6400 portable photosynthesis system (LICOR GmbH) at irradiances of 50, 100, 150, 200, 250, 300, 400, 500, 600, 800, 1000, 1250, 1500 and 2000µmol m$^{-2}$ s$^{-1}$ using a constant flow rate of 200µmol m$^{-2}$ s$^{-1}$, a sample CO$_2$ concentration of 400µmol m$^{-2}$ s$^{-1}$, and a block temperature of 28 °C with a relative humidity of at least 50%. Plants were allowed to acclimate to each subsequent irradiance level for 3 minutes. The average of four fluorescence measurements was then recorded at 30 second intervals and the average of up to 54 photosynthetic assimilation rate measurements recorded at 5 second intervals were taken at each irradiance level (see also Supplementary Material S1 of Chapter 2). The LICOR IRGAs were matched immediately prior to each measurement. All measurements were performed within the growth chamber plants were grown in.
3.4. Results

3.4.1. Gross morphology of early rice leaf development

In order to define the biological system used in this thesis, an initial characterization of early leaf development in rice was undertaken, with a focus on the development of those structural features important to photosynthetic performance. Figure 3.1A and B show the developmental plastochron stages P1 (youngest primordium) to P6 (mature leaf) of leaf 5, and Figure 3.2 provides an overview of the histology of the different plastochron stages of rice leaf development. Several observations of relevance to photosynthetic development and performance can be made without using a microscope. For example, primordia at the P1–P3 stages are not visibly green, whereas visible greening occurs at P4 stage. In addition, the gross morphology of veins is already visible to the naked eye at the P3 and P4 stages. As well as showing greening and vascular development, P4 stage is also a stage of rapid leaf blade elongation, which continues in P5 stage, and is then also accompanied by rapid elongation of the sheath. P6 stage, which is the mature leaf, shows little gross morphological change compared to P5 stage, except bending of the leaf at the lamina joint and a full greening of the leaf blade.

As choosing an appropriate leaf to study is key to repeatable measurements of development, I proceeded to compare the development of the first five leaves of a rice seedling. The first three leaves of a rice plant (P1–P3) are already initiated in the embryo prior to seed germination (Itoh et al., 2005). Following germination additional leaves are produced at regular intervals by the shoot apical meristem (Itoh et al., 2005). Thus, leaf five is produced from the shoot apical meristem rather than being present in the embryo. In addition, I found that leaf five showed a typical and reproducible growth pattern (Figure 3.1D). Therefore, leaf five was chosen for use in all subsequent analyses.

Observing developing leaves in rice is complicated by the fact that leaves at the P1-P4 stages are hidden from view by the sheaths of older leaves, as can be clearly observed in both longitudinal and transverse sections (Figure 3.2). Thus, a method of non-invasively determining the plastochron stage of leaf five was required. The development of new leaves (plastochron) in rice is correlated in time with leaf protrusion from the leaf sheaths of older leaves (phylochron). This phenomenon was used to develop a plastochron index by which the length of the third leaf could be used to estimate the plastochron stage (P1-P4) of the younger fifth leaf developing inside it, facilitating transfer of plants at particular leaf 5 plastochron stages (Narawatthana 2013, Thesis; van Campen et al., 2016) (Figure 3.1C).
Figure 3.1. Analysis of leaf growth. (A) Dissected apices reveal leaf primordia at P1, P2, P3 and P4-1 (<1 cm) stages. (B) Leaf primordia at stages P3, P4-2 (<2 cm), P4-12 (<12 cm), P5 and P6 stages. (C) Plastochron index showing the relationship between the length of leaf 3 and developmental stage (P1, P2, P3, P4) of leaf 5. (n=7). (D) Leaf blade elongation over time for the first 5 leaves of IR64 rice. Error bars show standard deviation (n=10). Scale bars in A= 0.25 mm (P1, P2, P3) or 1 mm (P4-1); B = 1 cm.
Figure 3.2. Anatomy of the rice shoot apical meristem and developing leaves. (A) Longitudinal section through rice shoot apical meristem (SAM) and P1, P2, P3, and P4 stage leaves, scale bar 300um. (B) SAM and P1 and P2 stage leaves, scale bar 100µm. (C) Cross section through the base of a rice plant, showing SAM, P2, P3, P4 and P5 stage leaves. Scale bar 100µm. (D) Diagram showing leaf primordia and P5 and mature leaf blades. Black bands indicate location of vascular development sections (Figure 3). P2 vascular development sections were taken from the middle of the primordium.
3.4.2. Vascular development in rice

Since veins are one of the most important determinants of leaf structure and function, I studied vascular patterning changes during the early leaf development of wild type IR64 rice plants in detail. Data presented here are the combined results of work by a masters’ student, Rona Costello, and myself. During small vascular bundle development (Figure 3.3 A-D) at P3 stage, a small patch of procambium (circled) can be seen as a darkly stained area in the cells of the ground tissue (A). This area grows larger in P4 (B), and by P5 (C) individual xylem and phloem cells of the vascular bundle are visible and bundle sheath formation has begun. Figure 3.3D shows the mature small vascular bundle. During large vascular bundle development (Figure 3.3 E-H), procambium formation can be seen at P3 stage (circled), with cells in the outer layer of procambium forming a ring around the developing bundle. At P4 stage (F), the area of procambial cells has grown and the formation of a protoxylem (arrow) can be seen on the adaxial side of the bundle. At P5 stage (G), two large metaxylem elements and a few phloem cells have formed, and the bundle and mestome sheath now surround the vascular tissue. The mature large vascular bundle (H) has slightly larger metaxylem and a few more phloem cells (circled) and the protoxylem has been replaced with the primary lacuna. During the formation of the midvein vascular bundle (Figure 3.3I-L), the procambium (circled) of the midvein at P3 stage is the largest of the three vein types and protoxylem formation (arrow) on the adaxial side can be seen (I). This becomes larger in P4 stage (J) and cells of the phloem begin to differentiate (circled). The P5 stage midvein much resembles the large vascular bundles at this stage (J). The P6 stage midrib is composed of one large vascular bundle and three small vascular bundles whose final structure is the same as that of other bundles in the leaf.
3.4.3. Acclimation of leaf morphology to altered irradiance

In order to define the developmental stage at which morphological features such as veins are set, I subjected rice leaves at different developmental stages to a change in their light environment. I hypothesized that at developmental stages at which leaf morphology was developing and still plastic, this would lead to acclimation to the new conditions, whereas at later developmental stages, acclimation would be limited because development of the structures involved had already been completed. Figure 3.4 A shows a schematic of the experiment, in which plants were moved from high light (700µmol m⁻² s⁻¹) to low light (250µmol m⁻² s⁻¹) at different plastochron stages of leaf five.

Figure 3.3. (sections by Rona Costello). Development of the small, large and midvein vascular bundles. Transverse sections of Lf5 showing development across plastochron stages P3 (A, E, I), P4 (B, F, J), P5 (C, G, K) and P6 (D, H, L) for the three types of longitudinal veins in rice. VBS, vascular bundle sheath; Ph, phloem; Xy, xylem; MX, metaxylem; MS, mestome sheath; SV, small vascular bundle; LV, large vascular bundle. Scale bars: 10µm.
Under my experimental conditions, entirely low light grown leaves were significantly thinner than those grown under high light (Figure 3.4B). I tested whether there is a developmental window within which transfer from high to low light leads to the development of a thinner leaf. Plants transferred from high to low light when leaf five was at early plastochron stages (P1 and P3) developed a mature leaf five that was fully acclimated to the low light environment it had been transferred to, being as thin as leaves grown in low light throughout (Figure 3.4B). However, leaves transferred to low light at the late P5 stage, when they had already protruded from the leaf sheath but were not fully expanded, displayed an intermediate thickness in between the thickness of leaves developed under high or low light (Figure 3.4B).

Having established that leaf morphology acclimates to light in rice, the acclimation of other structural features was studied. In addition to being thicker, high light grown leaves were also found to be wider than those grown in low light (Figure 3.5A). Leaves transferred from high to low light at P1 or P3 stage were significantly narrower than those grown under high light and the same width as those grown constantly under low light. In a similar pattern to our leaf thickness observations, leaves transferred from high to low light at P5 stage reached an intermediate width, and were not significantly different to fully high light grown or fully low light grown leaves.

Another structural feature of relevance to leaf performance is the vasculature. High light grown leaves had a slightly larger number of veins across the whole width of the leaf than low light grown leaves, but this difference was not significant (Figure 3.5B). Leaves transferred from high to low light at P1 or P5 stage contained a number of veins not significantly different to a high light grown leaf, whereas leaves transferred at P3 stage had a significantly smaller number of veins than fully high light grown leaves.

Figure 3.4. A. Schematic representation of light transfer experiment. HL, high light, LL, low light, das, days after sowing. B. Leaf thickness in mature leaf 5 after growth in continuous high light (HL), continuous low light (LL) or transfer from high to low light at P1, P3 or P5 stage. Error bars indicate standard error of the mean. Letters indicate statistically significantly different means (p<0.05; Tukey test, n≥6).
Similarly, no significant difference in vein density (number of veins per millimetre) is seen between high light grown leaves or low light grown leaves, or those transferred from high to low light at P3 or P5 stage (Figure 3.5C). Curiously, leaves transferred from high to low light at P1 stage have significantly higher vein density than any of the other categories considered.

Finally, vein spacing was studied, as the differences between C₃ and C₄ plants in this parameter are thought to be key to performance differences between these photosynthetic types. Intervernal distance was found to acclimate to the light environment, with high light grown leaves having significantly larger interveinal distances between small veins than low light grown leaves (Figure 3.5D). In a pattern reminiscent of that seen in leaf thickness and leaf width, leaves transferred from high to low light at P1 stage have interveinal distances that were the same size as those seen in fully low light grown leaves, whereas leaves transferred at the later P3 stage had interveinal gaps that are intermediate in size between high and low light grown leaves. Indeed, leaves transferred at P5 stage had significantly greater interveinal distances than leaves grown constantly under low light, with interveinal distances being the same as those seen in fully high light grown leaves.

Figure 3.5. Morphological acclimation to light in rice leaves. A. Leaf width, B. Number of veins, C. Veins per mm, and D. Intervernal distance between small veins. Parameters were measured in mature leaf 5 after growth in continuous high light (HL), continuous low light (LL) or transfer from high to low light at P1, P3 or P5 stage. Error bars show standard error of the mean. Different letters indicate statistically significantly different means (p<0.05), Tukey test (n≥6).
3.4.4. Acclimation of leaf physiology to altered irradiance

In addition to investigating the acclimation of leaf morphology to light, I investigated the photosynthetic acclimation to light of the rice leaf. Gas exchange analysis and chlorophyll fluorescence were used to probe the physiology of leaves grown at high light (700µmol m$^{-2}$ s$^{-1}$) and low light (250µmol m$^{-2}$ s$^{-1}$). As with my study of the acclimation of morphological parameters, transfers from high to low light were used to investigate whether acclimation ability was affected by the developmental stage at which a new environment was experienced.

Acclimation of photosynthetic capacity to the light environment was found to occur, with the maximum photosynthetic capacity achieved by plants grown under high light conditions found to be markedly higher than that of those grown under low light conditions (Figure 3.6A). However, no increased efficiency at lower irradiances was observed in low light grown plants compared to high light grown plants (Figure 3.6B). In contrast to our observations with regard to the acclimation of morphological parameters, no effect of transfer of plants at different developmental stages was observed. Indeed, photosynthetic performance in leaves transferred from high to low light at any plastochron stage was found to be indistinguishable from that of leaves developed under constant low light, both in terms of the dynamics of the assimilation rate under different irradiances and the maximum assimilation rate under light saturation. Although attempts were also made to study the acclimation of the dark respiration rate, due to the small leaf area used and the error associated with measurements, no statements about the dark respiration rates of any plants can be made.

In addition to these studies of photosynthetic gas exchange, the efficiency of the photosynthetic electron transport chain of leaves acclimated to different light environments was studied using chlorophyll fluorescence. Several parameters were calculated from chlorophyll fluorescence measurements, including $\Phi_{\text{PSII}}$ (the quantum efficiency of photosystem II), non-photochemical quenching (NPQ) and the electron transport rate ($\Phi_{\text{PSII}} \times \text{leaf absorbance} \times \text{irradiance}$). $\Phi_{\text{PSII}}$ and thus electron transport rate were found to be higher in high light grown leaves than in low light grown leaves at irradiances above around 300µmol m$^{-2}$ s$^{-1}$, below which there was no difference between high light and low light grown leaves (Figure 3.6A and B). As with photosynthetic gas exchange results, no effect of the plastochron age at which plants were transferred was seen, with leaves transferred from high to low light at different plastochron stages all acclimating to low light in terms of their $\Phi_{\text{PSII}}$ and electron transport rate. Electron transport rate seems to drop off above an irradiance of 1000µmol m$^{-2}$ s$^{-1}$ for all samples, but this is likely to be an artefact of increased measurement error when values of $\Phi_{\text{PSII}}$ are small, combined with the possibility that overheating of the fluorescence sensor influences measurements at high irradiances.
Next, the light acclimation of non-photosynthetic quenching (NPQ) in rice was assessed. NPQ reflects the dissipation of excess light energy. In all samples, NPQ was very low up to an irradiance of 300µmol m$^{-2}$ s$^{-1}$, after which it increased more rapidly for all samples (Figure 3.6C), indicating that leaves were increasingly needing to dissipate excess light energy above this irradiance. However, little acclimation of this dissipation was observed, with high light grown leaves having only slightly lower NPQ at all irradiances tested than low light grown leaves or leaves transferred to low light at different developmental stages. This difference in NPQ response between leaves acclimated to different environments was not found to be statistically significant. Following on from this, the relationship between Φ_{PSII} and NPQ was assessed at a range of irradiances. NPQ increased almost linearly with decreasing Φ_{PSII} from a Φ_{PSII} of around 0.5 (Figure 3.6D). In addition, the shape of the light response curve displayed by NPQ was similar for all samples, as was the relationship between NPQ and Φ_{PSII}. This indicates that leaves acclimated to high light and low light did not dissipate the light energy received at different irradiances in markedly different ways.
Figure 3.6. Physiological acclimation to light in rice leaves. A. Photosynthetic assimilation rate in response to changes in irradiance in high light and low light grown plants and plants transferred from high to low light at different developmental stages. B. Leaf absorbance of plants grown under different light treatments. C. \( \Phi_{PSII} \). D. Electron transport rate E. Non-photochemical quenching F. Non-photochemical quenching vs. \( \Phi_{PSII} \). HL, high light all along; LL, low light all along; P1>LL, P3>LL, P5>LL: transferred from high to low light at developmental stage P1; P3 and P5 respectively. Error bars represent standard deviation.
3.5. Discussion

3.5.1. Characterization of rice developmental stages

In this chapter, I set out to characterize the early development of the rice leaf in order to choose an appropriate study system and to guide further experiments. The overall anatomy of developing rice leaves has been previously characterized (Itoh et al., 2005; Kusumi et al., 2010). I found that the rice variety studied here (IR64, an ‘indica’ variety) showed a similar pattern of development to the variety used in these studies (Nipponbare, a ‘japonica’ variety). For further in depth study of leaf development in rice, it was also important to choose a particular leaf to work on. The fifth leaf of rice has previously been used in several studies that have looked in depth at rice leaf development (Murchie et al., 2005; Kusumi et al., 2010). Combined with the fact that the fifth leaf is not present in the embryo prior to germination and the robust growth pattern displayed by this leaf, this previous use in the literature makes it an ideal model for the development of other shoot apical meristem-derived rice leaves.

In rice, early leaf development largely occurs in an environment that is to some extent ‘sheltered’ from the outside environment by the sheaths of older leaves. The same can be said for leaf development in other grasses. Use of a plastochron index was found to be helpful for non-invasively sampling specific developmental stages that are obscured from view by the sheaths of older leaves. However, interestingly, the plastochron (time interval in between the initiation of successive primordia) in rice is longer than in maize (Xiaojia Yin and Peng Wang, personal communications). A different way of seeing this would be that there is one additional primordium developing inside the concentric whorls of developing leaves in maize compared to rice. Thus, plastochron indexes must be specific to the grass species studied. In addition, previous work in the Fleming lab has shown that the rice leaf plastochron index differs for high light and low light grown rice leaves (Narawatthana 2013, Thesis), so careful attention must be paid to the environment leaves develop in.

In addition to broadly characterising the different plastochron stages observed in rice, I studied the development of the vasculature in more detail, as vasculature is known to be key to many aspects of mature leaf function, including photosynthetic efficiency and drought and heat tolerance (Sack et al., 2013). Changes in vein number and size were found to occur at specific moments during rice leaf development. As in maize, the midvein is initiated early (by rice P2 stage, maize P1/2 stage), with development of lateral veins occurring at the next developmental stage (rice P3, maize P3/4 stages) (Wang et al., 2013). However, in maize, foliar leaves develop the spacing (by P4 stage) and characteristic Kranz anatomy (by P5 stage) of C₄ plants, which does not occur in our C₃ rice leaves. The venation pattern that develops in rice is instead broadly
similar to that of many other C₃ grasses (Nelson and Dengler, 1997; Sage and Sage, 2009; Sakaguchi and Fukuda, 2008).

3.5.2. Plasticity in leaf structure is limited to early developmental stages

In order to investigate at which developmental stages of the rice leaf certain morphological parameters were plastic, rice plants of which leaf five was at different developmental stages were exposed to a change in the light environment to see to what extent acclimation would occur. Though little studied in rice, acclimation to high or low light conditions has been studied in species as diverse as *A. thaliana*, *Chenopodium album*, cotton and barley (Kim et al., 2005; Kubinova, 1991; Oguchi et al., 2003; Pushnik et al., 1987; Yano and Terashima, 2001). Across these species, acclimation often results in plants allocating their resources to different growth strategies. In general, thick high light acclimated leaves have large internal mesophyll cell surface areas occupied by chloroplasts for CO₂ uptake, whereas thinner low light acclimated leaves use fewer resources to build leaf thickness and more to attempt shade avoidance and elongation growth (Terashima et al., 2006). Features of high light leaf anatomy are generally thought to be established at an early stage of leaf expansion, though the specific developmental stages involved in rice are unknown (Jurik et al., 1979; Sims and Pearcy, 1992).

From my observations, I conclude that rice leaves transferred from high to low light at different plastochron stages (P1, P3 and P5) fully acclimate to their new low light environment in terms of their photosynthetic capacity (maximum assimilation rate and electron transport under saturating light). No developmental ‘setting’ of the biochemical aspects of photosynthetic capacity thus occurs. Instead, leaf physiology is highly plastic even at later developmental stages. However, morphological parameters showed much more developmentally restricted acclimation. For example, although leaves transferred from high to low light at early developmental stages (P1 and P3) fully acclimate in terms of their thickness, leaves transferred to low light at the later P5 stage may become thinner than those that stay at high light, but not as thin as leaves grown at low light all along. A similar pattern of acclimation potential is seen in leaf width, and the interveinal distance in between small veins, but not in the number of veins or in the number of veins per millimetre. These latter structural parameters appear not to show much acclimation, though this may be confounded by the effect of counting large veins in this measure, which may themselves change in width and thus affect total leaf width. These results suggest that although morphological parameters such as thickness, interveinal distance and leaf width may be partly set by the time a leaf has protruded from the sheaths of older leaves, biochemical parameters that affect the maximum assimilation rate are still plastic, and these acclimate under low light.
conditions to give a leaf with a low maximum assimilation rate despite having intermediate morphological parameters.

Previous work in rice has also considered the developmental dynamics of leaf acclimation to light, though to a lesser developmental resolution. Murchie et al. (2005) showed that rice plants grown in high light developed thicker leaves with a higher maximum rate of photosynthesis ($P_{\text{max}}$), more Rubisco per unit leaf area, and a higher chlorophyll a:b ratio than those grown in low light. This response is similar to the previous results from other species mentioned above and to my results. Interestingly, differences were also seen between leaves transferred from low to high light at different developmental stages in this study, although leaves at early primordia stages were not probed (Murchie et al., 2005). Leaves transferred before expansion displayed full photosynthetic acclimation, whereas leaves transferred later showed a reduction in the chlorophyll a:b ratio and an increase in Rubisco content but no change in leaf thickness. Thus, thickness was set at a stage of development prior to leaf expansion. Despite thickness being set, leaves were still able to acclimate fully in terms of their photosynthetic capacity, with leaves transferred from low to high light displaying high photosynthetic capacity after two days in their new high light environment. Thus, it can be inferred that other factors, primarily the chlorophyll a/b ratio and the Rubisco content, are more important than leaf thickness in allowing acclimation to new irradiances (Murchie 2005).

Despite the fact that thickness is not as important for photosynthetic capacity as Rubisco content and the chlorophyll a/b ratio, my results on leaf thickness acclimation raise a number of interesting questions. The first of these concerns the nature of the signalling from mature to developing leaves in response to a change in light. Although some light undoubtedly reaches the developing primordia, the developing leaf is still very much shielded from the environment by the sheaths of older leaves. As leaves were found to be more able to acclimate in terms of thickness prior to protrusion from the leaf sheath, do they receive signals from older leaves that inform them on the light environment around the plant?

Secondly, the mechanisms by which this signal results in a response in leaf thickness and biochemical adaptation require further study. Since these responses trigger a long term physiological change that is maintained as the leaf grows, some form of memory is also likely to play a role. Long distance signalling within plants in response to light has previously been described (Coupe et al., 2006). Previous research has indicated that other environmental information can also be relayed from one part of the plant to another (Lake et al., 2001; Lake et
al., 2002). Thus, long distance signalling between plant organs is a growing field of study, to which light response-related studies in grasses could make important contributions.

Applications of this work could also lie in crop improvement. There is significant interest in modifying rice leaf structure for increased photosynthesis and ultimately yield (Hibberd et al., 2008; Long et al., 2006). The exploration of the limits of phenotypic plasticity in the normal process of leaf differentiation can inform on the boundaries of the system and identify whether particular stages of development should be targeted for modification. In particular, my data show that transfer of a rice leaf from a HL to LL environment at a very early stage of development (P1) leads to narrower leaves with an increased number of (but smaller) interveinal gaps. Vein density is one on the key properties that will need to be modified in order to introduce C₄ photosynthesis into rice (Feldman et al., 2014; Kajala et al., 2011; Smillie et al., 2012). Previous research has shown that variation in vein density can be created in rice through the use of a deletion mutant population (Smillie et al., 2012). I show that varying vein densities can also be observed in plants which have been grown at different irradiances, but that there is a specific developmental window within which acclimation can take place. Thus, modification of leaf structure may be facilitated by targeting alterations to particular developmental stages.

As well as being informative for engineering Kranz anatomy, this work is also informative for other aspects of monocot leaf development. In particular, vein density in monocots affects leaf water and CO₂ relations at a fundamental level through its influence on stomatal patterning. Due to the anatomy of rice leaves, the width of a stomatal complex will be influenced by the width of the epidermal cell file within which it arises. Coupled to the number of cell files within an interveinal gap and the number of interveinal gaps in a leaf (which will be set by the number of veins and the overall width of a leaf), a complex interaction of cellular and leaf-scale patterning and differentiation events will influence the final number and size of stomata. Recent work has successfully manipulated rice stomatal aperture to improve drought tolerance (Hu et al., 2006; Huang et al., 2009). Future work could employ modifications to stomatal density for similar purposes, or to enhance evaporative cooling in order to enhance heat tolerance. Pinpointing the developmental stages at which developmental plasticity is greatest could make any such modifications easier to implement, since altering properties throughout a plant or at all developmental stages may lead to unintended and complicated outcomes.
Chapter 4

The transition to photosynthetic competence
4. The transition to photosynthetic competence

4.1. Introduction

Of primary importance to the yield of cereal crops is the amount of carbon fixation they can carry out through photosynthesis (Furbank et al., 2015). Until recently however, cereal breeding programs have not focused on increasing yield through improving photosynthesis, instead often focusing on more easily measurable traits such as harvest index (Evans, 1997; Reynolds et al., 2011). It is now becoming clear that improving the photosynthetic performance of crops such as wheat and rice must be the focus of future work, since the limits of progress through breeding for improved harvest index are being reached (Mitchell and Sheehy, 2006; Parry et al., 2011). However, our understanding of the developmental aspects of photosynthesis in young leaf primordia of grasses is limited. Thus, the main aim of this chapter is to investigate the physiological aspects of early leaf development in rice (Figure 4.1), a grass that is vital to global food security.

![Leaf development in rice](image)

Figure 4.1. Leaf development in rice. Dissected apices reveal rice leaf primordia at P1, P2, P3 and P4-1 (<1 cm) stages. (B) Leaf primordia at stages P4-2 (<2 cm), P4-12 (<12 cm), P5 and P6. Scale bars in A= 0.25 mm (P1, P2, P3) or 1 mm (P4-1); B = 1 cm.
Photosynthesis in mature rice leaves of rice has been studied for decades. For example, photosynthetic capacity and the activity of key photosynthetic enzymes are known to increase rapidly from leaf emergence (from the sheath of the previous leaf) until the onset of leaf senescence 20 days later (Makino et al., 1983). More recently, it was found that the photosynthetic rate per unit area is very low in the mature blade of the 2nd leaf produced, and increases gradually in subsequently produced leaves (Itoh et al., 2005). However, neither of these studies considers early developmental stages before the leaf emerges from the sheath of the previous leaf. Thus, although the process of photosynthesis is very well studied, the development of this process in rice and its integration with leaf development are much less well understood.

In other plants, including Arabidopsis thaliana and the C₃ grass barley (Hordeum vulgare L.), the photomorphogenic process by which a functional chloroplast develops from a proplastid is well characterised (Jarvis and Lopez-Juez, 2013; Figure 4.2). To fulfil their photosynthetic function, chloroplasts need the tetrapyrrole chlorophyll, which is synthesized from Glu-tRNA in plastids (Mochizuki et al., 2010). This chlorophyll must be combined with various nuclear encoded proteins to form photosynthetic reaction centres and antenna complexes (Chi et al., 2012; Tanaka et al., 2011). Thylakoid membranes, which are the site of the energy transduction reactions of photosynthesis, must also be laid down in developing chloroplasts (Vothknecht et al., 2012). Finally, the organisation of the thylakoid membranes containing the photosynthetic complexes into stacked and unstacked grana occurs (Anderson et al., 2008; Armbruster et al., 2013; Chow et al., 2005).

Figure 4.2. Plastid development. A. Thylakoids in chloroplasts (photosynthetic plastids) develop from vesicles in proplastids (undifferentiated plastids). B. Sequential proplastid-to-chloroplast development in wheat mesophyll cells. Along the length of the leaf, a dramatic increase in plastid number and size accompanies chloroplast differentiation. C. Sequential proplastid-to-chloroplast development visualized by transmission electron microscopy in an Arabidopsis thaliana virescent mutant (in which this process is delayed). Small proplastids in leaf primordia (for a size comparison, see the mitochondrion indicated with a black arrow) possess very few internal thylakoid membranes. As leaf cells differentiate (from the leaf tip to basal margins), chloroplasts develop and the thylakoids form granal stacks. A fully developed A. thaliana mesophyll cell (viewed using Nomarski optics) is also shown (bottom right). Adapted from Jarvis and Lopez-Juez, 2013.
As plastids differentiate, extensive signalling between these organelles and the nucleus must occur. Although plastids do have their own plastome, most of the 2000-3000 plastid proteins are encoded by nuclear genes (Sakamoto et al., 2008). This includes key photosynthetic components, Calvin cycle enzymes and transporters. Thus, careful coordination between a plant cell’s nuclear and plastid genomes is needed. Several molecular mechanisms underlying signalling during plastid development have been identified. These are thought to be distinct from the plastid-nuclear signalling that occurs in mature tissues to cope with fluctuating environmental conditions (Jarvis and Lopez-Juez, 2013). The genomes uncoupled1 (gun1) mutant has defects in retrograde signalling from plastid to nucleus, which affect plastid differentiation (Cottage et al., 2007). The pentatricopeptide repeat-containing protein encoded by GUN1 was long thought to be an integrator of multiple retrograde signals (Koussevitzky et al., 2007). However, more recently it has been proposed that GUN1 might instead act mainly through directly regulating tetrapyrrole synthesis and signalling (Jarvis and Lopez-Juez, 2013; Terry and Smith, 2013). One key tetrapyrrole synthesized in plastids by ferrochelatase 1 is haem (Tanaka et al., 2011; Terry and Smith, 2013). Haem is thought to act as a signal of chloroplast readiness to receive nuclear encoded photosynthetic proteins, indirectly inducing their transcription through the GOLDENLIKE1 transcription factor (Pesaresi et al., 2006; Waters et al., 2009; Woodson and Chory, 2008). Countering this induction, the chloroplast envelope associated PLANT HOMEODOMAIN WITH TRANSMEMBRANE DOMAINS (PTM) transcription factor is activated upon photooxidative exposure, which may occur during failures in the final steps of chlorophyll biosynthesis (Sun et al., 2011). Through ABSCISIC ACID INSENSITIVE4 (ABI4), PTM blocks the transcription of key photosynthetic genes by competing with the light-induced transcription factor HY5 (Kakizaki et al., 2009). These regulatory networks point to careful integration of plastid tetrapyrrole biosynthesis, light signalling and the nuclear production of the protein components of photosynthetic complexes. Since many intermediates of the chlorophyll biosynthetic pathway are highly phototoxic and their accumulation must be prevented, it comes as no surprise that these processes are finely adjusted to each other (Jarvis and Lopez-Juez, 2013).

Although some or all of these mechanisms are likely to occur in rice, the onset of photosynthesis during rice leaf development is little studied. Recently, Kusumi et al. (2010) used chlorophyll fluorescence imaging to show that in rice, leaves at the P4-2 stage (around 2cm long; before emergence) have measureable electron transport rates. These leaves also express several genes involved in chloroplast development. However, whether this is the earliest stage at which these aspects of photosynthetic development occur is not clear.

Most recent work on photosynthesis at early stages of leaf development (both in monocots and dicots) has focussed on changes in the transcriptome (see chapter 1, section 1.3.6). However,
although gene expression changes can inform on the developmental timing of the onset of photosynthetic metabolism to a certain extent, changes in transcript level do not necessarily infer biochemical or physiological function (Amiour et al., 2012; Fernie and Stitt, 2012; Lan et al., 2012). This is a particularly important consideration in photosynthetic metabolism, where post-translational enzyme regulation is common (Geigenberger et al., 2005; Smith and Stitt, 2007; Stitt et al., 2010). Therefore, relating transcript data to the physiological function of the leaf is often problematic. A functional analysis of developmental changes in physiology during early leaf development can thus yield insights that cannot be provided by gene expression data alone.

In the case of photosynthesis, the quantitative analysis of chlorophyll fluorescence quenching has developed as a routine technique for the measurement of photosynthetic function (Krause and Weis, 1991; Maxwell and Johnson, 2000; Meng et al., 2001; Baker, 2008), however most equipment is designed for measurements in leaves which can be clamped within a chamber, greatly restricting the minimum size of the material that can be analysed. Microfluorescence techniques have been developed which provide the appropriate resolution for the analysis of small leaf primordia, however these have generally been applied to the analysis of plant/microbe interactions (reviewed in (Berger et al., 2007) and plant stress response (reviewed in (Baker and Rosenqvist, 2004) rather than used in the context of leaf development. Therefore, I decided to use chlorophyll fluorescence imaging microscopy to look at electron transport chain function in early developmental stages of rice leaves, namely the P3, early P4 (also known as P4-1; up to 1cm long) and P5 stages. Figure 4.1 shows leaf primordia in rice at various developmental stages. P3, P4-1 and P5 stage primordia were studied using chlorophyll fluorescence imaging in a time series, as these allowed me to measure dynamic functional parameters. Parameters calculated were:

\[
\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \quad \text{The initial maximum quantum efficiency of PSII in the dark.}
\]

\[
\Phi_{\text{PSII}} = \frac{F_m' - F_s}{F_m'} \quad \text{The effective quantum efficiency of PSII in the light.}
\]

\[
\text{NPQ} = \frac{F_m - F_m'}{F_m'} \quad \text{Non-photochemical quenching.}
\]

Where $F_v$ is the variable fluorescence, $F_m$ is the maximum fluorescence, $F_o$ is the minimum fluorescence, $F_m'$ is the maximum fluorescence in the light, and $F_s$ is the steady state fluorescence.
4.2. Aims

- To develop a protocol for imaging the efficiency of electron transport in rice leaf primordia using chlorophyll fluorescence microscopy
- To determine when functional electron transport starts to occur
- To characterize the light response of electron transport efficiency in developing leaf primordia
4.3. Brief methodology

General methods are described in Chapter 2; details are shown below of the specific methods used in this chapter.

4.3.1. Microscopy

For confocal microscopy, P4 stage leaf primordia were dissected, mounted in water, and imaged using an inverted LSM510 Meta confocal microscope (Zeiss, www.zeiss.co.uk). Excitation was with a 488 nm argon laser; detection of emitted light was at 650-710 nm. For transmission electron microscopy, primordia at different developmental stages were dissected into 3% (v/v) glutaraldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4). Further fixation and processing were as described (Wallace et al., 2015).

4.3.2. Chlorophyll fluorescence imaging

Mature fifth leaves and dissected P3 and P4-1 stage rice leaf primordia were imaged using a custom built chlorophyll fluorescence imaging system using a modified Olympus BX50WI microscope (Rolfe & Scholes, 2002). Leaves and leaf primordia were exposed to an initial dark period of five minutes, after which \( F_o \) and \( F_m \) measurements were recorded. Leaves and leaf primordia then went through photosynthetic induction over a total period of 10min12s (P3 and P4 stage primordia) or 15min12s (P5 stage and mature leaves) at an irradiance of 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (P3 and P4 stage primordia) or 200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (P5 stage and mature leaves). For light response curves, samples were then exposed to irradiances of 30, 50, 100, 150, 200, 300, 400 and 600 (and 800, 1500 for mature leaves) \( \mu \text{mol m}^{-2} \text{s}^{-1} \). After acclimation to each subsequent irradiance level (four minutes for leaf primordia, five minutes for mature leaves), four \( F_o/F_m' \) measurements were taken at 30 second intervals. Captured \( F_o, F_m, F_s \) and \( F_m' \) images were processed to calculate photosynthetic parameters (Rolfe & Scholes, 2002). Absorbance was imaged by measuring the relative reflection of red and near-infrared light as described in Rolfe and Scholes (2010). For the ‘induced photomorphogenesis’ experiment, primordia were exposed to a constant irradiance of 30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for three hours. In order to prevent desiccation in all these experiments, a polyethylene controlled environment shroud was used to keep samples at 100% humidity, with ambient CO\(_2\).
4.4. Results

4.4.1. Chlorophyll distribution and plastid development in rice leaf primordia

In order to investigate the distribution of chlorophyll in leaf primordia during development, confocal microscopy was carried out. Confocal microscopy is complicated by the fact that the chlorophyll autofluorescence signal is weak in developing leaves, and the chloroplasts are small. However, chlorophyll autofluorescence can be detected in the tip of late P3 stage leaves (Figure 4.3 A). In P4 stage primordia a clear maximum of signal intensity was observed near the tip of the leaf, with more proximal regions displaying a striated pattern of chlorophyll fluorescence (Figure 4.3 B). Figure C shows spectra recorded in three different locations on the leaf primordium. A clear chlorophyll $a$ emission peak was detected in bands of high fluorescence, and was absent in bands of low fluorescence.

Figure 4.3. Distribution of chlorophyll in rice leaf primordia at A. late P3 stage (tip only) and B. P4 stage (tip only) imaged using confocal microscopy with excitation at 488 nm. Chlorophyll autofluorescence is depicted in red. Scale bars 100µm. Arrows indicate locations at which a spectrum was recorded. C. Emission spectrum of three regions of interest indicated in B, recorded using confocal microscope. Blue: low chlorophyll region; red, green: high chlorophyll regions.
Since chlorophyll autofluorescence was detected in P3 and P4 stage leaf primordia, suggesting chlorophyll accumulated even at these early developmental stages, a structural analysis of plastids at these developmental stages was carried out to determine when plastids became mature at a structural level. Plastids in P3 primordia lacked any obvious granal structure (Figure 4.4 A) whereas by P4 stage plastids with occasional grana were observed (Figure 4.4 B). In contrast, P5 plastids had distinct grana (Figure 4.4 C) which looked very similar to those in mature leaves (Figure 4.4 D). It was noticeable that even in the P3 stage plastids distinct starch grains were observed (Figure 4.4 A).

![Figure 4.4. Plastid differentiation during early rice leaf development. (A-D) Transmission electron micrographs of plastids in leaves at (A) P3 stage; (B) P4 stage; (C) P5 stage; (D) mature leaves. (E) Locations sections were taken from (not to scale). Scale bars in A-C= 0.5 µm, D=1 µm. G: stacked grana, *: starch grain. Arrows indicate plastids.]
Having determined when and where chlorophyll accumulation and plastid differentiation occur, I proceeded to examine to what extent leaf primordium tissues could absorb light energy. In order to determine how much light primordia at different stages could absorb to use in photosynthesis, absorbance was imaged in P3, P4, P5 stage and mature leaves using the red/far-red method (Rolfe and Scholes, 2010). Absorbance was found to be low and fairly uniform for P3 and P4 stage leaves, reaching maxima of around 0.3 in most of these primordia (Figure 4.5). Some regions did not absorb light, particularly along the longitudinal axis of these primordia. P5 stage and mature leaves showed much higher and very uniform absorbance, with values of around 0.8-0.85, with P5 stage leaves occasionally showing higher absorbance than mature leaves. These differences in absorbance must be taken into account when comparing photosynthetic efficiency in leaves at different developmental stages.

![Figure 4.5. Absorbance of P3, P4, P5 and mature leaves. Colour scale indicates absorbance, with hotter colours indicating higher absorbance, on a scale from 0 (no light absorbed) to 1 (all light absorbed). Scale bars 0.25 mm.](image)
4.4.2. The development of electron transport chain function

As well as looking at the distribution of chlorophyll and the morphology of developing plastids, I considered whether functional electron transport could occur within the regions in which chlorophyll autofluorescence could be detected. \( \Phi_{\text{PSII}} \), which is defined as \( \frac{F_{m'}-F_{s}}{F_{m'}} \), represents the quantum efficiency of photosystem II (Maxwell and Johnson, 2000). As photosystem II is the most sensitive part of the electron transport chain, this parameter can be used as a measure of the efficiency of photosynthetic electron transport. Thus, values of \( \Phi_{\text{PSII}} \) at steady state were imaged using chlorophyll fluorescence microscopy at an irradiance of 50\( \mu \text{mol m}^{-2} \text{s}^{-1} \) in P3, P4, P5 stage and mature leaves.

Figure 4.6 shows a series of example images from leaves at P3, P4 and P5 stages, as well as mature leaf blades. Due to their relative size, only portions of the P5 and mature leaf blades are shown. For each sample the raw fluorescence output is shown adjacent to the calculated values and distribution of \( \Phi_{\text{PSII}} \). Although rice leaf primordia are encased by the sheaths of older leaves, chlorophyll autofluorescence could be detected in the tips of three out of eight P3 stage leaf primordia (Figure 4.6 A). The highest autofluorescence values observed in these primordia were around 0.1 (where 1 represents the maximum level of fluorescence detected in mature leaves). The other five P3 stage primordia tested showed a very low level of chlorophyll autofluorescence. In contrast, all eight P4 stage leaf primordia showed detectable chlorophyll fluorescence, with regions nearer the tip showing the highest levels of autofluorescence, which were up to around 0.17 (Figure 4.6 B- three representative primordia are shown). These values are still much lower than those observed in P5 stage and mature leaves (autofluorescence values up to 1), where chlorophyll autofluorescence is more uniformly distributed (Figure 4.6 C, D).
Figure 4.6. Chlorophyll fluorescence and photosynthetic efficiency during early rice leaf development. Raw chlorophyll fluorescence (left image) and $\Phi_{\text{PSII}}$ (right image) at 50 µmol m$^{-2}$ s$^{-1}$ in (A) P3, (B) P4, (C) P5 and (D) mature leaves. Images are shown for 3 biological replicates for each leaf stage. Scale bars 0.25 mm. $\Phi_{\text{PSII}}$ value is indicated by the scale adjacent to D.
In order to verify that reasonable measurements could be made on time series of chlorophyll fluorescence images taken using our approach, I recorded $\Phi_{PSII}$ measurements during induction and $\Phi_{PSII}$ and NPQ measurements during light response curves on mature high light grown (700µmol m$^{-2}$ s$^{-1}$) fifth leaves of rice. The resulting induction and light response curves showed that robust measurements which display patterns that match those seen in previous studies can be taken using my approach (Figure 4.7) (Makino et al., 2002). As expected, $\Phi_{PSII}$ rose rapidly in the initial three minutes of exposure to actinic light (after a five minute dark adaptation), before climbing steadily until around 10-15 minutes after exposure to light, when a steady electron transport rate was reached (Figure 4.7 A). During light response curves, $\Phi_{PSII}$ fell with increasing irradiance and NPQ rose rapidly from an irradiance of around 400µmol m$^{-2}$ s$^{-1}$ (Figures 4.7 B and C, respectively). These findings are consistent with measurements made in my light acclimation experiment (see Chapter 3).

Figure 4.7. Dynamics of chlorophyll fluorescence quenching derived parameters in mature leaves. Induction kinetics of $\Phi_{PSII}$ (A), light response of $\Phi_{PSII}$ (B) and light response of NPQ (C) of three high light grown mature fifth leaves. Colours represent different leaves; three regions of interest were used per leaf.
After concluding above that photosynthetic electron transport could occur in three out of the eight P3 stage primordia tested, we recorded dynamic chlorophyll fluorescence imaging measurements to further investigate the nature of the biochemical and physiological events underpinning the very early stages of the acquisition of photosynthetic potential. These included images of $\Phi_{\text{PSII}}$ during induction at an irradiance of 50$\mu$mol m$^{-2}$ s$^{-1}$ in P3 and P4 stage leaf primordia and at an irradiance of 200 $\mu$mol m$^{-2}$ s$^{-1}$ in P5 stage and mature leaves. Induction curves from regions along the length of all three P3 stage primordia and three representative P4 stage primordia, P5 stage leaves and mature leaves are shown in Figure 4.8.

The results indicated a high degree of variation both along individual primordia (from base to tip) and between primordia, both at the P3 and P4 stage, consistent with the idea that rapid changes were occurring in the capacity for electron transport around this transition.

In P3 stage primordia, only the tip regions of the three primordia that showed detectable electron transport showed induction kinetics. The highest $\Phi_{\text{PSII}}$ attained during induction in a P3 stage sample was around 0.31. Induction kinetics and the highest $\Phi_{\text{PSII}}$ attained were highly variable along the length of primordia and between primordia, although rapid induction kinetics were a shared feature of several P3 stage leaf regions examined.

All P4 stage primordia studied showed induction of photosynthetic electron transport. In addition, induction was observed in more basal regions in P4 stage primordia than in P3 stage primordia. The highest $\Phi_{\text{PSII}}$ attained during induction in P4 stage samples ranged from 0.41 to 0.73, and was again very variable along and between primordia. Slower induction kinetics were observed in some tip regions of P4 stage primordia than seen in P3 primordia (Figure 4.8 B). These data can be compared with those observed in P5 stage and mature leaves which showed slower induction kinetics but much higher values of $\Phi_{\text{PSII}}$ and overall more robust patterns of induction despite the higher irradiance used (Figure 4.8 C,D).
Figure 4.8. Induction kinetics of $\Phi_{\text{PSII}}$ during early rice leaf development. Induction of $\Phi_{\text{PSII}}$ at 50 $\mu$mol m$^{-2}$ s$^{-1}$ in different regions of (A) P3 stage and (B) P4 stage leaves and at 200 $\mu$mol m$^{-2}$ s$^{-1}$ in (C) P5 stage and (D) mature leaves. Images are shown for 3 biological replicates for each leaf stage. Regions of measurement, from tip to base, are indicated by the colour legend.
In addition to \( \Phi_{\text{PSII}} \), NPQ \( \frac{F_m - F_{mt}}{F_m} \) was also measured during induction (Figure 4.9 A-D). However, as initial Fm values were low when compared to mature leaves, the absolute NPQ values recorded may not be entirely accurate. As with \( \Phi_{\text{PSII}} \), NPQ was found to be very variable in P3 and P4 stage leaves, with P5 and mature leaves showing more repeatable kinetics. P3 stage primordia often showed higher NPQ than P4 stage primordia, but no clear pattern was observed in the spatial differences along individual primordia. There was a clear early peak of NPQ during induction in P5 stage and mature leaves (around 60-120 seconds after the actinic light was switched on), which was not observed in P3 stage or P4 stage leaves. In all primordia, NPQ reached a consistent level after a period of illumination, usually after 480-960 seconds.
Figure 4.9. Induction of NPQ during early rice leaf development. Induction of NPQ in different regions of (A) P3 stage primordia at 50 µmol m$^{-2}$ s$^{-1}$ and (B) P4 stage primordia at 50 µmol m$^{-2}$ s$^{-1}$ and in (C) P5 stage leaves at 200 µmol m$^{-2}$ s$^{-1}$ and (D) mature leaves at 200 µmol m$^{-2}$ s$^{-1}$.
After leaf primordia at different developmental stages had undergone induction and reached a steady state of electron transport, they were exposed to a series of increasing irradiances (Figure 4.10 A-D). At each irradiance, steady state $\Phi_{\text{PSII}}$ images were collected and data was extracted from regions along the length of leaf primordia.

In P3 stage leaf primordia a light response of $\Phi_{\text{PSII}}$ was only measurable in the regions nearest the distal tip, and $\Phi_{\text{PSII}}$ rapidly dropped to very low levels as the irradiance increased, with similar kinetics in different regions within primordia (Figure 4.10 A). In P4 stage primordia, a light response of $\Phi_{\text{PSII}}$ was measurable in regions further away from the tip than in P3 primordia. $\Phi_{\text{PSII}}$ was generally highest at all irradiances in regions near the tip and all 10 primordia analysed still showed a $\Phi_{\text{PSII}}$ of 0.2-0.1 in regions nearest the tip at the highest irradiance used (Figure 4.10 B). Generally, the heterogeneity seen in steady state $\Phi_{\text{PSII}}$ values after induction was also seen in the light response of $\Phi_{\text{PSII}}$ in P3 and P4 stage leaves.

This can be compared with the pattern of $\Phi_{\text{PSII}}$ during light induction seen in P5 and mature leaf samples (Figure 4.8 C, D) which showed relatively consistent induction kinetics along the length of the leaves analysed and higher consistency between different samples than the light response of $\Phi_{\text{PSII}}$. 
Figure 4.10. Light response kinetics of $\Phi_{\text{PSII}}$ during early rice leaf development. Light response of $\Phi_{\text{PSII}}$ in different regions of (A) P3 stage, (B) P4 stage, (C) P5 stage and (D) mature leaves. Images are shown for 3 biological replicates for each leaf stage. Regions of measurement, from tip to base, are indicated by the colour legend adjacent to A.
In addition, these $\Phi_{\text{PSII}}$ data can be compared to NPQ values also recorded during light response curves (Figure 4.11 A-D). Again, as with the induction kinetics of NPQ, initial $F_m$ values were low when compared to mature leaves, so the absolute NPQ values recorded may not be entirely accurate. NPQ was measurable further towards the base in P3 stage leaves than $\Phi_{\text{PSII}}$. In seven out of eight primordia, the two regions nearest the tip had the highest NPQ values throughout the light response curve (data not shown). The increase in NPQ followed similar dynamics in regions within the same primordium.

Figure 4.11. Light response of NPQ in different regions of (A) P3 stage, (B) P4 stage, (C) P5 stage and (D) mature leaves.
Next, I decided to investigate changes in $\Phi_{\text{PSII}}$ during photomorphogenesis. In order to determine the timescale over which photomorphogenesis can occur in rice leaf primordia, I imaged $\Phi_{\text{PSII}}$ in P3 and P4 stage leaf primordia exposed to an actinic irradiance of 50$\mu$mol m$^{-2}$ s$^{-1}$ at 45 minute intervals over a period of three hours (Figure 4.12). Of three P3 stage primordia examined, none showed a measurable level of $\Phi_{\text{PSII}}$, and of ten P4 stage primordia examined, data could be interpreted for two primordia, as growth of primordia resulted in them moving out of focus during imaging. In these two P4 stage primordia, little change in $\Phi_{\text{PSII}}$ was observed in regions already displaying a measurable level of $\Phi_{\text{PSII}}$, but a slight increase in $\Phi_{\text{PSII}}$ was observed in regions that displayed no measurable $\Phi_{\text{PSII}}$ at the start of the experiment.

Figure 4.12. Changes in $\Phi_{\text{PSII}}$ over three hours in two P4 stage primordia exposed to actinic light at 50$\mu$mol m$^{-2}$ s$^{-1}$. Scale bar 100$\mu$m. Arrowheads indicate regions in which a slight increase in $\Phi_{\text{PSII}}$ was observed. $\Phi_{\text{PSII}}$ is indicated by colour scale (0-1).
4.5. Discussion

4.5.1. The development of a functional electron transport chain in rice

The main structural and anatomical changes that occur during rice leaf development have been previously characterised (Itoh et al., 2005; Chapter 3 in this thesis). The results presented in this chapter provide an analysis of how this structure relates to function, i.e., when a leaf actually gains the capacity to perform an essential element of photosynthesis (absorption of light energy to generate an electron flow). The findings presented here show that a steep developmental gradient exists along the P3 stage leaf, with the tip being the first to develop a measurable $\Phi_{PSII}$. In addition, P4 stage leaves show a marked increase in the maximum $\Phi_{PSII}$ reached at an irradiance of 50 $\mu$mol m$^{-2}$ s$^{-1}$ compared to P3 stage leaves. This indicates that the development of a photosynthetic electron transport chain initiates at the tip of the P3 stage primordium. The transition to photosynthetic competence then happens rapidly during early P4 stage. Electron transport efficiency does not reach the level of that of a mature leaf until the P5 stage, while little additional photosynthetic electron transport efficiency is gained between P5 stage and the mature leaf.

The developmental timing indicated by my findings matches other work done on rice chloroplast development. Mullet (1993) identifies three stages of chloroplast development: stage 1, in which chloroplast replication and DNA synthesis occur; stage 2, in which the transcriptional and translational machinery is established; and stage 3, in which genes that encode parts of the photosynthetic apparatus are expressed and ‘photosynthetic competence’ is activated. More recently, Kusumi et al. (2010) found that genes necessary for stage 1 ($OsPOLP1$, $FtsZ$) were expressed maximally in tissue taken from the base of the plant containing P0 to P3 stage leaves, but that genes necessary for stage 2 ($OsRpoTp$, $V2$) were not expressed very highly until the P4-2 stage (P4 leaves smaller than 2 cm; using this naming scheme, the early P4 leaves used in this chapter- up to 1 cm long- are ‘P4-1’ stage). Genes encoding components of the light harvesting and electron transport machinery ($Lhcb$, $rbcS$) could not be detected in P0-P3 stage leaves, and were not expressed highly until the much later P5 stage (when leaves had protruded from older leaf sheaths). When combined with basic chlorophyll fluorescence imaging and TEM images of chloroplasts developing in P4-1 to P5 stage leaves, these findings led Kusumi et al. to propose that the majority of chloroplast development happens during the P4 stage. I show that this is indeed the case, but I also show that the first detectable electron transport occurs before this in the tip of P3 stage leaves, and that development of photosynthetic function occurs basipetally thereafter. This finding highlights the need to consider the tip-to-base developmental gradients within grass leaf primordia.
My interpretation that photosynthetic competence develops around the P3-P4 stage transition was supported by a structural analysis of plastid differentiation. Plastids prior to P3 stage lacked obvious grana whereas by P4 stage plastids had internal structure consistent with capacity for photosynthetic activity. Perhaps surprisingly, even P3 plastids frequently contained starch granules, suggesting that the plastids were importing carbon from source leaves and that starch grains were being used as a temporary carbon store, potentially enabling rapid growth during the fundamental biochemical switch from sink to source metabolism.

The micro-fluorescence technique used for this analysis places some constraints on data interpretation (for example, no absolute measures of electron transport rate or photosynthetic capacity were calculated since absorbance was found to be variable and other assumptions made in the comparison of these very different tissues may not be met). However, I am confident that true quenching of chlorophyll fluorescence was occurring in these primordia and the Φ_{PSII} measurements presented here are valid. I cannot rule out the presence of some cyclic electron flow, reduction of the plastoquinone pool in the dark (Corneille et al., 1998), or the presence of a small proportion of light harvesting complexes not connected to reaction centres. However, I did not observe the very high raw fluorescence signal that might be observed if a large proportion of light harvesting complexes were uncoupled, and the reasonable F_{v}/F_{m} values observed indicate non-zero amounts of quenching. Furthermore, the light response of Φ_{PSII} observed in primordia shows the expected pattern of decreasing Φ_{PSII} at increasing irradiances, consistent with a functioning electron transport chain.

4.5.2. Induction kinetics and plastoquinone pool size

After establishing that electron transport can occur in P3 and P4 stage leaf primordia, I imaged chlorophyll fluorescence quenching upon exposure to light after a dark adaptation period. One key feature of the kinetics of the photosynthetic induction observed was that it was very rapid compared to induction in mature leaves, although slower rates of induction were observed in some tip regions of P4 stage primordia. There are two possible explanations for this observation. The first is to do with the establishment of the electron transport chain.

Although the development of photosynthesis in rice leaf primordia is not very well studied, much work has been done on the formation of a functional electron transport chain after exposure to light of etiolated tissues in other plants. In barley, (Ohashi et al., 1989) used various electron donors and acceptors on isolated chloroplasts to show that photosystem I is the first to display activity, as early as one hour after exposure to light, followed by photosystem II, and the entire photosynthetic electron transport chain is established after around four hours. Although a crucial light-dependent enzyme in chlorophyll biosynthesis, protochlorophyllide reductase, is activated by light in milliseconds (Heyes et al., 2002), Ohashi et al. found that significant
chlorophyll accumulation is not seen until after both photosystems are established. Photosynthetic induction could first be measured after four hours, and was initially rapid. The authors suggest that this is a result of a small plastoquinone pool that is all rapidly reduced. As this pool grows and takes longer to fully reduce during development/greening, the induction rate slows. Thus, our fast induction rates could be a result of a small plastoquinone pool present in P3 and P4 stage primordia. The slightly slower induction seen in the tips of some P4 stage primordia could be a result of the increased plastoquinone pool size in these developmentally more advanced tissues.

However, a second result of an incomplete or not yet fully established electron transport chain would be a low maximum $\Phi_{\text{PSII}}$. Across all primordia observed, the maximum $\Phi_{\text{PSII}}$ was very variable. In one of the P3 stage samples, $\Phi_{\text{PSII}}$ reached 0.36 during a light response curve, whereas in one of the P4 stage samples, a high of 0.71 was seen (data not shown). Neither of these samples with relatively high $\Phi_{\text{PSII}}$ (compared to the other primordia) displayed the slowest induction kinetics observed. In addition, despite being surrounded by the leaf sheaths of older leaves, these primordia are likely to have experienced more than four hours of light, albeit at a very low irradiance. Although de-etiolation kinetics are species specific and experiments on isolated chloroplasts may not translate directly to those in entire leaf primordia, parts of our samples may thus be at a more advanced state of electron transport chain development than the samples of Ohashi et al. Thus, an incompletely developed plastoquinone pool may not be the explanation for the rapid induction kinetics observed.

4.5.3. Induction kinetics in sink and source leaves

Another explanation for rapid induction may come from the metabolism of the tissues studied. P3 and P4 stage primordia are sink tissues, net importers of carbon from more mature leaves. In a source tissue such as a mature leaf, the rate of induction may be slower due to a lag time associated with the buildup of Calvin cycle intermediates, the activation of Calvin cycle enzymes and the opening of stomata. Our developing primordia have no stomata; stomatal development is first seen in later P4 stage leaf primordia (Itoh et al., 2005). Since these primordia are sink tissues, they are also likely to have high levels of reduced metabolites (Turgeon, 1989). The notion that fast induction can be a result of the accumulation of these reduced metabolite pools is supported by evidence that inducing the oxidative pentose phosphate pathway through stimulating the cell cycle with exogenous cytokinin or by local pathogen infection results in fast induction kinetics in mature leaves (Esfeld et al., 1995; Prokopova et al., 2010). Meng et al. (2001) found that even sink regions at the base of young tobacco leaves (the youngest used was 3.9 cm long) have a measurable rate of electron transport, and induction in these tissues is more rapid than in mature leaves. However, in this system ‘fast’ induction kinetics take place over 3-4 minutes, which is a lot
slower than seen in P3 and P4 stage rice leaves. One limitation of our experiments, however, is that we cannot measure exactly how fast induction is, as this exceeds our speed of measurement.

Other than studies of isolated chloroplasts and the work by Meng et al. (2001) and Kusumi et al. (2010), there are few studies reporting photosynthetic measurements on very young leaves. Peterson et al. (2014) reported changes in the relative abundance of PSI and PSII along maize leaves (the third leaf of 12-day old seedlings was used), and conclude that redox mediation of chlorophyll biosynthesis may regulate PS assembly and thus ensure the development of a suitable PSI/PSII excitation balance. In the most immature (basal) leaf segment studied, Peterson et al. (2014) also report a larger non-variable component of fluorescence, which they attribute to a moderate accumulation of incompletely assembled, non-functional PSII complexes. However, no detailed measurements of $\Phi_{\text{PSII}}$ induction kinetics in at different developmental stages of the leaf are reported in these studies.

4.5.4. Light response of $\Phi_{\text{PSII}}$ and NPQ

One feature of the photochemical and non-photochemical quenching of light energy by plants is that both show a response to changing irradiance. Generally in mature leaves, $\Phi_{\text{PSII}}$ decreases with increasing irradiance as the electron transport chain becomes saturated, and NPQ increases with increasing irradiance as more light energy needs to be dissipated as heat because it is in excess of what can be used to drive electron transport. The shapes of the light response curves of $\Phi_{\text{PSII}}$ and NPQ differ depending on the plant species, the light environment the plant is acclimated to and the developmental stage of the leaf (Terashima and Takenaka, 1990; Murchie and Horton, 1997; Murchie et al., 1999; Terashima et al., 2006).

Here, I have shown that the regions of P3 and P4 stage leaves that show a measureable $\Phi_{\text{PSII}}$ also show a light response of this parameter. An electron transport rate cannot be calculated from this, as we do not know the differences in light absorbance at different irradiances and in different leaf regions. We can conclude, however, that the rate of decrease of $\Phi_{\text{PSII}}$ with increasing irradiance is highly variable between samples and between regions within P4 stage leaves. This indicates once again that these primordia are undergoing rapid development, and our samples thus include leaves with widely differing abilities to use increasing amounts of light to drive electron transport. Further analysis needs to be carried out to determine whether these differences in kinetics are related to the maximum $\Phi_{\text{PSII}}$ achieved and how this is related to the developmental gradient along the primordium.

Similarly, the variability of the light response of NPQ points to developmental differences between and within P3 and P4 stage primordia in the proportion of light energy that cannot be
used for electron transport at increasing irradiances. A general pattern that can be observed here is that NPQ rises faster in leaf regions near the tip of P3 stage leaves, and is higher throughout in regions near the tip of many P4 stage leaves. This might indicate that these developmentally more advanced regions are not, as might be expected, so much better able to use light energy that they do not need to dissipate it through NPQ. Instead, perhaps their increased chlorophyll content means that they absorb more light energy and thus need to dissipate more.

4.5.5. Changes in $\Phi_{\text{PSII}}$ during photomorphogenesis

In order to delimit the amount of time taken for leaves to develop photosynthetic competence upon illumination, I dissected out leaf primordia at P3/P4 stage and exposed them to a low level of actinic irradiance. Photomorphogenesis in planta happens rapidly at the P3 to early P4 stages of leaf development in rice, despite the fact that these developmental stages are surrounded by the sheaths of older leaves throughout their development. As the development of the photosynthetic machinery and the accumulation of chlorophyll are both known to be tightly correlated to the light environment, it would be interesting to know what the light environment around these leaf primordia in planta is. Although one limitation of this study is that leaf primordia were dissected under dim green light for around five to seven minutes, which may have led to limited additional chlorophyll biosynthesis during dissection, there is no doubt that some light is getting to these primordia in planta as they have a measurable electron transport rate, which is not usually seen until four hours after illumination of chloroplasts from etiolated tissues (Ohashi et al., 1989).

The irradiance level used in my experiment was carefully chosen so as not to induce any light stress responses, but was high enough to trigger photomorphogenesis- the photon flux required for this is known to be minimal (Bukhov et al., 1999; Chen et al., 2004). However, I encountered difficulties in measuring the change in $\Phi_{\text{PSII}}$ during photomorphogenesis as leaf primordia that were successfully dissected often moved out of focus under the microscope due to elongation growth. No signal was detected from any of the three P3 stage primordia imaged, which is consistent with the small number of P3 stage primordia that showed a detectable $\Phi_{\text{PSII}}$ in our initial experiments (three out of eight). However, the two P4 stage primordia that were successfully imaged showed slight basipetal development of photosynthetic capability within the small amount of time (three hours) over which they were imaged.
4.5.6. Photosynthesis in the context of other leaf developmental processes

My results in Chapter 3 and those presented here show that the development of vascular and photosynthetic competence occur during the same stages of rice leaf development. As such, the question arises of how the onset of photosynthetic competence is coordinated with vascular development and other processes occurring during the ontogeny of the rice leaf.

Much has been written about the role of chloroplast retrograde signaling during early leaf development (Larkin et al., 2003; Nott et al., 2006; Woodson and Chory, 2008). Most of this work has been done in *Arabidopsis thaliana*, and some has been done in the monocot maize (*Zea mays*). From studies of the development of light signaling mutant plants in the dark, it can be concluded that although plastid development is necessary for normal leaf development, plastid greening and the development of functional photosynthesis is not (Chory et al., 1989).

How differentiation of vascular and surrounding photosynthetic tissue is organised has been much debated (Berleth et al., 2000; Scarpella et al., 2003). The differentiation of photosynthetic tissue may be organised in reference to the network of developing vascular tissue, which provides a ‘scaffold’ from which positional signals inform the differentiation of the mesophyll. Conversely, Andriankaja et al. (2012) found that the transition from cell proliferation to cell expansion in *Arabidopsis* leaves happens rapidly and coincides with leaf greening, and suggest that chloroplast function and development may influence cell cycle gene down regulation upon the transition to cell expansion. In addition, Scarpella et al. (2004) demonstrate in Arabidopsis that mesophyll differentiation terminates the iterative process of vascular initiation. Which of these processes are at play in monocots, and rice specifically, is unclear. However, we provide evidence that vascular development and the initiation of photosynthetic differentiation are temporally and spatially coordinated in rice, with both occurring around the P3/P4 stages. In addition, the striated pattern of chlorophyll autofluorescence observed in P4 stage primordia is reminiscent of vascular patterning. Direct evidence for positional signals from the vasculature informing photosynthetic development in rice is not available, however in plants with very clear compartmentalisation of photosynthetic and non-photosynthetic function, such as the C₄ plant maize, such positional information is known to be key (Nelson and Langdale, 1989). The molecular nature of positional information derived from the vasculature has begun to be pinned down in root development (Sabatini et al., 1999; Nakajima et al., 2001; Wu et al., 2014), suggesting this is a tractable problem. Thus, further research into the links between photosynthetic development and other leaf developmental processes is necessary, and the developmental gradients seen in rice and other grass leaves offer a good system to study these.
Chapter 5

Gene expression during rice leaf development
5. Gene expression during rice leaf development

5.1. Introduction

The development of plant leaves has been well studied in model species such as *Arabidopsis thaliana* and maize (Braybrook and Kuhlemeier, 2010; Fleming, 2005; Freeling, 1992; Hay and Tsiantis, 2009; Leyser and Day, 2003; Tsukaya, 2002). Many aspects of leaf structure and function are regulated by changes at a transcriptional level during development. With respect to the analysis of these changes during the very earliest stages of grass leaf development, previous work has successfully used laser micro-dissected portions of maize shoot apical meristem domains to identify transcripts associated with leaf initiation (Ohtsu et al., 2007). This work was restricted to the extremely early processes of leaf determination and initiation (up to P1) and did not encompass subsequent stages of leaf development. This was achieved by Wang et al. (2013a) who recently provided a detailed transcriptomic analysis of early leaf development in maize using dissected primordia. These data provided the first analysis of a monocot leaf at this developmental resolution. To date a similar analysis has not been reported for rice.

As the second plant species to have its entire genome sequenced and one of the world’s most important crop plants, rice has gained interest as an alternative model species for the study of leaf development (Goff et al., 2002). Rice grain yield ultimately depends on how much carbon fixation mature leaves can carry out. Therefore, it is important to understand how the rice leaf develops its shape and metabolic capabilities if we are to engineer future crops to have improved yield. Several studies have yielded new insights into rice stomatal development (Kamiya et al., 2003; Kaplan-Levy et al., 2012; Luo et al., 2012), vascular development (Chu et al., 2013; Matsukura et al., 2000; Qi et al., 2008; Scarpella et al., 2002; Scarpella et al., 2003), and shoot architecture (Arite et al., 2007; Komatsu et al., 2003). Traits directly related to yield such as inflorescence development (important for panicle architecture) and endosperm development (important for grain filling) have also been studied (Ashikari et al., 2005; Gao et al., 2013; Jeon et al., 2000).

However, the gene expression patterns underlying several other processes are still not well understood in rice. In particular, vascular development and patterning in rice are not yet well described. In the dicot model species *Arabidopsis thaliana*, vascular development is thought to be regulated by polar auxin transport, positional information from polarity regulators such as *PHABULOSA* and *REVOLUTA*, and peptide signaling (Bonke et al., 2003; Fukuda et al., 2007; Ohashi-Ito et al., 2013). It is unclear how exactly these processes act during vascular development in rice. Several vascular development mutants in rice have been described of which the molecular nature is unclear. Most of these have abnormal leaf shape, with high vein density often resulting...
in a narrow leaf phenotype (Feldman et al., 2014; Qi et al., 2008; Scarpella et al., 2003; Smillie et al., 2012). The only well-described rice vein patterning mutant is in the HD-Zip transcription factor *OsHOX1*, which affects polar auxin transport (Scarpella et al., 2002). In addition, beta-glucuronidase expression driven by the auxin-responsive promoter DR5 is localized to provascular tissue in developing rice leaves, again lending credibility to the involvement of auxin in rice vascular patterning (Scarpella et al., 2002). Chapter 3 of this thesis described the morphological changes in rice leaves during vascular development, as well as the timing and limits of developmental plasticity in vascular patterning at different developmental stages. This chapter aims to identify novel regulators of rice vascular development, as well as characterizing the expression of rice orthologs of genes known to be involved in vascular development in other plants. As stomatal development also occurs during these developmental stages in rice (Yaapar 2016, Thesis), a brief investigation into genes potentially regulating this process was also carried out.

A second poorly understood process during rice leaf development is the onset of photosynthesis. In *Arabidopsis thaliana*, maize, spinach (*Spinacia oleracea*), and barley (*Hordeum vulgare*), studies into the genes underlying the development of photosynthetic capacity have been carried out since the 1980s (Babani et al., 1996; Kobayashi et al., 1980; Monte et al., 2004; Ohashi et al., 1989; Potter et al., 1996; Wollman et al., 1999). More recent studies have revealed details of the assembly of the photosynthetic apparatus (Cai et al., 2010; Minagawa and Takahashi, 2004; Peng et al., 2006) and the key plastid development genes *GOLDEN2-LIKE1* and *GOLDEN2-LIKE2* (*GLK1* and *GLK2*; Waters et al., 2009). Many of these studies were carried out on isolated chloroplasts or on etioplasts, which rarely occur in natural leaf development in the light. Intact leaf primordia developing naturally have also been studied, with a recent focus on retrograde signalling from the chloroplast to the nucleus during development (Fey et al., 2005; Mochizuki et al., 2001; Nott et al., 2006; Vinti et al., 2005) and on the links between the shift to photosynthetic metabolism and the transition from cell division to cell elongation (Andriankaja et al., 2012). Chapter 4 of this thesis described the onset of photosynthesis in developing rice leaves. Understanding the transcriptional changes governing this process is a major aim of this chapter.

A renewed interest in rice vascular and photosynthetic development has arisen from work to engineer C₄ photosynthesis into rice plants, which normally have C₃ photosynthesis (Hibberd et al., 2008). Increasing the efficiency of photosynthesis in rice in this way promises higher yields, higher water use efficiency and higher nitrogen use efficiency (Hibberd et al., 2008). For this project to succeed, an understanding of the exact timing and regulation of expression of vascular and photosynthetic genes in rice will be necessary such that these can be accurately manipulated. In addition, the differences between C₃ rice and C₄ maize leaf photosynthetic development may
point to novel ways of engineering a C₄ rice. To this end, several recent studies have used next generation sequencing to investigate gene expression changes during the development of photosynthesis in rice and maize. The natural gradient in photosynthetic development occurring in C₄ maize leaves from base to tip was explored by Li et al. (2010). These data from maize have also been compared to similar data from rice (Wang et al., 2014a), where the equivalent segments along the rice leaf developmental gradient were used. In contrast, Wang et al. (2013) used intact maize leaf primordia at different developmental stages, looking at biochemical and structural changes associated with C₄ photosynthetic development in the foliar leaf and C₃ photosynthetic development in the husk leaf.

Here, I set out to study the gene expression patterns underlying the processes of vascular and photosynthetic development in young rice leaf primordia. The rice developmental stages used in this work have been chosen to match those studied by Wang et al. (2013) in maize to facilitate the comparison of C₃ and C₄ leaf development. In addition, my analysis of both physiology and structure identified the P3/P4 transition as an important stage in rice leaf development with respect to the acquisition of photosynthetic capability. This tissue also has the ability to respond to environmental signals by altering aspects of cellular differentiation (chapters 3 and 4, this thesis). To investigate the gene expression changes underpinning the P3/P4 transition I performed RNA-Seq on leaf primordia at P3, P4-1 and P5 stage (blade only) (see Figure 3.1 in Chapter 3). The advantage of this type of material over segments of a mature leaf is that even in the youngest segments of a mature leaf, the patterning of many structures may already be laid down. Therefore, the genetic changes underlying the initial formation of these structures (including veins and stomata) may be absent from young segments of a mature leaf. In addition, the naturally occurring gradient in photosynthetic capacity in mature monocot leaves may not be the same as a naturally occurring gradient in photosynthetic development—after all, the base of a rice or maize leaf is arrested in the ‘immature’ photosynthetic state, rather than being on a trajectory to a higher photosynthetic capacity.

Broadly, the developmental stages used here (P3, P4, P5) capture the transition of the leaf from a sink to a source tissue, and the transition from growth through rapid cell division (P3 stage) to growth through cell elongation (P4 stage) and finally the end of major leaf growth (P5 stage). The most dramatic changes in photosynthetic capacity seen during rice leaf development occur during these stages. The leaf develops chloroplasts from proplastids, and goes from no ability to carry out any electron transport (P3 stage) to a quantum efficiency of electron transport (Φ_{psii}) in P5 stage almost matching that of a mature leaf (Figure 5.1; chapter 4; Kusumi et al. 2010). It is also during these developmental stages that formation occurs of other structures important for photosynthetic capacity, such as veins and stomata (Figure 5.2; chapter 3). Importantly, the leaf
also goes from developing entirely inside the sheaths of older leaves (P3 stage) to protruding above these leaf sheaths such that it is exposed to light (P5 stage). This means that there is a gradient in irradiance experienced by the developing leaf, with younger leaf primordia exposed to less light than older ones.

Figure 5.1. Photosynthetic development in stages studied using RNA-Seq (see also chapter 4). Top row: Transmission electron micrographs of plastids in A. P3, B. P4, C. P5 stage and D. mature leaves. Scale bars in A-C= 0.5 µm, D=1 µm, E= 100 µm. G: stacked grana, *: starch grain. Arrows indicate plastids. Bottom row: Quantum efficiency of electron transport in E. P3, F. P4, G. P5 stage and H. mature leaves at an irradiance of 30 μmol m⁻²s⁻¹ as visualized using chlorophyll fluorescence imaging. Scale bars 0.25 mm. ΦPSII value is indicated by the scale adjacent to E.

Figure 5.2. Development of veins and stomata in stages studied using RNA-Seq (see also chapter 3). A. Vascular development in A. P3, B. P4, C. P5 stage and D. mature leaves. Bottom row: stomatal development in E. a P3 stage leaf, F. a close-up of the P3 stage primordium surface, G. P4 stage surface, H. mature leaf surface (E, F and G are taken from Amin Yaapar, 2016 (thesis); H is taken from Woodward and Hetherington (2003)). Scale bars in E and F = 30 µm. Scale bar in G = 10 µm.
To investigate the gene expression changes underpinning these anatomical and physiological changes I performed RNA-Seq on dissected leaf primordia at three defined stages of development. RNA-Seq has become the tool of choice for investigating genome wide gene expression changes during plant development. Datasets are publicly available that describe the maize leaf developmental gradient (Li et al., 2010), the rice leaf developmental gradient (Wang et al., 2014a), maize leaf primordia (Wang et al., 2013), rice endosperm development (Gao et al., 2013), maize endosperm development (Li et al., 2014), and several other rice tissues (Fujita et al., 2010; Wang et al., 2010). Previously, microarrays were used for the study of gene expression, but RNA-Seq has several important advantages over this (Wang et al., 2009). Less input RNA is required, which is a distinct advantage when working with small tissues such as leaf primordia. Background noise is also lower, and the dynamic range much higher in RNA-Seq than in microarrays, both of which are useful when looking at the low expression level transcriptional regulators that are important in leaf development. In addition, RNA-Seq can be used to detect the expression of any RNA, regardless of its splice isoform, whether the gene it originates from is known, and whether it is mRNA or a (regulatory) non-coding RNA, whereas not all genes are on the available rice microarray chip. The use of next generation sequencing can thus provide a wealth of information about the gene expression changes underlying leaf development.
5.2. Aims

- To identify rice genes important for the formation of structural features established at the P3-P4 stage transition, such as stomata and veins
- To identify patterns of gene expression which can be associated with the development of photosynthetic capacity, in order to pinpoint key genes involved in this process
- To contribute to the identification of novel potential regulators of Kranz anatomy and C₄ photosynthetic development by comparing these data to similar data from a maize leaf primordium dataset (Wang et al., 2013).
5.3. Brief methodology

General methods are described in Chapter 2; details are shown below of the specific methods used in this chapter.

5.3.1. RNA-Seq

RNA-Seq was carried out as described in detail in chapter 2. Briefly, high quality (RIN≥8) RNA was extracted from 240 P3, five P4 and three P5 stage leaves per replicate (three biological replicates were used per stage) using TriZol and cleaned up and DNase 1 treated using the Sigma Plant Total RNA kit. RNA-Seq was carried out at the Liverpool Genomics Centre using RiboZero treated RNA with library construction following the Illumina TruSeq stranded mRNA protocol. Paired end reads with a read length of 100bp were trimmed and low-quality reads filtered out. RSEM was used to align the resulting reads to the Oryza sativa L. var. japonica MSU Release 7.0 genome. All pairwise comparisons between developmental stages were made using DESeq, using the default normalization method and identifying differentially expressed genes as those with a Benjamini-Hochberg corrected p-value ≤ 0.05. Genes were grouped into expression clusters (as described in Chapter 2), on which MapMan enrichment analysis was carried out (version 3.5.1R2; Usadel et al. 2005).

5.3.2. RT-PCR

RT-PCR was carried out as described in chapter 2 in an ABI StepOne Plus RT-PCR system using SYBR® Green reagents. The comparative C_T method was used to quantify gene expression changes, with dnaJ (LOC_Os03g57340) and Profilin (LOC_Os06g05880) used as endogenous control genes and P4 stage as the reference stage to which other stages were compared to calculate relative fold change. A Tukey test was used to assess significance of the fold change found.

5.3.3. In situ hybridisation

Detailed methods are provided in Chapter 2. Briefly, fragments of genes of interest were cloned into pBluescript, and DIG-labelled (Roche) riboprobes were transcribed from this plasmid using T3 (antisense probes) or T7 (sense probes) RNA polymerase. The base 5mm of 13 day old rice seedlings was embedded in Paraplast Xtra and sectioned at a thickness of 8µm. After tissue pre-treatment, probes were hybridised to the sections at 50°C overnight. Washing, RNase treatment and blocking steps were then used to limit background noise. An alkaline-phosphatase conjugated anti-DIG antibody (Roche) was then bound to the DIG-labelled probes, and the alkaline phosphatase substrate NBT/BCIP was used to visualise the presence of this antibody.
5.4. Results

5.4.1. Overview of gene expression changes

I generated an initial overview of gene expression changes during early rice leaf development. Out of 25768 protein coding genes of which expression was detected (including 89 plastidially encoded genes and 51 mitochondrially encoded genes; there are a total of around 39,045 annotated non-TE loci in rice; Ouyang et al., 2007), 14502 were significantly differentially expressed in at least one comparison between different developmental stages. 4596 were significantly up regulated between P3 and P4 stage, 3576 were significantly down regulated between P3 and P4 stage, 4310 were significantly up regulated between P4 and P5 stage, and 3671 were significantly down regulated between P4 and P5 stage (Figure 5.3).

Figure 5.3. A. Overview of developmental stages used for RNA-Seq. B. Overview of gene expression changes between developmental stages. Numbers indicate number of genes showing significant increase or decrease between two subsequent stages.
Genes were clustered into expression profiles defined by rules to which the direction of $\log_2$(Fold change) (positive or negative) and its significance (Benjamini-Hochberg adjusted p≤0.05) had to conform (see also methods in Chapter 2). Eleven clusters were formed in this way (Figure 5.4).

Of the 25768 protein coding genes of which expression was detected, 11266 showed no significant change in expression in any comparison of developmental stages (‘neutral’ cluster). 2274 genes had unchanged expression from P3 to P4 stage but were then up regulated to P5 stage (‘up 1’ cluster); 2560 genes were up regulated from P3 to P4 stage and then maintained the same level of expression to P5 stage (‘up 2’ cluster); 2036 genes were up regulated from both P3 to P4 and P4 to P5; and 686 genes were not significantly up regulated from P3 to P4 or P4 to P5, but did show a significant up regulation from P3 to P5 (‘up 4’ cluster).

Conversely, 1726 genes were stably expressed from P3 to P4 stage but down regulated from P4 to P5 (‘down 1’ cluster); 1631 genes were down regulated from P3 to P4 stage and then maintained the same level of expression to P5 stage (‘down 2’ cluster); 1945 genes were significantly down regulated from both P3 to P4 and P4 to P5 stage (‘down 3’ cluster), and 655 genes were not significantly differentially expressed between P3 and P4 or P4 and P5, but did show a significant down regulation from P3 to P5 stage (‘down 4’ cluster).

Finally, smaller numbers of genes with contrasting changes between P3 and P4 and P4 and P5 were found. 945 genes were up regulated from P3 to P4 but down regulated from P4 to P5 (‘peak’ cluster), and 145 genes were down regulated from P3 to P4 and up regulated from P4 to P5 (‘trough’ cluster).
Figure 5.4. Patterns of gene expression in different clusters. All protein coding genes of which expression was detected in my dataset were assigned to one of the above clusters based on the significance (Benjamini Hochberg adjusted \( p \leq 0.05 \)) and direction of the \( \log_2(\text{Fold Change}) \) in their expression.
5.4.3. Validation of RNA-Seq data using RT-PCR

In order to determine the accuracy of the RNA-Seq results, the expression levels of six genes at the three developmental stages used were evaluated using RT-PCR. These genes were chosen because their expression patterns either placed them in a cluster important for vascular development (e.g. cluster ‘down 2’- DROOPING LEAF and cluster ‘down 3’- MONOPTEROS) or in a cluster important for photosynthetic development (e.g. ‘peak’ cluster- IMMUNOPHILIN; ‘up 2’ cluster- NARA5; ‘up 3’ cluster- THF1), and because they or their Arabidopsis thaliana orthologues are known to play a role in one of these processes. The ‘neutral’ cluster gene dnaJ was selected because it has both one of the highest expression levels of ‘neutral’ cluster genes and a small fold change between P3 and P4 and between P4 and P5 stages. The selected genes were found to show similar patterns of expression in the RT-PCR and in the RNA-Seq results, with a relatively high $R^2$ value of 0.942 observed for the correlation between log$_2$(RNA-Seq Fold Change) and log$_2$(RT-PCR Fold Change) (Figure 5.5A). In addition, the expression patterns of individual genes studied by RT-PCR mimic those observed in the RNA-Seq data clusters they represent (Figure 5.5B).

![Figure 5.5](image-url)

Figure 5.5. Validation of RNA-Seq data using RT-PCR. A. Correlation of log2(fold change) in gene expression between developmental stages as calculated from RT-PCR data and RNA-Seq data for selected genes (see text). B. Expression patterns of selected genes evaluated by RT-PCR match those seen in the RNA-Seq results. Y-axis shows log$_2$(Fold Change) relative to P4 stage; data were normalized to the expression of the control gene Profilin in each sample. P3: n=2, P4: n=3, P5: n=3. Error bars show standard error of the mean. Different letters indicate a significant difference in mean level of expression between developmental stages for the same gene (p<0.05; Tukey test). Floating graphs indicate expression clusters in RNA-Seq data (see Figure 5.4).
5.4.4. Enrichment analysis and expression of TF families

Following on from this, I used these clusters to identify functional categories of genes that were significantly overrepresented in each group of genes with a similar expression profile (using MapMan; Usadel et al., 2005). As expected, functions related to photosynthesis were overrepresented in clusters showing increasing expression during development, including the ‘peak’ cluster (Figure 5.6). Functions related to mitochondrial respiration, DNA synthesis, the cell cycle and regulation of transcription were overrepresented in clusters with a downward trend (Figure 5.7). The ‘neutral’, ‘trough’ and ‘up 4’ clusters were not significantly enriched in any functions.

<table>
<thead>
<tr>
<th>Up 1 cluster</th>
<th>Up 2 cluster</th>
<th>Up 3 cluster</th>
<th>Peak cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450</td>
<td>Cell wall proteins</td>
<td>Calvin cycle</td>
<td>Tetrapyrole synthesis</td>
</tr>
<tr>
<td>WRKY domain TF family</td>
<td>Phospholipid synthesis</td>
<td>Light reactions</td>
<td>Protein</td>
</tr>
<tr>
<td>UDP glucosyl and glucuronyl transferases</td>
<td>Lipid Transfer Protein (LPT) family protein</td>
<td>ATP synthase, subunit B</td>
<td>- folding</td>
</tr>
<tr>
<td>DUF 26 receptor kinases</td>
<td>Plastocyanin-like</td>
<td>- Cyclic electron flow</td>
<td>- targeting to the chloroplast</td>
</tr>
<tr>
<td>Cp 30S rib. prot., SU S7</td>
<td>H+ transporting two-sector ATPase</td>
<td>- Cytochrome b6/f complex</td>
<td>- chloroplast synthesis</td>
</tr>
<tr>
<td></td>
<td>Cp 30S rib. prot., SU S3</td>
<td>- NADH DH</td>
<td>Proline rich cell wall proteins</td>
</tr>
<tr>
<td></td>
<td>(and three more SUs)</td>
<td>- PSI polypeptide subunits</td>
<td>Lipid metabolism</td>
</tr>
<tr>
<td></td>
<td>Cp 50S rib. prot., SU L14</td>
<td>- PSII polypeptide subunits</td>
<td>Amino acid activation</td>
</tr>
<tr>
<td></td>
<td>(and two more SUs)</td>
<td>- LHC-II</td>
<td>Signalling: G-proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ascorbate and glutathione</td>
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<tr>
<td></td>
<td></td>
<td>Carotenoid biosynthesis</td>
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<td>Lignin biosynthesis</td>
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<td>- Dirigent proteins</td>
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<td>Protein</td>
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<tr>
<td></td>
<td></td>
<td>- Cp 30S rib. prot., SU S14</td>
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<td></td>
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<td>- Protein assembly and cofactor ligation</td>
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<td>- Amino acid transport</td>
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<td></td>
<td></td>
<td>Phosphatidylethanolamine O-acyltransferase</td>
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<tr>
<td></td>
<td></td>
<td>Constans-like ZNF TF family</td>
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</tr>
</tbody>
</table>

Figure 5.6. Functional enrichment analysis of genes in clusters showing upward trends. In green are terms related to photosynthesis; in purple are categories containing transcription factors. Rib. prot., ribosomal protein; Euk., eukaryote; Mt., mitochondrial; Cp., plastid, SU, subunit. The ‘neutral’, ‘trough’ and ‘up 4’ clusters were not significantly enriched in any functions.
<table>
<thead>
<tr>
<th>Down 1 cluster</th>
<th>Down 2 cluster</th>
<th>Down 3 cluster</th>
<th>Down 4 cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA -Synthesis- Histones</td>
<td>Pentatricopeptide (PPR) repeat-containing protein</td>
<td>Pentatricopeptide (PPR) repeat-containing protein</td>
<td>Pentatricopeptide (PPR) repeat-containing protein</td>
</tr>
<tr>
<td>Mitochondrial electron transport - ATP synthesis - F1-ATPase - NADH-DH - Carbonic anhydrase</td>
<td>Regulation of transcription- Zinc Finger homeodomain TF family</td>
<td>Regulation of transcription- chromatin remodeling</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Euk. 40S rib. prot., SU S15A (and one more SU)</td>
<td>Euk. 60S rib. prot., SU L24 (and two more SUs)</td>
<td>Proteasome function</td>
<td>Proteasome function</td>
</tr>
<tr>
<td>Protein targeting to the secretory pathway- Endoplasmic Reticulum</td>
<td>Protein targeting to the secretory pathway- unspecified</td>
<td>Protein targeting to the secretory pathway- unspecified</td>
<td>Protein targeting to the secretory pathway- unspecified</td>
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<tr>
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</tr>
</tbody>
</table>

Figure 5.7. Functional enrichment analysis of genes in clusters showing downward trends. In red are terms related to respiration; in purple are categories containing transcription factors. Rib. prot., ribosomal protein; Euk., eukaryote; Mt., mitochondrial; Cp., plastid, SU, subunit. The ‘neutral’, ‘trough’ and ‘up 4’ clusters were not significantly enriched in any functions.
The expression patterns of all annotated rice transcription factors were examined and sorted into those with broadly up regulated expression patterns (transcription factors in clusters ‘up 1’, ‘up 2’, ‘up 3’ and ‘up 4’), those with broadly down regulated expression patterns (transcription factors in clusters ‘down 1’, ‘down 2’, ‘down 3’ and ‘down 4’) and those with ‘peak’ expression patterns (transcription factors in the ‘peak’ cluster). The distribution of differentially expressed transcription factors across these expression patterns is shown in Figure 5.8. Transcription factor families of which many members are down regulated (highest in P3) during rice leaf development include the GRF, C3H and ZF-HD families. Transcription factor families of which many members are up regulated (highest in P5) during rice leaf development include the NAC, C2C2/CONSTANS-like and WRKY families. Transcription factor families of which relatively many members show a ‘peak’ expression pattern (highest in P4) during rice leaf development include the TCP, SBP, GATA and Trihelix families.

5.4.5. Identification of genes underlying morphological differentiation

In addition to looking at enriched functional categories of genes and transcription factor families in each cluster, the expression of individual genes underlying specific developmental processes can be investigated in more detail using these data. In particular, I was interested in the development of photosynthetic function, vascular development, and stomatal development. A two-pronged approach was taken to find individual genes that may be involved in these processes. Firstly, rice orthologs of Arabidopsis thaliana genes known to be involved in developmental processes occurring at these stages were examined. Secondly, I used the gene expression clusters above (Figure 5.4) to identify novel developmental regulators expected to be expressed at times when differentiation was visible. Together, these approaches yielded several lists of genes which may merit further investigation.
As the development and patterning of veins is key to the functioning of leaves, but our understanding of these processes in grasses is limited, I started by investigating gene expression changes potentially underlying vein development. Vascular development regulators identified by Scarpella and Meijer (2004) in Arabidopsis thaliana, Zea mays and Petunia hybrida and the expression clusters of their putative rice orthologs are listed in Supplementary Table S 1. The expression clusters of an additional list of 15 genes identified through a literature search as being involved in vascular development in rice are shown in Supplementary Table S 2. In addition, the genetic mechanisms regulating stomatal development in rice are poorly understood. Thus, transcription factors thought to be involved in stomatal development in rice and orthologs of transcription factors such as VAS, AGO1, WOL, COV1, Oshox1, PIN1, APL, VH1, VEP1, CPD, ISR, FAMA, ZmROP2, HYD1, BDL, CVP1, FAMA, ZmROP2, PHV, PHB, DWF7, SMT1, ZLL, PHF2, YODA, ZmPAN1, ZmROP2, EPFL1, APL, VH1, VEP1, CPD, ISR, FAMA, ZmROP2, HYD1, BDL, CVP1, FAMA, ZmROP2, PHV, PHB, DWF7, SMT1, ZLL, PHF2, YODA, ZmPAN1, ZmROP2, and others are listed in the figure.

Figure 5.9. Expression patterns of rice orthologs of known vascular development regulators from Scarpella and Meijer 2004 (purple) and stomatal development regulators (red). Genes can be found in several clusters if several rice orthologs exist. Clusters ‘Trough’, ‘Up 3’ and ‘Down 4’ did not contain any rice orthologs of known vascular or stomatal development regulators, except SCREAM2, which is in the ‘Trough’ cluster.

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factors involved in stomatal development in *Arabidopsis* and maize were also identified from the literature. The expression patterns of these genes across the rice developmental stages P3, P4 and P5 were also evaluated (Figure 5.9).

In addition, novel potential regulators of vascular development in rice were identified according to their expression patterns. These genes were most highly expressed at P3 stage; not expressed in mature leaves or endosperm (data taken from the MSU Rice Genome Annotation Project Resource; Ouyang et al., 2007); rapidly down regulated from P3 to P4 stage (log2 (Fold Change) ≥2); and annotated as being transcription factors. This yielded a list of 39 genes (Supplementary Table S3). These include LOC_Os04g35660.1, which is the rice ortholog of *Arabidopsis thaliana* ‘XYLEM NAC DOMAIN 1’, a gene involved in vascular cell differentiation in *Arabidopsis thaliana* and *Populus trichocarpa* (Grant et al., 2010); and LOC_Os09g36160.1, of which the *Arabidopsis thaliana* ortholog is implicated in auxin homeostasis as well as xylem and phloem pattern formation (Baylis et al., 2013). In addition, the list includes the known rice vascular development regulator DROOPING LEAF (LOC_Os03g11600.1; Itoh et al., 2005). None of the other genes are known to be involved in vascular patterning.

### 5.4.6. Genetic changes underlying the acquisition of photosynthetic function

As well as being important for the development of many morphological features (chapter 3, this thesis), the developmental stages studied here are crucial for the development of photosynthesis in rice (chapter 4, this thesis). Thus, this material is useful for studying the gene expression changes underlying the acquisition of photosynthetic function. Several genes involved in photosynthesis (of a total of 351 annotated with ‘PS’ (photosynthesis) in rice using MapMan) (Usadel et al., 2005) are already expressed to 50% of their maximal expression level at the earliest developmental stage studied (P3 stage). These 34 genes, which include several involved in the light reactions of photosynthesis, photorespiration, and the Calvin-Benson cycle (Supplementary Table S4), may be important for the onset of photosynthesis. However, the most change in the expression of photosynthesis-related genes occur after P3 stage, as can be seen when looking at functional enrichment analysis of clusters showing upward trends, which contain many terms to do with photosynthesis (Figure 5.6), but also when genes coding for electron transport chain components and Calvin cycle enzymes are focused on (Figure 5.10).

This knowledge of the global expression patterns of photosynthesis-related genes in early leaf development combined with my finding that photosynthetic capacity increases throughout early rice leaf development (chapter 4, this thesis) was used to formulate expectations of the expression patterns in these data of novel photosynthesis-related genes. These genes were expected to be most highly expressed at P5 stage; relatively highly expressed in mature leaves
(over 25 counts; Ouyang et al., 2007); rapidly up regulated from P3 to P4 stage (log2 (Fold Change) ≥1); and annotated with the GO-term GO:0015979 (Photosynthesis) (Carbon et al., 2009) but not known to be ‘core photosynthetic machinery’ (i.e. not Calvin cycle enzymes or known electron transport chain components). These criteria yielded a list of 17 genes (Supplementary Table S 5), of which seven are annotated only as ‘expressed protein’ in rice. Four of these seven had putative orthologs in *Arabidopsis thaliana* that are known to be involved in assembly of the photosynthetic apparatus. In order to find genes more likely to have a regulatory function in the establishment of photosynthesis, transcription factors were investigated. If the same expression criteria are applied to all known rice transcription factors (none of which are annotated with GO:0015979), 51 genes show the same expression pattern (Supplementary Table S6). These include known chloroplast development regulators *GLK1* and *GLK2* (Waters et al., 2009) and several members of the MYB related, *CONSTANS*-like and GATA families.

![Expression of genes involved in photosynthesis during early leaf development in rice](image)

**Figure 5.10.** Expression of genes involved in photosynthesis during early leaf development in rice. Expression level (normalized counts) of genes involved in photosynthetic electron transport (left) and the Calvin-Benson cycle (right) at the P3, P4 and P5 stages of rice leaf development. Each small square represents the expression level of one gene. Visualized using MapMan (Usadel et al., 2005).
5.4.7. Comparison to gene expression during the development of C₄ photosynthesis

The results generated here represent the first RNA-Seq data to be available of the P3, P4 and P5 stages of early rice leaf development. Maximum benefit can be gained from these data by comparing them to other available transcriptomic datasets, particularly a recent experiment describing early maize leaf development (Wang et al., 2013). Here, I use this publicly available data to (1) find the expression patterns during early rice leaf development of genes identified as putative positive regulators of Kranz anatomy; (2) find the expression patterns at these stages of genes present in rice that may be co-opted for C₄ photosynthetic metabolism; and (3) broadly compare the transcriptome of the developing C₄ maize foliar leaf and the developing C₃ rice leaf.

One of the key requirements for engineering a C₄ rice is thought to be a C₄-like Kranz anatomy (Kajala et al., 2011; Leegood, 2013). Wang et al. (2013) identified a number of transcription factors as putative positive regulators of the development of Kranz anatomy in maize. Here, I have identified 21 rice orthologs of these genes. Of the 21 genes studied here (Supplementary Table S7), one was not expressed during early leaf development in rice (FPKM≤1), and of the rest, only 11 were significantly differently expressed between at least two stages. Most of these significantly differently expressed genes were down regulated during rice leaf development.

In addition to the requirement for anatomical modifications, successful implementation of C₄ photosynthesis in C₃ grasses also requires changes to metabolism. However, despite the differences between C₃ and C₄ metabolism, C₃ rice has several genes that code for enzymes or transporters known to be required for C₄ photosynthesis. Here, I consider 16 genes which encode components which may be co-opted for C₄ photosynthetic metabolism in rice. All of these genes were expressed during at least one of the developmental stages considered (FPKM≥1), and 13 were significantly differently expressed between at least two developmental stages (Supplementary Table S8; Figure 5.11).
Figure 5.11. Expression during early rice leaf development of genes that may be co-opted for C₄ metabolism. Summary of NADP-ME subtype C₄ biochemistry (as seen in maize). Numbers indicate enzymes (green) and transporters (blue) encoded by rice genes that may be co-opted for C₄ metabolism (see Supplementary Table S8). Graphs indicate expression patterns during early leaf development. Modified from Kajala et al. 2011.

Finally, in light of the recent interest in manipulating C₃ leaf anatomy to facilitate C₄ photosynthesis, I sought to identify genes whose expression level correlates with the differences in development between C₃ and C₄ leaves. In order to identify genes that may have conserved roles in maize and rice, I was interested in finding genes that showed similar expression patterns during early leaf development in rice and maize. In particular, genes that enable photosynthetic development in both rice and maize can be expected to show broadly the same expression pattern, since leaves of both plants need to develop a functional photosynthetic apparatus during the same developmental stages. In contrast, vascular development and tissue patterning are different in maize and rice: rice plants develop a ‘standard’ C₃ monocot venation, whereas maize plants develop the denser venation and Kranz anatomy necessary for C₄ photosynthesis (Leegood 2011). Therefore, I was interested in potential regulators of vascular patterning and Kranz anatomy that showed contrasting expression patterns in rice and maize, as these might enable the development of the denser venation and Kranz anatomy seen in maize.
Wang et al. (2013a) studied the development of the C₄ maize foliar and husk leaf at comparable stages to the rice developmental stages studied here. However, identifying exactly which developmental stages of rice and maize correspond is not trivial, since the length of the plastochron of maize and rice is slightly different, and as such the number of plastochron stages between leaf initiation and the mature leaf stage is also different. However, consideration of the morphology of the primordia in these two investigations suggested that the maize FP3/FP4 stages were most similar to rice P3 stage, that maize FP5 was comparable to rice P4 stage, and that the maize FI stage was similar to rice P5 stage (Figure 5.12).

![Figure 5.12. Comparison between maize and rice developmental stages (maize images taken from Wang et al. 2014). FP3, FP4, FP5 and FI are maize developmental stages, whereas P3, P4 and P5 are rice developmental stages.](image)

Rice and maize developmental stages can also be tested for similarity on the basis of gene expression data. Thus, the expression of 7613 rice genes for which one-to-one orthologs could be found in maize was correlated with the expression of the rice ortholog. This correlation was carried out for every comparison of developmental stages using either a Spearman or a Pearson correlation analysis (Figure 5.13). Figure 5.13 shows that in terms of gene expression, C₄ rice foliar leaf samples (P3, P4, P5) are more similar to maize foliar leaf samples (FP, FP34, FP5, FI, FE) than to maize husk leaf samples (HP, HP34, HP5, HI and HE). There is little difference among maize stages FP, FP3/4 and FP5 in their similarity to rice P3 stage, whether assessed by Spearman or by Pearson correlation. Rice P4 stage shows most similarity to maize FI according to Spearman correlation, but is almost equally similar to maize FP5 and FI according to Pearson correlation results. Rice P5 stage is almost equally similar to maize FI and FE according to Spearman Correlation, and most similar to FI according to Pearson correlation.
Thus, the leaf staging suggested by morphology was generally substantiated by the correlation of gene expression data, although it was clear that the delineations were somewhat fuzzy based purely on molecular data. However, the gene expression correlation results were not inconsistent with comparing rice P3 to maize FP3/4, rice P4 to maize FP5 and rice P5 to maize FI stage. Taking these stagings as best estimates, I compared the early leaf primordia datasets from rice and maize.

In order to compare gene expression during early rice and maize leaf development, maize expression clusters across FP3/4, FP5 and FI stages were formed in the same way as rice clusters across P3, P4 and P5 stages. Broader expression clusters were then designed in both maize and rice, which contained all genes that were up regulated from any one stage to the next or down regulated from one stage to the next (rice/maize ‘up’ and ‘down’ clusters). To identify genes that might be conserved across maize and rice, clusters showing the same general pattern were compared across maize and rice and the overlap lists tested for overrepresented MapMan terms (Figure 5.14). In order to identify genes that might contribute to the differences between maize and rice in later leaf development, the comparison between maize ‘general up’ and rice ‘general down’ clusters is of particular interest. I observed two enriched functional terms associated with the set of genes that were downregulated during rice development but upregulated during maize development. These were ‘protein import to the chloroplast’ and ‘amino acid biosynthesis’. The former comprised TIC21, TIC22, TOC64, an outer membrane protein in the OMP85 family, a signal recognition particle protein and a secA family translocase. The latter comprised several enzymes associated with aspartate metabolism and may reflect differences in the accumulation of C4 cycle metabolites between rice and maize.

Figure 5.13. Correlation between gene expression in rice and maize leaf development. A. Spearman correlation and B. Pearson correlation between expression of 7613 one-to-one orthologs in maize and rice. Numbers are correlation coefficients ($r$); orange indicates high correlation, whereas green indicates low correlation. Black outlines indicate comparisons discussed in the text. FP, FP34, FP5, FI, FE indicate progressively older maize foliar leaf samples; HP, HP34, HP5, HI and HE indicate progressively older maize husk leaf samples; P3, P4 and P5 indicate progressively older rice leaf samples.
Transcription factors that are down regulated during early rice leaf development but up regulated in maize may also be of particular interest, as they may contribute to the higher vein density seen in maize. I found eight rice transcription factors that decreased in expression during rice leaf development (down regulation between at least two of three developmental stages; no up regulation between any stages) and for which the maize orthologs increased in expression during maize foliar leaf development (up regulation between at least two of three developmental stages; no down regulation between any stages) (Figure 5.15). Of these eight genes four have very low expression in the mature leaf mesophyll and bundle sheath but the other four have been reported to be differentially expressed between mature maize mesophyll and bundle sheath cells, the key cell types of C₄ maize Kranz anatomy (Li et al., 2010).

### Figure 5.14. Enriched functional categories in lists of genes found to be:

<table>
<thead>
<tr>
<th>A. Maize up</th>
<th>B. Maize down</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice up</td>
<td>Rice down</td>
</tr>
<tr>
<td>Lipid metabolism - lipid transfer proteins</td>
<td>Cell cycle, Cell division</td>
</tr>
<tr>
<td>Protein synthesis - prokaryotic ribosomal protein, chloroplast, 30S and 50S subunits</td>
<td>DNA, DNA repair, DNA synthesis/ chromatin structure</td>
</tr>
<tr>
<td>Photosynthesis - Calvin cycle</td>
<td>Pentatricopeptide (PPR) repeat-containing protein</td>
</tr>
<tr>
<td>- Light reactions</td>
<td>Protein synthesis, ribosomal protein, eukaryotic and prokaryotic; Ribosome biogenesis; Pre-rRNA processing and modifications</td>
</tr>
<tr>
<td>- NADH DH</td>
<td>Protein targeting to the nucleus</td>
</tr>
<tr>
<td>- PSII, PSII-LHC-II, PSII polypeptide subunits</td>
<td>Regulation of transcription: Chromatin Remodeling Factors; General Transcription; Putative transcription regulator; zf-HD TFs RNA binding</td>
</tr>
<tr>
<td>RNA- Regulation of transcription: C2C2(Zn) CONSTANS-like zinc finger family</td>
<td></td>
</tr>
</tbody>
</table>

### C. Maize down | D. Maize up |
| Rice up | Rice down |

None | Protein targeting to the chloroplast |

Amino acid metabolism - synthesis
Figure 5.15. Transcription factors which may be putative regulators of Kranz anatomy. Transcription factors that increase in expression during maize leaf development (data from Wang et al. 2013a) but decrease in expression during rice leaf development and their expression pattern in maize bundle sheath and mesophyll cells (data from Li et al. 2010).
5.4.8. Visualisation of gene expression patterns using in situ hybridisation

In order to determine the spatial distribution of genes deemed of interest from their temporal expression patterns, I carried out in situ hybridisation. In particular, I was interested in using this technique to examine whether any of the potential vascular development regulators identified are expressed specifically in the vasculature (or excluded from this domain). Using cross sections from the base of young seedlings allowed me to image several leaves at different developmental stages at the same time. I used HISTONE H4, a gene highly expressed in dividing cells, as a positive control. This gene is known to show a ‘dotty’ expression pattern, which was clearly observed in my samples (Figure 5.16A), and no signal was seen in the negative control (a sense probe for HISTONE H4, which does not hybridise to the HISTONE H4 mRNA as it has the same sequence; Figure 5.16B), validating the functionality of my in situ method.

![Figure 5.16. A. Expression pattern of HISTONE H4, as revealed using an antisense probe (a). B. No signal is observed in tissues hybridised using a control HISTONE H4 sense probe (s). Scale bars 500µm.](image)

Having validated my in situ hybridisation technique, I proceeded to investigate the expression patterns of three genes shown in my RNA-Seq data to be expressed at times coinciding with the development of the vasculature in rice. These genes included DROOPING LEAF (DL), MONOPTEROS (MP) and FACKEL (FK), which have all been previously shown to affect vascular development in rice (DL) or Arabidopsis (MP, FK), and are expressed early in leaf development (highest at P3 stage; see Figure 5.9, this chapter), making them likely candidates for vascular development regulators. However, no vascular-specific expression pattern of these genes was observed (Figure 5.17). In addition, I investigated the expression pattern of two other known regulators of vascular development in Arabidopsis, namely CONTINUOUS VASCULAR RING1 (COV1) and DWARF7 (DWF7). Again, neither of these genes showed vascular specific expression patterns (Figure 5.18; images by Rona Costello).
Figure 5.17. A-C, antisense probes, D-F, sense control probes for DL (A,D), MP (B,E) and FK (C,F). Scale bars 500µm.

Figure 5.18. A-B, antisense probes, C-D, sense control probes for COV1 (A,C) and DWF7 (B,D). Scale bars 250µm. Images by Rona Costello.
In addition to examining the expression patterns of genes potentially involved in rice vascular development, I was also interested in the patterns of expression of genes necessary for photosynthesis. Thus, I examined the pattern of expression of *THYLAKOID FORMATION1* (*THF1*), which is necessary for appropriate plastid development, as well as the photosystem II subunit (and putative oxygen evolving complex) *PsbP* and a *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*), which are both known to be central components of the higher plant photosynthetic machinery. Both *THF1* and *PsbP* were found to be highly and ubiquitously expressed even in early developmental stages (Figure 5.19). However, the probe I used for *CAB* unfortunately did not hybridise (Figure 5.19 C, F).

**Figure 5.19.** A-C, antisense probes, D-F, sense control probes for *THF1* (A,D), *PsbP* (B,E) and *CAB* (C,F). Scale bars 250µm.
5.5. Discussion

Here, I have described the changes in gene expression underlying early leaf development in rice. The stages studied encompass the formation of several key structures in the leaf, such as stomata, veins and functional chloroplasts. As well as considering the changes in gene expression between each developmental stage, I have grouped genes into clusters based on the direction of the expression change and its significance, and identified enriched functional categories of genes in each cluster. In addition, I investigated the expression changes of specific genes thought to underpin morphological and physiological differentiation. Finally, a comparison between gene expression during C$_3$ rice leaf development and C$_4$ maize leaf development was carried out.

5.5.1. Functional enrichment in gene expression clusters

First, after sorting all genes for which expression was detected into clusters, I identified functional categories of genes which were overrepresented in each cluster. The general metabolic trends expected in this developmental series are easily identified in different expression clusters. Genes involved in mitochondrial electron transport (particularly those related to ATP synthesis, and coding for the F1-ATPase, NADH-DH and carbonic anhydrase) are found in the ‘down 1’ cluster, whereas three of the four clusters containing genes with an upward trend in their expression patterns (‘up 1’, ‘up 2’ and ‘up 3’) contain an overrepresentation of genes involved in photosynthesis (see Figures 5.6 and 5.7). These include genes coding for Calvin cycle enzymes and for chloroplast electron transport chain components such as ATP synthase, the cytochrome b6/f complex, NADH DH, PSI, PSII and LHC-II. Interestingly, ascorbate and glutathione as well as carotenoid metabolism-related genes are also upregulated in this way, suggesting that plants develop protective mechanisms to prevent oxidative photodamage as they develop the photosynthetic machinery, rather than up regulating the required genes only when in stressful environments (Foyer, 2011; Demmig-Adams 1996; Ruban et al 2007). Interestingly, three out of four downward clusters are enriched in pentatricopeptide repeat (PPR) containing proteins. This large family of relatively poorly understood genes was first discovered in 2000 and is thought to be involved in RNA editing in mitochondria and chloroplasts in plants (Lurin et al., 2004; Small and Peeters, 2000). One of the best studied members of this family is the plastid-nucleus retrograde signalling gene GUN1 (Cottage et al., 2007). Some members of this family are thought to promote RNA cleavage, whereas others may stabilise RNAs (Barkan and Small, 2014). It is possible that the down regulation of these genes during rice leaf development allows for greater stability of chloroplast transcripts and thus facilitates photosynthetic development.

In addition to the presence of categories reflecting metabolic processes and their development, the expected trends reflecting the changing growth mechanisms in a developing leaf are also
present. Cell division and DNA replication are important in earlier developmental stages, and the downward expression patterns of the genes associated with this (e.g. cell cycle genes in the ‘down 3’ cluster and DNA synthesis genes in the ‘down 1’ and ‘down 3’ clusters) reflect the decrease in cell division in older developmental stages. In contrast, genes required to make more cell membrane and cell wall material are up regulated in later stages as growth continues through cell elongation. This can be seen in the overrepresentation of cell wall proteins, phospholipid synthesis, and Lipid Transfer Protein (LTP) family proteins in the ‘up 2’ cluster. The latter have been shown to play a role in facilitating cell wall extension in tobacco (Nieuwland et al., 2005). The presence of many phosphatidylethanolamine O-acyltransferases in the ‘up 3’ cluster, which are known to be involved in the hydrolysis of galactolipids, the major constituent of chloroplast membranes, may reflect the need for chloroplast membrane remodelling during photosynthetic development (Yoon et al., 2012). In the same cluster, lignin biosynthesis genes are found, which fit with the expected cell wall deposition occurring during later developmental stages.

Of the three clusters not showing a general downward or upward trend (‘peak’, ‘trough’, or ‘neutral’), perhaps the most interesting clusters is the ‘peak’ cluster, which contains genes that are up regulated from P3 to P4 stage, and immediately down regulated again to P5 stage. P4 stage has previously been identified as the stage at which photosynthetic capacity changes the most (Kusumi et al., 2010). In addition, this is a stage of rapid growth through cell elongation (Itoh et al., 2005). As such, P4 stage is a pivotal point at which the leaf transitions from a sink tissue in which rapid cell division occurs driven by energy from mitochondrial respiration, to a source tissue that is growing almost exclusively through cell elongation and starting to derive most of its energy from photosynthesis in its own chloroplasts. This cluster contains relatively few genes compared to the other main clusters (945 genes compared to 1600-2500 genes in ‘up 1-3’ and ‘down 1-3’), but these genes are enriched in several interesting functions. For example, P4 stage is when tetrapyrrole synthesis is at a peak, which likely reflects the need for chlorophyll precursors. Also in preparation for photosynthesis, a relatively large proportion of genes up regulated specifically in P4 stage but then down regulated are involved in chloroplast protein synthesis but also protein folding and targeting of proteins to the chloroplast. At the same time, several functional categories to do with rapid cell expansion and cell wall synthesis and remodelling are found in the ‘peak’ cluster. These include the proline rich cell wall proteins, which in the ‘peak’ cluster are all annotated as extensin family protein precursors. The overrepresentation of genes involved in lipid metabolism may also point to a need for the biosynthesis of cell wall and cell membrane components in rapidly elongating cells.

There are no significantly enriched functional categories in the ‘up 4’ cluster, the ‘trough’ cluster, or the ‘neutral’ cluster. Only the category ‘pentatricopeptide repeat containing protein’ is enriched
in the ‘down 4’ cluster. This lack of enriched functions likely reflects the fact that these clusters do not reflect any particular developmental processes happening at these times. For example, no known developmental process in rice happens at P3 stage and P5 stage but not P4 stage, and as such, the ‘trough’ gene expression cluster may be expected not to contain any enriched functions. However, this cluster may contain diverse individual negative regulators of processes happening only during P4 stage, which might be missed if only the MapMan analysis is considered. As such, these clusters may contain individual genes of interest, but are not of primary interest for functional enrichment analysis.

5.5.2. Identification of genes underlying specific developmental events

Although studying enriched functional categories can inform on general processes, I was also interested in pinpointing genes involved in more specific processes, such as the development of veins, stomata and functional chloroplasts. In combination with my detailed studies of morphological and physiological changes in rice leaf primordia (Chapters 3 and 4; see also Itoh et al., 2005; Kusumi et al., 2010), the clustering described above can facilitate the identification of transcriptional regulators with expression patterns consistent with a role in regulating such specific developmental processes. In addition, information gathered through studies of *Arabidopsis thaliana* can be translated into rice by identifying rice orthologs of genes of interest in this model plant that show relevant expression patterns in this dataset.

I was most interested in photosynthetic development, as this is well captured by the developmental series studied here and is a major target for improving rice yield (Evans, 1997; Horton, 2000). The earliest signs of photosynthetic electron transport capacity in developing rice leaves are seen at P3 stage (Chapter 4). Out of 351 genes in rice that are annotated with the MapMan term ‘Photosynthesis’, only 34 are already at over half their maximal expression level at this stage. This indicates that although photosynthesis is initiated at the tip of P3 stage primordia, most genes involved in this process do not reach a high level of expression until later in leaf development. Among those genes that are expressed early are several core enzymes of the Calvin-Benson cycle, as well as several components of the photosynthetic electron transport chain. A small number of genes involved in photorespiration also show a relative high level of expression during early rice leaf development.

However, several other genes which are not core elements of photosynthesis are also expressed at these early developmental stages, and may be key regulators of early photosynthetic development. Foremost among these is the pentatricopeptide repeat (PPR) containing protein LOC_Os02g49830. PPR proteins (477 in rice, which show remarkably little redundancy) are thought to be involved in RNA editing in chloroplasts and mitochondria (Barkan and Small,
A mutant in the maize ortholog of LOC_Os02g49830 has an ‘ivory leaves’ phenotype but has not been described further (Photosynthetic Mutant Library; Alice Barkan lab; http://pml.uoregon.edu/pml_table.php). It is unclear which (if any) of the 21 edited sites in the chloroplast transcriptome (Corneille et al., 2000) is edited by this PPR protein, and whether the editing stabilises the RNA or makes it more prone to degradation. However, this protein may play a regulatory role in photosynthetic development. Phytochrome B is also already highly expressed in P3 stage and has been found to regulate the development of chloroplasts and expression of photosynthetic enzymes in rice (Zhao et al., 2012). Other genes in this list may not have regulatory functions but could nevertheless be necessary for early photosynthetic development. These include two copper transporters (LOC_Os08g37950 and LOC_Os07g43040), which may be required for the assembly of the copper-containing thylakoid membrane electron transfer agent plastocyanin. The hydrolase present is a putative ortholog of Arabidopsis thaliana AT1G68890, an essential gene for the biosynthesis of the PSI-associated electron transfer agent phylloquinone (Gross et al., 2006). Digalactodiacylglycerol synthase is involved in synthesis of chloroplast membrane lipids, further indicating that chloroplast assembly is already occurring at P3 stage (Wang et al., 2014b). At the same time as building photosynthetic capacity, a capacity for redox regulation may need to be developed, a core component of which is glutathione (Foyer and Noctor, 2011). The precursor for this molecule is cysteine, which could explain the high expression of a cysteine synthase early in development. Of the other genes in this list, several have very low levels of expression (e.g. ferritin-1, oxygen evolving enhancer protein 3), have diverse possible functions in metabolism (e.g. 4-hydroxy-2-oxovalerate aldolase) or are members of very large families (e.g. protein phosphatase 2C - part of a family of 73 genes), complicating further interpretation.

Genes important for further photosynthetic development were expected to be most highly expressed in P5 stage, as this stage shows the highest photosynthetic capacity. Thus, I next focused on the ‘up 1’, ‘up 2’ and ‘up 3’ clusters. Within these clusters, a further 17 genes were identified as key to photosynthetic development. Several of these are annotated in rice and have already been described in rice or other species to have functions in photosynthetic development. These include PROTON GRADIENT REGULATION5 (Nishikawa et al., 2012) and THYLAKOID FORMATION1 (Yamatani et al., 2013). The serine/threonine-protein kinase stt7 was previously described in Chlamydomonas reinhardtii to be involved in state transitions and LHCII phosphorylation (Depege et al., 2003). The Arabidopsis thaliana orthologs of the pkB family kinase identified are known to be crucial to leaf development (Gilkerson et al., 2012) and one putative ortholog has been found to regulate the bulking up of photosynthetic enzyme essentials such as Rubisco subunits (Ogawa et al., 2009). Four further genes are annotated only as
‘expressed protein’ in rice, but have putative orthologs in *Arabidopsis thaliana* that are known to be involved in assembly of the photosynthetic apparatus. These orthologs are *LOW QUANTUM YIELD OF PSII* (Lu et al., 2011); *CHLOROPLAST PROTEIN ENHANCING STRESS TOLERANCE* (Yokotani et al., 2011); *LOW PSII ACCUMULATION3* (Cai et al., 2010); and *THYLAKOID RHODANESE-LIKE* (Juric et al., 2009). Of the remaining nine genes, six have unknown functions and no known orthologs, but are annotated with various subcellular localisation descriptors, namely ‘membrane protein’ (one gene), or ‘thylakoid lumen(-al) protein’ (five genes). Three others are annotated only as ‘expressed protein’ in rice. These nine genes have all been associated with the GO term ‘photosynthesis’, but their exact biological function remains to be elucidated.

No transcription factors in rice are annotated with the GO term ‘Photosynthesis’, but 33 were found to show the same expression pattern as the genes above and might thus be regulating this process. The fact that these clusters contain the known chloroplast development regulators *GLK1* and *GLK2*, which are part of the G2-like family of transcription factors, validates our approach (Waters et al., 2009). One additional G2-like transcription factor (LOC_Os03g55590) may be involved, since it shows a similar expression pattern to *GLK1* and *GLK2*. Six MYB-related transcription factors are also present in this list of putative photosynthetic development regulators, as are five TALE-family and four CONSTANS-like transcription factors. The latter have been found to regulate photomorphogenesis in *Arabidopsis thaliana*, and may play a similar role in rice (reviewed by Gangappa and Botto, 2014). Further investigation of these genes through *in situ* hybridization to determine their spatial expression patterns may provide insight into their function, although the study of the expression patterns of *THYLAKOID FORMATION1*, a *CHLOROPHYLL A/B-BINDING* gene and *PsbP* here did not show any spatially restricted expression patterns. As it is little known what expression patterns to expect from genes involved in photosynthetic development (in contrast to genes involved in the development of specific structures such as veins or stomata), perhaps other approaches are more promising. This may include the protoplast isolation and transformation system recently used to study the function of *GLK1* in rice (Zhang et al., 2011).

In addition to the development of photosynthesis, the development of the vasculature is important to leaf performance and occurs during the rice leaf developmental stages studied here. Thus, gene expression changes possibly relating to the development of veins were also studied. The youngest rice developmental stage studied here (P3) already contains a midvein, but no histologically distinguishable other (pro-)vascular tissue. Large veins are formed during P4 stage. In P5 stage, most large and small veins are fully formed, and their phloem and xylem are fully differentiated. The distribution in the leaf primordium of pre-provascular tissue that cannot be
distinguished histologically is the first determinant of vein spacing in the mature leaf (Scarpella et al., 2004). Thus, transcription factors expressed in P3 stage but rapidly down regulated to P4 stage are prime candidates for markers of early vascular differentiation.

39 transcription factors that are potential regulators of vascular development were identified. These come from diverse families. None are well studied in rice, but several have putative *Arabidopsis thaliana* orthologs. Thus, these genes are obvious targets to be followed up. However, there are 23 genes out of my list of 39 potential vascular development regulators that have not been studied in rice and for which an ortholog in another species can also not be identified. Clarifying whether they play a role in vascular development in rice remains a formidable challenge, particularly due to the fact that these previously unstudied transcription factors are often members of larger families of genes with high sequence similarity and possibly high redundancy.

Scarpella and Meijer (2004) comprehensively reviewed vascular development in dicots and monocots, and offer detailed lists of some of the best studied vascular development regulators in *Arabidopsis thaliana*, maize and rice. The expression patterns of the rice orthologs of these genes during early leaf development gives insight into their likely roles in rice. For example, cluster ‘down 2’, which likely contains genes important for early events in vascular development, contains the well-studied rice gene *DROOPING LEAF* (Yamaguchi et al., 2004), the rice orthologs of the auxin efflux carrier *PIN1* (Xu et al., 2005), the known vascular development regulator *AXR6* (Hobbie et al., 2000), and the auxin responsive gene *ZLL* (Tucker et al., 2008). The ‘down 3’ cluster, in which genes are further down regulated from P4 to P5 after having been down regulated from P3 to P4 stage, may also contain genes important for early events in the formation of veins. This cluster contains the auxin responsive rice gene *OsPNH*, as well as rice orthologs of *EMB30*, weak mutant alleles of which show vascular patterning effects attributed to polar auxin transport issues; the known vascular development regulators *MP*, *VCS* and *FK*; and genes involved in cell or organ polarity such as *TAN1*, *PHV* and *PHB* (reviewed by Scarpella and Meijer, 2004).

In contrast, some rice orthologs of *Arabidopsis thaliana* genes involved in vascular development have expression patterns in rice that do not reflect the patterns of vein development observed in this thesis (Chapter 3). This may make them less likely candidates for early vascular development regulators in rice. These include genes that are up regulated in developmental stages (P4, P5) in which the basic vascular pattern is already established, such as those in clusters ‘up 1’ (*VAS*, *AGO1*, *WOL*, *COV1*, and *Oshox1*), ‘up 2’ (*PIN1*, *APL*, *VH1*, *VEP1*, *CPD* and *ISR*) and ‘up 3’, as well as those that are down regulated in later developmental stages, but not until the P4 to P5
stage transition (‘down 1’ cluster, which contains orthologs of Arabidopsis thaliana PHV, PHB, DWF7, SMT1, and ZLL). Those that show no change in their level of expression across the developmental stages studied (i.e. those in the ‘neutral’ expression cluster, namely rice orthologs of EMB30, AXR6, KNAT1, FZY, APL, Athb8, VCS, REV, AMP1 and KN1) may also be of less interest as likely regulators of early vascular development in rice. However, it is also possible that these upregulated or neutral genes are active in vascular patterning in stages prior to those in which gene expression was measured in this work (P1, P2). Alternatively, their increasing or constant expression during leaf development may be to do with functions other than vein development, in addition to which their absolute level of expression in early stages is still high enough to regulate vein development.

The study of potential regulators of vascular development through in situ hybridization has the potential to reveal both whether they are expressed in patterns of relevance to vascular development, and at what developmental stage their expression is highest. The cross sections used here capture several developmental stages of the rice leaf, enabling the comparison of tissues with different levels of vein differentiation. However, none of the genes I studied showed expression patterns restricted to or excluded from the vasculature. This may indicate that they do not play a role in vascular differentiation, or that they interact with other factors that do have specific expression patterns, or that their spatial regulation is not at the transcriptional level.

5.5.3. Comparisons with maize leaf development

A major theme in efforts to improve rice photosynthesis has been to identify the genetic differences which might account for the differentiation of C₃ or C₄ leaf anatomies. Leaves of C₄ plants develop a high vein density and the specialized arrangement of mesophyll and bundle sheath cells known as Kranz anatomy. In contrast, C₃ rice leaves develop lower vein density and lack Kranz anatomy. With the advent of next generation sequencing and gene expression technologies, comparison of genome-scale data has become practical and a series of insightful analyses of C₄ leaf development has ensued (Li et al., 2010; Braeutigam et al., 2011; Wang et al., 2013a; Wang et al., 2014). My data indicate that the key steps in the establishment of fundamental aspects of rice leaf photosynthesis occur early in development (P3/P4), thus comparison of the relevant gene expression patterns at this specific stage may provide novel leads as to which of the myriad genes expressed in C₃ and C₄ leaves might be appropriate targets for manipulation.

One initial finding from my correlation of gene expression in developing maize and rice leaves is that rice primordia show a more similar pattern of gene expression to maize foliar primordia than to maize husk primordia, despite the fact that maize husk leaves also have C₃ photosynthesis whereas maize foliar leaves carry out C₄ photosynthesis. This may indicate that the primary
function of the rice leaves studied, namely to carry out photosynthesis, is shared with the maize foliar leaf and is the most important determinant of gene expression pattern, whereas maize husk leaves may be more distinct due to their role in the floral transition and in protecting the reproductive organs from biotic and abiotic stress (Wang et al., 2014a).

Functional enrichment testing on the overlap between clusters reveals that some genes may have conserved roles in maize and rice during early leaf development. These genes are up regulated early in leaf development and down regulated in older leaf primordia in both maize and rice. Not only do they include genes with functions in the cell cycle, cell division, DNA and protein synthesis, as might be expected in young rapidly dividing cells, several other interesting functions are also enriched in early leaf development in both maize and rice. These include categories with regulatory functions such as pre-rRNA processing and modification, protein targeting to the nucleus, chromatin remodeling factors, zinc finger-HD transcription factors, RNA binding proteins and pentatricopeptide (PPR) repeat-containing proteins.

Among the genes showing an increase in expression from younger to older leaves in both maize and rice, many are part of the core photosynthetic machinery, as might be expected. In addition, the CONSTANS-like family of C2C2 transcription factors are up regulated during development in both rice and maize. These genes are known to have a role in photomorphogenesis, and several are light inducible (Gangappa and Botto, 2014). As such, it is likely that they are a central part of the regulatory network that forms a photosynthetic mature leaf in both maize and rice. A second enriched category of genes that is up regulated in both maize and rice is that of the lipid transfer proteins. The biological relevance of the presence of lipid transfer proteins in both the maize and the rice up regulated transcriptomes is unclear.

Genes that are down regulated during rice leaf development but up regulated during maize leaf development may enable the development of ‘C₄’ structure and function in maize (Wang et al., 2013). Thus, I identified all rice genes that show a general downward trend in expression during development of which the maize orthologs show an upward trend in expression. Within the resulting list of genes, only two functions are enriched, namely protein targeting to the chloroplast and amino acid synthesis. The finding that one enriched functional term was ‘protein targeting to the chloroplast’ is consistent with the fact that appropriate spatial separation of different enzymatic functions is a key aspect of C₄ photosynthesis (Sheen, 1999; Majeran et al., 2005; Hibberd and Covshoff, 2010). Thus, my data support the idea that a chloroplast protein import based mechanism for the compartmentation of photosynthesis between mesophyll and bundle sheath cells active during the later stages of maize leaf development may be lacking in rice leaf development (Langdale, 2011). The significance of the enrichment in genes involved in
amino acid synthesis in the list of genes that are up regulated in maize but down regulated in rice is less obvious.

The comparison of the data set reported here with that of Wang et al. (2013a) also allowed me to identify eight rice transcription factors that decrease in expression during rice leaf development of which the maize orthologs increased in expression during maize foliar leaf development. Four of these genes have been reported to have very low expression in the mature leaf mesophyll and bundle sheath in maize but the other four have been reported to be differentially expressed between mature maize mesophyll and bundle sheath cells, the key cell types of C₄ maize Kranz anatomy (Li et al., 2010). These eight transcription factors represent novel targets for the manipulation of the rice leaf to produce a more C₄-like architecture.

5.5.4. Conclusions and future work

This dataset provides an overview of gene expression changes in early leaf development in rice plants. It can guide efforts to identify novel regulators of vascular, stomatal and photosynthetic development in rice, and thus help translate the work done in model species such as *Arabidopsis thaliana* into this vital crop plant. In addition, it can be compared to recent datasets on early maize leaf development and the maize and rice leaf gradients (Wang et al., 2014a; Wang et al., 2013). Such comparisons can be used to identify both conserved patterns of gene expression in monocots and patterns that may have diverged to facilitate the development of C₄ structures.

In studying this dataset, I have focused partly on the development of photosynthesis in rice leaf primordia. The P3, P4 and P5 stages of rice leaf development show a rapid increase in photosynthetic capacity (Chapter 3, this thesis). In this chapter, I have demonstrated that the genes coding for the core photosynthetic machinery in rice (which are largely known) show a concurrent increase in expression. I have identified a list of 51 transcription factors that are poorly studied in rice that may play a role in the acquisition of photosynthetic capacity. In addition, the comparison of this dataset with an earlier equivalent maize dataset revealed that members of the *CONSTANS*-like transcription factor family in particular are overrepresented in the expression clusters that contain many photosynthetic components in both maize and rice. The expression patterns of these potential regulators of photosynthetic development can be further investigated, and mutants may elucidate their exact biological function. Identifying whether any of these genes share downstream targets with known regulators of plastid development such as *GLK1* and *GLK2* may confirm their role as regulators of photosynthetic differentiation.

In addition, the genetic mechanisms underlying vascular patterning in rice were investigated. Several novel potential regulators of early vascular development identified here merit further
study. A small number can be pinpointed as being of particular interest from orthology with *Arabidopsis thaliana* genes known to regulate vascular development or due to their auxin responsiveness. Studying the expression patterns of the unknown rice transcription factors identified here as possible regulators of rice vascular development in the developing vasculature further may also provide more specific insight. In addition, several of the orthologs of the *Arabidopsis thaliana* vascular development regulators described by Scarpella and Meijer (2004) have never been described in rice before, and these data could help translate findings from this model species into a vital crop plant.

Another morphological parameter of interest for crop improvement is stomatal patterning, and thus, the expression of genes putatively involved in stomatal development was investigated. Stomatal differentiation occurs during P4 stage (Amin Yaapar, thesis, 2016) and the analysis presented in this chapter revealed the expression during this phase of a number of genes associated in other systems with processes of stomatal patterning and differentiation. Previous attempts to detect the expression of some of these genes in rice (e.g., *OsSPCH1* and *OsSPCH2*) have failed (Liu et al., 2009), suggesting that the highly targeted and staged RNA-Seq approach taken here was essential for the detection of these transcripts. The overall pattern of stomatal associated gene expression was consistent with the observed limitation of stomatal differentiation to the P3/P4 stage. Expression of most of these genes was minimal in P5 stage leaves and the others tended to show a gradual decline in expression from P3 to P5 or some showed a peak in expression at P4 (e.g., *OsFAMA*, *OsEPFL9*). In light of the role of these genes in controlling stomatal patterning and differentiation in other systems, these rice genes likely play a vital role in setting parameters of stomatal pattern and number. Studying whether these genes are expressed in developing stomata is technically challenging, but may be feasible in the future if whole mount in situ hybridization techniques in rice are further developed (Amin Yaapar, thesis, 2016).

Finally, I was interested in the differences in gene expression during C₃ and C₄ leaf development. Many comparisons of C₃ and C₄ leaf gene expression are now available, and the timing and location of expression of many of the core elements of C₄ photosynthetic metabolism are known (Langdale et al., 1988; Li et al., 2010; Wang et al., 2014a). However, the transcription factors regulating the development of Kranz anatomy and cell-specific expression in C₄ leaves remain elusive (Hibberd and Covshoff, 2010). Here, I suggest a list of eight transcription factors which are upregulated during maize leaf development but down regulated during rice leaf development. Overexpression of these maize transcription factors in rice may help elucidate their exact function, particularly if promoters can be identified that enable targeted expression during the later stages of rice leaf development. However, it is likely that Kranz anatomy, higher vein density, and other morphological differences between C₃ and C₄ plants are regulated by a suite of
genes (Brown and Bouton, 1993; Leegood, 2013). Therefore, the overexpression of informed combinations of these transcription factors and others may be the way forward (Wang et al., 2014a).

5.5.5. Final remarks

The work presented here describes the expression patterns of genes involved in the key processes occurring during the early development of the rice leaf. As such, it provides insight into the genetic networks underlying vascular, stomatal and photosynthetic development in rice. As well as identifying key processes occurring at each developmental stage, the work pinpoints several individual genes that may merit future work. In addition, the overwhelming majority of data generated in this study has not yet been analyzed. These data include immature mRNA transcripts, which may give insight into which genes are actively being transcribed at a particular developmental stage and which have been up regulated since earlier in development. They include all alternatively spliced transcripts of each known gene, provided that they are expressed at some point during early leaf development, as well as transcripts originating from previously completely unknown genes. Transcripts originating from the mitochondrion or from the plastid are also available. The stranded nature of the library generated has created a dataset that is ideal for the analysis of genes that may be coded for on opposite strands but in overlapping reading frames. Finally, even long non-coding RNAs can be detected in these data, and the stranded library preparation can reveal their expression pattern at the same time as that of any protein-coding genes on the opposite strand that may or may not be regulated by them. Thus, the publicly available data generated here has the potential to reveal much more about early rice leaf development in future work.
## 5.6. Supplementary material

<table>
<thead>
<tr>
<th>Gene ID</th>
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<th>Description</th>
<th>Rice orthologs(s)</th>
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<td>AT1G20330.1</td>
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<td>AT1G73590.1</td>
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<td>Ortholog</td>
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<td>LOC_OS</td>
<td>Expression</td>
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<td>Cytochrome P450</td>
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<td>GRMZM2G039113</td>
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</table>

Supplementary Table S1. Arabidopsis thaliana and Zea mays genes known to regulate vascular development (Scarpella and Meijer 2004), their putative rice orthologs, and expression clusters of these orthologs. not ex., not expressed. N/A, not applicable.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene name</th>
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<tbody>
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<td>LOC_Os06g12610</td>
<td>OsPIN1a</td>
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</tr>
<tr>
<td>LOC_Os06g02180</td>
<td>NARROW AND ROLLED LEAF 1; CSLD2</td>
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<tr>
<td>LOC_Os07g03770</td>
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<tr>
<td>LOC_Os08g34380</td>
<td>COE1 receptor-like kinase</td>
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</tr>
<tr>
<td>LOC_Os03g33650</td>
<td>OsAGO7</td>
<td>down 3</td>
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<tr>
<td>LOC_Os04g52479</td>
<td>NAL1; peptidase, trypsin-like serine and cysteine proteases</td>
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</tr>
<tr>
<td>LOC_Os08g41720</td>
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<td>LOC_Os11g01130</td>
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</tr>
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<td>LOC_Os12g01120</td>
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<tr>
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<td>cytochrome P450 (CYP90D2)</td>
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<td>OsPIN1b</td>
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Supplementary Table S 2. *Oryza sativa* genes known to be involved in vascular development and their expression during early leaf development.
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<tr>
<td>LOC_Os03g11600</td>
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</tr>
<tr>
<td>LOC_Os01g45570</td>
<td>homeobox associated leucine zipper</td>
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<tr>
<td>LOC_Os09g25040</td>
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<td>LOC_Os07g03250</td>
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<td>LOC_Os08g36700</td>
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Supplementary Table S 3. Oryza sativa genes identified as potential novel regulators of vascular development in rice.
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<td>LOC_Os07g01480</td>
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<td>LOC_Os04g53612</td>
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<td>'PS.calvin cycle.aldolase'</td>
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<td>'PS.calvin cycle.aldolase'</td>
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<td>fructose-bisphosphate aldolase isozyme</td>
<td>'PS.calvin cycle.aldolase'</td>
</tr>
<tr>
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<td>transketolase, chloroplast precursor</td>
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<td>ribulose-phosphate 3-epimerase</td>
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Supplementary Table S 4. Photosynthetic genes that are among the first to be expressed to at least 50% of their maximal expression level at the earliest developmental stage studied (P3 stage).
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<th>Cluster</th>
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<td>up 3</td>
</tr>
<tr>
<td>LOC_Os02g51020</td>
<td>expressed protein</td>
<td>up 2</td>
</tr>
<tr>
<td>LOC_Os05g28090</td>
<td>expressed protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os03g14040</td>
<td>expressed protein; putative ortholog of <em>Arabidopsis</em> LOW QUANTUM YIELD OF PSII1</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os01g58470</td>
<td>expressed protein; putative ortholog of <em>Arabidopsis</em> CHLOROPLAST PROTEIN ENHANCING STRESS TOLERANCE</td>
<td>up 2</td>
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<td>LOC_Os02g02520</td>
<td>expressed protein; putative ortholog of <em>Arabidopsis</em> LOW PSII ACCUMULATION 3</td>
<td>up 2</td>
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<td>expressed protein; putative ortholog of <em>Arabidopsis</em> THYLAKOID RHODANSE-LIKE</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os10g42240</td>
<td>kinase, pfkB family; putative ortholog of <em>Arabidopsis</em> NECESSARY FOR THE ACHIEVEMENT OF RUBISCO ACCUMULATION 5 (NARA5)</td>
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</tr>
<tr>
<td>LOC_Os04g33830</td>
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</tr>
<tr>
<td>LOC_Os08g45190</td>
<td>PROTON GRADIENT REGULATION 5</td>
<td>up 3</td>
</tr>
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<td>LOC_Os05g40180</td>
<td>serine/threonine-protein kinase stt7, chloroplast precursor</td>
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<tr>
<td>LOC_Os07g37250</td>
<td>THYLAKOID FORMATION1, chloroplast precursor</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os08g40160</td>
<td>thylakoid lumen protein, chloroplast precursor</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os05g33280</td>
<td>thylakoid lumen protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os08g39430</td>
<td>thylakoid luminal 19 kDa protein, chloroplast precursor</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os01g59090</td>
<td>thylakoid luminal 20 kDa protein</td>
<td>up 3</td>
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<tr>
<td>LOC_Os12g08830</td>
<td>thylakoid luminal 29.8 kDa protein</td>
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Supplementary Table S 5. Non- transcription factor genes identified as important to photosynthetic development in rice.
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<td>LOC_Os02g29550</td>
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</tr>
<tr>
<td>LOC_Os02g39360</td>
<td>B-box zinc finger family protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os06g15330</td>
<td>CCT/B-box zinc finger protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os03g50310</td>
<td>CCT/B-box zinc finger protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os02g39710</td>
<td>CCT/B-box zinc finger protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os04g42020</td>
<td>CCT/B-box zinc finger protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os09g13570</td>
<td>CPuORF2 - conserved peptide uORF-containing transcript</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os02g49440</td>
<td>dof zinc finger domain containing protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os03g07360</td>
<td>expressed protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os05g02420</td>
<td>expressed protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os06g24070</td>
<td>GLK1; myb-like DNA-binding domain containing protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os01g13740</td>
<td>GLK2; myb-like DNA-binding domain containing protein</td>
<td>up 3</td>
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<td>LOC_Os10g40740</td>
<td>helix-loop-helix DNA-binding domain containing protein</td>
<td>up 3</td>
</tr>
<tr>
<td>LOC_Os03g29970</td>
<td>histone-like transcription factor and archaeal histone</td>
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</tr>
<tr>
<td>LOC_Os02g49700</td>
<td>homeobox associated leucine zipper</td>
<td>up 2</td>
</tr>
<tr>
<td>LOC_Os12g43950</td>
<td>homeobox domain containing protein</td>
<td>up 2</td>
</tr>
<tr>
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</tr>
<tr>
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<td>up 3</td>
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<td>LOC_Os01g34060</td>
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Supplementary Table S 6. Transcription factors identified as potential novel regulators of photosynthetic development in rice.

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<tr>
<td>LOC_Os09g36730</td>
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</tr>
<tr>
<td>LOC_Os03g25550</td>
<td>myb-like DNA-binding domain containing protein</td>
<td>peak</td>
</tr>
<tr>
<td>LOC_Os08g33660</td>
<td>MYB family transcription factor</td>
<td>peak</td>
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<td>LOC_Os01g68700</td>
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<tr>
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Supplementary Table S 7. Expression profiles of rice orthologs of putative regulators of Kranz anatomy in maize (gene list from Wang et al., 2013). NS, not significantly differently expressed between any two stages; NE, not expressed.
<table>
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<th>O. sativa annotation</th>
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<tr>
<td>LOC_Os09g12600</td>
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<td>phosphate/phosphate translocator</td>
<td>down 2</td>
</tr>
<tr>
<td>LOC_Os07g42600</td>
<td>3</td>
<td>aminotransferase, classes I and II</td>
<td>down 3</td>
</tr>
<tr>
<td>LOC_Os08g33720</td>
<td>4</td>
<td>lactate/malate dehydrogenase</td>
<td>neutral</td>
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<tr>
<td>LOC_Os01g09320</td>
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<td>LOC_Os09g02214</td>
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<td>Sodium-proton antiporter (putative)</td>
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<td>up 2</td>
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<td>LOC_Os07g31380</td>
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Supplementary Table S 8. Expression profiles of rice genes encoding orthologs of enzymes and transporters involved in C₄ photosynthesis in maize across three developmental stages of the rice leaf (gene list from Wang et al., 2013).
Chapter 6

Discussion
6. Discussion

6.1. When does a rice leaf gain capacity for photosynthesis?

A key aim of this thesis was to identify the point at which developing rice leaves gain the ability to carry out photosynthetic electron transport. In order to do this, I developed a protocol to image the chlorophyll fluorescence dynamics of rice leaf primordia. My results show that photosynthetic electron transport first occurs in the tip of P3 stage primordia. A steep developmental gradient exists along the leaf at this developmental stage, with only regions nearest the tip showing photosynthetic function. In P4 stage leaves, the region in which photosynthetic electron transport can occur is much larger, and the absolute level of $\Phi_{PSII}$ is also much higher in these regions. These results, along with my finding that the complexity of chloroplast structure increases from P3 to P4 stage, indicate that rice leaves gain photosynthetic competence around the P3-P4 stage transition. They also emphasise the need to study developmental processes in grass leaves at a high resolution to adequately capture the spatial heterogeneity from tip to base.

The chlorophyll fluorescence imaging technique developed here may also prove useful for the high spatial resolution study of physiological processes in other developing systems. Specifically, two possibilities could include studying the relationship between the onset of photosynthetic function and cell cycle arrest front formation in dicot leaves (Andriankaja et al., 2012), or studying the timing and effect on photosynthetic function of differentiation of the bundle sheath in C4 plants to have an altered photosystem II: photosystem I ratio compared to the surrounding mesophyll (Pfuendel and Neubohn, 1999; Peterson et al., 2014).

A second finding was that at these early stages of leaf development the dynamics of photosynthetic electron transport in response to light are different to those seen in mature leaves. For instance, induction of electron transport upon exposure to light is much faster in P3 and P4 stage primordia than in P5 stage and mature leaves, and primordia lose photosynthetic efficiency rapidly at increasing irradiances. These results inform on both the developmental status of the plastid electron transport chain and the metabolic status of the primordial tissues in which photosynthetic electron transport efficiency is being measured. In particular, the plastoquinone pool in these primordia is likely to be small and rapidly reduced, leading to fast induction kinetics (Ohashi et al., 1989). This small plastoquinone pool may be one of the factors limiting the capacity of the developing electron transport chain. A rapid decrease in photosynthetic efficiency is seen at higher irradiances in P3/P4 stage leaves, again confirming the hypothesis that the capacity of the electron transport chain is low at early developmental stages. However, the rapid induction observed may also reflect the presence of high levels of reduced metabolites in these
tissues (Turgeon, 1989; Meng et al., 2001). The low absorbance and low electron transport efficiency seen in these primordia confirms their status as sink tissues that are incapable of surviving on autotrophic metabolism, instead being reliant on photosynthate imported from mature leaves.

A question that remains is why these developing leaf primordia carry out photosynthetic electron transport. Little light is available to them, absorbance is low as little chlorophyll is present, and as a result the reducing power generated by photosynthetic electron transport at these stages is likely to be negligible compared to that generated in a mature leaf. This early onset of photosynthesis has also been observed in other systems at the level of gene expression and structural differentiation of plastids. In maize, genes encoding Calvin cycle enzymes and photosystem II subunits are already expressed in the L1 layer of the shoot apical meristem (SAM); in tomato (*Solanum lycopersicon*), Rubisco is upregulated from the SAM to the P1 stage primordium; and in *Arabidopsis*, fairly developed chloroplasts are seen in the L1 and L3 layers of the SAM (Fleming et al., 1993; Ohtsu et al., 2007; Charuvi et al., 2012). One hypothesis is that the establishment of high photosynthetic capacity requires a certain amount of time, and an early onset of photosynthetic differentiation is necessary to allow the synthesis of sufficient amounts of pigments, electron carriers and enzymatic machinery. However, chlorophyll biosynthesis is a very rapid process, with protochlorophyllide reductase (POR) known to be one of the fastest enzymes on earth (Masuda and Takamiya, 2004). In addition, biosynthesis of the required protein complexes is also relatively fast, since the genes involved in this can be upregulated rapidly and to a very high level when necessary, as is observed in our gene expression data comparing stages P3, P4 and P5. Although gene expression does not always translate directly to an increase in functional protein complex abundance, previous work has shown that in the case of photosynthetic protein complexes such as photosystem II, full function can be acquired as little as four hours after the illumination signal necessary for chlorophyll biosynthesis, which is coordinated with the expression of chlorophyll binding proteins (Ohashi et al., 1989). Therefore, why doesn’t a rice leaf initiate photosynthetic development four hours before it is first outside the developing sheaths of older leaves, where sufficient light is available for a meaningful amount of photosynthesis?

One possibility is that although the small amount of photosynthesis carried out by developing leaves may not be meaningful in terms of leaf energy provision, it may be very meaningful in terms of the developmental signals it provides. Photosynthetic metabolism at early developmental stages may be necessary to produce specific metabolites that cannot be synthesized from
imported sugars and are necessary signals for appropriate development. This may happen at a cellular level, with the onset of photosynthetic metabolism required to allow an individual cells to end their proliferative state or to enter the appropriate differentiation pathway. Alternatively, the developing photosynthetic electron chain may amplify signals received from the environment through generating reactive oxygen species upon photo-oxidative stress induced by the small amount of light it receives. This could play a role in acclimation to the environment at early developmental stages, as reactive oxygen species generated during photosynthetic electron transport are quantitatively affected by many environmental stressors, such as desiccation, cold stress and nutrient limitation (Wise, 1995; Fey et al., 2005; Takahashi and Murata, 2008; Lawlor and Tezara, 2009; Pfannschmidt et al., 2009). In this scenario, the limited capacity of the developing electron transport chain may allow for increased responsiveness to the dampened environmental signals primordia receive as a result of being surrounded by the sheaths of older leaves: small changes in efficiency induced by environmental inputs may rapidly affect the reduction state of the entire plastoquinone pool as there is little excess capacity to buffer these changes. Experiments investigating whether etiolated (non-photosynthesising) leaves fully acclimate structurally to different non-light related environments (for example temperature or CO₂ changes) may inform on this further. However, studies in other systems have implicated long distance signalling from mature to developing leaves in the acclimation of ‘sheltered’ leaves to their environment, which may negate the need for this function of the early electron transport chain (see below).

An alternative explanation for the early onset of photosynthetic electron transport may be that it is not adaptive, instead being an evolutionary ‘spandrel’: an unavoidable by-product of the evolution of photosynthesis (Gould and Lewontin, 1979; Pigliucci and Kaplan, 2000). Intriguingly, although the key chlorophyll biosynthesis enzyme protochlorophyllide reductase (POR) requires light in angiosperms, chlorophyll biosynthesis can occur in the dark in photosynthetic bacteria, algae and gymnosperms, as the protochlorophyllide reductase in these systems (DPOR) is light independent (Adamson et al., 1997; Schoefs and Franck, 2003). A possibility is that the loss during angiosperm evolution of DPOR (likely because it is moderately oxygen sensitive; (Schoefs and Franck, 2003)) left in place the mechanisms inducing the onset of other components of photosynthesis very early in development. Thus, it would be interesting to see whether expression of this light independent DPOR at very early stages of grass leaf development would promote the biosynthesis of chlorophyll at stages where little light is present (pre-P3). It would remain to be seen whether this would ultimately contribute to leaf photosynthetic efficiency, since DPOR is moderately oxygen-sensitive and requires NADPH for
catalysis, and the synthesized chlorophyll may cause photooxidative stress due to the absorption of more light energy than the underdeveloped electron transport chain could dissipate (Schoefs and Franck, 2003). However, if the photosynthetic machinery is all ‘set’ to go upon sufficient light for chlorophyll biosynthesis being available, as in etiolated tissues, one might expect that rapid differentiation of the photosynthetic machinery might also occur if the light-dependent step of chlorophyll biosynthesis is bypassed.

6.2. How well does the onset of photosynthesis correlate with morphological aspects of leaf differentiation?

In addition to the onset of photosynthetic electron transport, the developmental stages studied here also encompass the development of key morphological features such as vasculature and stomata. In chapter 3, I characterised morphological changes during rice leaf development in detail by performing histological analyses of rice leaves at different stages of development. Using the highly predictable plastochron intervals observed in rice allowed me to stage the fifth leaf produced by measuring the length of leaf three (van Campen et al., 2016). This was key to studying the development of the vasculature in rice, as the early primordial stages important to this process are obscured from view by the sheaths of older leaves. I found that the midvein in rice is initiated before P2 stage, and the patterning of lateral veins develops by P3 stage. Although further differentiation of xylem and phloem occur from P3 stage up until leaf maturity, the spacing of veins is established early and no new veins are initiated after P4 stage. This spacing of veins is a key difference between C\textsubscript{3} and C\textsubscript{4} plants, and its development is thus of interest for improving rice photosynthetic performance (Smillie et al., 2012; Leegood, 2013; Wang et al., 2013; Feldman et al., 2014).

Vascular patterning in plant leaves is known to involve a range of molecular signalling pathways (Nelson and Dengler, 1997; Scarpella and Meijer, 2004). In particular, the involvement of auxin in both the initiation of primordia and the formation of the first vascular trace (the midrib) is well documented in both dicots such as Arabidopsis thaliana and monocots including maize and rice (Nelson and Dengler, 1997; Qi et al., 2008; Baylis et al., 2013). The auxin canalization hypothesis suggests that auxin then continues this role in the further patterning of veins; polar flow of auxin from the tip of the leaf to the shoot apical meristem via PIN proteins is thought to be a key process leading to the formation of continuous vascular strands (Berleth et al., 2000; Scarpella et al., 2006; Qi et al., 2008). The resulting auxin drainage from primordia to the shoot apical meristem is also thought to be important for establishing polarity in the leaf, which in turn is necessary for the planar structure of leaves: polarity defects lead to radialized leaves with an
almost tubular structure (Eshed et al., 2001; Eshed et al., 2004; Smith et al., 2006; Kuhlemeier, 2007; Zhang et al., 2009; Moon and Hake, 2011; Deb et al., 2015). Given that leaf width (a result of planar growth) and vascular spacing are closely linked in monocot leaves (mutant screens for narrow vein spacing in both rice and maize have revealed many narrow leaf mutants; Smillie et al., 2012; Feldman et al., 2014; Rizal et al., 2015; Marja Timmermans, personal communication), it is possible that polarity signals facilitating the wide, planar structure of leaves are also involved in vascular spacing. Indeed, many of the described polarity mutants in *Arabidopsis* have defects in both vascular patterning and vascular differentiation, as well as having planar growth defects (Emery et al., 2003; Juarez et al., 2004). However, further study of polarity networks and their relation to early vascular development in monocots is necessary, since monocot and dicot vascular patterning are very different and the failure to identify high vein density monocot mutants with no leaf width phenotype is intriguing.

My data show that vascular patterning is established at developmental stages physically close to the meristem (P1-P3). Development at these stages is strongly affected by signals from the meristem and auxin drainage to this tissue (Juarez et al., 2004; Smith et al., 2006). However, I found that the end of initiation of new vasculature occurs later, at around the P4 stage. At this stage, my results show that the differentiation of key photosynthetic tissues occurs, and may thus take over from the meristem as a key source of developmental information. It is unknown whether mesophyll tissues are arranged around the ‘scaffolding’ of the developing vasculature, or whether the photosynthetic differentiation of the mesophyll shapes vascular patterning by ending vascular initiation (Langdale et al., 1988; Berleth et al., 2000; Scarpella et al., 2004; Andriankaja et al., 2012). This has been much debated, but it is possible that both processes are involved, with the second process reinforcing the tissue arrangements initiated by the first. The coordination of vascular and photosynthetic tissue development is of importance to the performance differences between C₃ and C₄ plants, since the primary photosynthetic tissue in C₃ plants is the mesophyll, whereas in C₄ plants it is often the bundle sheath of the vasculature (Leegood, 2008; Sage and Sage, 2009; Wang et al., 2013). However, how this coordination differs between C₃ and C₄ plants is an unsolved question.

Debate also still exists around how plastid development and other aspects of leaf development are coordinated (Aluru et al., 2001; Tsukaya, 2002; Andriankaja et al., 2012; Lin et al., 2015). Fundamentally, plant cells need plastids to replicate if they are to replicate. Plastid DNA replication happens before nuclear DNA replication, and defects in plastid biogenesis or plastid DNA replication can inhibit nuclear DNA replication, hampering cell cycle progression and
leading to severe growth defects (Blamire et al., 1974; Rose et al., 1975; Garton et al., 2007; Hudik et al., 2014). Kobayashi et al. (2009) showed that in both photosynthetic unicellular algae and non-photosynthetic tobacco BY-2 cells (which may be analogous to root cells; Kobayashi et al., 2009), the chlorophyll precursor Mg-ProtoIX (which requires no light for synthesis) induces nuclear DNA replication after plastid DNA replication in the absence of light. This would provide a mechanism to ensure coordination of plastid and cell proliferation in both leaf and root cells, and could potentially contribute to maintaining appropriate plastid volume in cells. In addition to this, mutants in the nuclear and chloroplast DNA pre-replication factor AtCDT1 have severe developmental phenotypes combined with inhibited plastid division (Raynaud et al., 2005). Thus, AtCDT1 has been implicated in the coordination of plastid division and nuclear DNA replication (Raynaud et al., 2005). This gene may act through the well-studied plastid division protein ARC6, mutation of which leads to chloroplast replication, but interestingly has no obvious effect on cell division (Pyke and Leech, 1992). Thus, plastid replication and cell division (and thus development) are intimately related but also governed by at least partly independent pathways.

In addition to this coordination of organellar DNA replication with nuclear DNA replication (and thus the cell cycle), several authors have also linked the later onset of cell cycle arrest in plants with the switch from division to differentiation of chloroplasts, although work has been largely restricted to the dicot Arabidopsis thaliana (White, 2006; Kazama et al., 2010; Andriankaja et al., 2012). An abrupt ‘cell cycle arrest front’ has been shown to exist in developing Arabidopsis leaves, which coincides with leaf greening and thus potentially with signals from chloroplast differentiation (Andriankaja et al., 2012). Some authors have described the presence of two arrest fronts- a primary arrest front ending the general proliferation of cells, and a secondary arrest front slightly later which ends the proliferation of stomatal and vascular precursors (White, 2006).

However, the transition between cell proliferation and differentiation is much less well studied in monocots. I found no evidence of an abrupt onset of photosynthetic differentiation or an abrupt cell cycle arrest front spatially limiting vascular patterning along rice leaf primordia. Instead, a gradient of photosynthetic differentiation is seen, with leaves rapidly acquiring photosynthetic function from tip to base at the P3/P4 stage transition. Although I did not study in detail the development of the vasculature at different locations along these leaf developmental stages using histology, instead focusing on snapshots along leaf primordia, I observed a striated pattern of chlorophyll fluorescence (likely in between vascular strands) throughout the leaf at P4 stage, when photosynthetic function is present in a gradient from tip to base. In addition, stomatal
differentiation does not display a sudden onset at a particular ‘front’ in rice leaves (van Campen et al., 2016). These findings argue against an abrupt secondary cell cycle arrest front of vascular and stomatal precursor cells in rice leaves. In addition, I did not observe a sudden shift in overall cell size at any particular point along the rice leaf. If this were observed, it may indicate that there are indeed two cell cycle arrest fronts in monocot leaves, with a primary arrest front limiting the proliferation of most cells in a local coordinated manner and a secondary arrest front leading to the limitation of vascular and stomatal precursor proliferation in a more diffuse gradient along the leaf. However, inferences about the primary cell cycle arrest front of non-vascular/stomatal precursors would require more in-depth study of cell size along developing rice leaves, possibly alongside an analysis of cell proliferative state along the leaf using S-phase fluorescent markers such as EdU, or marking dividing cells using histone H4 mRNA in situ hybridisation (Itoh et al., 1998; Kazda et al., 2016).

Despite the lack of an obvious localised cell cycle arrest front, I found that the timing of the onset of photosynthetic function does correlate very much with the timing of the end of initiation of new vascular strands and with the rapid stomatal differentiation (P3/P4 stage transition). Thus, I suggest there is not a locally coordinated secondary cell cycle arrest front in rice (and perhaps other monocot leaves), with the developmental switch from proliferation to differentiation instead happening in a more diffuse way at a cellular level. This could result in the gradient of development observed along the monocot leaf, perhaps with separate signalling pathways involved for vascular precursors, stomatal precursors and other cells. Inputs into these pathways from the onset of photosynthetic function (P3/P4 stage) may come in the form of light-dependent tetrapyroles or signals directly derived from electron transport chain function (e.g. reactive oxygen species) rather than from earlier aspects of plastid differentiation such as plastid division or the development of the plastid genetic machinery, since these are already established at a time when cell proliferation is still very much driving leaf development (pre-P3 stage).

6.3. How flexible are rice leaf developmental processes, and when is developmental plasticity lost?

Despite the fact that canopy crop plants such as rice are known to show a relatively weak acclimation response to light, morphological and physiological traits in rice have been previously shown to acclimate to the environment (Murchie et al., 2005). Thus, in order to determine the limits of flexibility of the normal process of leaf development, I performed light environment transfer experiments at different stages of rice leaf development. This allowed me to identify
whether there is a developmental window within developmental plasticity can occur. Such developmentally restricted acclimation ability has been previously observed in other species in root/shoot partitioning and in reproductive strategy choice (Gedroc et al., 1996; Vogler et al., 1998). In rice, I found that leaf structural parameters acclimate to the external light environment, but only within a defined developmental window. Leaf width and the distance between small veins showed acclimation which was limited to pre-P5 stages, supporting the idea that leaf width and vascular patterning are closely linked in monocots, although little acclimation in the number of veins or the number of veins per millimetre was observed. Biochemical aspects of photosynthesis also acclimated, but this was not limited to particular developmental stages. Thus, the different timescales of development of leaf morphology and physiology are reflected in different potential to acclimate to novel environments. In addition, the ability of photosynthetic capacity and electron transport rate to acclimate even at late developmental stages (both in terms of decreasing, as shown here, and increasing performance; (Murchie et al., 2005; van Campen et al., 2016) confirms the suggestion above that physiological function may not require a long time to establish or change (Ohashi et al., 1989; Murchie et al., 2005).

The differential morphological acclimation potential of leaves at particular developmental stages may indicate that their amenability to manipulation may also differ. In particular, stages at which vascular patterning is still flexible may need to be targeted if higher vascular density is to be achieved, for example for the establishment of a C₄-like Kranz anatomy in rice (Hibberd et al., 2008; Sage et al., 2014). In addition, targeting transgene overexpression to specific developmental stages may help avoid unintentional effects on other developmental processes and thus other aspects of leaf structure and function, and may make it easier to gain regulatory approval for transgenic varieties (Bajaj and Mohanty, 2005). However, a challenge in this area is that developmental-stage specific promoters are poorly characterized in rice (Jeong and Jung, 2015).

Developmental plasticity itself has been shown to be genetically determined in rice and other systems, and may thus represent a useful target for breeding (Eiguchi et al., 1993; Shimizu et al., 2010; Niones et al., 2015; Pieper et al., 2016). However, if morphological acclimation can be limited by the developmental stage of the leaf, this may have implications for the relationship between leaf growth and the ability of plants to respond appropriately to environmental stimuli. It is possible, for example, that rice varieties with rapid early leaf growth (and thus a short time spent by the leaf at each developmental stage) are more robust to rapidly fluctuating environmental conditions because they do not acclimate unnecessarily, or conversely that the short time they have for acclimation limits their ability to acclimate sufficiently to short term
environmental perturbation. Implications of this may lie in breeding trade-offs between growth rate, resilience to short term environmental perturbation, and adaptability to multiple growing environments.

Since the developmental stages at which morphological parameters are plastic are shielded from the external environment by sheaths of older leaves, long distance signalling may play a part in morphological acclimation to the environment (Long et al., 2015; Notaguchi, 2015; Okamoto et al., 2015). Rather than detecting limited environmental signals themselves and amplifying them through electron transport chain function as suggested above, developing leaves may respond to mobile signals indicating the environment perceived by older leaves. Indeed, this has been shown to occur in Arabidopsis acclimation to a changed ambient carbon dioxide concentration (Lake et al., 2001; Lake et al., 2002; Coupe et al., 2006). A wealth of plant mobile signals informing development are known. Small RNAs, carbohydrates, phytohormones, peptides and mobile transcription factors are all possible candidates (Lough and Lucas, 2006; Chuck et al., 2009; Benkovics and Timmermans, 2014; Okamoto et al., 2015). Most of these long distance signals have been shown to move through the phloem (Lough and Lucas, 2006; Ruiz-Medrano et al., 2012). Therefore, experiments combining grafting (in non-grass species) and transfer between different environments may provide further insight (Jaeger and Wigge, 2007; Pant et al., 2008; Bai et al., 2011). Alternatively, in the case of acclimation to light, the sugar status of the entire plant may be affected by the environment it is grown in, allowing acclimation of developing leaves upon detection of a systemic shift in carbon status (Paul and Pellny, 2003; Geigenberger et al., 2005). However, given that there are large circadian changes in the carbon status of the plant, which are not a suitably specific signal for developing leaves to acclimate morphologically, a more specific signal is likely to be involved (Blasing et al., 2005). Recently, the transcription factor HY5 was found to move from the shoot to the root in Arabidopsis to coordinate carbon and nitrogen metabolism systemically in response to fluctuating light conditions (Chen et al., 2016). This transcription factor may also play a role in the structural alterations observed here in response to light.

6.4. Can genes regulating rice leaf developmental processes be identified from their expression patterns during early leaf development?

Having defined the timing and nature of the development of vasculature, leaf architecture and leaf physiology in rice, I proceeded to identify the gene expression changes underlying specific events in rice leaf morphological and physiological development. In order to do this, I carried out a targeted RNA-Seq analysis on developing rice leaves. In addition, I compared this RNA-Seq
data on developing rice leaf gene expression to equivalent data from maize to identify conserved and diverged gene expression patterns during leaf development in the C₃ grass rice and the C₄ grass maize (Wang et al., 2013).

Gene expression changes associated with metabolic shifts occurring during early leaf development were clearly observed. Notably, PRR proteins were heavily implicated in playing a role in this through modifying organellar transcription/translation dynamics (Barkan and Goldschmidt-Clermont, 2000; Barkan and Small, 2014). During stages of rice leaf development studied here, changes in many growth and development related genes were also observed, including those involved in cell division, the laying down of cell and chloroplast membranes, and cell wall modifications. In addition to confirming that the developmental stages observed here capture a key metabolic and developmental switch in rice leaf development, these data thus have the potential to contribute to the development of a systems model of this switch and to reveal the temporal interactions between key genetic players.

The dataset presented here are also of use for translating knowledge from Arabidopsis thaliana into the crop plant rice. Particularly for the study of transcription factors and other regulatory genes involved in the development of specific structures such as vasculature and stomata, Arabidopsis knowledge is far ahead of our understanding of rice. However, knowledge on when during leaf development certain transcription factors are expressed must be combined with spatial expression pattern investigation using in situ hybridisation and with studies of mutants to pinpoint the exact function of genes of interest. Notably, the lists of genes identified here as potentially involved vascular and photosynthetic development include multiple genes of likely small effect, rather than a small number of master regulators. Although such ‘master regulators’ are perhaps more common in, e.g., stomatal development, where genes such as FAMA, MUTE and SCREAM1/SCREAM2 are necessary and sufficient to induce specific cell state changes in the stomatal differentiation pathway, there are indications that there are unlikely to be master regulators for other aspects of plants development, such as vascular patterning (Kanaoka et al., 2008; Liu et al., 2009). One such argument against the existence of a vascular patterning ‘master regulator’ is the previously mentioned absence of monocot mutants with perturbed vascular patterning but no severe leaf shape defect, despite extensive searches (Smillie et al., 2012; Feldman et al., 2014; Rizal et al., 2015). Another is the long list of transcription factors identified here whose expression coincides with vascular patterning combined with the absence of vascular-specific expression in any of the genes studied through in situ hybridisation. Thus, the robust relationship between vascular patterning and leaf shape in monocots seems likely to be an
emergent property of a network of genes products, some of which may show high redundancy. Although master regulators able to induce chloroplast development have been identified (e.g., GLK1 and GLK2), these are not a master regulator of the spatial regulation of photosynthetic tissue differentiation (Fitter et al., 2002; Waters et al., 2009). This apparent lack of master regulators makes it even more remarkable that the evolution of C₄ photosynthesis, of which one of the first steps is thought to have been a modified leaf layout with high vein density and enlarged, photosynthetic bundle sheath cells, has occurred on as many as 22-24 occasions in grasses (Grass Phylogeny Working, 2012; Sage et al., 2014).

6.5. Concluding remarks and future perspectives
The formation of a planar structure in which light energy can be efficiently harvested and appropriate transport of water, carbon dioxide, oxygen and sugars can occur to enable capture of and export of this energy requires a high degree of coordination. Despite being key to the crop plants on which we most rely, monocot leaf development is understudied compared to dicot leaf development. Several key questions arise from the work in this thesis. In particular, the factors affecting the coordination of the onset of photosynthetic metabolism with the transition from proliferation and patterning to differentiation of morphological features requires further exploration. In addition, the properties of electron transport chain dynamics in developing and mature leaves could be further investigated to yield insights into what makes an efficient electron transport chain, and what factors are required to establish this at an appropriate time during development. The gene expression data in this thesis provide a wealth of information, particularly on the expression of transcription factors correlated with the timing of developmental events. However, improved mutant resources for rice are required for further characterisation of these genes. In the long term, this knowledge may be utilised to improve rice photosynthetic efficiency, either through, for example, the C₄ rice project or through other crop breeding approaches. In either case, an improved understanding of early leaf development is indispensable.
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