Leaf Rubisco turnover variation in a perennial ryegrass (*Lolium perenne* L.) population: Analysis of quantitative trait loci, implications for productivity, and potential for manipulation

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
in
Plant Science

Massey University
Institute of Natural Resources
College of Sciences
Palmerston North, New Zealand

Edith Nanjala Khaembah
2009

Abstract

The Grasslands II perennial ryegrass (*Lolium perenne* L.) mapping population comprising F_1 progeny with the two parents (one plant each from the cultivars Samson and Impact) (Crush et al., 2007) was used to detect putative quantitative trait loci (QTL) for leaf Rubisco turnover and herbage yield traits. Rubisco turnover was described by three mathematical parameters: *d* (maximum Rubisco content), *g* (time of *d*) and *f* (a measure of curve width). All three parameters exhibited continuous variation among the F_1 progeny. Sixteen QTL were detected, seven for Rubisco turnover and nine for herbage yield traits. Support interval overlap on linkage group (LG) 1 and close location on LG2 for plant dry weight (DW) QTL in this study and in a previous analysis (Sartie, 2007) of the same mapping population suggests DW QTL stability across environments. Some QTL identified by Sartie (2007) were not re-confirmed in this study, but new QTL were identified. This suggests genotype x environment interaction generated by variable expression of genes in different environments. Clusters of QTL with overlapping support intervals were found on LG2 and LG5. The cluster on LG2 included QTL for herbage yield traits leaf lamina length (LL), tiller number (TN), productivity index (PI) and DW. The cluster on LG5 included QTL for DW, PI, TN, and *d*. These two regions offer potential for plant breeding applications. Apart from the QTL for *d* on LG5, there was no co-location of Rubisco turnover and herbage yield QTL. However, principal component analysis indicated plants with lower *d* tended to have higher DW; thus Rubisco turnover effects on plant productivity may relate to energy cost of Rubisco synthesis rather than photosynthetic capacity. DW was generally unrelated to *f* and *g;* therefore, hypothesised nitrogen use inefficiencies arising from premature Rubisco degradation, or retention of Rubisco at leaf senescence, were not confirmed. LG5 and LG7 on which QTL for *d* were located have conserved syntenic regions with rice chromosomes 8 and 9 where QTL for Rubisco content at different stages during heading were mapped by Ishimaru et al (2001a).

Acknowledgements

I would like to thank my supervisors Associate Professor Cory Matthew, Dr. Louis John Irving and Dr. Errol Thom for the guidance, support and encouragement they have provided throughout my PhD programme. Your wealth of insights, new ideas and discussion topics at every phase of the programme were an inspiration for me. I thank you for your fruitful feedback during the writing of my thesis.

Thanks to the Massey University Plant Growth Unit staff Steven Ray, Lindsay Silva and Lesley Taylor for the construction of the hydroponic unit and for keeping an eye on the health of the plants. I am indebted to Mark Osborne, Peter Jessop, James Slater, Julia Collins, and Scott Avery for their willingness and commitment to help during planting preparation, the actual planting, and harvesting of experimental samples. I would also like to thank James Mwendwa and Hasinur Rahman for assisting with laboratory analysis.

I would like to thank Dr. Alan Stewart of PGG Wrightson for providing seeds for my first experiment. Thanks to Dr. Syd Easton of AgResearch for providing plants for my QTL analysis and for the useful suggestions during the experiment. Thanks to Mr. Michael Hickey of AgResearch for his help in preparing and taking care of the plants before they were ready for transplanting.

My gratitude also goes to Dr. Siva Ganesh for the training in SAS that I received from his block courses and for attending to my impromptu consultation needs during my data analysis. Thanks to Dr. Marty Faville of AgResearch for his assistance with QTL analysis, and for providing useful comments regarding the analysis. Thanks to Dr. Zhao Xiong He and Ms Denise Stewart for assisting with formatting, and to my colleague, Arif Robin for reading through the thesis.

I thank Dr. Francois Gastal of INRA, Lusignan, for the opportunity to collaborate on the analysis of Rubisco turnover. I greatly enjoyed the experience of working within the stimulating environment of INRA, and appreciate the support from the technical and scientific community. Special thanks to Serge Carre for organising the required laboratory equipment and chemicals for analysis and for helping with data collection and laboratory analysis. I am also thankful to Annie Enprinchard for taking care of the plants and for her assistance with physical data collection.

Finally, I would like to thank the Trevor Ellett Agricultural Research Trust for providing funding for my PhD programme including travel expenses to INRA, France. I am also thankful for funding received during the course of my PhD programme provided by the Helen Akers PhD Scholarship and the John Hodgson Pastoral Science Scholarship.

Dedication

To my parents, brothers and sisters

Table of Contents

CHAPTER 1

CHAPTER 2

CHAPTER 4

CHAPTER 5

CHAPTER 7

List of Tables

List of Figures

List of Appendices

Glossary of Abbreviations

AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
C	Carbon
CAPS	Cleaved amplified polymorphic sequences
CBB	Coomassie Brilliant Blue
CIM	Composite interval mapping
cM	Centimorgan
CO_2	Carbon dioxide
DH	Doubled haploid
DM	Dry matter
DMY	Dry matter yield
DNA	De-oxyribonucleic acid
DW	Dry weight
EST	Expressed sequence tags
FCC	Fluorescent chlorophyll catabolite
Fd	Ferredoxin
GDH	Glutamate dehydrogenase
GISH	Genomic *in situ* hybridization
GOGAT	Glutamate synthetase
GS	Glutamine synthetase
LHCII	Light harvesting complex of photo-system II
LD	Linkage disequilibrium
LDS	Lithium dodecyl-sulphate
LRS	Likelihood ratio statistic
LOD	Logarithm-of-odds
LPSSR	*Lolium perenne* simple sequence repeats
LSU	Large subunit
MAS	Marker assisted selection
MIM	Multiple interval mapping
MQM	Multiple QTL mapping
mRNA	Messenger RNA
N	Nitrogen

NADH	Nicotinamide adenine dinucleotide
NCC	Non-fluorescent chlorophyll catabolite
NH_3	Ammonia
NH_4^+	Ammonium
NIL	Near-isogenic line
NiR	Nitrite reductase
NIRS	Near infra-red spectrometry
NO_3^-	Nitrate
NR	Nitrate reductase
NRE	Nitrogen remobilisation efficiency
NUE	Nitrogen use efficiency
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PS	Photo-system
PVE	Phenotypic variation explained
QTL	Quantitative trait loci/locus
RAPD	Randomly amplified polymorphic DNA
RBC	Rubisco containing bodies
RCBD	Randomised complete block design
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred line
RCC	Red chlorophyll catabolite
RCCR	Red chlorophyll catabolite reductase
ROS	Reactive oxygen species
SAG	Senescence-associated genes
SCAR	Sequence characterised amplified region
SDC	Size/density compensation
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SIM	Simple interval mapping
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
SSU	Small subunit
STS	Sequence tagged site

Ub	Ubiquitin
UV-B	Ultraviolet B
WSC	Water soluble carbohydrate

Trait Abbreviations

Alf	Leaf appearance interval (days)
Alg	Ligule appearance interval (days)
CP	Crude protein content
d	Rubisco peak (mg/leaf, mg/g leaf dry weight, mg/cm^2)
DW	Plant dry weight (g)
f	Rubisco curve width (log of days)
g	Time of Rubisco peak (days)
LED	Leaf elongation duration (days)
LER	Leaf elongation rate (cm/day)
LL	Leaf length (cm)
PI	Productivity index
TW	Tiller weight (mg, g)
TN	Tiller number

Definition of Terms

Allele	An alternative form of a gene (one member of a pair) that is located at a specific position on a specific chromosome.
Epistasis	An interaction between nonallelic genes, especially an interaction in which one gene suppresses the expression of another.
Gene	A hereditary unit consisting of a sequence of DNA that occupies a specific location on a chromosome and determines a particular characteristic in an organism.
Heritability	The proportion of phenotypic variance accounted for by additive genetic effects.
Linkage	The tendency of two or more genes to remain together in the original combination in the same chromosome during the process of inheritance for a number of generations.
Locus	The specific site/place of a gene on a chromosome.
Pleiotropy	The control of more than one phenotypic characteristic by a single gene or set of genes.
Polymorphism	A difference in DNA sequence among individuals, groups, or populations.
Recombination	The process by which new combinations of parental genes or characters arise by independent segregation of unlinked loci or by cross-over between loci that are linked.
Trait	A genetically determined characteristic or condition.

CHAPTER 1

Introduction

1.1. General background

Perennial ryegrass (*Lolium perenne* L.) is a dominant forage grass species in the temperate regions of the world. Its popularity in temperate pastoral agriculture is due to its superior agronomic characteristics which include rapid establishment and growth (Daepp et al., 2001), high herbage yields and adequate seed production (Tas, 2006; Wilkins, 1991), excellent forage quality and good tolerance to grazing (Wilkins, 1991). Perennial ryegrass has a high nitrogen (N) requirement (Daepp et al., 2001) and therefore use of N fertiliser is considered an essential management tool for perennial ryegrass-based intensive systems. N fertiliser use allows high levels of dry matter (DM) production, sustenance of high stocking rates and high profit margins (Jarvis, 1996; McGrath et al., 1998; Monaghan et al., 2005). However, dependence on fertiliser N input to maximize yield potential is unsustainable. The production of N fertilisers by Haber-Bosch process is energy intensive, uses finite fossil energy and leads to large emissions of greenhouse gases (Vitousek et al., 1997). Also, extensive use of fertiliser N has increased leaching of nitrate-N that has caused pollution of ground and surface waters (Beman et al., 2005; Giles, 2005; Heisler et al., 2008). Many studies have confirmed that the amount of leached nitrate-N increases with an increase in fertiliser N application (Ledgard et al., 1998; Scholefield et al., 1993; Watson et al., 1992). These environmental and economical constraints suggest that modern agriculture can only marginally depend on N fertiliser to provide yield increases required to keep pace with the dietary demand of the increasing human population. Several mitigation options exist, including grass breeding to develop cultivars that utilise N more efficiently as a sustainable solution to soil N deficiency.

Methods developed to define and estimate nitrogen use efficiency (NUE) differ in a few basic ways, but most research has focused on the efficiency of extracting N from the soil; the efficiency with which applied N is used to produce grain in cereals (Good et al., 2004) or forage DM in grasses (Zemenchik and Albrecht, 2002). There have been developments in these methods to include genetic manipulation of plant characteristics linked to N utilisation (e.g. Andrews et al., 2004). As more and more studies have demonstrated the importance of remobilised N for new tissue growth in cereals (Good et al., 2004; Kichey et al., 2007; Mae and Ohira, 1981; Mickelson et al., 2003; Yoneyama and Sano, 1978) and forage crops (Kim et al., 1991; Lattanzi et al., 2005; Louahlia et al., 2000), there has been growing interest in understanding the processes involved in N recycling and how they relate to NUE in the plant. Remobilisation of N from vegetative parts allows the plant to re-use this essential nutrient and is therefore an exceedingly important process for the N economy of the plant. Improving the efficiency of N remobilisation would reduce the plant's N demand from uptake, and in turn reduce the amount of fertiliser N application.

Chloroplastic proteins are the predominant source of N recycled during leaf senescence in C_3 species (Feller and Fischer, 1994; Peoples and Dalling, 1988). The stromal enzyme Rubisco (E.C.4.1.1.39) represents the largest fraction of chloroplastic N, and is potentially an N storage protein (Friedrich and Huffaker, 1980; Mae et al., 1983; Millard, 1988) in addition to its catalytic function in photosynthesis. Rubisco turnover is therefore related to the carbon (C) and N economy of the plant. Despite this unique characteristic, there are a limited number of studies focussed on understanding Rubisco turnover and its effect on plant performance. Since Rubisco represents a large fraction of the plant N budget, it is hypothesized that understanding the regulation of Rubisco turnover would help us understand the regulation of N transition from assimilation to remobilisation. This knowledge is important if genetic manipulation of this process is to improve N remobilisation efficiency, improve plant N economy, and reduce the excessive input of inorganic N fertilisers without reducing crop yield and quality.

To understand how knowledge of Rubisco turnover might be used for agronomic advantage, there is need to study genetic variation in patterns of Rubisco synthesis and degradation and to determine the association with other plant yield characteristics. Previously, attempts to evaluate the genetic basis of plant traits (most of which are complex in nature) were restricted to simple genetic models. At present, the use of quantitative genetic studies associated with molecular markers is a powerful tool because it (1) enables identification specific quantitative trait loci (QTL) on the genome, and (2) reveals the nature of genetic relationships between different traits. With this new strategy, there is great potential for breeders to carry out marker assisted selection (MAS) for improvement of NUE and yield.

1.2. Summary of objectives

The genetic material used in this study is the Grasslands II mapping population developed by AgResearch to establish MAS capability for ryegrass breeding in New Zealand. The primary objective of the work reported here was to characterize regions of the genome that have basic control over Rubisco turnover to give insight into its regulation and its potential usefulness as an indicator of genotype differences between perennial ryegrass plants in the a physiological process of N cycling (i.e. remobilisation from senescent organs and redistribution to growth zones) that might be linked to differences in plant performance. The specific objectives were to:

1. Quantify change over time of Rubisco concentration in the leaves of the mapping population plants to identify variation in Rubisco turnover characteristics.

2. Study in parallel, Rubisco turnover and agronomic traits, and identify relationships between specific Rubisco turnover patterns and plant morphological characteristics.

3. Simultaneously detect QTL for Rubisco turnover and QTL for morphological characteristics to explore the genetic relationships between these traits.

4. With the knowledge above, assess the potential of Rubisco turnover data as a diagnostic tool for predicting inefficiencies in plant NUE and in N remobilisation from old tissue and redistribution to growth zones.

1.3. Thesis structure

The thesis is comprised of seven chapters. Chapter 1 gives the general introduction, the background of the project and defines the research question and objectives to be addressed. Chapter 2 reviews literature relevant to this research project. Chapter 3 examines Rubisco turnover in the leaves of barley plants growing under two N nutrition levels, primarily to establish a methodology for subsequent studies in perennial ryegrass. Chapter 4 assesses a sub-sample of the Grasslands II perennial ryegrass mapping population (16 selected genotypes and the two parental lines) for genotypic variation in Rubisco turnover. Chapter 5 assesses Rubisco turnover and physical data in 137 genotypes (135 full-sibs of the Grasslands II perennial ryegrass mapping population and their two parent plants) for QTL discovery. Chapter 6 assesses a link between Rubisco turnover and plant morphogenetic characteristics in long-leaved and short-leaved perennial ryegrass breeding populations developed by the French National Institute for Agricultural Research (INRA). Chapter 7 provides the general discussion, conclusions, and recommendations from the research.

CHAPTER 2

Literature review

2.1. Grassland farming and forage crop improvement

Grasslands occupy a very large portion of the earth's surface, and are the predominant form of land use (Hopkins and Wilkins, 2006; White et al., 2000). Grassland denotes all plant communities on which animals are fed, with the exception of crops sown annually (e.g. wheat, corn) that may also be used as forage (Barnes and Nelson, 2003). Grassland agriculture as a farming system emphasises the importance of forages (grasses and/or legumes) as a feed base for ruminant livestock and in land management. In the temperate regions of the world, grassland feeds provide more than 50% of the energy and protein consumed by ruminant livestock, with some systems totally reliant on grassland (Hopkins and Wilkins, 2006; Wilkins and Humphreys, 2003). These grasslands are also responsible for the production of 80% of the world's cow milk and 70% of the world's beef and veal and meat (Wilkins and Humphreys, 2003).

In addition to being the major source of feed for livestock, grasslands also contribute to complementary environmental objectives. These include; soil erosion control, improvement of soil structure, water conservation and protection, *in situ* conservation of plant genetic resources, environmental protection, habitation for wildlife, outdoor recreation and pleasure, forage biomass for conversion to energy as a renewable resource, and potential feedstock for manufacturing products (Barnes and Nelson, 2003; Humphreys, 2005; Sohl, 2005). There has been increasing recognition of the need to approach grasslands from the viewpoint of multi-functionality especially in the later part of the 20th century (Hervieu, 2002; Hopkins and Holz, 2005; Hopkins and Wilkins, 2006). The changing considerations concerning the use and management of grasslands have been covered in a recent review (Kemp and Michalk, 2007).

Significant changes have taken place in temperate grasslands over the last century as a result of man's intervention through research and the development of new technologies for plant and animal sciences (Hopkins and Wilkins, 2006; Wilkins and Humphreys, 2003). For much of the century, understanding and improving the production potential of grassland has been of primary concern to grassland scientists and practitioners

(Hopkins and Holz, 2005). Increase in grassland productivity and nutritive value that have occurred over the last half of the century owe a great deal to successful plant breeding (Frame et al., 1995).

Breeding of forages began more recently than that of some major arable crops. Dedicated breeding to improve landraces commenced early in the 20th century (Humphreys, 2005). In the UK, systematic forage grass breeding began in 1919 with production of the first *L. perenne* variety S-23 in 1931(Humphreys et al., 2006). In New Zealand, pasture breeding began in 1927 (Rumball, 1983). Many notable advances have been made in forage cultivar development equivalent to those achieved in many other crops (Hayward and Fageria, 2001; Humphreys, 2005; Wilkins and Humphreys, 2003). There have been improvements made in most species in forage yield (Barnes et al., 1988; Casler et al., 1996; Wilkins and Humphreys, 2003), persistence (Boschma et al., 2008; Brummer and Moore, 2000), insect and disease resistance (Barnes et al., 1988; Casler et al., 1996; Wilkins and Humphreys, 2003) and quality (Casler, 2001; Wilkins and Lovatt, 2004).

Much of the breeding work before the 1980s aimed at increasing animal production per unit area, and therefore dry matter yield and persistence were the main breeding targets (Wilkins and Humphreys, 2003). Use of improved forage cultivars also involved a move to higher inputs/outputs that relied on increased chemical usage (fertilisers and herbicides) and high stocking rates. Energy input into grassland, particularly fertiliser nitrogen (N), resulted in increased herbage production (Sodin, 1991). Intensive grassland livestock systems also resulted in overproduction, excessive costs, pollution of soil, water and the atmosphere, reduced botanical diversity, and overall landscape quality (Nösberger and Staszewski, 2002).

The fate of applied nutrients was given little attention until after the work of Ryden and his team (Ryden et al., 1984) which showed higher N leaching losses from grazed swards than cut swards receiving the same fertiliser input (Hopkins and Wilkins, 2006). Other studies conducted in the Netherlands highlighted sward deterioration effects and subsequent herbage yield loss associated with very high N inputs (Prins, 1984). Later, other studies especially targeting nitrate-N leaching from fertilised grasslands presented evidence that nitrate leaching from sites with similar soils and environmental conditions increased with increasing fertiliser-N applied (Jemison and Fox, 1994; Scholefield et al., 1993; Watson et al., 1992). Increasing awareness of the economic and environmental consequences of N loss led to broadening of breeding objectives to accommodate those that safeguard environmental integrity.

Human population growth which has been the main driver of increased production in the past is continuing and the world population is projected to reach about 10 billion by 2050 (Cakmak, 2002). The resultant increase in demand for animal protein means that greater output of milk and meat is continuously required from forage-based animal production systems (Hayward and Fageria, 2001; Kasha, 1999; Rae, 1999). Increasing concern by consumers about food quality, including food safety and animal welfare, especially in the developed world (Wright, 2005) will also put pressure on these systems to increase production. With the apparent environmental damage associated with the fertiliser N use, forage-based grassland agriculture is faced with the challenge to reduce excessive input of fertilisers and improve the quality of forages without affecting yield. Use of efficient farming techniques is one way to meet this challenge. Another approach is to lower crop demand for N by breeding for increased N use efficiency (NUE). NUE is a quantitative trait, and its improvement by conventional methods is a complex objective given that genetic improvement of forage species in itself presents unique challenges.

2.2. Conventional forage breeding methods and challenges

The majority of perennial forage species are allogamous (Lübberstedt, 2002), which determines the breeding methods employed for their improvement. Most gains in grass breeding have been achieved by accumulating desirable genes through sexual recombination and selection (Humphreys et al., 2006). All the major perennial forage grasses are out-breeders and therefore population improvement based on recurrent (mainly phenotypic) selection of individual spaced plants or half-sib/full-sib families is the main method of improvement (Connolly, 2001; Humphreys et al., 2006; Wilkins and Humphreys, 2003). Selection often takes multiple cycles of breeding to combine desirable agronomic or quality characteristics from different sources into a crop variety. For example, it took four generations of combined phenotypic and full-sib family selection within a perennial ryegrass population over 12 years to improve dry matter yield (DMY) and water soluble carbohydrate (WSC) of diploid perennial ryegrass (Wilkins and Humphreys, 2003). In addition to the high number of cycles required to produce a variety, another limitation of recurrent family selection is the large number of families required for evaluation at each generation which in itself is a major cost and a logistical challenge.

Both perennial grasses and cereals belong to the family Poaceae, but there are difficulties facing breeders of perennial forage grasses that are not encountered by breeders of cereal crops. The perennial nature of these grasses means that improvement strategies need to balance increases in usable production with maintenance of persistency (Pollock et al., 2005). Perennials must sufficiently maintain their performance into the second and subsequent harvest years, meaning each generation of selection for all-round performance takes at least 3 years compared with only 1 year with cereal crops (Wilkins and Humphreys, 2003). Also, as reviewed by these authors, perennial grasses are designed primarily for forage use, and it is difficult to obtain high yields of seed making it rather expensive. This has precluded the use of male sterile lines to produce F_1 hybrid seed, which has been pivotal in improving the yield of maize forage and grain. Due to these complexities, progress toward the generation of improved varieties in perennial ryegrass has been slow.

It is worth noting that besides recurrent selection, other methods or techniques have been instrumental in the improvement of forage grasses. Tetraploidy which involves chromosome doubling of parents by colchicine treatment, has been widely used to create tetraploid *Lolium* and *Festulolium* varieties, and to facilitate introgression from *Festuca* species into *Lolium* (Humphreys et al., 2006). In addition, technology has also played a vital role in enabling progress in forage grass breeding (Wilkins and Humphreys, 2003). As indicate by these authors, direct selection for plot yield and persistence facilitated by the development of plot harvesters as well as electronic field data collection and immediate analysis of the results (possible through the development in computing technology) have helped breeders reduce the time required for each generation of selection. Increased computing power also has enabled the application of near infra-red spectroscopy (NIRS) to the analysis of herbage samples simultaneously for several different components of nutritional value both quickly and accurately

(Bluett, 1999; Brown et al., 1990). Another development in forage grass improvement has been the multi-disciplinary approach: In contrast to early plant breeding, modern programmes are supported by teams comprising plant physiologists, and plant/animal nutritionists, thus extending the range of selection criteria (Frame et al., 1995).

2.3. DNA technology in forage grass breeding

Conventional breeding methods have been applied successfully in the past and will still have a major role to play in future forage crop improvement programs. Since conventional breeding involves crossing whole genomes followed by selection for superior recombinants from the segregation products, the procedure is both laborious and time consuming (Kumar, 1999). The entry of crop production into the genome era means there is promise for more targeted development of forage cultivars and faster achievement of breeding objectives. The major development in plant biotechnology has been the development of DNA (molecular) markers. Molecular markers are segments of DNA that breeders use to detect the presence or absence in experimental plants of specific alleles of interest and thus use them as selection tools (Beckmann and Soller, 1983; Darvasi and Soller, 1994). The association of easily screened DNA markers with traits of interest provides opportunities to supplement, and maybe to reduce the reliance on long term field selection trials by indirectly selecting for the marker. Before the discovery of DNA markers, morphological and isozyme genetic markers were important tools in genetic studies and plant breeding (Kumar, 1999). The major disadvantage of morphological and biochemical markers are that they are limited in number and are influenced by environmental factors or the developmental stage of the plant (Winter and Kahl, 1995). In contrast, DNA-based markers are virtually unlimited in number allowing dense coverage of whole genomes, are easy to apply using any kind of tissue, and have phenotypic stability (Lübberstedt, 2002; Winter and Kahl, 1995). With the advent of DNA marker technology, several types of DNA markers and molecular breeding strategies are now available to plant breeders and geneticists, helping them to overcome many of the problems faced during conventional breeding (Kumar, 1999).

Molecular marker systems fall into two categories a) hybridisation-based or polymerase chain reaction (PCR)-based) depending on how polymorphism is revealed (Collard et al., 2005). The hybridisation-based procedure involves the fragmentation of the genomic DNA by restriction enzymes that recognize specific DNA sequences/motifs (~4 -10 bp in length). The restricted fragments are separated by gel electrophoresis and transferred onto a membrane by Southern blotting (Southern, 1979). Hybridisation of the membrane to a labelled DNA probe determines the size of the fragments that are complementary to the probes. Sequence changes in the restriction sites, or insertions/deletions in the restriction fragments give rise to polymorphism. PCR procedure (e.g. for Random Amplification of Polymorphic DNA (RAPD)) involves use of primers (~10 nucleotides) to amplify random locations across the genome (Williams et al., 1990). Annealing at a number of locations in the genome is possible because of the short length of the primers. The number of PCR products correlates with the number and orientation of the sequences that are complementary to the primer in the genome. Molecular markers can exhibit two modes of inheritance, i.e. dominance/recessive or co-dominance. Co-dominant markers can clearly discriminate between homozygotes and heterozygotes whereas dominant markers do not (Collard et al., 2005).

2.3.1. Molecular marker systems in perennial ryegrass

Several molecular marker systems have been developed for studies of perennial ryegrass and other related species. They include Restriction Fragment Length Polymorphisms, RFLP (Hayward et al., 1994), Randomly Amplified Polymorphisms DNA, RAPDs (Stammers et al., 1995), Amplified Fragment Length Polymorphisms, AFLPs (Bert et al., 1999), Simple Sequence Repeats, SSRs (also known as microsatellites) (Jones et al., 2001). Each system has specific advantages and disadvantages. The decision to use a particular system depends on a wide range of factors including cost of development, breeding objectives, transferability within varieties of the same species or between species, ease of use, and whether they are dominant or co-dominant (Humphreys et al., 2006; King et al., 2008). RFLPs are hybridisation-based and are good anchor probes for map alignment and are co-dominant, but require large amounts of DNA and are time-consuming to use (Humphreys et al., 2006). As indicated by these authors, both RAPDs and AFLPs are PCR-based, quick, and easy to use and only require small amounts of DNA, but they are dominant and have poor transferability across mapping families. Microsatellites and tagged sites such as sequence tagged sites (STSs), expressed sequence tags (ESTs) and sequence characterised amplified regions (SCARs) are high-throughput PCR-based markers which may be associated with known functions. They require small amounts of DNA and are usually co-dominant. Single nucleotide polymorphisms (SNPs) are allele specific, and their wide genome coverage and assaying methods make them markers of choice (Doveri et al., 2008; Humphreys et al., 2006). Microsatellites have also become one of the most widely used molecular marker systems in plant genetics and breeding because they are highly polymorphic, co-dominant, ubiquitous in eukaryotes and prokaryotes, and can be found in coding and non-coding regions of the genome (Asp et al., 2007; Powell et al., 1996; Varshney et al., 2005).

2.3.2. Application of DNA marker systems

2.3.2.1. Tagging economically important traits

DNA markers offer the opportunity to locate and track genes of economic importance in a genome, thereby aiding selection. Traits of agronomic importance such as dry matter yield, forage quality, NUE and environmental stress tolerance are quantitative in nature (Yamada and Forster, 2005). Quantitative traits are governed by two or more genes and are also affected by the environment. These traits cannot be determined by normal phenotypic measurements made on individuals (Humphreys, 2001; Kumar, 1999; Mauricio, 2001). The regions within a genome containing these genes that control quantitative traits are referred to as quantitative trait loci (QTL) (Collard et al., 2005). QTL are identified by genetic mapping using DNA markers (described in Section 2.4). Prior to the advent of DNA marker technology, the techniques used to estimate the minimum number of QTL affecting a trait and the average gene action based their description on the average properties of a group of QTL (Paterson et al., 1991; Tanksley, 1993). With the aid of DNA markers, it is possible not only to assign chromosomal positions to individual QTL, but also to determine (1) the types and magnitude of gene effects of individual QTL, and (2) the parent that possesses the positive allele at each QTL (Kumar, 1999; Lander and Botstein, 1989; Paterson, 1995).

DNA markers and dense marker-based genetic maps developed for *L. perenne* (Armstead et al., 2002, 2004; Faville et al., 2004; Jones et al., 2002a, b) have been used in the analysis of the p150/112 reference population and other mapping populations to tag QTL for economically important traits in perennial ryegrass. There are numerous reports of DNA markers linked to QTL for a range of important economic traits in

L. perenne. QTL have been reported for resistance to crown rust (Dumsday et al., 2003; Muylle et al., 2005; Schejbel et al., 2007), resistance to powdery mildew (Schejbel et al., 2008), flowering time (Armstead et al., 2004; Jensen et al., 2005; Yamada et al., 2004), digestibility (Cogan et al., 2005), water soluble carbohydrates (Humphreys and Turner, 2001), herbage and seed production (Sartie, 2007), and components of NUE (Dolstra et al., 2007). Although research associated with the identification of QTL is expanding, examples of the application of marker assisted selection (MAS) methods in forage breeding programs are limited (Kölliker et al., 2005; Pollock et al., 2005).

2.3.2.2. Marker assisted selection of quantitative traits

When DNA markers that reliably predict a trait phenotype have been identified, they may be used for MAS (Collard and Mackill, 2008; Hospital, 2003). Among the emerging genetic biotechnologies, MAS is the most likely to have an immediate impact on plant breeding (Pollock et al., 2005). The application of MAS is designed to improve the efficiency of conventional breeding (Williams et al., 2007). Indirect selection based on the marker would increase the efficiency in breeding programs in several ways; (a) segregants can be selected at seedling stage for traits that are expressed late in plant development, (b) it is possible to screen, more economically and precisely, traits that are extremely difficult, expensive or time consuming to measure, (c) several traits can be selected simultaneously, (d) heterozygotes and homozygotes can be identified without resorting to progeny evaluation and (e) it is possible to select traits that are controlled by recessive alleles (Humphreys et al., 2006; Varshney et al., 2004; Williams et al., 2007). Most traits manipulated in modern grass breeding programmes belong to one or more of these categories.

2.3.2.3. Marker assisted gene introgression

Another application for molecular markers in forage breeding is to enable introgression of valuable genes controlling quantitative traits either into advanced populations or directly into synthetic varieties (Wilkins and Humphreys, 2003). The out-breeding nature of grasses means that highly heterogeneous ecotypes are available to provide a vast array of genetic variation, including adaptations to most climatic and edaphic conditions (Humphreys et al., 2006). Gene introgression from species of related grasses means that this range of genetic variation can be dissected and manipulated (Humphreys et al., 2006). The main targets for introgression have been the introduction of biotic and abiotic tolerance from *Festuca* species into *Lolium* species. High levels of recombination occur between homologous chromosomes in the *Festuca-Lolium* complex making it unique among crop species (Humphreys, 2005). Also, with the aid of DNA markers and genomic *in situ* hybridization (GISH), it is possible to distinguish between *Lolium* and *Festuca* chromosomes (King et al., 2002). Using introgression techniques, chromosome segments have been transferred from *F. pratensis* into *L. perenne* to improve winter hardiness, crown rust resistance and retention of green leaf colour (Humphreys, 2005).

2.3.2.4. DNA profiling/DNA finger printing

DNA profiling involves the use of marker loci to evaluate genetic diversity within and between natural and synthetic populations (Forster et al., 2001; Kölliker et al., 1999). Population and synthetic breeding are prevalent in the improvement of out-breeding forage grass species. In general, the scheme involves three steps; (i) generation of genetic variability, (ii) selection of parental genotypes, and (iii) testing of experimental populations or cultivars (Lübberstedt, 2002). Genetic diversity among parental genotypes is critical as it may substantially influence the success of a population or synthetic cultivar through mechanisms such as heterosis, general combining ability, inbreeding depression and self-incompatibility (Kölliker et al., 2005). In the development of parental genotypes for synthetics, for example, markers can be applied to help select genetically divergent parents and/or genes that increase general combining ability for desired traits. Selection at genome level will dramatically accelerate the evaluation of the experimental populations. Successful use of molecular markers to analyse genetic variability within and among perennial ryegrass cultivars and accessions has been reported in a number of studies (Barcaccia et al., 1998; Guthridge et al., 2001; Huff, 1997; Kölliker et al., 1999, 2005). Other applications of DNA profiling include; certification of seed batch purity (e.g. Yashitola et al., 2002), monitoring the genetic stability of clonally propagated species evaluation of the dynamics of population structure in natural and managed pastures, and underpinning plant variety rights (e.g. Forster et al., 2001).

2.3.2.5. Construction of genetic linkage maps

Molecular markers provide the opportunity to quickly develop very detailed genetic linkage maps for diverse crop species. During reproduction, genes (or markers) are often transmitted together from the parent to the offspring. Sometimes, however, groups of genes originally located on one chromosome may be separated during cross-over. The farther apart the genes are on a chromosome, the greater the chances they will be separated. By studying how often two genes are transmitted together, scientists can estimate how close they are on the chromosome and create a linkage map.

The construction of a linkage map requires a segregating population (Collard et al., 2005). The goal of the mapping project (for example, identification and orientation of DNA markers near a target gene for map-based cloning, QTL mapping or monitoring of disease resistance loci for pyramiding) will influence the choice of parents for crossing, the size of the mapping population, how the cross is advanced, and which generations are used for DNA and phenotypic measurements (Jones et al., 1997; Young, 1994).

Sufficient DNA marker polymorphism between parents is critical for linkage map construction. Out-breeding species have a higher level of DNA polymorphism than in inbreeding species; for adequate polymorphism, mapping in inbreeding species generally requires the selection of parents on the basis the level of genetic diversity (Collard et al., 2005; Humphreys et al., 2006). In some cases use of new technologies such as electrophoresis systems capable of separating DNA molecules with only a single base pair, probes based on minisatellites, or simple tetranucleotide motifs, can be used to uncover polymorphism within narrow-based crosses (reviewed by Young, 1994).

After suitable parents have been identified, the next step is to consider the type of genetic population for linkage mapping. Populations such as F_2 populations, F_2 derived F_3 populations and backcrosses are often used, but populations that can be maintained and produced permanently, such as doubled haploids (DHs) and recombinant inbred lines (RILs) are preferred because they allow replicated and repeated experiments (Collard et al., 2005). In self-pollinating species, mapping populations originate from highly homozygous (inbred) parents. Most out-breeding species, however, are inbreeding-intolerant, and many are polyploid. Mapping populations for out-breeding plant species may be derived by pair-crossing heterozygous parents (e.g. Forster et al., 2001) or crossing a heterozygous parent and a haploid or homozygous parent (e.g. Jones et al., 2002b; Wu et al., 1992).

The resolution of a map and the ability to determine marker order depends on the number of individuals evaluated; hence the importance of the size of the mapping population. The larger the mapping population the better, but this may be limited by factors such as the number of seeds available or the number of DNA samples that can be reasonably prepared (e.g. Young, 1994). Populations used for genetic studies range from 50 to 250 individuals (reviewed by Collard et al., 2005). If the goal is high resolution mapping in specific genomic regions or QTL mapping of minor effect, larger populations are required (Darvasi and Soller, 1995; Young, 1994). Mapping populations consisting of at least 1000 individuals have been used for high resolution mapping (e.g. Messeguer et al., 1991) although there is no universal number for the appropriate population size (Collard et al., 2005).

The next step involves using DNA markers that reveal differences between parents (i.e. polymorphic markers) to genotype the entire mapping population, including parents. Therefore, DNA must be extracted from each individual of the mapping population when DNA markers are used (Collard et al., 2005; Young, 1994). In a given population type (e.g. F_2, backcross, RILs or DHs), markers will segregate in a Mendelian manner although segregation distortion may occur (Sayed et al., 2002; Xu et al., 1997). Testing of the segregation pattern is done by pair-wise chi-square (X^2) tests of independent assortment (e.g. Hoi-Shan and Hai-Lou, 2002).

The final step of the construction of a linkage map involves coding data for each DNA marker on each individual of a mapping population and conducting linkage analysis using computer software (Collard et al., 2005). Genetic mapping computer software used for linkage analysis include Mapmaker/EXP (Lincoln et al., 1993), MapManager QXT (Manly et al., 2001) and JoinMap (Stam, 1993). Linkage between markers is usually calculated using the logarithm of odds (LOD) score, which defines the ratio of linkage versus no linkage (Collard et al., 2005; Risch, 1992). A LOD score of 3 between markers, for example, indicates that linkage is 1000 times more likely (1000:1) than no linkage. LOD scores of >3 are typically used to construct linkage maps although the score may be lowered to detect a greater level of linkage or to place additional markers within maps constructed at higher LOD values (Collard et al., 2005). Linked markers are placed in groups and then ordered within those groups. The criteria used for linkage grouping are usually recombination fraction, significance level of the recombination fraction and the known genome information. If many markers are used, a relatively high genome coverage is achieved, the data are highly informative, the genetic model for data analysis is adequate, then the number of linkage groups should be close to the haploid number of chromosomes for the organism (Liu, 1998). The distance between marker loci or genes is measured in centimorgan (cM) (Haldane, 1919; Kosambi, 1943), which is based on recombination frequency (1 cM = 1% recombination).

Linkage analysis, and complete genetic linkage maps consisting of co-dominant DNA markers was a major development in resolving the conflict between the Mendelian theory of particulate inheritance and the observation in the early 20th century that most traits exhibit continuous variation (Lander and Botstein, 1989; Liu, 1998). Although pioneering work showed that linkage could occasionally be detected for QTL, accurate and systematic mapping was not possible because the inheritance of an entire genome could not be studied with genetic markers (Paterson et al., 1988). Such studies became possible with the advent of RFLPs as genetic markers and the increasing availability of RFLP maps in many organisms (Botstein et al., 1980; Lander and Botstein, 1989). Many linkage maps based on other marker systems (e.g. SSRs, RAPDs, AFLPs) have been developed over years and are useful for genetic dissection of quantitative traits, MAS, gene isolation and cloning (Jones et al., 1997).

In general, genome mapping of forages has lagged behind that of major cereal crops, but good progress has been made in the production of a reference genetic map for ryegrass. A molecular-marker linkage map of perennial ryegrass ($2n=2x=14$) was developed by the International *Lolium* Genome Initiative (ILGI), using the p150/112 one-way pseudo-testcross population (Jones et al., 2002a; Jones et al., 2002b). It comprises 240 loci covering 811 cM on seven linkage groups (Pollock et al., 2005). The map was constructed using predominantly heterologous RFLP anchor probes derived from wheat, barley, oat and rice, and also AFLP markers (Jones et al., 2002b). There is a general agreement between the maps of perennial ryegrass, meadow fescue and *Triticeae* cereals (Jones et al., 2002b; Pollock et al., 2005). This relationship has allowed the inference of comparative relationships between perennial ryegrass and other *Poaceae* species, defining regions of conserved synteny useful in predicting the location of genes for comparable agronomic or physiological traits. The linkage map has been further developed through the mapping of a set of perennial ryegrass SSR (LPSSR) loci (Jones et al., 2002a).

Other genetic mapping populations of perennial ryegrass have been independently developed as successors to the p150/112 population, and have been aligned to the reference map using common markers (Armstead et al., 2002; 2004; Faville et al., 2004; Gill et al., 2006). These genetic maps contain functionally-associated molecular marker information through the inclusion of gene-associated cleaved amplified polymorphic sequences (CAPS) markers, and both RFLPs and SSRs markers from ESTs (Yamada and Forster, 2005). The development of genomic and EST libraries (Jones et al., 2001; Sawbridge et al., 2003) in perennial ryegrass, and their recent enhancement (King et al., 2008; Studer et al., 2008) has contributed to the dramatic increase in genomic information in this and related species.

2.4. Quantitative trait locus analysis

QTL analysis (also known as QTL mapping) is a procedure of identifying a region of a genome that is responsible for variation in the quantitative trait of interest. Appropriate mapping families, suitable marker maps, reliable measures of the phenotype, and appropriate statistical packages to analyse the genotypic information in combination with phenotypic information, are necessary for QTL analysis (Turner et al., 2006; Zeng et al., 2008).

QTL mapping is based on the co-segregation of different but linked genes, which is reflected in the co-transmission of genes from a parental to progeny population (Zeng et al., 2008). In a given mapping population, the idea is to detect an association between phenotype and genotype markers (Collard et al., 2005). Using markers, the mapping population is first partitioned into different genotypic groups based on the presence or absence of a particular marker locus (Tanksley, 1993; Young, 1996). Depending on the marker system and type of population, a significant difference between phenotypic means of the genotypic groups indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait (Collard et al., 2005). Generally, the chances of recombination are lower when the QTL and the marker are close together, and therefore the two will be inherited together in the progeny. The mean of the group with the tightly-linked marker will be significantly different to the mean of the group without the marker (Collard et al., 2005). Conversely, when a marker is loosely-linked or un-linked to a QTL, there is independent segregation of the marker and QTL, and the difference between means of the genotype groups based on the loosely/unlinked marker will not be significant (Collard et al., 2005).

2.4.1. Methods for QTL detection

A variety of methods are available for detecting QTL (Michelmore et al., 1991; Tanksley, 1993). Single-marker analysis is the simplest method, and is based on a simple t-test, ANOVA, or linear regression. This method does not require a complete linkage map and can be performed with basic statistical software programs. The major limitation of this method is that the further a QTL is from a marker the less likely it will be detected (Collard et al., 2005; Tanksley, 1993). Simple interval mapping (SIM) (Lander and Botstein, 1989) requires a linkage map and simultaneously analyses intervals between adjacent marker loci. Thus, it is possible to detect QTL in each interval lying between any two flanking markers that individually may show no association with the trait (Gupta, 2002). Composite interval mapping (CIM) combines interval mapping with linear regression using marker cofactors, which increases the power of QTL detection and allows simultaneous study of more than one QTL in the genome (Collard et al., 2005; Soler et al., 2006; Zeng, 1994). The main advantage of CIM is that it is more precise and effective at mapping QTL compared to single-marker analysis and SIM. Multiple-QTL mapping (MQM) is a type of CIM specific to MapQTL software (Van Ooijen and Maliepaard, 1996). Multiple interval mapping (MIM) uses multiple marker intervals simultaneously to fit multiple putative QTL directly in the model of QTL mapping (Kao et al., 1999). Compared to SIM and CIM, MIM tends to be more powerful and precise in detecting QTL. In addition, MIM can readily search for and analyse epistatic QTL and estimate the individual value and heritability of quantitative traits (Kao et al., 1999).

The most likely position of a QTL in relation to the linkage map can be determined by using LOD (see Section 2.3.2.5) or likelihood ratio statistic (LRS) (Collard et al., 2005). There is a direct one-one transformation between LOD and LRS scores calculated as: LRS = 4.6 x LOD (Collard et al., 2005; Liu, 1998). The most likely position for a QTL in relation to the linkage map is where the highest LOD score is obtained. However, the peak must exceed a specified level for it to be declared as statistically significant or real. Significance threshold is commonly done by permutation testing (Churchill and Doerge, 1994). As reviewed by Collard et al (2005), this procedure involves shuffling phenotypic values while keeping marker genotypic values constant (i.e. breaking all marker-trait associations). QTL analysis is then carried out to evaluate the level of false positive marker-trait associations. The process is repeated several times (e.g. 1000 times) and the significance level is then determined by the level of false positives. Before permutation tests were accepted as an appropriate method for determination of significant thresholds, a LOD score of 2.0 – 3.0 was usually chosen as the significance threshold. QTL positions are defined by the map position of the peak LOD score, and confidence interval (approximately 95%) is commonly defined by the peak ± 2 LOD (Van Ooijen, 1992).

There are several computer software packages that may be used for QTL analysis. These include MapQTL (Van Ooijen and Maliepaard, 1996; Van Ooijen et al., 2002), GMendel (Liu and Knapp, 1990), Mapmaker (Lincoln et al., 1993), Mapmanager (Manly et al., 2001), QGene (Nelson, 1997), PLABQTL (Haley and Knott, 1992), SAS (Knapp and Bridges, 1990), and Joinmap (Stam, 1993).

2.4.2. Experimental design factors

The power, precision, and accuracy of QTL results can be affected by experimental factors such as number of progeny, type of progeny, or precision of phenotypic measurement (Beavis, 1998; Mackay, 2001). The number of individuals and the nature of the genetic cross govern the sample of meoitic cross-over sites between markers and QTL (Xu et al., 2005). Simulation and experimental studies have indicated that the power of QTL detection is low with the commonly used population sizes of <200 progeny (Beavis, 1998; Kearsey and Farquhar, 1998; Young, 1999). Use of small population sizes may result in large confidence intervals for regions containing QTL, even for QTL with large effects (Collard and Mackill, 2008). In addition, sampling bias can lead to a large bias in estimates of QTL effects, especially in relatively small populations (Collard and Mackill, 2008; Melchinger et al., 1998). It has also been found that the type of progeny developed in the experiment will affect the power to identify QTL using mathematical transformation methods. DH are the most powerful at estimating additive effects, while backcross progeny are the least powerful (Beavis, 1998). Experimental error arising from errors in marker genotyping and inaccurate phenotypic evaluation may also affect QTL detection. For instance, genotyping errors and missing data can affect the order and distance between markers within linkage maps (Hackett, 2002). Also, construction of a reliable QTL map will depend on the reliability of the phenotypic data used. The expression of quantitative traits is also subject to environmental effects. It is therefore important that phenotypic measurements are replicated (e.g. across locations, years or seasons) to evaluate QTL x environment effects. The use of replicated progeny in mathematical transformation evaluations also have been shown to increase the power of QTL detection and precision of estimated genetic effects (Beavis, 1998). Experimental design factors have important implications for MAS, since the success of MAS depends on the accuracy of the QTL mapping (Collard and Mackill, 2008).

2.4.3. QTL validation

As described above (Section 2.4.2), QTL detection is influenced by a number of factors and therefore before being used for MAS, a QTL needs to be independently verified (e.g. Langridge et al., 2001). Confirmation studies may involve independent populations constructed from the same parental genotypes or closely related genotypes used in the primary QTL study (Collard et al., 2005; Melchinger et al., 1998). The populations will then be replicated across sites and/or years. This tests whether the same QTL appears when the material is grown in other locations and/or years, and whether its effect can still be detected when introduced into a series of different genetic backgrounds (e.g. Brown et al., 2003; Langridge et al., 2001). Sometimes larger populations may be used (Collard et al., 2005). Another way of validating putative QTL is by creating a specific type of population called near isogenic lines (NILs) (e.g. Landi et al., 2005). A NIL is a new line that differs from its parent in only one genomic location: where the QTL is located. Using the marker identified for QTL, backcrosses are made to the recurrent parent until the entire genome of the line is exactly like the recurrent parent except in the region around the marker locus. Any phenotypic difference between these two lines is then probably due to the QTL linked to the marker locus, thereby validating the QTL.

2.4.4. Heritability

Heritability is often used by plant breeders and geneticists as a measure of precision of a trial or series of trials (Piepho and Mohring, 2007). There are two approaches to heritability; narrow sense heritability which is the proportion of the phenotypic variance that is due to additive genetic effects only, and broad sense heritability which measures the proportion of phenotypic variance that is due to all genetic effects (additive, dominance and epistasis) (Liu, 1998; Pan et al., 2007). Heritability is a key parameter in quantitative genetics because it determines the response to selection (Piepho and Mohring, 2007). Traits with high heritabilities are more effectively improved by phenotypic selection. However, most quantitative traits have low heritability (generally <50%) with individual QTL having only a fraction of the heritability of the trait (Kearsey, 1998). MAS is more efficient than phenotypic selection to improve such traits (Melchinger et al., 1998; Ruane and Colleau, 1995). Estimates of heritability vary from one population to another and would also vary with types of progeny, number of replications and environments used (Hallauer, 2007). The major problem with all QTL analyses is that small effect QTL are difficult to detect, and this gives a biased result of few large effect QTL in a population. Low individual QTL heritabilities also cause the estimates of QTL location to have large confidence intervals (Hyne et al., 1995). Confidence intervals can be reduced by using more dense maps, large numbers of genotypes to be tested and many replications (Kearsey, 1998). Minimising environmental variation by having many replicates of each individual (as can be easily achieved with RILs or DH lines) can enhance heritability of individual QTL (Kearsey and Farquhar, 1998).

2.4.5. QTL mapping *versus* Association mapping

For decades, linkage analysis has been the most commonly employed tool for dissecting complex traits in plants. Limitations of linkage analysis include low recombination events within mapping populations and the cost associated with propagating and evaluating a large number of genotypes (Doerge, 2002; Holland, 2007). In recent years, there has been tremendous interest in an alternative approach called association mapping (e.g. Zhu et al., 2008). This approach involves searching genotype-phenotype correlations in unrelated individuals (Myles et al., 2009; Risch, 2000). It utilises historical recombination events and natural genetic diversity to allow higher resolution genetic maps to be generated (Breseghello and Sorrells, 2006; Zhu et al., 2008). In addition to increased mapping resolution, association mapping is more rapid and cost effective. A group of unrelated individuals normally presents variation for many phenotypic aspects, thus several traits can be studied in the same population using the same genotypic data (Breseghello and Sorrells, 2006). Also, if elite lines (e.g. core collection from a gene bank) are used for the study, multi-year and multi-location phenotypic data may be available at no additional cost (Rafalski, 2002). Association mapping, however, requires extensive knowledge on SNPs within the genome of the organism, and is therefore difficult to perform in species that have not been well studied or do not have well-annotated genomes (Yu et al., 2008). In literature, association mapping has often been used interchangeably with linkage disequilibrium (LD). However, it has been pointed out that while association mapping refers to significant association of a molecular marker with a phenotypic trait, LD refers to non-random association between two markers or genes or QTL or between a gene/QTL and marker locus (Gupta et al., 2005).

2.5. Improvement of perennial ryegrass for sustainable pastoral systems

2.5.1. Molecular approach for the improvement of NUE in perennial ryegrass

Forage yield is still the major target of perennial ryegrass breeding programs. This is because of the slow increase in forage yield of less than 6% per decade during the latter half of the 20th century (Wilkins and Humphreys, 2003). Application of N fertilisers is considered fundamental to pastoral farming productivity, especially intensive dairy. Due to the environmental and economic constraints associated with N fertiliser use, it has become important that genetic approaches for grass improvement address nitrogen use efficiency (NUE) and other traits that would improve sustainability. NUE defined as the amount of forage dry matter for each unit of N applied (Zemenchik and Albrecht, 2002) is a complex polygenic trait. The complexity of NUE makes it difficult to evaluate reliably and then improve it based on phenotypic selection. Therefore, MAS, based on molecular genetic markers, provides an attractive option for its improvement.

Perennial ryegrass lags behind major agricultural crops in QTL studies relating to NUE but preliminary work has been reported (Dolstra et al., 2007; Van Loo et al., 2003). The work of Dolstra et al (2007) reported at a conference in 2003, was the first attempt to use molecular markers in the improvement NUE in perennial ryegrass. In this report QTL for seven component traits of NUE were mapped in an F_2 population originating from an F_1 plant (progeny of a cross between two genotypes contrasting in NUE) crossed with a doubled haploid plant. Positive alleles located on five chromosomal regions were selected, discerned by markers that co-located with 1-5 NUE related traits. Markers were selected based on a summation index which proved effective for complex traits such as NUE.

2.5.2. Agronomic to physiological basis of NUE

Development of cultivars of perennial ryegrass with improved NUE has the potential to enhance yield under low N and thereby reduce environmental pollution while maintaining a sufficient net income for the farmer. Improved plant NUE can be either in strict physiological sense such as increased C gain per unit plant N per unit time, or in an agronomic sense such as greater dry matter or protein yield per unit plant N, or per unit N applied /available to the crop (Andrews et al., 2004). Previously, evaluations of plant genotypes for greater efficiency for N were carried out primarily for responsiveness to applied N. This used agronomic approaches whereby genotypes were compared based on the yield response to applied N (Andrews et al., 2004; Vinod, 2007). Valuable selection parameters in this case were traits related to efficient N uptake and metabolism such as the N uptake characteristics of seedlings (Teyker et al., 1989) and plant nitrate content (Mollaretti et al., 1987). Differences in N uptake are likely to be related to the quantity and quality of the root system (Gallais and Hirel, 2004). High yielding crop cultivars used in intensive agricultural systems typically respond favourably to increased N inputs (e.g. Andrews et al., 2004). Application of N would generally be at levels required to saturate the response of the crop to N, and consequently there was the risk of excess N loss to the environment.

The need for environmental protection and the emphasis on sustainability of agriculture has led to research concentrating on adapting crops to N limiting conditions. In addition, there is a change in focus from purely agronomic approaches to physiological approaches targeting regulatory mechanisms involved in the steps of N acquisition, primary N assimilation and the subsequent biochemical pathways involved in secondary N metabolism (e.g. Andrews et al., 2004; Gallais and Hirel, 2004; Hirel et al., 2001; Mickelson et al., 2003; Obara et al., 2001). The use of N by plants involves several steps, including uptake, assimilation, translocation, and remobilisation. It has also been established that better N utilisation is a function of better N assimilation and remobilisation efficiencies within genotypes (Good et al., 2004). Genotypes play a major role in determining how efficient the nutrient assimilation in the presence of external factors (Singh et al., 1998). Efficient genotypes are those that produce high yield under sub-optimal N levels due to utilisation efficiency. On the other hand, remobilisation of N from senescing plant organs contributes to the N economy of the plant. The target is therefore to understand the factors/processes linked to N utilisation and remobilisation and genetically manipulate these factors in such a way that the need for N fertilisers is reduced.

2.5.3. QTL analysis of physiological traits related to NUE

Biochemical and physiological traits such as the levels and activities of enzymes involved in plant N metabolism are determined by an interaction of multiple gene functions (Obara et al., 2004). A number of studies in different crops have used marker-based QTL analysis to map QTL associated with NUE and its components using these biochemical/physiological traits. The major focus has been on enzymes specifically activated during N assimilation and N remobilisation (Gallais and Hirel, 2004; Hirel et al., 2001; Obara et al., 2001, 2004; Yamaya et al., 2002).

Ammonium (NH_4^+) and nitrate (NO_3^-) are the main forms of N taken up and assimilated by most non-N fixing crops (Raven et al., 1992). Inorganic N assimilation involves three major reactions (see review by Masclaux et al., 2001). The first reaction is the reduction of nitrate to nitrite by nitrate reductase (NR) enzyme. This is followed by further reduction of nitrites into ammonia in a reaction catalysed by nitrite reductase (NiR). Ammonia (NH_3) from this reaction is converted by either cytosolic form (GS1) or the plastidic form (GS2) of the enzyme glutamine synthetase (GS) to form glutamine. Glutamate synthetase (GOGAT), subsequently, catalyses the reductant-dependent conversion of glutamine and 2-oxaloglutarate to two molecules of glutamate and occurs as two distinct isoforms, one ferredoxin dependent (Fd-GOGAT) and the other NADH-dependent (NADH-GOGAT) (Andrews et al., 2004; Lea and Ireland, 1999; Obara et al., 2001). The GS/GOGAT pathway is considered the main pathway for inorganic N assimilation and re-assimilation, although other potential enzymatic reactions exist, for example, those catalysed by glutamate dehydrogenase (GDH) and asparagines.

QTL approaches have been performed on some model cereal species to show the importance of N metabolism enzymes in plant N management and performance. A study in rice (Obara et al., 2001) identified seven QTL for GS1 and six for NADH-GOGAT some of which were located in QTL regions for various biochemical and physiological traits affected by N recycling. These authors suggested that variation in GS1 and NADH-GOGAT protein contents was related to the changes in the rate of N recycling from senescing organs to developing organs, leading to changes in physiological traits. In further work involving a transgenic variety over-expressing NADH-GOGAT, about 80% increase in grain weight was observed indicating that NADH-GOGAT was indeed a key step for N utilisation and grain filling in rice (Yamaya et al., 2002). Functional genomics studies confirmed that GS1 played a key role in normal growth and grain filling in rice (Obara et al., 2004; Tabuchi et al., 2005) . Also, a 50 cM fragment on chromosome 2 was found to contain a regulatory gene for GS1 protein content, panicle weight and panicle number (Obara et al., 2004).

A study of maize recombinant inbred lines (RILs) under low and high N nutrition revealed consistent coincidences between genes encoding for enzymes of N and C metabolism and QTL for vegetative development and grain yield and its components (Bertin and Gallais, 2001). Another study by Hirel et al. (2001) reported a positive correlation of yield and its components with GS1 activity and nitrate content. Coincidences of QTL for yield and its components with genes encoding GS1 and the corresponding enzyme activity were also detected. Further work confirmed that the GS locus on chromosome 5 (*gln4*) could be considered a good candidate gene influencing grain filling (Gallais and Hirel, 2004). These authors also reported that two other GS genes (*gln1* and *gln2*) on chromosome 1 and the GS gene on chromosome 4 (*gln3*) could be involved either in N mobilisation or in translocation during the grain filling process. Knock-out mutation of two GS1 isoenzymes resulted in impaired GS mRNA expression, a reduction in GS1 protein and activity, and a reduction in kernel size and number in maize (Martin et al., 2006), further confirming the importance of the two GS1 isoenzymes in N remobilisation and yield. Coincidences of QTL for yield components and the location of GS1 genes indicated that GS1 might be a key component of NUE and yield.

In barley, mapping of QTL associated with N storage and remobilisation (Mickelson et al., 2003) reported that chromosomes 3 and 6 were probable location of genes that were directly involved in N recycling or genes regulating N recycling. These authors found that alleles associated with inefficient N remobilisation were also associated with depressed yield and higher levels of total or soluble organic N during grain filling and vice versa. In this study, the most prominent QTL for protein concentration (on chromosome 6) did not co-localize with QTL for N remobilisation, but QTL peaks for nitrate and soluble organic N were detected at this locus. Alleles associated with low grain protein concentration were associated with higher soluble N levels in leaves during grain filling indicating that genes found at this locus might influence sink strength of developing barley grains.

2.6. Nitrogen remobilisation and NUE

Much of the research on NUE has focused on N uptake from the soil and its metabolism and transport to leaves (Good et al., 2004). However, it has been demonstrated in several studies that new tissue growth in crops is based not only on nitrate uptake but also on remobilisation of N from vegetative tissues (Lattanzi et al., 2005; Louahlia et al., 1999; Mae, 1997; Mickelson et al., 2003; Peoples and Dalling, 1988). These studies have also demonstrated that remobilised N contributes more to new tissue growth than N uptake from the soil. Remobilisation has been considered an effective strategy evolved by both annual and perennial plants to conserve N (Peoples and Dalling, 1988). Physiological studies in cereal crops including barley (Mickelson et al., 2003), corn

(Eghball and Maranville, 1991), sorghum (Youngquist and Maranville, 1992), and wheat (Kichey et al., 2007; Papakosta and Gagianas, 1991; Vansanford and Mackown, 1987) have shown that the ability to remobilise N is subject to genetic variability, and therefore amenable to genetic improvement. To address the question of N remobilisation efficiency (NRE), efforts have been made to study the physiological and biochemical mechanisms involved in N metabolism in order to detect the limiting factors that can be manipulated (Masclaux-Daubresse et al., 2008). Improving NRE from vegetative tissues would increase plant N economy and limit the N demand from exogenous sources, thus reducing the need for N fertiliser application.

Developing leaves constitute significant net importers (‘sinks’) for N and other nutrients, which are used to build the organ’s cellular components (Fischer, 2007; Hirel et al., 2001). Leaves then go through a sink-source transition becoming net exporters (‘sources’) of N and amino acids for the formation of newly developing leaves and/or storage organs (Fischer, 2007; Hirel and Gallais, 2006; Ishimaru et al., 2004). In most plant tissues, the largest fraction of organic N, which is potentially available for remobilisation during senescence, is contained in proteins (Fischer, 2007).

2.7. Leaf development

The lifespan of a leaf comprises three distinguishable developmental phases; growth with rapid protein synthesis, maturity with protein turnover at a consistently low level, and a senescence phase in which protein degradation is predominant (Buchanan-Wollaston, 1997). In grass species, leaf growth is normally unidirectional, resulting primarily in increase in length (Macadam et al., 1992a; Macadam et al., 1992b). Leaf growth is initiated by the division of cells at the base of leaf primordial near the apical meristem, and is a linear process in which cells are displaced in parallel longitudinal rows by continuous production and expansion of cells (Macadam et al., 1989). The growth zone, a short zone at the base of growing leaves, comprises three zones in sequential pattern; a cell production zone, a cell expansion zone, and a cell maturation zone (Gastal and Nelson, 1994; Lattanzi et al., 2004). The continuous production of cells at the basal position and their subsequent expansion gives rise to a flux of tissue-bound mass out of the growth zone (Lattanzi et al., 2004). These processes are associated with the consumption of substantial amounts of organic substrate within the leaf growth zone (Schnyder and de Visser, 1999).

The growth zone may be regarded as a place where substrates are imported, transformed and exported as structurally and functionally differentiated tissue (Lattanzi et al., 2004). The length of the growth zone and the relative elongation rate of the tissue in the growth zone determine leaf elongation rate (Arredondo and Schnyder, 2003). As the leaves develop from the basal meristem, there is a clear gradation within the first few millimetres of the leaf from undifferentiated meristematic cells to fully differentiated, photosynthetically competent cells (Shaikh et al., 2000). The gradient of development provides a model where the sink and the source are simultaneously located in the same organ with the emerged leaf tip already mature and photosynthetically active while the growing base is acting as a sink (Bregard and Allard, 1999). In perennial ryegrass, Schaufele and Schnyder (2001) found that N content decreased with increasing distance from the base. The high rate of N deposition near the base has been interpreted in terms of a high demand for synthesis of proteins and nucleic acids in cells undergoing cell division (Schaufele and Schnyder, 2001).

When a leaf has attained full length and expansion has ceased, it exhibits a reduced capacity to photosynthesize and enters a senescent phase when it becomes a major source of N and other minerals as its reserves are depleted and transferred to other parts of the plant (Gan and Amasino, 1997; Hirel and Gallais, 2006; Ishimaru et al., 2004). Changes taking place during senescence form a genetically programmed sequence of events, with close coordination at the cell and tissue levels (Gan and Amasino, 1997; Smart, 1994).

2.7.1. Senescence and nutrient remobilisation

Leaf senescence involves degradation of chlorophyll, macromolecules (e.g. proteins, nucleic acids, lipids) and membranes (Guo and Gan, 2006; Liu et al., 2008). Chlorophyll degradation is the first visible sign of senescence but by the time yellowing of the leaf can be seen, the majority of the senescence process has occurred (Buchanan-Wollaston et al., 2003). Chloroplasts undergo changes involving the distortion of grana stacks, accumulation of osmiophillic globuli, a decrease in chloroplast volume and, eventually the complete loss of chloroplast component (Martinez et al., 2008). The breakdown of the mitochondria/nucleus is a relatively late event (Buchanan-Wollaston et al., 2003; Hörtensteiner and Feller, 2002).

Senescence can be genetically controlled, thus occurring at a given time in the life of the leaf even when growth conditions are near optimal (Buchanan-Wollaston et al., 2003; Huffaker, 1990). Differential gene expression is believed to play a role in leaf senescence, and many genes that are expressed in green leaves, including those involved in photosynthesis, are down-regulated, whilst a subset of genes called senescence-associated genes (SAGs), are up-regulated (Buchanan-Wollaston et al., 2003; Guo and Gan, 2006). Like many other genetically programmed developmental processes, leaf senescence is also subject to regulation by environmental and internal factors. Environmental factors include stresses such as extreme temperature, pathogens, drought, nutrient limitation, oxidative stress caused by UVB irradiation and ozone, while internal factors include age, reproductive development, and phytohormone levels (Gan and Amasino, 1997; Houtz and Portis, 2003).

2.7.1.1. Chlorophyll degradation

Chlorophyll degradation is vital during leaf senescence, as it allows for recycling of nutrients and for protection from a build-up of phototoxic chlorophyll intermediates (Eckardt, 2009; Hörtensteiner, 2006). Significant progress in the understanding chlorophyll degradation has been achieved in recent years (Hörtensteiner, 2006; Martinez et al., 2008) and a number of genes in the pathway have been cloned (Buchanan-Wollaston et al., 2003). The initial steps of chlorophyll degradation take place within the plastid (Hörtensteiner, 2006). The first step involves two subsequent reactions catalysed by chlorophyllase and Mg-dechelatase, respectively, and phytol and the central Mg atom are removed (Hörtensteiner, 2006; Pružinská et al., 2003). This is followed by oxygenolytic cleavage of the porphyrin ring by pheophorbide *a* oxygenase (Martinez et al., 2008; Mühlecker and Kräutler, 1996; Pružinská et al., 2003; Vicentini et al., 1995) which results in the yellowing of senescent leaves. The product, red chlorophyll catabolite (RCC), is rapidly converted to a primary fluorescent chlorophyll catabolite (pFCC) by the reduction of the C20/C1 double bond (Rodoni et al., 1997). Two isomeric forms of pFCC (pFCC-1 and pFCC2) exist, but within a given plant species, only one of the two isomers is formed (Pružinská et al., 2003). The specificity is determined by stereospecifity of the respective RCC reductases (RCCRs) (Hörtensteiner et al., 2000; Pružinská et al., 2003). In the final step of the chlorophyll breakdown pathway, FCCs are hyroxylated and in some cases conjugated with glucosyl or malonyl moeity and then exported into the vacuole by a primary active ATPase (Mühlecker and Kräutler, 1996; Vicentini et al., 1995). In the vacuole, FCCs are non-enzymatically tautomerized to the respective nonfluorescent chlorophyll catabolites (NCCs) (Hinder et al., 1996; Pružinská et al., 2003).

NCCs are deposited in the vacuole with no recycling of any of the N contained within them (Buchanan-Wollaston et al., 2003; Hinder et al., 1996). Thus, the ability of the plant to degrade chlorophyll during senescence seems vitally important for plant development and survival in order to prevent the accumulation of phototoxic intermediates (Pružinská et al., 2003). The importance of chlorophyll degradation has been demostrated by the isolation of the *Arabidopsis accelerated cell death 2* gene (*Acd*2) that develops a light-dependent mimic phenotype (Mach et al., 2001). *acd*-2 is deficient in RCCR, and in the absence of this enzyme activity, the accumulation of phototoxic RCC causes rapid cell death (Buchanan-Wollaston et al., 2003; Mach et al., 2001).

One mole of chlorophyll contains four moles of N, but chlorophyll contributes only about 2% of cellular N (Hörtensteiner, 2006). Chlorophyll is required for stabilisation of chlorophyll-binding proteins such as LHCIIb polypeptides, and it can be argued that that removal of chlorophyll is a prerequisite for the degradation of these proteins (Horn and Paulsen, 2004; Hörtensteiner, 2006). In a non-yellowing *Festuca* mutant, Bf 993, LHCIIb undergoes some proteolytic cleavage, although it is not degraded, pointing to an interplay between proteolytic and chlorophyll catabolic activities during senescence (Hörtensteiner, 2006). Chlorophyll(ide) *b* reductase could play a critical role because *in vitro* assembly of LHCIIb requires the presence of chlorophyll *b* in stoichiometric rates (Horn and Paulsen, 2004), and chlorophyll *b*-less mutants have been shown to experience a rapid turnover of LHCIIb (Harrison et al., 1993). It is possible that senescence-specific activation/synthesis of the thylakoid located Chlorophyll(ide) *b* reductase lowers the amount of chlorophyll b of individual chlorophyll-protein complexes, which may destabilise the complexes and make them accessible for proteases (Hörtensteiner, 2006).

Proteases responsible for degradation of chlorophyll-binding proteins are largely unknown except for D1 protein of the PS II reaction centre (Hörtensteiner, 2006). D1 is degraded in two steps; the first step is the cleavage into two fragments by DegP-type protease, followed by complete degradation through members of chloroplast localised FtSH protease (Adam and Clarke, 2002; Hörtensteiner, 2006). On the other hand, knowledge of the degradation of LCHIIb is limited. Studies have shown the involvement of serine/cysteine-type proteases in the degradation of LCHIIb, and a chloroplast-localised endopeptidase was identified, which cleaved a N-terminal peptide of a defined length from the LCHII subunit, Lhcb1 (Forsberg et al., 2005; Tziveleka and Argyroudi-Akoyunoglou, 1998). Another study identified a zinc-binding metalloprotease, possibly from the FtsH family, acting toward Lhcb3 (Hörtensteiner, 2006; Zelisko and Jackowski, 2004).

2.7.1.2. Protein degradation pathways

In most plant tissues, the largest fraction of organic N, which is potentially available for remobilisation during senescence, is contained in proteins (Fischer, 2007), and the mechanisms involved protein degradation during senescence have been discussed by several authors. Three major protein degradation pathways (discussed below) have been identified.

2.7.1.2.1. The ubiquitin/26S proteasome pathway

The ubiquitin/26S proteasome system (Ub/26S) is one of the most important proteolysis systems in eukaryotes and is important for targeted protein degradation during normal development and in response to environmental factors (Sullivan et al., 2003; Vierstra, 1987; Vierstra, 2003). It involves the small protein ubiquitin (Ub, a 76-amino acid globular protein) and the 26S proteasome (a 2-MDa protease complex). The Ub/26S pathway consists of concerted actions of enzymes that link chains of polypeptide co-factor, Ub, onto proteins to mark them for degradation (Glickman and Ciechanover, 2002; Pickart, 2004). This tagging process is ATP-dependent and three enzymatic components; ubiquitin- activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) are involved (Lecker et al., 2006). All E1s form thiolester bonds with the C-terminal glycine of Ub through conserved cysteine residue. E2 accepts Ub from E1 through a thiolester via a conserved ubiquitin conjugating domain with a cysteinyl sulfhydryl group (Kraft et al., 2005). Finally, E3 recognises a specific protein substrate and catalyses the transfer of activated Ub to it (Kraft et al., 2005; Lecker et al., 2006). The resulting Ub-protein conjugates are then recognised and degraded by the 26S proteasome (Smalle and Vierstra, 2004). A chain of five Ub molecules attached to the protein substrate is sufficient for the complex to be recognised by the 26S proteasome (Lecker et al., 2006). In all components of Ub/26S, approximately 90% of the genes encode elements of E3 ubiquitin ligases, and it has been proposed that the larger number of E3s determines substrate specificity (reviewed by Liu et al., 2008).

2.7.1.2.2. The chloroplast degradation pathway

Chloroplasts contain over 70% of total leaf N mainly in the form of proteins (Hörtensteiner and Feller, 2002; Makino and Osmond, 1991). Degradation of chloroplast proteins within the organelle is supported by the observation that chloroplasts contain a number proteases of the ChlP, FtsH, DegP and Lon families (Martinez et al., 2008). Three types of proteases have been categorised based on their localisation in cellular compartments. The ATP-dependent Chl proteases are found in the stroma, FtsH and Lon proteases in the thylakoid membrane proteases, and the ATP-independent Deg proteases in thylakoid membrane and lumina (Adam, 2001; Gregersen and Holm, 2007; Guo et al., 2004). All these proteases have been extensively characterised in senescing leaves (Adam and Clarke, 2002; Sakamoto, 2006). Chloroplast proteases are the most diverse in higher plants, but knowledge of their functioning is limited (Liu et al., 2008; Zheng et al., 2002). Some of FtsH and DegP proteases have been implicated in the breakdown of chlorophyll-binding proteins as discussed in Section 2.7.1.1. More recently, a chloroplast aspartic protease with DNA-binding domains (CND41) has been implicated in the degradation of Rubisco, the most abundant chloroplast protein (Kato et al., 2004). This is discussed further in Section 2.8.3.2.

2.7.1.2.3. The autophagic pathway

Autophagy is a process involving the degradation of a cell's own components through the lysosomal mechanism. This process which is important for non-specific protein and organelle turnover can be induced by leaf senescence or by stress factors such as cellular damage, nutrient deficiency, or pathogen attack (Liu et al., 2008). Different autophagic pathways exist. In microautophage, the tonoplast membrane invaginates, enclosing a portion of the cytoplasm, and the invaginated tonoplast eventually pinches off, releasing a vesicle containing cytosol into the central vacuole (Martinez et al., 2008). Macroautophagy is a sequestrating of organelles and long-lived proteins, and is initiated in the cytoplasm by the formation of a cup-shaped double membrane vesicle, known as an autophagosome, that encloses the material to be degraded (Liu et al., 2008; Martinez et al., 2008). The outer membrane of the autophagosome eventually fuses with the tonoplast, releasing a single membrane-bound autophagic vesicle into the vacuole (Liu et al., 2008; Martinez et al., 2008). In the vacuole, acid hydrolases degrade the autophagic body, and the degradation products are transported back to the cytosol. It has also been observed that autophagosomes can fuse with small lysosomes or endosomes, in which the contents can be degraded before eventual fusion with the vacuole (Liu et al., 2008).

2.8. Rubisco

2.8.1. Rubisco as a photosynthetic catalyst

Rubisco is a key enzyme of photosynthesis that catalyses the first major step of C fixation. This enzyme is important because it provides a link between the pools of inorganic and organic C in the biosphere. Rubisco is responsible for an estimated uptake of 120 gigatons of C per year (Bowes, 1991). At ambient CO_2 partial pressure and light-saturated conditions, the rate of CO_2 assimilation correlates well with the total amount of Rubisco throughout the leaf's life (Evans, 1986; Makino et al., 1985).

2.8.2. Rubisco as a potential N storage protein

Rubisco occurs at very high levels in photosynthesizing cells and is therefore considered as the most abundant protein in the world (Ellis, 1979). In photosynthetically active tissues of C_3 plant species, over 50% of this N is found in soluble (Calvin cycle) and insoluble (thylakoid) chloroplast proteins (Fischer and Feller, 1994; Peoples and Dalling, 1988; Spreitzer and Salvucci, 2002). N invested in Rubisco is considerably high, accounting for 12-35% of total leaf N in C_3 plants (Evans, 1989; Makino et al., 1992). The proportion of Rubisco within the soluble protein pool is a large reserve of stored N (Hirel and Gallais, 2006; Millard, 1988). Rubisco proteolysis during leaf senescence supports the idea that this enzyme also represents an important cellular component, in which N is stored during leaf expansion and which can be rapidly remobilised to sustain growth of the young tissues (Mae et al., 1983). The large amount of Rubisco protein partially compensates for its inefficiency as a photosynthetic catalyst (e.g. Spreitzer and Salvucci, 2002; Stitt and Schulze, 1994; Warren et al., 2003).

2.8.3. Rubisco turnover

Most proteins are in a continuous state of turnover where, at any one time, the absolute amount of a particular protein species is a function of the rate of synthesis and the rate of degradation (reviewed by Peoples and Dalling, 1988; Vierstra, 1993). Protein turnover has several important functions in regulating the plant’s metabolism. One physiological function attributed to this process and relevant to this study, is the provision of free amino acids during periods of growth (Vierstra, 1987). In the particular case of Rubisco, studies using [^{3}H] acetic anhydride and tritiated water in maize leaves (Simpson, 1978; Simpson et al., 1981) and ^{15}N tracers in rice leaves (Mae et al., 1983; Makino et al., 1984a) have shown that Rubisco was degraded while being synthesised. Rubisco content increases rapidly during leaf expansion, reaches a maximum around full leaf expansion and then declines as the leaf ages (Mae et al., 1983). Rubisco synthesis has a greater influence on leaf Rubisco content during leaf expansion while degradation becomes more important than synthesis during senescence (Suzuki et al., 2001).

2.8.3.1. Rubisco synthesis

Rubisco from higher plants is a hexadecamer composed of eight small subunits (SSU, ca 15 kDA) and eight large subunits (LSU, ca 55 kDA) encoded by the nuclear *rbcS* and chloroplast *rbcL* genes, respectively (Spreitzer, 1999). The SSU are synthesized in the cytosol with signal peptides and incorporated into the chloroplasts. Signal peptides are then removed from the chloroplasts and the SSU are assembled with the LSU to support the formation of the holoenzyme by chaperonin and ATP (Imai et al., 2005). The expression of *rbcS* and *rbcL* is coordinated by the adjustment of subunit stoichiometries in response to the abundance of unassembled subunits (Rodermel, 1999). When SSU accumulation is limiting (as in antisense mutants), LSU levels are primarily adjusted to those of the SSU at the level of *rbcL* mRNA translation initiation. On the other hand, when LSU accumulation is limiting (as in some *rbcL* nonsense mutants), SSU levels are adjusted to those of the LSU at the level of protein degradation. Studies of transcripts for the SSU and LSU of Rubisco have suggested that regulation at the transcriptional level occurs during the early stages of leaf development, but at later stages post-transcriptional regulation may be the operational mechanism (Lozatavera et al., 1990; Nikolau and Klessig, 1987; Suzuki et al., 2001). Rubisco synthesis is induced by light; light stimulates the accumulation of LSU and SSU mRNAs (Brady, 1988; Nikolau and Klessig, 1987). It has also been suggested that internal stimuli such as developmental and hormonal signals control the synthesis of LSU and SSU (Rodermel, 1999).

Investigations in rice leaves by Suzuki et al (2001) reported a correlation between the amount of Rubisco synthesised and the levels of *rbcS* and *rbcL* mRNAs during leaf expansion, suggesting that the amount of Rubisco during this period is primarily determined by its transcript abundance levels. However, a later study showed than N influx into leaf blades (N availability), rather the transcript levels, is more closely related to the amount of Rubisco synthesized in the leaf blade of rice throughout the life span of a leaf (Imai et al., 2005). Earlier studies (Mae et al., 1983; Makino et al., 1984a) also showed a correlation between the amount of Rubisco and N availability. N influx is highest during leaf expansion (Imai et al., 2005; Makino et al., 1984a) and a ^{15}N tracer study demonstrated that growing leaves exhibited a more extensive incorporation of N into Rubisco (Mae and Ohira, 1981). Rubisco content has been shown to correlate with leaf N content in expanding leaves (reviewed by Makino, 2003).

2.8.3.2. Rubisco degradation

Rubisco undergoes degradation during senescence, and its N is incorporated into growing tissues (Albuquerque et al., 2001; Feller and Fischer, 1994; Feller et al., 2008b; Ferreira et al., 2000; Mae et al., 1987). Senescence and Rubisco degradation already occur during early phases of vegetative growth (Feller and Fischer, 1994). Analysis of the relationships between the levels of *rbcL* and *rbcS* mRNAs and the amount of Rubisco during leaf development in a number of crops e.g. *Amaranthus hypochondriacus* (Nikolau and Klessig, 1987), wheat (Crafts-Brandner et al., 1998), *Phaseolus vulgaris* L. (Bate et al., 1991; Crafts-Brandner et al., 1996) and rice (Imai et al., 2005; Suzuki et al., 2001) showed a decline of Rubisco and its transcript abundances during senescence. The relationship of *rbcS* mRNAs, *rbcL* mRNAs, and the amount of Rubisco during senescence differed from that during leaf expansion. For example, in wheat, Bate et al (1991) observed a faster decline of the *rbcS* mRNAs in relation to *rbcL* mRNAs indicating a differential regulation of the nuclear and chloroplast genes in senescing leaves. In rice, a dramatic decline in Rubisco synthesis did not parallel the decline in its transcript abundance levels (Imai et al., 2005). Another study (Brady, 1988) indicated a faster decrease in Rubisco compared to leaf N, and also the ratio of synthesis of Rubisco to that of other proteins was found to decline as the leaves aged. The amount of Rubisco in senescent leaves is therefore much lower than predicted by leaf N (Makino, 2003). N removal from the growth medium at the time of full leaf elongation has been shown to enhance the rate of senescence and Rubisco degradation in wheat (Crafts-Brandner et al., 1998). In rice leaves, however, subjection of the plants to two N nutrient regimes soon after the leaf tip emergence showed that Rubisco was degraded more actively during the early stages of senescence in the N-sufficient leaf, whereas its degradation proceeded almost constantly in the N-deficient leaf during senescence (Makino et al., 1984a).

Rubisco has been widely studied but the mechanism of its degradation in leaves is still poorly understood (Diaz et al., 2008; Houtz and Portis, 2003). Rubisco degradation may occur within and/or outside the chloroplast. Model systems for studying the mechanism of Rubisco systems have used isolated chloroplasts or chloroplast lysates. In these systems, it has been reported that Rubisco degradation is often stimulated by light or oxidative stress (Ishida et al., 1998; Mitsuhashi et al., 1992; Roulin and Feller, 1997). Reactive oxygen species (ROS) which are readily generated by the photosynthetic apparatus under stress or senescence conditions may also directly cleave Rubisco or modify it in a manner making it more susceptible to proteolytic cleavage (Desimone et al., 1996; Desimone et al., 1998; Feller et al., 2008a; Ishida et al., 1998). It is unlikely that ROS are the only factors that may trigger *in vivo* degradation of Rubisco, because degradation has been shown to be stimulated when plants are placed in darkness

(Kokubun et al., 2002). It is now known that chloroplasts contain a number of different types of protease (Adam, 2001). In relation to Rubisco, a stromal metallo-endopeptidase known as EP1 partially purified from pea chloroplasts was able to degrade the LSU of Rubisco (Bushnell et al., 1993). A recent study has implicated a chloroplast DNA-binding protease, CND41, in Rubisco degradation during tobacco leaf senescence (Kato et al., 2005; Kato et al., 2004). *In vitro* analysis indicated that CND41 shows proteolytic activity against denatured inactive Rubisco, while native active Rubisco is resistant to proteolysis by CND41 (reviewed by Martinez et al., 2008). Rubisco is protected against proteolytic degradation under catalytic conditions or in the presence of an inhibitor binding to the active site in the LSU (Feller et al., 2008b). Active Rubisco in the chloroplast would be resistant to CND41 catalysed degradation until leaf senescence is underway (Diaz et al., 2008). As noted by these authors, it is likely that the deleterious effects of ROS initiate Rubisco proteolysis, and this is followed by degradation by CND41. It has been reported that accumulation of CND41 correlates with Rubisco loss, and antisense suppression of CND41 increases the amount of Rubisco in older leaves of N-depleted tobacco plants (Kato et al., 2005; Kato et al., 2004). CND41 antisense lines also show reduced levels of gibberelins, and a dwarf phenotype with reduced leaf expansion, suggesting that increased levels of Rubisco in lower leaves of antisense CND41 plants might point either to a direct involvement of CND41 in chloroplast protein degradation, or an indirect effect through regulation of plant development and the correlative controls of senescence (Martinez et al., 2008).

Other studies have indicated Rubisco degradation outside the chloroplast by autophagy. A study involving naturally senescing wheat leaves found that Rubisco is released from the chloroplast into the cytoplasm and transported to the vacuole for subsequent degradation in small spherical vesicles, called Rubisco containing bodies (RCBs) (Chiba et al., 2003). Similar structures were reported in soybean, Arabidopsis, and tobacco (Otegui et al., 2005; Prins et al., 2008). However, a recent study has found that senescence was promoted similarly in both wild-type Arabidopsis and an autophagy-defective mutant, *atg4a4b-1* (Wada et al., 2009). These reports indicate that Rubisco degradation may be effected through multiple pathways.

Both chloroplastic and autophagic Rubisco degradation pathways are enzyme-mediated. In general, enzymatic degradation is highly controlled implying that Rubisco degradation as part of leaf senescence is genetically regulated. Recently, some workers, notably Irving and Robinson (2006) proposed that Rubisco degradation occurs exponentially in accordance with first-order kinetics, suggesting a simple process that is not tightly regulated. This new model has its basis on several previous data as described in the next section.

2.8.4. Rubisco in leaf ontogeny

Whole leaf analyses have shown that Rubisco content increases rapidly during leaf expansion reaches a maximum just before full leaf expansion and then declines during senescence (Friedrich and Huffaker, 1980; Mae et al., 1983; Mae et al., 1989). The amount of Rubisco declines rapidly in the early phase of senescence, and more slowly in the later phase (Friedrich and Huffaker, 1980; Mae et al., 1984). Also, the decline of Rubisco during senescence is faster than that of other leaf proteins (Brady, 1988; Miller and Huffaker, 1985). In relation to leaf development in ryegrass, it has been observed that Rubisco biosynthesis takes place within the leaf at 5 – 8 cm distal from cell division zone (Skinner and Nelson, 1995). In tall fescue, Rubisco concentration has reached a maximum when a segment of the emerging leaf is 11 cm from the ligule (Gastal and Nelson, 1994). A model developed by Irving and Robinson (2006) describes the time course of Rubisco concentration by a log-normal curve, and assumes exponential decay of Rubisco, once produced. In this model, the rate of Rubisco biosynthesis, like leaf elongation rate is described by a normal distribution curve (Fournier et al., 2005). After full leaf expansion, the model predicts an exponential decline in the rate of Rubisco synthesis. This is supported by the corresponding exponential decline in *rbc* transcript abundances in senescence reported in a number of studies (Crafts-Brandner et al., 1998; Ishizuka et al., 2004; Suzuki et al., 2001).

This new model is not universally accepted since literature (as described in Section 2.8.3.2) pointing to enzyme-mediated degradation would favour tight regulation, for example, the control of CND41 as observed in sense and antisense tobacco leaves (Kato et al., 2005; Kato et al., 2004). This also raises the question of the difference in the pattern of Rubisco protein concentration during the leaf’s life span. While the new model describes Rubisco concentration by a log-normal curve, enzymatic degradation starts later in leaf’s life implying that Rubisco protein would increase and plateau before declining drastically as the leaf senesces. However, comparative studies have shown that change in Rubisco concentration during leaf development differs from other proteins (e.g. LHCII) and chlorophyll (Chiba et al., 2003). As described by these authors, Rubisco, LCHII and chlorophyll was shown to increase in a similar manner during leaf expansion. However, after reaching a maximum, Rubisco started to decline rapidly while LCHII and chlorophyll remained at the same level for eight more days before starting to decline. Therefore, even though Rubisco degradation has been studied by many researchers, its degradation mechanism remains elusive.

Since Rubisco is the most abundant protein and a high amount of N is invested in it, many researchers have suggested or discussed its potential as an N storage protein (Friedrich and Huffaker, 1980; Mae et al., 1983; Racusen and Foote, 1965; Wittenbach, 1979). However, the usefulness of the N storage function in relation to plant productivity has not been clarified. Since the C assimilation role requires active Rubisco enzyme while the N remobilisation role requires that the enzyme is degraded, the efficiency of the enzyme would be reflected in its ability to balance these functions in such a way that one role does not compromise that of the other with consequential implications on plant performance.

QTL and physiological approaches have shown variation in N remobilisation and its effect on plant yield especially in cereal crops (e.g. Gallais and Hirel, 2004; Hirel et al., 2001; Obara et al., 2001). Similarly, variation in the turnover of Rubisco reflected by differences in the rate of synthesis and degradation is expected to have an effect on C assimilation and N remobilisation, thus influencing plant yield. The possibility that the pattern of Rubisco turnover may influence NUE is therefore of interest. Moreover the opportunity to research this question for a mapping population and potentially identify QTL for Rubisco turnover could shed light on the genetic control of physiological processes and provide an avenue for plant improvement. One way to understand this physiological process and how it can be used for agronomic application is to study by way of quantitative genetics Rubisco accumulation and degradation in leaf ontogenesis in relation to other agronomic characteristics. The determination of Rubisco turnover in this study will be based on the dynamic model of Rubisco turnover in cereal leaves proposed by Irving and Robinson (2006). Therefore, as a preliminary step towards the QTL study, the next chapter describes the model, the development of the technique for measuring Rubisco, and testing of the model using leaves of barley plants growing under two N concentration levels.

CHAPTER 3

Rubisco turnover in barley (*Hordeum vulgare* L.) leaves at two levels of nitrogen nutrition

3.1. Introduction

Quantification of Rubisco during leaf development has been a subject of many scientific studies (Mae et al., 1983; Makino et al., 1984a; Simova-Stoilova et al., 2001; Suzuki et al., 2001; Takeuchi et al., 2002). Leaf Rubisco content is the result of the balance between its synthesis and degradation (Suzuki et al., 2001). Therefore, a change in the amount of Rubisco can be the result of a change in either the rate of synthesis or degradation or both. While many studies have been successful in showing net changes in leaf Rubisco content between treatments/genotypes, few have been able to resolve these changes in terms of the contributing processes of synthesis or degradation. Irving and Robinson (2006) proposed a new model to estimate Rubisco content, synthesis, and degradation at any point during the lifespan of the leaf. According to this model, the time course of leaf Rubisco content is described by a log-normal curve which represents the combined effects of simultaneous synthesis and degradation. It assumes Rubisco degradation occurs exponentially according to first-order kinetics. The log-normal curve is described by three mathematical parameters *d*, *f*, and *g* as summarised in Fig. 3.1. The curve parameters provide a means to quantify how Rubisco dynamics differ among treatments or genotypes.

Using data from previous studies, Irving and Robinson (2006) demonstrated the capability of the model to uncover treatment and genotypic effects. In rice plants growing under different nitrogen (N) nutrition levels (Makino et al., 1984a), they showed a significantly higher *d* in high N than low N plants. They also observed significant genotypic differences in *g* in two rice genotypes exposed to supplementary UV-B radiation (Takeuchi et al., 2002). These examples, particularly the latter, illustrate that stress will cause alteration in the plant’s mode of N (Rubisco-N) management, and this can be detected by the model. On this basis, the model can be used for assessment of genetic material for variation in Rubisco turnover in relation to plant yield characteristics. This should give insight into the link between the physiological function

(Rubisco turnover) and plant yield and yield components.

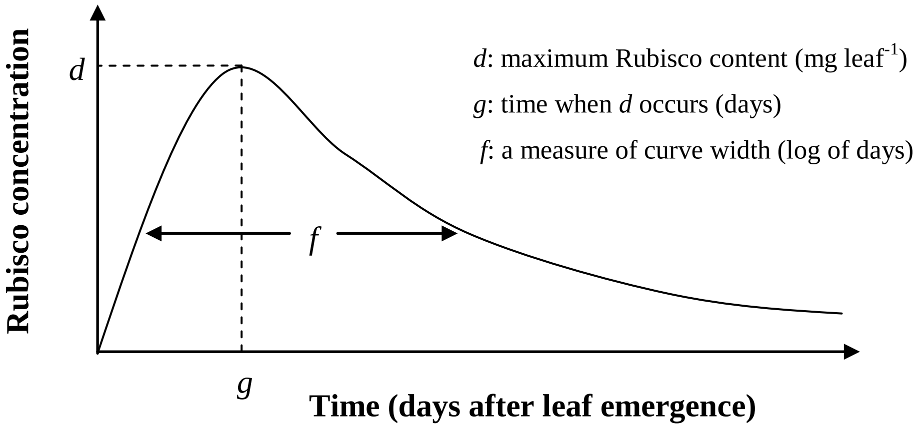


Figure 3.1: Log-normal model for the time course of leaf Rubisco concentration (Irving and Robinson, 2006). Under this model, the rate of change in Rubisco concentration at any point in time is the balance between the rate of synthesis and the rate of degradation (assumed to be continous and concentration-dependent). The model is under debate because many researchers believe Rubisco degradation would be enzyme-mediated. In that case the expectation would be for leaf Rubisco concentration to plateau, and then fall suddenly late in the life of the leaf.

3.1.1. Hypothesis

Rubisco represents a large proportion of leaf N, and therefore it is expected that the remobilisation characteristics of Rubisco-N would have an effect on yield. Based on the model, variation in Rubisco-N remobilisation will be reflected in Rubisco turnover curve patterns with consequential effects on the magnitudes of *d*, *f*, and *g*. As a dual function protein, Rubisco concentration needs to follow a time course that provides a balance between N removal via remobilisation and the maintenance of photosynthesis to produce assimilate during senescence. Therefore, this study was built on the hypothesis that a turnover rate that is too fast would reduce total photosynthesis over the lifetime of a leaf. Similarly, a turnover rate that is too slow would result in N being trapped and lost in dying leaves. It is assumed that a strategic balance whereby the efficiency of one physiological process does not compromise that of the other is what contributes to high yields and N use efficiency (NUE) in crop plants. Therefore, studying leaf Rubisco turnover will not only contribute to our knowledge about this fundamental physiological process, but may also lead to ways of manipulating Rubisco turnover for improvement of plant productivity.

3.1.2. Experiment objectives

This experiment was set up as a first study to establish a foundation for the evaluation of Rubisco turnover by quantitative genetics in a perennial ryegrass (*Lolium perenne* L.) mapping population. Technique development was the main objective and therefore the first step was to build a new hydroponic culture system and confirm that it was working satisfactorily. Secondly, we needed to use this experiment to test Rubisco assay techniques. Lastly, we needed to test the application of the model using a limited number of sampling dates over the leaf life span.

For initial technique development it was decided to use plants of a commercial barley variety subject to contrasting levels of N nutrition, rather than directly attempt detection of postulated genotype differences in Rubisco turnover in perennial ryegrass. It was reasoned that since the model development work was carried out on cereals including barley (Friedrich and Huffaker, 1980; Irving and Robinson, 2006), an appropriate first step would be to reproduce results similar to those of previous authors. The breeding system of commercial barley varieties ensures a high degree of genetic similarity between individuals in a population, meaning that individual seedlings can be used for different time points on a curve and the creation of clonal replicates is not necessary. The effect of the level of N nutrition on Rubisco synthesis and degradation has been studied in rice (Makino et al., 1984a) but the pattern of Rubisco turnover as defined by the Irving & Robinson model has not so far been studied. Barley has comparatively large leaves, and it was felt prudent to establish the methodology for a larger-leaved plant before working with a smaller-leaved plant like perennial ryegrass.

3.2. Materials and Methods

3.2.1. Plant materials and growth conditions

Malt barley (*Hordeum vulgare* L. cv Optic) seeds were weighed to create a seed line of relatively uniform weight and approximately 5 times the required number of seeds were then germinated on a saran net floating on tap water for 8 days. Selected seedlings were then transferred into the hydroponic growth system (Fig. 3.2) in a vented glasshouse under natural sunlight at the Massey University Plant Growth Unit, Palmerston North. The outside mean temperature during the experiment (May 19, 2006 – August 24, 2006) ranged between 3.7˚C – 15.2˚C. The relative humidity ranged between 51.6% – 100% (Source: Meteorological dept., AgResearch, Palmerston North). In the hydroponic system, a continuous flow solution culture was used. The hydroponic system consisted of two 200L tanks each delivering nutrient solution to 10 polypropylene trays of approximately 15L capacity. One tank supplied a high N solution (N+) containing 2 mM NH_4NO_3 while the other supplied low N solution (N-) containing 0.5 mM NH_4NO_3 throughout the experiment. The 20 trays were arranged in two rows of 10 with the nutrient supply from each of the tanks supplying alternate trays in each row, and returning to the same reservoir tank. Continuous aeration was provided by a compressed air line to each tray, and each tray was fitted with a lid drilled with 24 uniformly spaced holes in a 6 x 4 grid pattern. During the experiment, plants were suspended through these holes so that their roots were fully submerged in the nutrient solution. Each plant was secured by a foam rubber plug to hold the stem in an upright position.

The solution used for hydroponic culture contained the following nutrient concentrations: 2 mM NH_4NO_3 (for the N+ treatment) or 0.5 mM NH_4NO_3 (for the N- treatment), 0.6 mM $NaH_2PO_4.H_2O$, 0.6 mM $MgCl_2.H_2O$, 0.3 mM $CaCl_2.H_2O$, 50 µM H_3BO_3, 45 µM Fe-EDTA, 9 µM $MnSO_4.5H_2O$, 0.7 µM $ZnSO_4.7H_2O$, 0.3 µM $CuSO_4.5H_2O$, 0.1 µM $NaMoO_4.2H_2O$, 5 mM MES dissolved in tap water. The solutions with different NH_4NO_3 concentrations were replaced once a week. The pH of the solutions was maintained at 5.5 using 6 M HCl. The trays were moved around every two days to minimise any position effects within the glasshouse.

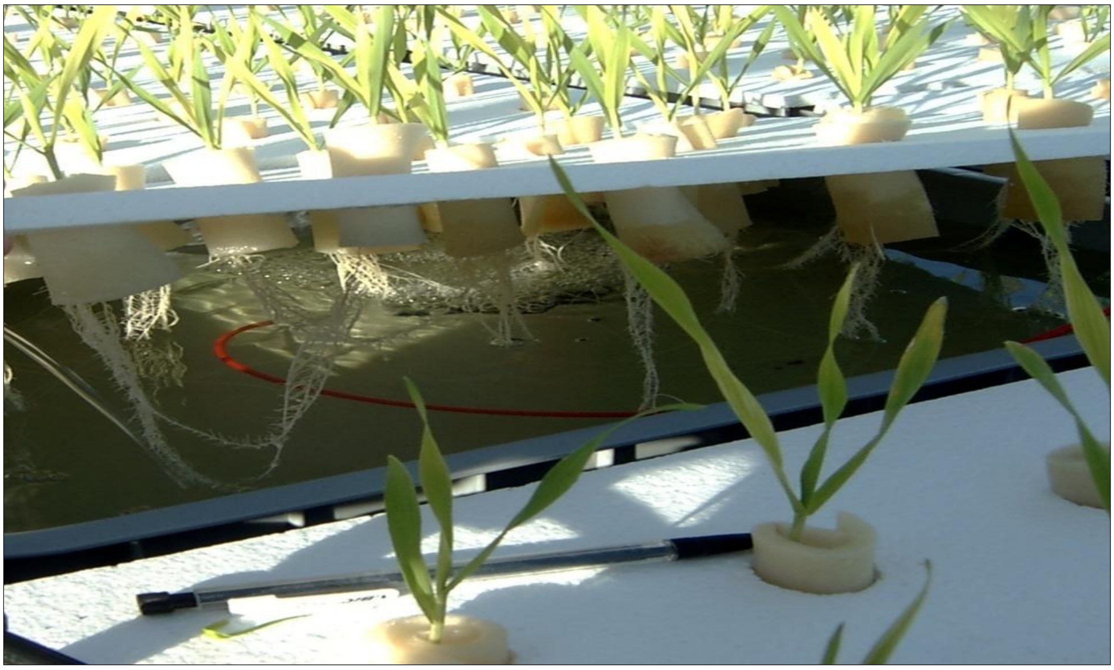


Figure 3.2: Experiment 1 barley plants growing under two N treatments in the hydroponic system. Massey University Plant Growth Unit, Palmerston North, New Zealand, June 2006.

3.2.2. Sampling

Leaf 4, leaf 6, leaf 8, and leaf 10 were sampled for Rubisco analysis. A set of 48 plants (2 trays) was assigned to each leaf category in each N treatment. Leaf appearance was monitored, and the day when most target leaf tips had emerged from the previous leaf sheaths was recorded as the leaf tip emergence day (day 1). For each leaf category and N treatment, the first sampling was 4 days after leaf tip emergence, and thereafter at specified intervals for a period ranging from 31 to 40 days after leaf tip emergence (Appendix 3.1). On each sampling day three plants were randomly picked from the two trays. Randomisation was achieved by use of a random number generator for plants assigned to each leaf category in each N treatment. For each of the three plants, the target leaf was excised at the ligule, weighed and immediately frozen and stored until further analysis.

3.2.3. Determination of total leaf N and Rubisco

Frozen leaf blades were homogenized in 50 mM sodium phosphate buffer (pH 7.5) containing 2 mM iodoacetic acid, 0.8% (v/v) β-mercaptoethanol and 5% (v/v) glycerol at a leaf buffer ratio of 1:5 (g:ml) in a chilled mortar and pestle and acid washed quartz sand to ensure complete trituration.

3.2.3.1. Determination of N

Part of the crude extract was used for total N determination with Nessler's reagent after Kjedhal digestion of samples. To 100 µl of homogenate in a digest tube, 100 µl of digest reagent (60% H_2SO_4 and 40% H_2O_2) was added. The mixture was heated at 140˚C for 30 minutes. A few drops (3 - 4) of H_2O_2 were added and the tubes re-heated for a further 30 minutes at 160˚C. Lastly, 3-5 drops of H_2O_2 were added and the heating repeated at 180˚C for another 30 minutes. This final step was repeated until the digest was clear. The clear sample was diluted with 5ml of water and centrifuged at 10,000 x g for 5 minutes. A 250 µl sample was drawn from the supernatant and further diluted with 2ml of water. Lastly, 50 µl of 10% potassium-sodium +/- tartrate tetrahydrate, 25 µl of 2.5 M sodium hydroxide and 50 µl of Nessler's reagent were added and the mixture was vortexed thoroughly. The mixture was allowed to stand for 10 minutes for colour development. Absorbance was read against digested standards (0, 10, 50, 100, 150 and 200µl of $(NH_4)_2SO_4$ (1000mg N/L)) in a spectrophotometer at 420 nm.

3.2.3.2. Determination of Rubisco

To a 200 µl aliquot of the homogenate, 2 µl of 25% Triton-X was added and thoroughly mixed. The mixture was centrifuged at 10,000 x g for 15 minutes. The temperature in the centrifuge was maintained at 4˚C. ß-Mecarptoethanol (5.3 µl) and 8.6 µl of LDS (25% (w/v)) were added to the supernatant and the mixture boiled for 90 seconds at 100˚C, pulse-centrifuged and then stored in a freezer for separation by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The denatured proteins were resolved at room temperature by SDS-PAGE. The running gel and stacking gel contained 12% and 5% acrylamide, respectively. The samples were re-boiled for 30 seconds before being applied to the gel. After electrophoresis, the gels were subjected to staining with Coomassie Brilliant Blue R-250 (CBB). Gels were stained in 0.25% (w/v) CBB, 20% (w/v) methanol and 20% (w/v) acetic acid at room temperature for 3-5 hours, and de-stained with successive changes of 20% (w/v) methanol and 20 % (w/v) acetic acid until the gel background was clear. The section of the gel corresponding to the large subunit (LSU) of Rubisco (Fig. 3.3) was cut out. The amount of Rubisco was determined spectrophotometrically (at 595 nm) after formamide extraction of Coomassie Brilliant Blue R-250 stained subunit bands separated by SDS-PAGE (Makino et al., 1986). Calibration curves were made using bovine serum albumin standards (Seikagaku Co., Tokyo, Japan).

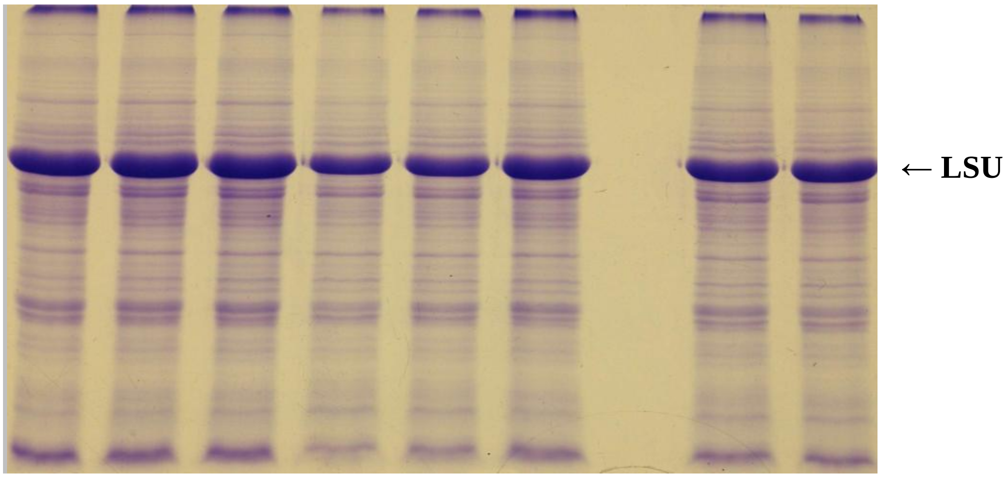


Figure 3.3: Gel showing protein bands separated by SDS-PAGE and stained by Commassie brilliant blue (CBB). LSU represents Rubisco's large subunit

3.2.4. Statistical analysis

Rubisco turnover curve parameters *d*, *f*, and *g* were determined in Sigma plot (version 11) using non-linear regression. Data points for the log-normal curve for the 8th and 10th leaves were the mean of the three replications while the 4th and 6th leaves were based on single leaf measurements. Total N and total fresh weight data for independent replications were analysed in SAS (SAS Institute Inc., Cary, NC, USA). Mean separations between the N treatments for each leaf position were tested using Student's t-test.

3.3. Results

3.3.1. Change in fresh weight and total leaf N

While the pattern varied with leaf position and level of N nutrition, leaves generally attained their greatest fresh weight or N content at around 10 days of age and a gradual decline then followed (Figs. 3.4A, B). Both leaf size (as measured by fresh weight) and total N per leaf were visibly increased in N+ plants (Figs. 3.4A, B) although variability between individual seedlings was such that statistically significant ($P < 0.05$) differences between N treatments were observed only in the 8th and 10th leaves. The tendency to reduced leaf size under reduced N supply was first evident in a faster loss of fresh weight and total N/leaf lamina at leaf 6 (Figs. 3.4A, B) and thereafter successive leaves were progressively more reduced in size at N- nutrition level (Fig. 3.5A). Results for total N/leaf lamina approximately reflected those for leaf fresh weight, with reduction in leaf N content at reduced N supply first seen in results for leaf 6 but with less indication of a progressive decline in later-formed leaves (Fig. 3.5B).

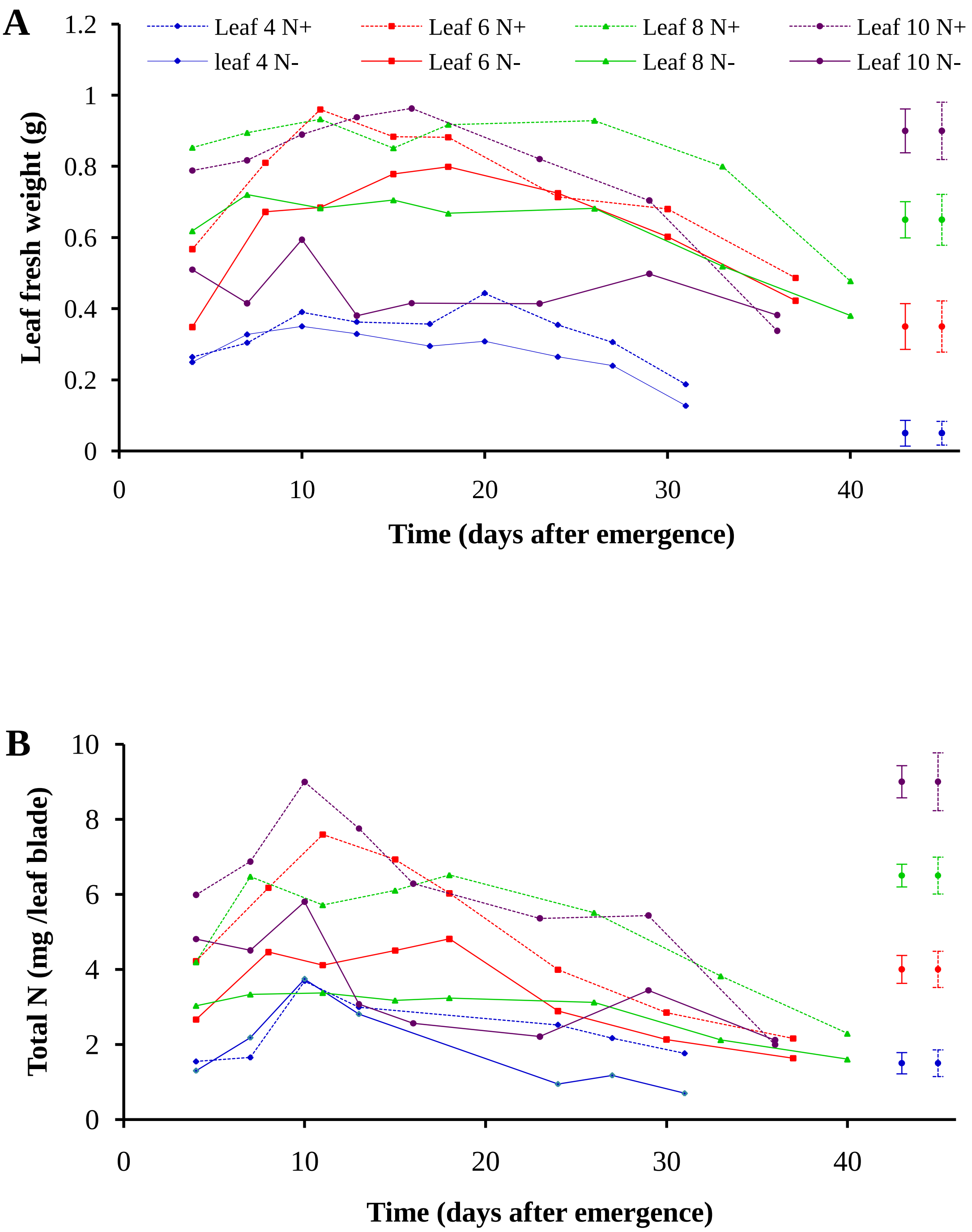
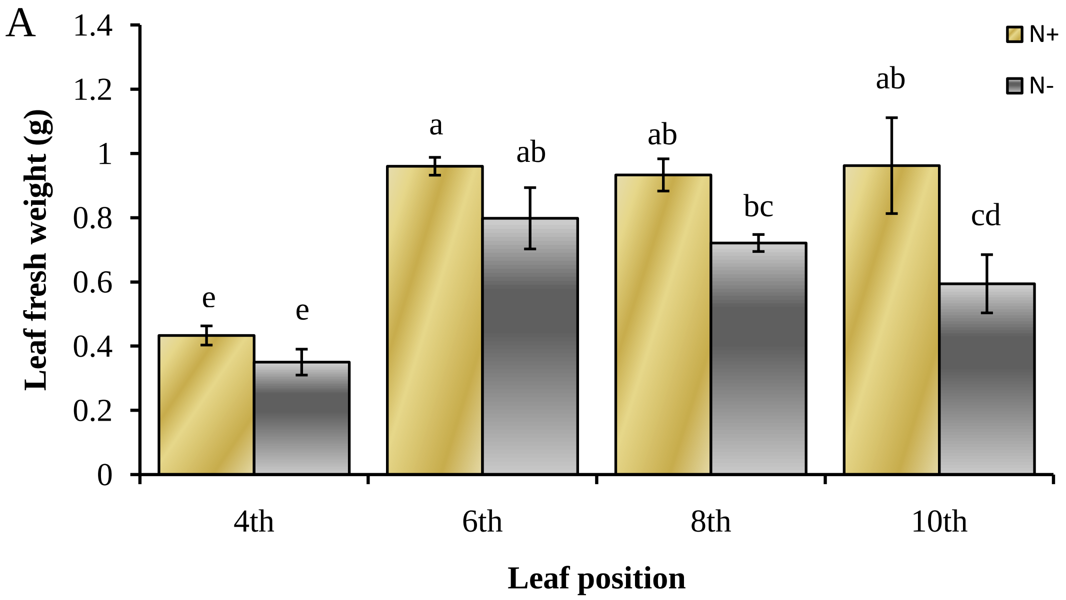


Figure 3.4: Changes in fresh weight (A) and total N (B) of leaves at different positions in barley plants growing at low (N-) and high N (N+) nutrition levels. Points in each graph are means of 3 replicates (n=3). Bars at the right side of the graphs are the mean SE.



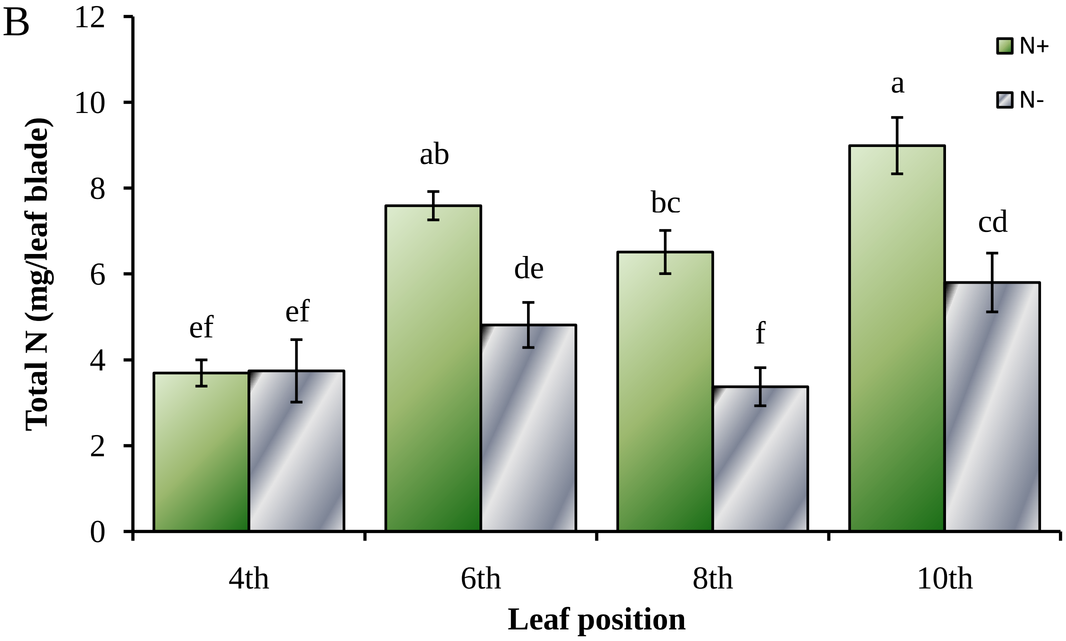


Figure 3.5: The maximum fresh weights (A) and the maximum amount of total N (B) in leaf blades at different positions on the main tiller of barley plants growing at low N (N-) and high N (N+) nutrition levels. Each bar graph represents a mean value ± SE (n=3). Error bars represent 95% confidence interval estimates; bars with different letters represent significantly different means (Student's t-test, $P < 0.05$).

3.3.2. Change in Rubisco content and model-derived Rubisco turnover characteristics

Data points with superimposed log-normal curves presented in Figs. 3.6 & 3.7 showed that leaf Rubisco content was also lower under reduced N supply. Variation in the actual data points around the log-normal curve was observed in all leaves at the different positions on the tiller, and in the two N treatments (Figs. 3.6 & 3.7). Modelled Rubisco peak was consistently lower than the measured Rubisco peak (Figs. 3.6 & 3.7). Late in the lifespan, the N+ 6th and 8th leaves showed higher-than modelled amounts of Rubisco (Figs. 3.6B & 3.7A). There was an increase in the amount of Rubisco from the 4th leaf to the 6th leaf and later-formed leaves tended to have lower Rubisco levels (Fig 3.8). When Rubisco was at its maximum content in the leaves, there was no evidence that reduced N supply altered the ratio of Rubisco N:total leaf N (Table 3.1).

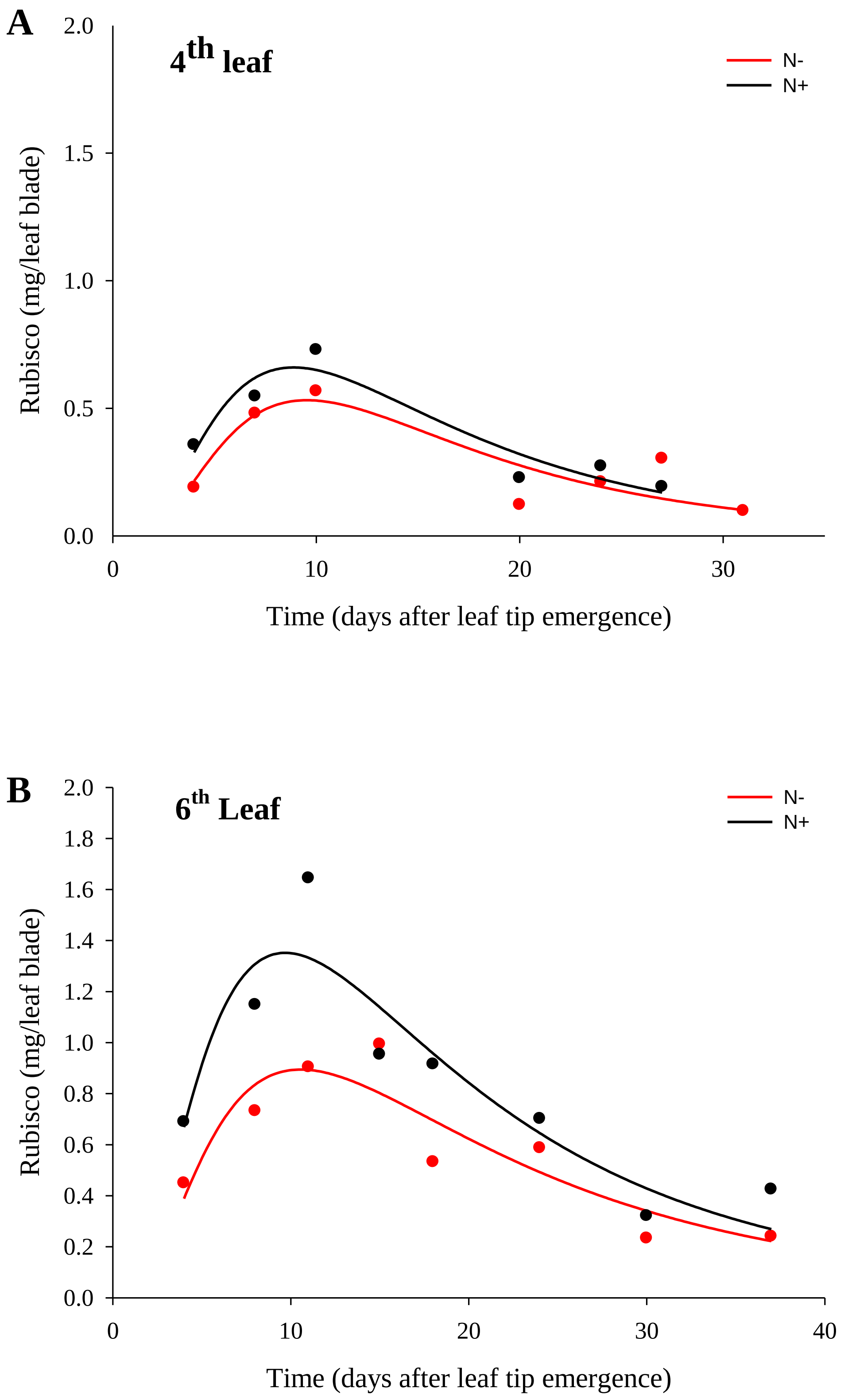


Figure 3.6: Model-derived curves of changes in the content of Rubisco in the 4th (A) and 6th (B) leaf blades of barley plants growing at low N (N-) and high N (N+) nutrition levels. Data points in each graph are based on single leaf measurements.

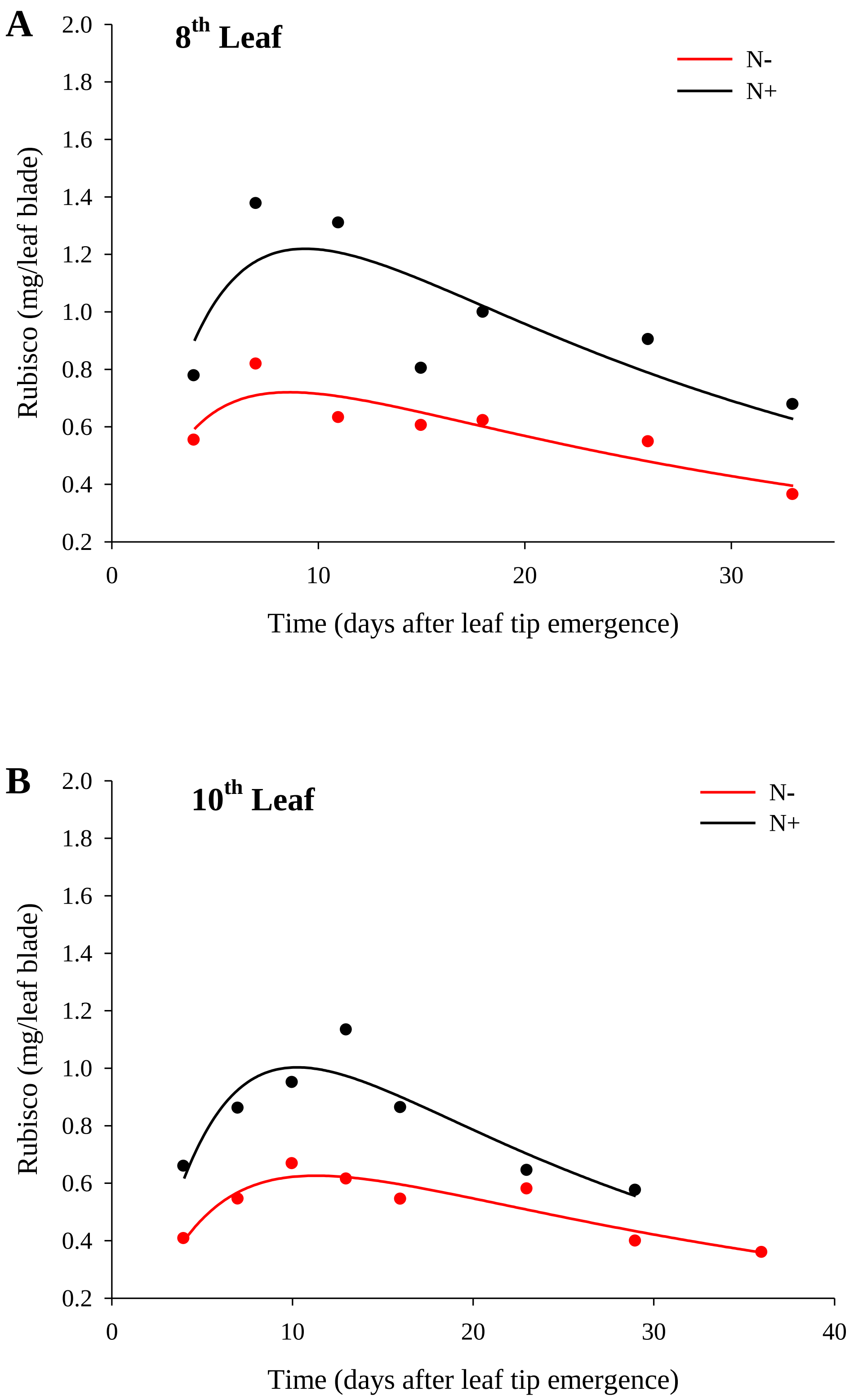


Figure 3.7: Model-derived curves of changes in the content of Rubisco in the 8th (A) and 10th (B) leaf blades of barley plants growing at low N (N-) and high N (N+) nutrition levels. Data points in each graph are means (n=3).

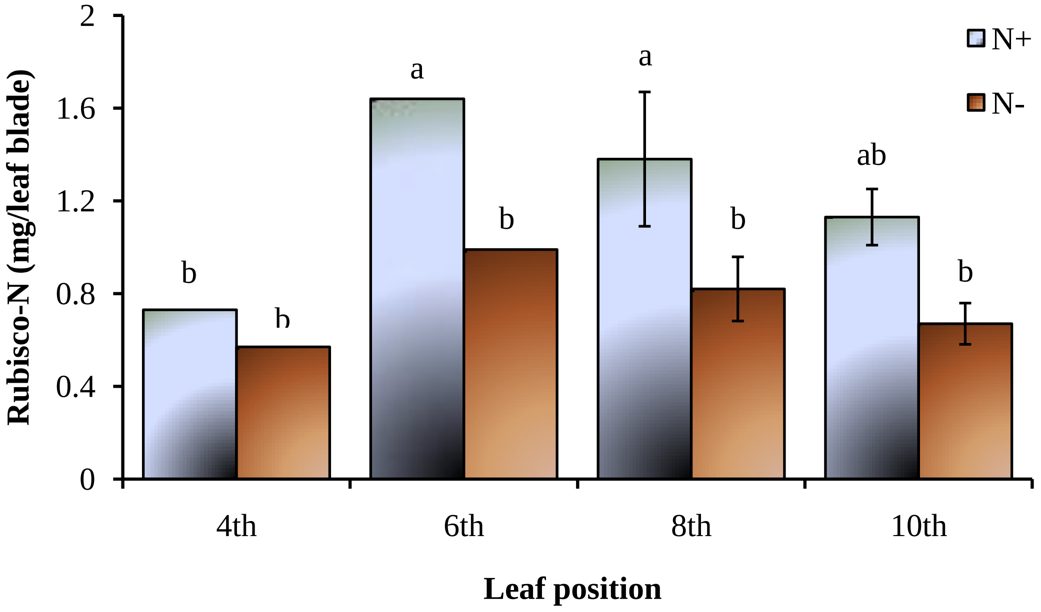


Figure 3.8: The maximum amount of Rubisco N in leaf blades of barley plants growing at low N (N-) and high N (N+) levels of nutrition. Each bar in the 8th and 10th leaves represents a mean value ± SE (n=3). Error bars represent 95% confidence interval of estimates; bars with different letters are significantly different (Student's t-test, $P < 0.05$).

Table 3.1: Ratio of Rubisco to total leaf N when Rubisco had reached a maximum; N- and N+ denote low N treatment and high N treatment, respectively.

Leaf position	N treatment	Maximum Rubisco-N (mg N/leaf)	Ratio of Rubisco to total leaf N (%)
4th	N-	0.57	15
	N+	0.73	24
6th	N-	0.99	22
	N+	1.64	22
8th	N-	0.82	25
	N+	1.38	21
10th	N-	0.67	12
	N+	1.13	15

Values of Rubisco concentration in the 8th and 10th leaves are means (n=3) while in the 4th and 6th leaves, they are single leaf measurements.

All non-linear regressions to determine Rubisco turnover model parameters were statistically significant at $P < 0.10$, and most were significant at $P < 0.05$ or $P < 0.01$

(Table 3.2). Rubisco peaks (*d*) were consistently higher in the N+ leaves than in the N- leaves. The time of the peak (*g*) and curve width (*f*) did not differ significantly between the N+ and N- plants at any of the leaf positions tested (Table 3.2). There was no evidence of the Rubisco turnover model fitting better for leaves at a particular position on a tiller or under a particular N regime (Table 3.2).

Table 3.2: Irving and Robinson (2006) Rubisco turnover model parameters for leaves of barley plants grown hydroponically under low (N-) and high (N+) N nutrition levels. Values were estimated from log-normal regression in Sigma plot. Values are means (n=3) except for Rubisco in the 4th and 6th leaves which were based on single leaf measurements.

Leaf position /N treatment	*d*	*f*	*g*	R^2	*P* value
4th					
N-	0.57 ± 0.01	0.62 ± 0.01	9.99 ± 0.07	0.99	<0.01
N+	0.70 ± 0.11	0.65 ± 0.11	9.35 ± 0.91	0.82	0.08
Significance	ns	ns	ns		
6th					
N-	0.93 ± 0.08	0.73 ± 0.09	11.1 ± 0.10	0.89	0.01
N+	1.35 ± 0.13	0.75 ± 0.10	9.67 ± 0.95	0.84	0.01
Significance	*	ns	ns		
8th					
N-	0.72 ± 0.05	1.23 ± 0.24	8.61 ± 1.32	0.75	0.06
N+	1.34 ± 0.09	0.98 ± 0.11	10.1 ± 0.81	0.89	0.01
Significance	*	ns	ns		
10th					
N-	0.63 ± 0.03	1.10 ± 0.11	11.3 ± 0.80	0.87	0.01
N+	1.00 ± 0.06	0.95 ± 0.12	10.3 ± 0.85	0.83	0.03
Significance	*	ns	ns		

d, maximum Rubisco concentration (mg/leaf blade); *g*, time in days after leaf emergence when *d* occurs; *f*, a measure of curve width. For *d*, *f* and *g*, values are regression estimates ± SE. Significance (*; $P < 0.05$, ns; non-significant). Differences in curve parameters between N treatments were tested by Student’s t-test.

3.3.3. Decline in leaf N with age

Since both total N and Rubisco content decreased during senescence (Fig. 3.4B, Fig. 3.7), it was of interest to quantify the total N and Rubisco N remaining late in the leaf life span (although while leaves were still green) as a proportion of that found in younger leaves (Table 3.3). For the majority of the sampling dates considered the amount of total N remaining in the leaf was greater than that of Rubisco-N. There was no indication that the proportion of the maximum amount of Rubisco-N lost at specified leaf ages was higher in the N- than in the N+ leaves.

Table 3.3: The amount of total N and Rubisco-N retained in older leaves expressed as a percentage of the maximum measured in younger leaves at approximately 10 days of age. Quantities were calculated at a specified age (*) in each leaf category.

			N remaining (%)	
Leaf position	N level	Leaf age (days)*	Total N	Rubisco-N
4th	N-	27	31.3	52.6
	N+	27	58.8	26.1
6th	N-	37	33.9	24.2
	N+	37	28.5	25.6
8th	N-	33	62.9	43.9
	N+	33	59.0	49.3
10th	N-	37	59.3	59.7
	N+	37	61.0	50.4

Values of total N are means (n=3). Values of Rubisco concentration in the 8th and 10th leaves are means (n=3) while in the 4th and 6th leaves, they are single leaf measurements.

3.4. Discussion

Whole leaves and not sections of leaves were sampled in this experiment since this was standard procedure for data (Friedrich and Huffaker, 1980; Mae et al., 1983; Mae et al., 1989) used in model development. Also, numerous other studies have used whole leaves to evaluate Rubisco content during the leaf's lifespan (Makino et al., 1984a; Suzuki et al., 2001; Takeuchi et al., 2002).

The increase in leaf size and leaf N content with the increase in N supply paralleled the increase in leaf Rubisco content. This was consistent with previous studies in rice which have shown a correlation of the amount of Rubisco synthesized with N availability (Imai et al., 2005) and with N content in leaves (Mae et al., 1983; Makino et al., 1984a). The greater fresh weight in N+ plants was first evident in the 6th leaf. This suggests that at the 4th leaf stage, exogenous N supply was in excess, and therefore there was no growth benefit from the N+ over the N- treatment. By the time the 6th leaf started forming, plant size had increased, and so did the N demand. This resulted in reduced size of the 6th and successive leaves in the N- plants.

The approximate time of full leaf expansion and maximum leaf N (10 d, Fig 3.4) was within the modelled time range (9.35 – 11.3 d) for peak Rubisco content (Table 3.3). During senescence the decrease of Rubisco in proportion to the peak content was more pronounced than the decrease in total N (Table 3.2). This result is consistent with the observations that Rubisco content decreases faster than leaf N or other proteins during senescence (Albuquerque et al., 2001; Mae et al., 1983; Makino et al., 1984b; Nakano et al., 1995). These results also support suggestions that Rubisco acts as an easily remobilisable N source in the leaf, a role that has been widely suggested/discussed by a number of researchers (Friedrich and Huffaker, 1980; Mae et al., 1983; Makino et al., 1984a; Racusen and Foote, 1965).

Although two of the fitted curves were of marginal statistical significance (P = 0.08 and P = 0.06 for the N+ 4th leaves and the N- 8th leaves, respectively, Table 3.2) and there was noticeable variation around the curves (Fig. 3.6 & 3.7), the clear statistical significance of the other six fitted curves and the pattern of data points of Rubisco content over time gave an indication that Rubisco declined more quickly immediately after full leaf expansion, and then more slowly thereafter. This finding supports the exponential decay characteristics of Rubisco in senescence as described by the model (Irving and Robinson, 2006) and related studies that have reported a similar pattern in the decline of Rubisco content (Chiba et al., 2003; Friedrich and Huffaker, 1980; Mae et al., 1983; Mae et al., 1984) as well as in *rbc* transcript abundances (Crafts-Brandner et al., 1998; Ishizuka et al., 2004; Suzuki et al., 2001). Plant to plant variation as observed in total N and leaf fresh weights during leaf development (see Fig. 3.4) may have contributed to the variation around the log-normal curves. Variation of the points around the curves raises the question of the model's systematic lack of fit. However, it was decided that this question would be better investigated in one of the later experiments with a larger data set and hopefully with reduced plant-to-plant variability in leaf size.

Rubisco degradation has been found to follow the chloroplastic pathway or autophagic pathway or both, and subject to enzymatic activity. Physiological studies have identified some proteases that can degrade Rubisco within the chloroplast or in the vacuole (Feller et al., 2008a; Kato et al., 2005; Kato et al., 2004; Kokubun et al., 2002; Roberts et al., 2003) although the regulation of these proteases is still unknown. Other studies have indicated non-enzymatic Rubisco degradation initiated by ROS under photo-oxidative conditions (Martinez et al., 2008). It was beyond the scope of this study to resolve the mechanism or pathway involved in Rubisco degradation. However, data from this study indicated a Rubisco time course different from a typical enzyme-mediated degradation model like that described by Chiba et al (2003) for other proteins (e.g. LHCII) or chlorophyll.

An increase in N supply increased Rubisco peak but had no measurable effect on the time of the peak and the curve width (Table 3.3). These results were in agreement with the previous finding in rice plants (Irving and Robinson, 2006; Makino et al., 1984a). However, differences have been observed in Rubisco turnover characteristics that suggest that the N nutrition status of a plant may influence *g* and *f*. For example, Imai et al. (2005) reported a delay in both the time taken for Rubisco to peak (i.e. a later *g*) and a delay in the decline of Rubisco (i.e. a wider *f*) with an increase in N supply. Also, a number of studies have reported accelerated senescence to increase N recycling and remobilisation under N depletion conditions (Lattanzi et al., 2005; Schulze et al., 1994; Terce-Laforgue et al., 2004). Another previous study has shown that N nutrition during leaf development greatly influences both the lifespan and the rate of senescence (Makino et al., 1984b). It is argued that by recycling the endogenous nutrients from senescing leaves, plants can better support the growth of younger leaves and reproductive organs under nutrient-limiting nutrition (Diaz et al., 2008; Hirel and Gallais, 2006). There is need for further studies to show if differences in *f* and *g* are genotype-based, or if the model is sensitive enough to detect these differences in plants growing different N nutrition levels.

The results in Fig. 3.4B and Fig. 3.6 showed that both total N and Rubisco started to decline as early as 10 days after leaf emergence. This means that loss of N and Rubisco and consequently reduction in photosynthetic function occurred early in the life of the leaf before any visible signs of senescence. It has been reported that leaves of many annual crops become predisposed to senesce near the time of maximum leaf size (Crafts-Brandner et al., 1996; Mae et al., 1983). The subsequent rate of senescence and remobilisation of leaf N depends on many factors including source:sink ratio, growth regulator metabolism and plant nutrition (Crafts-Brandner et al., 1996; Feller and Fischer, 1994; Masclaux et al., 2000; Smart, 1994). In relation to this, it would be of interest to see the role played by the plant genotype in the Rubisco turnover characteristics. The model not only predicts the contribution of synthesis/degradation at any stage of the leaf development, but also period of functional photosynthesis (indicated by the measure of *f*). It is then economically significant to the plant if the curve width does not match the lifespan of the leaf, in which case there is potential inefficiency since the energy cost of Rubisco synthesis is not fully compensated for in photosynthesis. As stated in the hypothesis in the introduction to this chapter, such a Rubisco turnover characteristic would be a cost to the plant and lead to reduced yields.

3.5. Conclusions

Technique development: The hydroponic culture unit built expressly for this study performed to specification and the quantification of leaf Rubisco and modelling of the time course of leaf Rubisco turnover was successful although there was some variation in the data points around the curve. Eight to nine data points were sufficient to fit the log-normal curve. However, there was need for increased sampling intensity (≤ 4 days) around the time of peak leaf Rubisco content for a more accurate prediction of *d*. There is every reason to expect that a follow up experiment using perennial ryegrass would be successful.

Nitrogen turnover: Reduced N supply in the malting barley cultivar ‘Optic’ reduced leaf size (as measured by leaf fresh weight) for measured leaves appearing after leaf 4, and hence also reduced the amount total N and Rubisco per leaf blade for those leaves. Reduced N supply did not measurably alter the time when peak Rubisco content occurred, or the curve width.

Leaf fresh weight and total N per leaf in malt barley peaked about 10 days after leaf emergence. This is consistent with the Irving and Robinson (2006) model which predicted the amount of Rubisco per leaf reaching its peak very early in the life of the leaf and then declining through the remaining life of the leaf. These results suggest that substantive loss of Rubisco in leaves precedes the visible senescence symptoms, and that some Rubisco remains at least until senescence is visibly advanced. Assuming the same is true for perennial ryegrass experimental investigation of genetic variation in Rubisco turnover and the related cost/benefit for potential production would be of interest.

CHAPTER 4

Assessment of genotypic variation of Rubisco turnover in a sample of the Grasslands II perennial ryegrass mapping population

4.1. Introduction

In Chapter 3 we observed that Rubisco turnover determination by the model detected N treatment differences in Rubisco peak (*d*) but not in the time of the peak (*g*) or curve width (*f*). This result in barley reproduced the turnover pattern of Irving and Robinson (2006) indicating that technique development was successful and it was now feasible for Rubisco quantification procedures and model to be used for evaluation of Rubisco turnover in perennial ryegrass. Rubisco synthesis and degradation as predicted by the model covers a large part of the leaf's life. Since photosynthetic capacity is closely associated with leaf N concentration (most of which is found in Rubisco), the variability of Rubisco turnover characteristics in relation to plant performance will be a point for follow-up in this and subsequent chapters.

This chapter moves on to use the model to analyse Rubisco turnover characteristics in a small sample of a perennial ryegrass mapping population. The focus is to confirm the existence of genotypic variation of this physiological process as a basis for a more detailed analysis of the regulation of Rubisco turnover by a quantitative genetic approach. The availability of genetic variation is the basis of breeding new cultivars (McKersie, 1997) and QTL analysis (Turner et al., 2006). For perennial ryegrass, its out-crossing and highly self-incompatible nature (Thorogood et al., 2002) means that there is usually high heterogeneity available to provide genetic variation in a wide range of traits. Genetic variation in Rubisco turnover is of interest in perennial ryegrass breeding because of its possible correlation with forage yield.

4.2. Objectives

This experiment was set up with the following objectives:

1. To use the dynamic model to test for the existence of genetic variation in Rubisco turnover patterns in a sub-sample of the mapping population.

2. To confirm that there was evidence of a relationship between specific patterns of Rubisco turnover and forage yield characteristics in a smaller pilot study before investing resources in a larger study.

4.3. Materials and methods

4.3.1. Plant material

The mapping population (Grasslands II; n=200) used was that previously described by Crush et al (2007). The F_1 population plants were produced from a cross between two diploid ryegrass plants in summer 2001-2002. The pollen parent was from the perennial ryegrass cultivar Grasslands Samson and the seed parent was from the hybrid ryegrass (*L. boucheanum syn. L. hydridum*) cultivar Grasslands Impact. The cultivar Impact differs from cultivar Samson in shoot morphology, characterised by a higher number of smaller tillers per plant on average, and also a tendency to longer leaf sheaths and narrower leaves than the cultivar Samson (Sartie et al., 2009). The Samson parent was infected with the *Neotyphodium lolii* strain AR6 endophyte, and the Impact parent was infected with the wild-type *N. lolii.*

The aim was to include a variety of plant types based on morphology and productivity. Therefore, a principal component analysis (PCA) was performed on herbage yield trait data collected on the mapping population in autumn 2003 (Sartie, 2007). Based on the PCA results (see Appendix 4.1), sixteen F_1 plant genotypes were selected for this experiment. On the basis of PC1 that accounted for 30% of the variation in the data, three plant genotypes (62, 63, and 181) were selected for their higher tiller population and productivity index (PI), and leaf characteristics including longer leaf appearance intervals and longer leaf elongation duration. Four genotypes (68, 111, 134 and 149) were selected for having the opposite behaviour as measured by PC1 scores (Appendix

4.1). Similarly, plants with contrasting scores for PC3 (explaining about 15% of data variation) were selected for inclusion in this experiment. Plant dry weight (DW) and leaf elongation rate (LER) had the largest contribution to PC3 and genotypes 1, 7, 51, 59 and 70 were selected based these characteristics. Genotypes 29, 67, 141, and 165 were selected for having the opposite behaviour (Appendix 4.1).

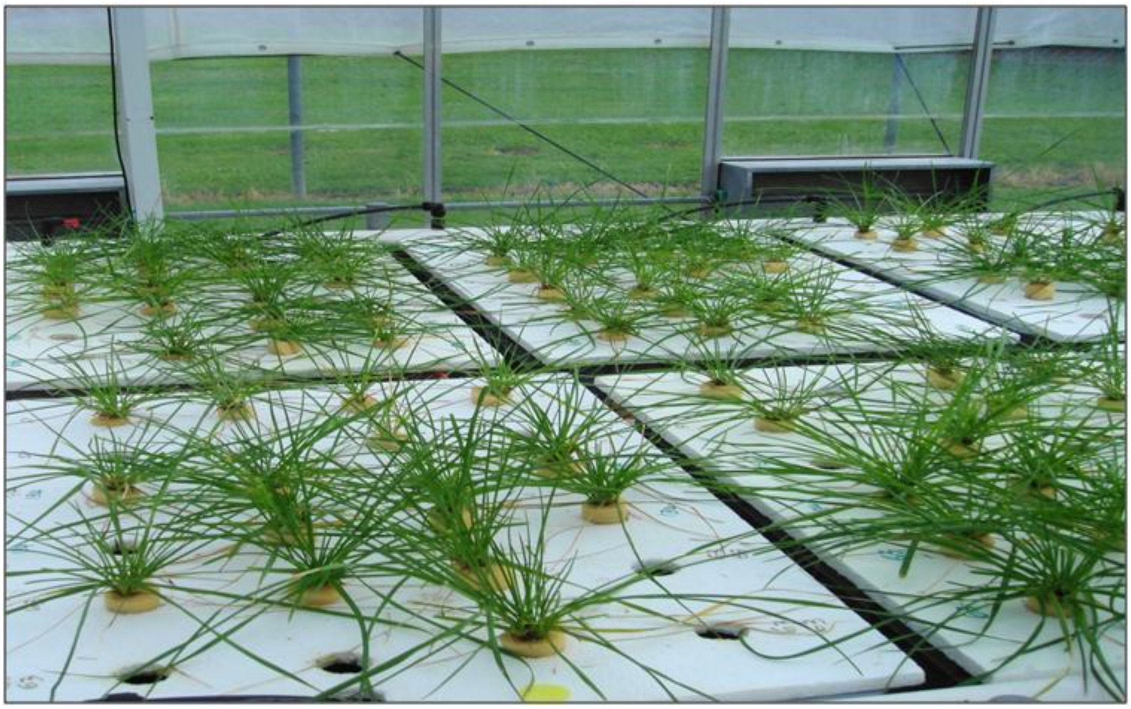


Figure 4.1: Grasslands II mapping population plants growing in the hydroponic system. Massey University Plant Growth Unit, Palmerston North, New Zealand. Experiment 2, November 2006.

4.3.2. Growth conditions

The sixteen genotypes and their parental plants (Grasslands Samson & Grasslands Impact) were grown in a glasshouse (between October 6 2006 and December 27 2006) at the Massey University's Plant Growth Unit, Palmerston North (Fig. 4.1). For each plant genotype, sixteen uniform tillers were obtained from a subdivision of four plants. The tillers were trimmed to a uniform height and then transplanted into a specially built hydroponics growth system described in Chapter 3 (Section 3.2.1). The glasshouse was vented, and the temperature during the experiment ranged between 15° C and 25° C.

The nutrient solution was similar to that described in Chapter 3 (Section 3.2.1) except that the NH_4NO_3 concentration used in this experiment was 0.3 mM NH_4NO_3. The nutrient solution was renewed weekly and its pH adjusted to 5.5 with 1 M HCl. The trays were moved around weekly to minimise any position effects within the glasshouse.

4.3.3. Sampling

For each genotype, when plant size had reached about 10 tillers per plant, 1 – 2 young, medium sized tillers per plant were identified and marked. The marked tillers were then trimmed to a uniform height and observed daily to identify the date of the 6th leaf tip emergence. On days 4, 7, 11, 15, 22, 29, 36 and 41 after the emergence of the 6th leaf tip, leaf blades were excised from the plant at the ligule, weighed and immediately frozen and stored until analysis. In each sampling, 3 leaves were harvested from 3 randomly selected plants for each genotype.

4.3.4. Determination of Rubisco

Rubisco was determined using the procedure outlined in Section 3.2.3.2.

4.3.5. Morphological traits

In this study, morphological traits were not measured in order to focus on determination of the change in level of Rubisco with leaf age. Data on morphology and plant growth used in PCA analysis with Rubisco data from this study were genotype means from a previous experiment conducted in the autumn 2003 (Sartie, 2007).

4.3.6. Statistical analysis

Rubisco turnover for independent leaf samples of each genotype were treated as independent replicates, and an analysis of variance (ANOVA) to determine genotype differences for curve parameters was performed in SAS (SAS Institute, Cary, North Carolina) using a completely randomised design model. Curve parameters averaged across three replicates for each genotype were combined with morphology and growth data obtained in a previous experiment (Sartie, 2007). Correlation coefficients for all traits in the combined data were determined and the data further analysed by PCA using the covariance matrix method of Minitab version 10.51 (Minitab Inc., State College, Pennsylvania), to test for associations between Rubisco turnover parameters and genotype morphology traits. Broad sense heritability (H_b) was calculated using ANOVA mean squares as variances (Appendix 4.3) as;

$$H_b = \delta^2_g/\delta^2_g + \delta^2_\varepsilon$$

Where, δ^2_g = genotypic component of variance, δ^2_ε = residual variance of genotypes (Liu, 1998).

4.4. Results

4.4.1. Genotypic variation in Rubisco turnover

There was a wide range of values for curve parameters with the progeny showing transgressive segregation in both directions (Table 4.1). An ANOVA indicated highly significant ($P < 0.01$) differences among genotypes for all curve parameters.

Table 4.1: Genotype effects on Rubisco turnover curve parameters for 16 plant genotypes of the Grasslands II perennial ryegrass mapping population and the two parent plants of the population. Data are the range of genotype means and their SEM: *d* = curve height (mg Rubisco/leaf); *f* = measure of curve width; *g* = time of curve peak (days).

Curve Parameter	Range	P1	P2	SEM	*P* value	Broad sense heritability
d	0.12 – 0.43	0.24	0.40	0.01	<0.01	0.97
f	0.81 – 1.40	0.86	1.03	0.07	<0.01	0.68
g	7.96 – 16.32	8.00	13.5	0.76	<0.01	0.73

P1 and P2 indicate values for the two parent plants, from Grasslands Samson and Grasslands Impact, respectively (see ANOVA table in Appendix 4.3).

Progeny leaves took between 7.96 to 16.32 days to attain the maximum Rubisco content (expressed on per leaf basis). Maximum Rubisco content varied from 0.12 mg per leaf to 0.43 mg per leaf (Table 4.1). The curve width, which measures Rubisco retention, varied from 0.81 to 1.40. Leaves of the plant from Grasslands Impact had a significantly ($P < 0.01$) higher and later Rubisco peak than the plant from Grassland Samson. The mapping population parents did not differ significantly in curve width. Broad sense heritability estimates were high (Table 4.1).

4.4.2. Trait correlation analysis

Correlation analysis of the various traits of the 18 tested genotypes (Table 4.2) showed significant (negative and positive) correlations between morphological and growth parameters as observed in the previous experiment (Sartie, 2007). In relation to Rubisco turnover parameters, a significant ($P < 0.05$) negative correlation was observed between d and TW (r = -0.58). There were also near-significant correlations between d and TN ($P = 0.097$) and f and LED ($P = 0.056$) (Table 4.2). For the rest of the combinations of Rubisco turnover and morphological and plant growth traits, correlations were non-significant (Table 4.2). No correlations were observed among Rubisco turnover parameters themselves as their P values were always greater than 0.27. However, the direction of two relationships namely, the negative correlation of f with d (r = -0.27) and the positive correlation of g with f (r = 0.27) were noted.

Table 4.2: Correlation of Rubisco turnover and morphological and plant growth parameters for the two parent plants and 16 F_1 progeny of the Grasslands II perennial ryegrass mapping population: Mean values for morphological traits were taken from the autumn data of Sartie (2007). Alg, ligule appearance interval (days/leaf); Alf, leaf appearance interval (days/leaf); LER, leaf elongation rate (cm/day); LED, leaf elongation duration (days); LL, leaf lamina length (cm); TN, tiller number; TW, tiller weight (g); DW, herbage dry weight (g); *d*, curve height (mg Rubisco/leaf); *f*, measure of curve width; *g*, time of curve peak (days).

Trait	Alg	Alf	LER	LED	LL	TN	TW	DW	*d*	*f*
Alf	0.88**									
LER	-0.40	-0.62**								
LED	0.89**	0.94**	-0.56*							
LL	0.22	0.15	0.55*	0.13						
TN	0.32	0.33	-0.43†	0.29	-0.58*					
TW	-0.43†	-0.24	0.26	-0.25	0.25*	-0.80**				
DW	-0.02	0.18	-0.27	0.17	-0.10	0.46†	0.15			
d	0.20	0.14	-0.13	0.09	-0.27	0.40†	-0.58*	-0.27		
f	0.22	0.36	-0.27	0.46†	0.02	-0.01	0.09	0.15	-0.27	
g	0.12	0.03	0.02	0.01	0.19	-0.14	-0.06	-0.33	0.02	0.27

†,*, ** Significant at $P < 0.1$, $P < 0.05$ and $P < 0.01$, respectively

4.4.3. Principal component analysis of Rubisco turnover, plant morphology and growth

The first five principal components (PCs) explaining 91% of the variation in the data are presented in Table 4.3. PC1 explaining 35.6% of the data variation separated plants based mainly on morphological characteristics as indicated by their large coefficients (Table 4.3). The separation was based on a contrast of Alg, Alf, LED and TN with LER and TW (Table 4.3). Parent plants were clearly separated by PC1 (Appendix 4.3). Also, the contrasting behaviour of the progeny plants based on PC1 for plant material selection (see Section 4.1.1) was maintained by PC1 in this study (Appendix 4.3).

Table 4.3: Principal component structure for the first five PCs of eleven variables of the two parent plants and 16 F_1 progeny of the Grasslands II mapping population: *d*, curve height (mg Rubisco/leaf); *f*, measure of curve width; *g*, time of curve peak (days); Alg, ligule appearance interval (days/leaf); Alf, leaf appearance interval (days/leaf); LER, leaf elongation rate (cm/day); LED, leaf elongation duration (days); LL, leaf lamina length (cm); TN, tiller number; TW, tiller weight (g); DW, herbage dry weight (g).

Parameter	PC1	PC2	PC3	PC4	PC5
d	-0.18	0.33	-0.42	-0.17	0.09
f	-0.17	-0.32	0.17	0.61	-0.01
g	0.01	-0.20	-0.42	0.57	-0.32
Alg	-0.41	-0.24	-0.22	-0.23	-0.06
Alf	-0.44	-0.28	-0.02	-0.17	0.09
LER	0.37	-0.07	-0.27	-0.13	-0.53
LED	-0.43	-0.30	-0.02	-0.06	0.03
LL	0.14	-0.50	-0.18	-0.39	-0.29
TN	-0.35	0.37	0.11	0.03	-0.45
TW	0.31	-0.37	0.29	-0.08	0.21
DW	-0.12	0.01	0.61	-0.11	-0.25
Variation (%)	35.60	22.20	15.90	10.10	7.40

PC2 (22.2% of data variation) had a stronger influence of Rubisco turnover components with a link to morphological measures (Table 4.3). The coefficients indicated that plants with a high and earlier *d* tended to have a low *f*, LED, LL and TW and a high TN (Table 4.3). PC3 (16% of the variation) had a strong DW contribution arising from a high TW (Table 4.3). These plants tended to have a lower and earlier Rubisco peak with a sustained Rubisco retention (Table 4.3). There was a distinct separation of the parent plants by PC3 (Appendix 4.3). PC4 (10% of data variation) had very high coefficients for *f* (0.61) and *g* (0.51) and therefore associated a very late Rubisco peak and an over-extended Rubisco retention (Table 4.3). Plants exhibiting such Rubisco turnover characteristic had very short leaves. PC5 accounted for only 7% of the data variation. However, it is included here because it is the first PC to indicate a strong link between increased LER and increased DW as shown by similar signs of their coefficients (Table 4.3). In addition to increased LER which resulted in increased LL, increased TN also enhanced DW (Table 4.3). TW had a moderate contrasting contribution (coefficient = 0.21) (Table 4.3). These plants were characterised by an earlier Rubisco peak (Table 4.3).

4.5. Discussion

There was high genotypic variation for all the three Rubisco turnover parameters suggesting that Grasslands II mapping population could be used for genetic analysis of Rubisco turnover. Conventional correlation analysis (Table 4.2) reflected the strong correlations among morphological variables from the Sartie (2007) study. Increased TW and decreased TN were associated with a high *d* and a high *f* with a high LED but there was little evidence of a link between Rubisco turnover and plant productivity. However, PCA identified links (albeit weakly) between Rubisco turnover and previously measured morphological traits of the selected genotypes (Table 4.3).

PCA details showed that both PC1 and PC2 displayed strong size/density compensation (SDC), a phenomenon that has been described in both undefoliated (Lonsdale and Watkinson, 1982) and defoliated (Hernández Garay et al., 1999; Matthew et al., 1995) grass swards. These PCs also indicated two alternative strategies by which a grass plant may form a heavier tiller or a longer leaf. In PC1, plants achieved heavier and/or longer leaves through a higher LER, while in PC2 increased LED was the contributing factor

(Table 4.3). The parent plants were separated in scores for PC1 reflecting their contrasting morphology as earlier described (Crush et al., 2007; Sartie, 2007).

It has been shown in previous studies that an increase in herbage yield of pastures can result from either an increase in TN or TW or both (Bircham and Hodgson, 1983; Grant et al., 1983; Volenec and Nelson, 1983). The increase in TW and TN shown in PC1 and PC2, respectively, did not result in an increase in herbage yield. The decreased LED and Alf was a indication that the interval between the initiation of successive leaves was reduced (Robson, 1967; Skinner and Nelson, 1994) reflecting a fast leaf appearance rate for these plants. While this is expected to lead to the production of a high number of small tillers (Lemaire and Chapman, 1996), this was not so in PC1, possibly because of reduced site filling (Davies, 1974). In PC2, reduced plant yield despite the high tiller population was possibly due to the “trade-off” relationship between photosynthetic capacity and N remobilisation during leaf senescence. The association of a low Alf and short LER is a reflection of reduced leaf life span and therefore a high leaf tissue turnover (Mazzanti and Lemaire, 1994). In this case therefore, the leaves senesced very quickly and Rubisco was degraded very rapidly. A rapid loss of Rubisco would be expected in such plants because of the susceptibility of Rubisco to degradation following the onset of senescence (Feller and Fischer, 1994; Grover, 1993; Mae et al., 1993). Although the degradation products were exported to sink organs, this was at the expense of photosynthetic capacity because a close relation between the decline in Rubisco activity and content and photosynthetic function has been demonstrated (Jiang et al., 1999; Mae et al., 1993). Accordingly, the result was a marked reduction in plant size as shown by reduced LL and TW. This also confirms our hypothetical mechanism that rapid protein turnover would cost the plant by reducing the time of leaf capture of solar energy and its conversion to plant-usable assimilates during photosynthesis.

PC3 associated an earlier and lower Rubisco peak with an extended Rubisco retention time (Table 4.3). This Rubisco turnover pattern favoured yield (high DW) which was contributed to by the large tiller size (high TW) in the plants. This is the first PC to have a sizeable coefficient for DW, and DW in this PC has the largest coefficient (Table 4.3). It was also in this PC that the significant negative correlation of TW with *d* (Table 4.2) was reflected. Comparing the Rubisco turnover characteristics displayed in the first three PCs, it appears that an extended Rubisco retention (even under conditions of low leaf Rubisco peak) is what favoured high yield in these plants. Yield enhancement from a lower *d* and a wider *f* Rubisco pattern was not envisaged at the beginning of Experiment 1. These results suggest that a wider *f* (extended duration over which photosynthesis is maintained) is critical for yield while *d* is less critical. These results correlate well with other results from previous studies which have shown a correlation between sustained photosynthesis and crop yield. Osaki et al (1993) for example, found that high yield in rice, winter wheat, maize, soybean and potato was achieved by maintaining high foliar concentrations of Rubisco (and chlorophyll) until late ripening stage. In a study involving two rice cultivars, it was shown that the high yielding cultivar experienced a slower decrease in light saturated photosynthesis and a slower loss of proteins than the standard yielding cultivar (Jiang et al., 1999). Further analysis of these cultivars revealed that the partitioning of N to ears was lower (tantamount to sustained Rubisco retention in this study) in the high yielding cultivar and this proved effective in maintaining a high rate of photosynthesis during ripening (Ookawa et al., 2003). Another study involving barley forage varieties differing in Rubisco content in expanded leaves also found that productivity of the low leaf Rubisco variety was enhanced by maintenance of a high level of photosynthetic components (including Rubisco) in the phases critical for grain filling (Simova-Stoilova et al., 2001). Prolonged Rubisco retention is useful only if it is accompanied by leaf longevity. Previous studies in maize have indicated that leaf longevity, leading to extension of leaf metabolic activity improved the ratio between the assimilate supply from source leaves and demand in sink leaves, and was the main factor in yield increase (Ma and Dwyer, 1998; Tollenaar, 1991). Even though longevity was not evaluated in the Sartie (2007) study, there is every indication that extended leaf life span and Rubisco were coupled, otherwise a shorter life span would have resulted in the loss of Rubisco via senescence and reduced plant yield.

PC scores revealed that PC4 largely arose from a very long Rubisco retention time in one particular genotype (165) and therefore did not represent a general relationship among the eighteen genotypes evaluated in this experiment (Appendix 4.3). Since Rubisco retention time could potentially interfere with N recycling from aging leaves, there is a logical reason why such genotypes would not necessarily be high yielding and therefore unfit as candidates in a breeding program. Genotype 165 in fact had a DW near the average for the 18 genotypes tested in this experiment. PCA without genotype

165 eliminated PC4 altogether and showed four PCs (84.1% of data variation) with similar coefficient configuration as PC1, PC2, PC3 and PC5 in Table 4.3. The PCA results (Table 4.3) were kept because genotype 165 may be representative of many other genotypes in the mapping population which can only be realised when a larger sample of the mapping population is evaluated (e.g. in Experiment 3, Chapter 5).

The link between LER and DW first displayed in PC5 has been found in previous morphological studies in perennial ryegrass (Bahmani et al., 2000; Chapman and Lemaire, 1993; Horst et al., 1978; Van Loo, 1992). From the PCA coefficients, plants with increased LER resulted in long leaves, as expected. There was a SDC mechanism operating, and productivity of these plants was achieved by an increase tiller density and leaf length. In relation to Rubisco turnover, productivity was enhanced by a later Rubisco peak (Table 4.3). It is likely that increased photosynthetic activity for assimilate production was achieved mainly before full leaf expansion because Rubisco reaches its maximum concentration in the leaves just before full leaf expansion (Mae et al., 1983).

Rubisco concentration in this study was expressed as mg per leaf blade, implying that large leaves being larger sinks would have more Rubisco. It has been shown that the content of Rubisco per unit area is a rate limiting factor for photosynthesis (Hudson et al., 1992; Makino et al., 1997). It is therefore likely that that the expression on leaf weight or leaf area basis would change the results. This is a point to be followed up in the next experiment.

By using the methodology described in Chapter 3, this work has confirmed genotypic differences in Rubisco turnover and also identified some links (albeit somewhat weakly) between the measured Rubisco turnover and previously measured morphological traits of the selected plant genotypes. The ‘signal’ relating to the effect of Rubisco turnover on morphological traits is remarkable given that data were measured in two different experiments and environments. Since there is often difficulty correlating photosynthetic rate to plant performance because of the complexity with which the multiple factors interact to regulate photosynthesis (Sane and Amla, 1990; Stitt and Schulze, 1994), a strong link between Rubisco turnover and plant performance would not have been expected either. Therefore, the present results were considered to be confirmation of the experimental hypothesis, and justification for continuing to a full quantitative genetic analysis of the Grasslands II perennial ryegrass mapping population for Rubisco turnover.

4.6. Conclusions

The conclusions drawn from this experiment are:

- Significant genotypic variation in Rubisco turnover characteristics was observed among the eighteen perennial ryegrass genotypes evaluated in this experiment;
- It was possible to associate variation in Rubisco turnover characteristics with variation in morphological traits although this association was only weak to moderate;
- The particular patterns of interest observed were: A high and early Rubisco peak and a rapid Rubisco degradation was associated with a lower TW, a high DW was associated with an earlier and lower Rubisco peak and a sustained Rubisco retention, and increased plant DW and LER were associated with a later Rubisco peak;
- Based on the genotypic variation in Rubisco turnover characteristics observed in this experiment, a larger experiment to measure genetic differences in Rubisco turnover in Grasslands II perennial ryegrass mapping population would be a feasible and worthwhile project.

CHAPTER 5

Analysis of quantitative trait loci (QTL) for Rubisco turnover and herbage production traits in the Grasslands II perennial ryegrass mapping population

5.1. Introduction

The results of Chapter 4 showed genotypic differences in all three (*d*, *f*, and *g*) model parameters for quantification of Rubisco turnover, and some relationship between Rubisco turnover and herbage yield characteristics in the sample plants of Grasslands II mapping population. This was an indication that genetic analysis of Rubisco turnover in the Grasslands II mapping population would be a feasible project. This chapter describes an experiment to evaluate Rubisco turnover in that mapping population by marker-based quantitative genetics approach. Quantitative trait loci (QTL) analysis of herbage yield traits of this mapping population has been carried out previously (Sartie, 2007), but it was envisaged that where possible, morphological traits would be measured again in this study to overcome the possibility of genotype x environment interaction (as reported in Chapter 4) affecting the results. A parallel analysis would also reflect more accurately the correlation of physiological and agronomic characteristics. Repeated measurements also provide an opportunity to measure variation of these traits across the two environments.

A QTL study requires an accurate phenotypic evaluation of an adequately large population, the profiling of that population by way of molecular markers, and a statistical test to work from the phenotype to the genotype to localise chromosomal regions that might contain genes contributing to the phenotypic variation in a complex trait of interest (Mauricio, 2001; Tuberosa et al., 2002). A parallel DNA marker-based genetic analysis of morphological and physiological traits enables the determination of regions of the chromosome involved in the variation of these traits and their genetic effects. This sheds light on the bases of genetic correlations and contributes to our understanding of the physiological and morphological bases of yield. Key physiological components that influence plant productivity may provide markers for selection of NUE to optimise plant performance and rationalise the future use of N fertilisers.

5.2. Objectives

The main objective of this experiment (Experiment 3) was to detect QTL for Rubisco turnover and herbage yield in the Grasslands II perennial ryegrass mapping population. The specific objectives were:

1. To measure Rubisco turnover parameters for the plants of the mapping population;

2. To assess correlations between morphological traits and Rubisco turnover parameters;

3. To perform QTL analysis of Rubisco turnover parameters and also reconfirm QTL for herbage yield/agronomic traits in the Grasslands II perennial ryegrass mapping population;

4. To evaluate potential for use of leaf Rubisco N turnover data in plant improvement programmes

5.3. Materials and methods

5.3.1. Cultivation of plants

The experiment was carried out between May and August 2007 at the Plant Growth Unit, Massey University in Palmerston North. The mapping population consisted of full-sib F_1 plants (n=200) and one plant each from the pollen parent (Samson) and the seed parent (Impact) used in the generation of Grasslands II mapping population described in Chapter 4. The individual genotypes of the mapping population were maintained outdoors on a concrete pad at AgResearch Grasslands Research Centre in Palmerston North, New Zealand. For each genotype, one selected plant was subdivided on April 13 2007 to provide 4 uniform tillers which were transplanted into ¾ litre planter bags (PB ¾) and returned to the concrete pad. These plants were allowed to grow until they had produced several tillers. From these plants, all 18 genotypes used in the pilot experiment in Chapter 4 and 142 additional genotypes were selected for QTL analysis. The experimental sample size of 160 plants was determined based on what was logistically possible in relation to the resources available. Selection of genotypes for inclusion was by visual appraisal; a genotype was eliminated if it did not provide enough tillers that were approximately uniform in size. On 23 May 2007 the selected plants were further subdivided to provide 12 clonally replicated tillers per genotype. The twelve tillers of 158 F_1 plants and the two parent plants were each transplanted into 1.5 litre planter bags (PB 1½) containing a potting mixture of Manawatu B horizon soil (45%), builder's sand (55%), and osmocote (300g/100L sand/soil mix). The planting operation (of 1920 plants) was completed in one day by a team of 5 technical staff, including the author.

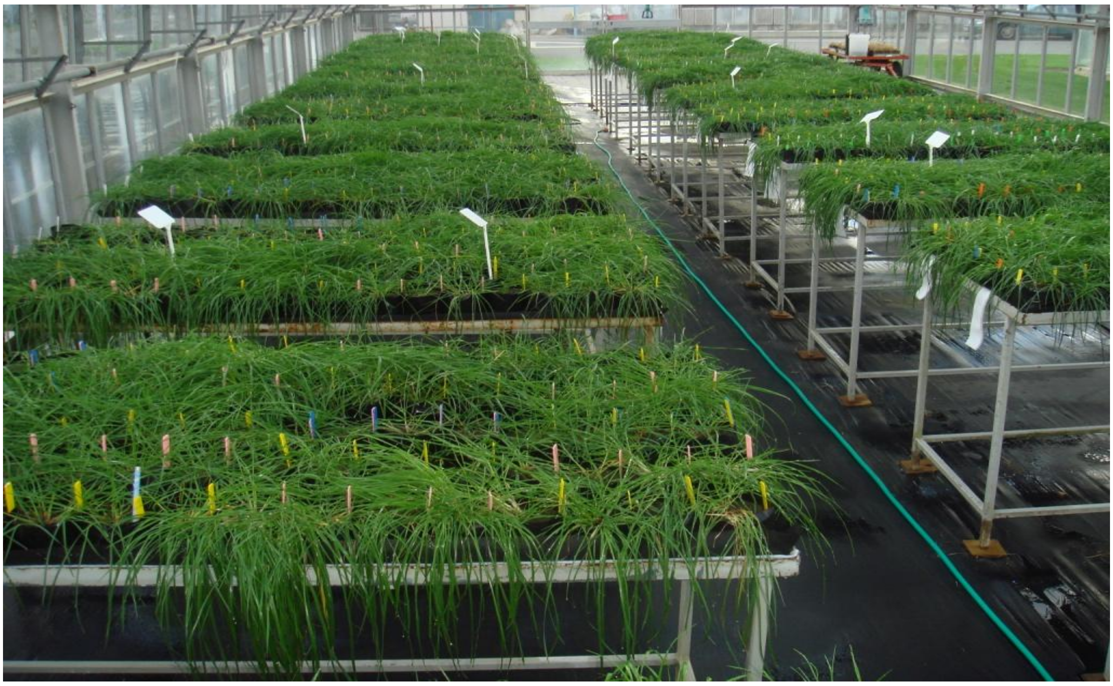


Figure 5.1: Grasslands II mapping population plants growing in the glasshouse 81 days after transplanting – Massey University Plant Growth Unit, Palmerston North, New Zealand (August 2007).

The twelve tillers per genotype were then grown on in a glasshouse under natural conditions in a randomised complete block design (RCBD) with three replicates. A guard row consisting of a mixture of the mapping population genotypes was arranged on all tables in such a way that it enclosed the experimental plants away from the glasshouse wall. Figure 5.1 shows the experimental layout.

5.3.2. Growth conditions

The temperature and solar radiation during the experiment was monitored using a data logger (Skye DataHog 2, Skye Instruments Ltd, Llandrindod Wells, Powys LD1 6DF, UK) linked to two temperature and two light sensors. The temperature in the glasshouse ranged between 2˚C and 24.6˚C (mean = 13.6˚C). Solar radiation ranged from 0.3 MJ/m^2/day to 10.6 MJ/m^2/day (mean = 4.96 MJ/m^2/day). Detailed data on temperature and solar radiation during the evaluation period are shown in Appendices 5.1 and 5.2.

5.3.3. Management and chemical input for weed, disease, and pest control

The plants were watered by a capillary system. The planter bags were arranged on tables overlaid with fibrous matting material. Water to the matting material was supplied by a network of 0.89 mm bore micro-tubes connected to a main tube that was in turn connected to a water tap. The experiment was watered automatically for 5 minutes at 9:00 am and 12:45 pm each day. The pots were kept weed free by hand weeding. Leaf rust was controlled by spraying with Systhane (2 x 10^{-4} ml/L) and Orthene (4 x 10^{-3} ml/L) two times (10 July 2007 and 13 August 2007) during the experiment.

5.3.4. Tiller/leaf marking

Eight Rubisco content determinations over the leaf lifespan were carried out for Rubisco turnover curve fitting for each genotype. Rubisco analysis was performed initially at 4 day intervals (day 4, 8, 12, 16 and 20 after the emergence of the target leaf tip) followed by 7 day intervals (day 27, 34 and 41 after the emergence of the leaf tip). Three replicate samples of each genotype were analysed on each occasion, plants for each replicate being spatially separated in the glasshouse. The short sampling interval early in the life of the leaves was to increase the sampling intensity around the time of the maximum Rubisco content. Therefore, on each plant, 2 average-sized tillers were identified and marked with rings made from 3 mm diameter plastic tube. Marking was completed over two days; commencing on 3 July 2007 for plants in the first replicate and on 4 July 2007 for plants in the second and third replicates. Each marked tiller served as an experimental unit. On each marked tiller, the new expanding leaf was identified as the experimental leaf and the leaf immediately below on the same tiller axis was marked with a correction pen for identification (the experimental leaf was not marked in order to eliminate any possible interference of the marker ink with leaf metabolic processes). To estimate the approximate age of the experimental leaves at the start of sampling, the length of the experimental leaf and the adjacent marked leaf were measured. In this way the approximate age in days of the experimental leaf when marked could be estimated as:

$$LED * (Le/Lm*1.2)$$

Where; LED = the leaf elongation duration measured as the average time (days) from the time of leaf tip appearance to full leaf elongation in the leaves of 10 non-test plants; Le = length (mm) of the experimental leaf; Lm = length (mm) of the marked leaf; 1.2 = a constant indicating expectation of 20% increase in leaf length for successive leaves (Sartie et al., 2009; Verdenal et al., 2008).

Ultimately, samples were collected from 137 of the 160 established genotypes. Twenty three genotypes showed low survival and could not provide enough plants for Rubisco analysis and were therefore eliminated. Each marked tiller provided only one leaf for analysis. On each sampling day, one leaf per genotype per replicate was excised at the ligule, weighed and immediately snap-frozen in liquid N, and stored at -80˚C until further analysis.

5.3.5. Measurement of morphological traits and dry weight

Following leaf sampling for Rubisco determination, selected morphological traits were measured in two replicates of the experiment as follows:

Leaf lamina length (LL): Three of the larger tillers (excluding the largest) were selected for one pot of each plant genotype in each of the 2 replicates. For each tiller, the youngest fully expanded leaf with a visible ligule was identified and its length measured as the distance (mm) from the ligule to the tip. LL was recorded as the average length of the three leaves. The rationale of this methodology was to obtain a measure of LL that reflected the potential rather than the phenotypic average of each genotype.

Tiller number (TN): The number of tillers in each of the three clonally replicated plants of a genotype was counted and averaged. Where 4 plants of a genotype were present within a replicate the smallest plant as judged by visual inspection, was rejected.

Plant dry weight (DW): For each plant for which a tiller count had been taken, herbage was harvested by cutting at soil level. Cut herbage was placed in a paper bag, dried for 48 hours in an oven set at 80˚C, weighed, and the weights averaged.

Tiller weight: This was measured in two ways: The first measurement (TW) was based on plant average and was estimated as

$$TW = DW/TN \qquad (1)$$

The second measurement (TW_{large}) was based on the three large tillers used to measure LL. For each genotype TW_{large} was estimated as the average weight of three largest oven-dried (80˚C, 48 hours) tillers.

Productivity index (PI) – This was derived based on TW (g) and TN as follows:

$$PI = Log(TW) - [-1.5 \text{ x } Log(TN)/A] \qquad (2)$$

Where: -1.5 = constant assumed for size/density compensation (SDC, Matthew et al., 1995); A = surface area of the planter bag (m^2).

5.3.6. Rubisco measurements

Determination of *d, f,* and *g* – Rubisco was extracted from frozen leaves and quantified according to the procedure outlined in Chapter 3 (Section 3.2.3.2). Rubisco concentration determined as milligrams per leaf at each point was converted to milligrams Rubisco-N per gram of leaf dry weight (mg Rubisco-N/g leaf DW) as follows: Leaf dry weights at each point were estimated from the average dry weight of four replicate leaves from non-test plants harvested on the same days as those for Rubisco analysis. These leaves were oven dried at 80˚C for 48 hours and then weighed for determination of DW at each time point. A graph of leaf dry weight against time was plotted and used to estimate leaf dry matter of the Rubisco sample leaves for concentration conversion. Log-normal curves were fitted in Sigma Plot (version 11) to derive the curve parameters *d*, *f*, and *g*. There were also derived variables as follows:

Peak (PK) – During preliminary data analysis a systematic lack of fit of the log-normal curves was suspected for some genotypes. To measure the extent to which *d* fully described the maximum Rubisco content in the leaf, PK Rubisco was determined as the average of the two Rubisco determinations closest to the curve maximum, *d*. PK had a mean value of 43.2 (± 0.7) mg Rubisco-N/g of leaf DW. The mean value of *d* was 45.6 (± 0.6) mg Rubisco-N/g of leaf DW.

Tail (TL) – This represented residual leaf Rubisco content late in the life of the leaf, and was calculated as the average amount of Rubisco of the last two sampling dates. The derivation of TL was also based on observations in preliminary data analysis of systematic lack of fit of the log-normal curves, with data points often appearing above the fitted curve in late leaf senescence, indicating greater-than-modelled Rubisco retention. In some cases the final value for leaf Rubisco content was higher than that obtained in the penultimate measurement.

d/f **-** This ratio was derived to identify the genotypes which theoretically would be expected to have rapid Rubisco turnover, which would imply a larger cost of Rubisco synthesis and/or less photosynthetic contribution over the leaf lifespan.

5.3.7. Statistical analysis

Log-normal curves were fitted using all three points (replicates) at each sampling time. Values obtained for Rubisco turnover parameters *d*, *f*, and *g* together with the mean values of all herbage yield parameters were subjected to correlation analysis and PCA, using SAS (SAS Institute Inc., Cary, NC, USA). Frequency distribution graphs were constructed using Microsoft excel software (Microsoft 2007).

5.3.8. QTL analysis

The IxS consensus genetic linkage map developed for perennial ryegrass by the AgResearch project (e.g. Sartie, 2007) was used for genetic analysis. This map is based on simple sequence repeat (SSR) and sequence-tagged site (STS) markers derived from a proprietary perennial ryegrass expressed sequence tag (EST) resource (Faville et al., 2004; Sawbridge et al., 2003). QTL analysis was carried out using MapQTL 4.0 (Van Ooijen et al., 2002) with the population structure set as CP (cross pollinated). The procedure for QTL detection in the IxS mapping population of herbage and seed production traits by Sartie (2007) was adopted for this experiment. Each trait was first analysed by SIM using the phenotypic mean value of each genotype. The declaration of the presence of a putative QTL in a given genome region was based on a minimum LOD threshold of 2.5 and if the QTL position was supported by Kruskal-Wallis nonparametric single locus analysis. Declared QTL were further resolved by MQM in MapQTL 4.0, using cofactors chosen with the help of the automatic cofactor selection option (Van Ooijen et al., 2002). Cofactors were identified by initially selecting six to eight markers at approximately 20 cM intervals (including QTL markers identified by SIM) on a linkage group. Automatic cofactor selection was then executed. This process was repeated on a linkage group-by-linkage group basis with the identified cofactors from previous linkage groups included when computational limitations allowed (no more than nine cofactors could be included in the automatic cofactor selection at one time). MQM was performed on trait by trait basis using all identified cofactors for each trait. Permutation analysis (1000 iterations) was used to establish a genome-wide significance ($P < 0.05$) value defined as a minimum threshold for each trait in MQM (Churchill and Doerge, 1994; Doerge and Churchill, 1996). Since a mapping population of 135 members would be considered small for a QTL analysis, genome-wide values were also established at the 90% confidence level. For each form of QTL analysis, the maximum LOD value associated with the most closely linked marker and the proportion of the phenotypic variance attributable to the QTL were tabulated. Map positions were defined by the peak ± 2 LOD (Van Ooijen, 1992). QTL nomenclature consisted of the trait it associated with followed by the linkage group.

The allelic effects at the detected QTL were estimated using phenotypic trait means for the four QTL genotypes (ac, ad, bc, and bd) automatically calculated in MapQTL 4.0. Maternal effect (difference in effect of the alleles (a and b) inherited from the Impact parent (I)) and paternal effect (difference in effect of the alleles (c and d) inherited from Samson parent (S)) were estimated as follows (after Knott et al., 1997; Sewell et al., 2000; 2002):

Maternal effect (I) = (ac + ad) – (bc + bd)

Paternal effect (S) = (ac + bc) – (ad + bd)

5.4. Results

5.4.1. Phenotypic variation for traits in Experiment 3

The mapping population exhibited a wide range of values for all measured traits (Table 5.1). An analysis of variance (ANOVA) indicated significant ($P < 0.01$) differences for all the herbage yield traits measured in this experiment. Among the herbage yield traits, only PI differed significantly ($P < 0.1$) between the two parent plants, being higher in the Impact than the Samson parent plant. Differences among genotypes existed for all

Rubisco turnover parameters (t-test using means and standard error obtained for each parameter in Sigma plot). The progeny showed continuous variation in all traits (Fig. 5.2), suggesting quantitative inheritance. The variation among the progeny plants significantly exceeded that between the parental plant accessions possibly due to transgressive segregation and/or the age difference between the parent plants and progeny. For TN, PI, *d*, *f* and *g*, the population progeny mean values were between the parental plant values (Fig. 5.2). The progeny means were higher than the parental values for TW and lower for LL and DW (Fig. 5.2A, B &D). TN showed an unbalanced segregation, with only two progeny plants exceeding the Impact parent plant value (Fig. 5.2C). For this trait also, approximately 63% progeny had their mean values between the two parental values. A very strong segregation was observed in some traits. For instance, the parental phenotypes for TW were not so different (Impact plant TW = 80mg; Samson plant TW = 94mg), but progeny TW values were found as extreme as 8mg and 157mg.

Table 5.1: Genotype effects on Rubisco turnover and herbage yield traits for 135 genotypes of the Grasslands II perennial ryegrass mapping population and the Samson (P1) and Impact (P2) parent plants. Data are the range of genotype means and their SEM: TW = tiller weight (mg); LL = leaf lamina length (mm); TN = tiller number; DW = herbage dry weight (g); PI = productivity index; *d* = maximum Rubisco content (mg/g leaf dry weight); *f* = curve width measure; *g* = time of *d* (days).

Trait	Range	P1	P2	SEM	*P* value
TW	8.0 – 224.5	94.00	80.00	20.80	<0.01
LL	178 – 433	321.00	336.00	32.70	<0.01
TN	13 – 106	52.00	104.00	13.70	<0.01
DW	0.2 – 12.6	4.80	6.70	1.65	<0.01
PI	3.2 – 5.2	4.50	4.90	0.18	<0.01
d	31.6 – 64.3	42.80	49.40	3.73	<0.05
f	0.6 – 1.2	0.84	0.77	0.11	<0.05
g	9.0 – 17.9	13.50	12.20	1.16	<0.05

Genotypic differences in herbage yield traits tested by ANOVA, differences in Rubisco turnover tested by Student’s t-test ($P < 0.05$) using mean differences and SE derived by Sigma plot (see Appendix 5.4 for ANOVA table of herbage yield traits).

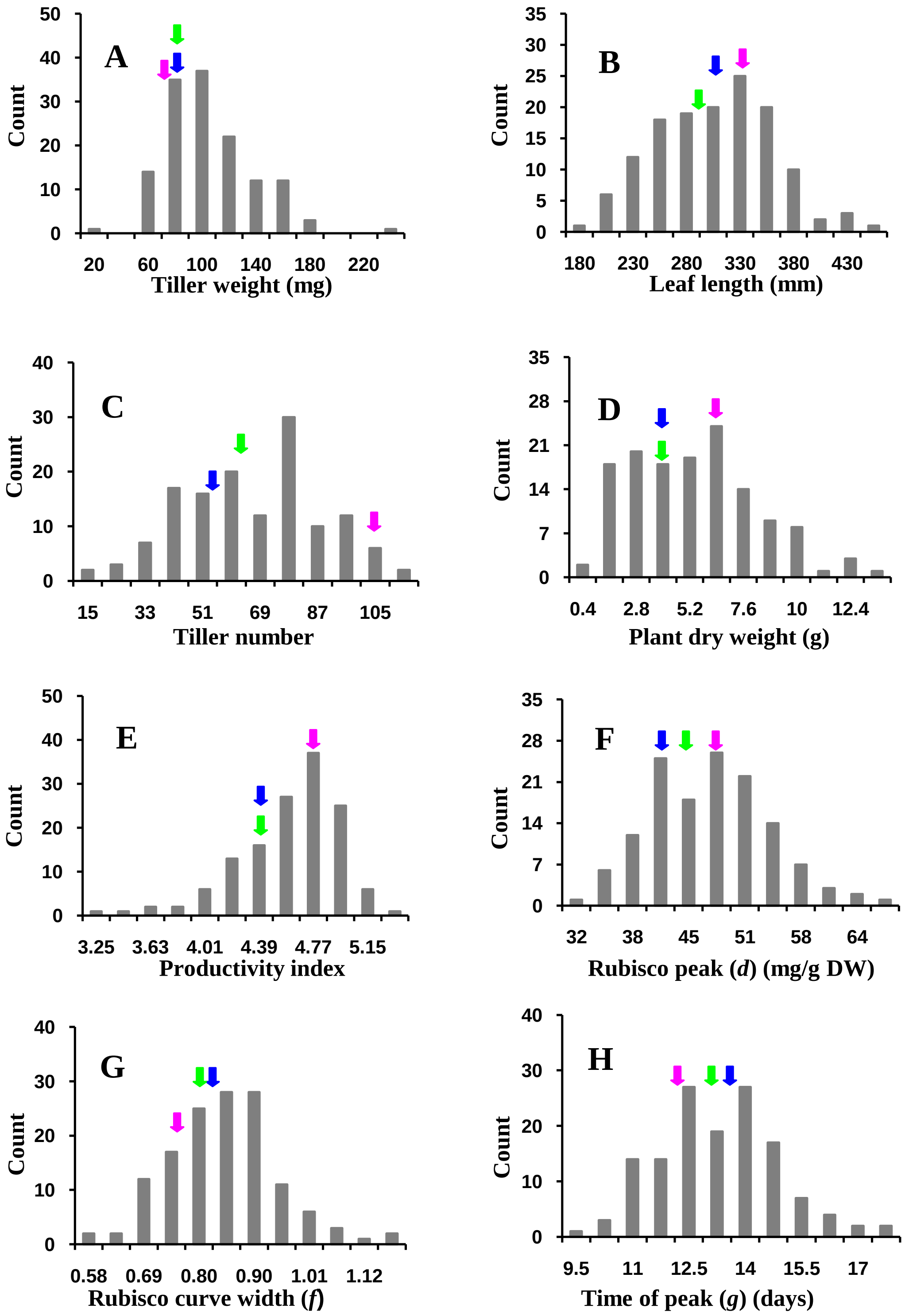


Figure 5.2: Frequency distribution of the traits measured in the 135 Grasslands II mapping population progeny and the two parent plants. A = tiller weight; B = leaf lamina length; C = tiller number; D = plant dry weight; E = Productivity index; F = Rubisco peak; G = Rubisco curve; H = Time of Rubisco peak. The arrows show the F_1 progeny mean (green) and parental values, Impact (pink) and Samson (blue).

5.4.2. Correlations among traits measured in Experiment 3

The herbage yield traits measured in this experiment showed significant positive correlations ($P < 0.01$, range of r was between 0.32 and 0.87) (Table 5.2). Of the Rubisco turnover parameters, *d* correlated significantly and negatively with all herbage yield parameters ($P < 0.01$ for LL; ($P < 0.05$ for TW, DW and PI; $P < 0.1$ for TN), while *f* correlated negatively ($P < 0.1$) with only DW (Table 5.2). A higher value for *d* was associated with a narrower curve width, *f*, indicating shorter Rubisco retention. A similar but weaker pattern of association was noted for the curve PK. The derived measure *d*/*f* correlated marginally ($P < 0.1$) and negatively with only LL among the morphological measures. Rubisco retention as measured by TL also showed a significant negative correlation with the measures of plant size (Table 5.2).

It was of interest at this stage to evaluate the repeatability of measurement of Rubisco turnover characteristics for the genotypes evaluated in both Chapter 4 and this chapter. The analysis of Rubisco turnover parameters (estimated when Rubisco was expressed as mg/leaf blade) showed that values of *g* showed a tendency towards negative correlation between experiments, albeit marginally ($r = -0.442$, $P = 0.09$). On the other hand, values of *d* ($r = -0.16$, $P = 0.56$) and *f* ($r = -0.15$, $P = 0.57$) were uncorrelated between the two experiments.

Table 5.2: Coefficients of phenotypic correlations of the traits measured in the135 F_1 plants of the Grasslands II perennial ryegrass mapping population and the Impact and Samson parent plants. TW = tiller weight (mg); LL = leaf lamina length (mm); TN = tiller number; DW = herbage dry weight; PI = productivity index; *d* = maximum Rubisco content (mg/g of leaf dry weight); *f* = curve width measure; *d/f* = *d*:*f* ratio; *g* = time of *d*; PK = average of the two Rubisco content determinations closest to *d* (mg/g of leaf dry weight); TL = residual Rubisco content measured of the average of the last two time points (mg/g of leaf dry weight).

Trait	LL	TN	TW	DW	PI	*d*	*f*	*d/f*	*g*	PK
TN	0.45**									
TW	0.57**	0.32**								
DW	0.62**	0.80**	0.66**							
PI	0.58**	0.87**	0.67**	0.86**						
d	-0.23**	-0.15†	-0.20*	-0.21*	-0.17*					
f	0.01	-0.10	-0.04	-0.14†	-0.12	-0.24**				
d/f	-0.16†	-0.04	-0.08	-0.07	-0.03	0.81**	-0.74**			
g	-0.05	0.05	-0.02	0.03	0.03	-0.20*	-0.39*	0.10		
PK	-0.22**	-0.10	-0.13	0.15†	-0.11	0.78**	-0.09	0.57**	-0.21*	
TL	-0.28**	-0.18*	-0.25**	-0.31**	-0.21*	0.29**	0.27**	0.02	0.17*	0.36**

†,*, ** indicate $P < 0.1$, $P < 0.05$ and $P < 0.01$ significance levels, respectively

5.4.3. Principal component analysis of Experiment 3 traits

When the various data from Experiment 3 were subjected to PCA, the first four PCs from the eleven available, cumulatively explained 83% of data variation (Table 5.3). PC1 (36% of data variation) reflected the positive correlation between the morphological traits (Table 5.3), and indicated that large plants tended to have a lower Rubisco concentration (negative coefficients for *d*, PK and TL) (Table 5.3). A notable feature of this PC was that the influence of Rubisco turnover on plant productivity was predominantly by the effect of variables related to Rubisco concentration (*d*, *d*/*f*, PK and TL) and that *f* and *g* did not feature (Table 5.3). The parent plants were not separated by this PC (Appendix 5.4).

Table 5.3: Principal component analysis structure of the final data for 135 genotypes of Grasslands II perennial ryegrass mapping population and the Impact and Samson parent plants: TW = tiller weight; LL = leaf lamina length; TN = tiller number; DW = herbage dry weight; PI = productivity index; *d* = maximum Rubisco content; *f* = curve width measure; *d*/*f* = *d*:*f* ratio; *g* = time of *d*; PK = average of the two Rubisco content determinations closest to *d* (mg/g of leaf dry weight); TL = residual Rubisco content measured as the average of the last two time points (mg/g of leaf dry weight).

Trait	PC1	PC2	PC3	PC4
LL	0.38	0.05	0.11	-0.09
TN	0.38	0.18	0.09	0.23
TW	0.36	0.10	0.11	-0.06
DW	0.45	0.18	0.07	0.08
PI	0.44	0.21	0.11	0.17
d	-0.25	0.47	0.25	-0.04
f	0.01	-0.42	0.55	0.15
d/*f*	-0.16	0.57	-0.16	-0.12
g	0.02	0.04	-0.60	0.55
PK	-0.22	0.40	0.38	0.09
TL	-0.22	-0.01	0.25	0.74
Variation (%)	35.90	23.30	13.90	10.20

PC2 (23% of the variation) highlighted the negative correlation between *d* and *f*, and the associated index of *d*/*f* ratio (Table 5.3). The involvement of morphological traits was much smaller, but indicated that plants with a higher *d*/*f* ratio also tended to have a higher DW, TN, and PI (coefficients 0.18, 0.18, 0.21, respectively). This PC discriminated between the two parent plants (Appendix 5.4). The salient associations captured in PC3 (14% of data variation) were an early Rubisco peak and comparatively high levels of Rubisco at all stages of leaf development (Table 5.3). The morphological traits were at best only weakly associated with this Rubisco turnover pattern (Table 5.3). PC4 (10% of data variation) described plants with more Rubisco later in the leaf's life span (*g* coefficient = 0.55 and TL coefficient = 0.74) (Table 5.3). Plants exhibiting this Rubisco turnover pattern showed a modest tendency to higher TN (coefficient = 0.23) (Table 5.3). This trait association was not found in simple correlations (Table 5.3), presumably because of masking by data variation extracted in the higher-order PCs.

5.4.4. Cross-experiment correlations between morphological traits

One point of consideration in data analysis was the correlation of the measures of plant morphology traits from Experiment 3 with similar data collected for the same genotypes in earlier experiments. Therefore, data from Experiment 3 were compared with data from Sartie's autumn experiment (Sartie, 2007). Three issues were explored in relation to comparing data between these experiments: (i) the extent of the correlation among genotype means when the same variables were re-measured in a new experiment, (ii) whether or not any relationship between Rubisco turnover measurements in Experiment 3 and morphological data from the Sartie (2007) experiment would be detected, and if so, the commonality or otherwise with those relationships for morphological data from Experiment 3, and (iii) whether or not introducing the Sartie (2007) information relating to genotype differences in plastochron and LER, not measured in the current experiment, would provide any additional insight into the nature of links between Rubisco turnover and morphological traits.

Some other unpublished data from prior research with the mapping population at AgResearch were also explored for possible correlation with Rubisco turnover data. These included a near infrared spectroscopy assessment of foliar crude protein and short term ^{15}N uptake by potted plants in sand culture. However, no correlation was found and these investigations are therefore not reported.

The herbage yield traits common to both experiments were LL, TN, TW, DW, and PI. Both LL (r = 0.28, $P < 0.01$) and TW (r = 0.31, $P < 0.01$) were correlated in the two experiments. For the remaining variables, data from the two experiments were uncorrelated (for TN, r = -0.07, for DW, r = 0.01 and for PI, r = -0.06, with *P* always greater than 0.43). Also, data for the two measures of tiller weight, TW and TW_{Large} (see Section 5.3.5), were compared with tiller weight data from the autumn and spring experiments of Sartie (2007) (Table 5.4). Both TW and TW_{Large} correlated significantly with tiller weight data from the spring and autumn experiment (Table 5.4).

Table 5.4: Coefficients of correlations among tiller weight data measured in 135 F_1 plants of the Grasslands II mapping population and the parent plants (Impact and Samson). TW = tiller weight (as in Table 5.2, 5.3), TW_{Large} = average weight of three largest tillers, TW_a and TW_s = tiller weight from Sartie's (Sartie, 2007) autumn and spring data, respectively.

Trait	TW	TW_{Large}	TW_a
TW_{Large}	0.731**		
TW_a	0.306**	0.186*	
TW_s	0.236**	0.278**	0.227**

*, ** indicate $P < 0.05$ and $P < 0.01$ significance levels, respectively

Within Experiment 3, there were significant negative correlations of LL with *d*, *d*/*f*, PK and TL, and also of TW with *d* and TL (Table 5.2). These correlations were largely seen when LL and TW data of Sartie (2007) was analysed with Rubisco turnover data from the current experiment (Table 5.5). LER correlated significantly ($P < 0.05$) with *d*, PK and TL (Table 5.5). Rubisco curve width, *f*, correlated negatively with both DW and PI (Table 5.5).

Table 5.5: Cross experiment correlations between Rubisco turnover and herbage yield traits of 135 progeny of the Grasslands II perennial ryegrass mapping population and the two parent plants: Agronomic traits were measured in a previous experiment (Sartie, 2007)[1]. DW_a = herbage dry weight; LL_a = leaf lamina length; TN_a = tiller number; TW_a = tiller weight; PI_a = productivity index; Alf_a = leaf appearance interval; Alg_a = ligule appearance interval; LED_a = leaf elongation duration; LER_a = leaf elongation rate; *d* = maximum Rubisco content; *f* = curve width measure; *d*/*f* = *d*:*f* ratio; *g* = time of *d*; PK = average of the two Rubisco content determinations closest to *d* (mg/g of leaf dry weight); TL = residual Rubisco content measured as the average of the last two time points (mg/g of leaf dry weight).

Trait	LL_a	TN_a	TW_a	DW_a	PI_a	Alf_a	Alg_a	LED_a	LER_a
d	-0.30**	0.02	-0.15†	-0.12	-0.09	-0.12	-0.11	-0.13	-0.20*
f	-0.05	-0.09	0.00	-0.17*	-0.16†	0.09	-0.09	0.08	-0.10
d/*f*	-0.17*	0.07	-0.09	0.04	0.04	-0.13	-0.13	-0.12	-0.08
g	0.04	-0.03	0.04	0.07	0.04	0.03	-0.01	0.10	0.00
PK	-0.28**	-0.07	-0.11	-0.21*	-0.19*	-0.13	-0.12	-0.12	-0.17*
TL	-0.22*	0.06	-0.15†	-0.10	-0.04	0.05	0.04	0.07	-0.25**

†, *, ** $P < 0.1$, $P < 0.05$ and $P < 0.01$ significance levels, respectively

[1], the agronomic traits used were from the autumn data collected in the Sartie (2007) experiment (see Section 5.5.1).

Table 5.6: PCA structure involving a combination of traits measured in this study and those measured in the Sartie (2007) autumn study (coefficients in bold italics) for 135 progeny of the Grasslands II perennial ryegrass mapping population and the two parent plants. TW = tiller weight; LL = leaf lamina length; TN = tiller number; DW = herbage dry weight; PI = productivity index; Alf_a = leaf appearance interval; Alg_a = ligule appearance interval; LED_a = leaf elongation duration; LER_a = leaf elongation rate; *d* = maximum Rubisco content; *f* = curve width measure; *d*/*f* = *d*:*f* ratio; *g* = time of *d*; PK = average of the two Rubisco content determinations closest to *d* (mg/g of leaf dry weight); TL = residual Rubisco content measured as the average of the last two time points(mg/g of leaf dry weight).

Trait	PC1	PC2	PC3	PC4	PC5
TW	0.35	0.04	0.12	0.11	-0.06
LL	0.34	0.11	0.12	0.13	-0.10
TN	0.36	0.01	0.22	0.12	0.24
DW	0.43	0.01	0.18	0.09	0.08
PI	0.44	0.01	0.24	0.14	0.17
Alf_a	***-0.16***	***0.41***	***0.35***	***0.03***	***-0.07***
Alg_a	***-0.13***	***0.38***	***0.33***	***0.06***	***-0.10***
LED_a	***-0.18***	***0.39***	***0.31***	***-0.02***	***-0.02***
LER_a	***0.29***	***-0.15***	***-0.23***	***-0.11***	***-0.10***
d	-0.19	-0.39	0.30	0.25	-0.07
f	0.05	0.24	-0.35	0.54	0.14
d/*f*	-0.09	-0.40	0.41	-0.15	-0.14
g	0.01	0.03	0.11	-0.58	0.57
PK	-0.16	-0.35	0.24	0.37	0.06
TL	-0.21	-0.06	0.01	0.26	0.71
Variation (%)	28.40	20.70	14.50	10.20	7.50

Data for plastochron and LER from the Sartie (2007) autumn experiment were combined with data from Experiment 3 and subjected to PCA. The first five PCs of the available fifteen explained 81.4% of the data variation (Table 5.6). PC1 was largely unchanged between the two analyses (Tables 5.3, 5.6). LL and LER had similar coefficients in PC1, in keeping with the similarity in their relationship with Rubisco turnover parameters in cross-experiment correlations (Table 5.5). PC2 (21% of data variation) associated a longer interval between successive leaves (coefficients for Alf, Alg and LED 0.41, 0.38 and 0.39, respectively), with a lower *d* and a greater *f*, but not with morphological characteristics (Table 5.6). Conversely, PC3 (14.5% of data variation) associated higher values of Alf, Alg and LED with higher *d* and reduced *f* (Table 5.6). This pattern of association tended to be associated with increases in TN, and PI, and possibly DW (Table 5.6). PCs 4 and 5 in Table 5.6 resembled PCs 3 and 4 in Table 5.3.

5.4.5. QTL identification

QTL detected for Rubisco turnover parameters and herbage yield traits are presented in Table 5.7. The positions of the different significant ($P < 0.05$) QTL detected by MQM are shown on the IxS perennial ryegrass linkage map in Fig. 5.3. SIM identified 20 significant QTL (maximum LOD $\geq$ 2.4) and 19 suggestive QTL (2.0 < LOD > 2.4) for all traits across six of the seven linkage groups (LGs) (Table 5.7). Fifteen of these QTL were confirmed by MQM (based on overlap of peaks or support intervals). MQM identified two significant QTL (one each for *g* and PK) previously identified as suggestive by SIM (Table 5.7). No significant QTL were detected on LG3 and LG6 (Table 5.7, Fig. 5.3). There was at least one significant QTL identified for each trait except for Rubisco curve width (*f*). Three QTL (qd-4.2, qPK-2 and qPI-1.2) detected at the 90% confidence level in MQM were lost when the confidence level was increased to 95% (Table 5.7).

5.4.5.1. QTL detection for Rubisco turnover parameters

Seven significant QTL were detected for measured and derived Rubisco turnover parameters (Table 5.7, Fig. 5.3). These QTL were distributed on four LGs (LG2, LG4, LG5, and LG7). The trait phenotypic variation explained (PVE) by individual QTL ranged from 8.7 % to 24.1% (Table 5.7).

Two QTL of similar effect were detected for *d* on LG5 and LG7. On LG5, qd-5.1 explaining 12.5% of the PVE was located near marker rv0340 (Table 5.7). This QTL was mapped to the same region of LG5 as the QTL associated with TN (qTN-5, marker pps0718) as shown by the overlap of the support intervals (Table 5.7, Fig. 5.3) and close to the QTL for TW (qTW-5). Flanking markers rv0340 and pps0718 are located next to each other on the chromosome, separated by a distance of only 3 cM. The second QTL for *d*, qd-7, on LG7 (marker pps0065, PVE = 12.3%) overlapped with qTL-7.1 (PVE = 24.1%), the sole significant MQM-detected QTL for TL (Table 5.7, Fig. 5.3). The markers flanking qd-7 and qTail-7 on LG7 are less than 1 cM apart. At both qd-5.1 and qd-7, an allele from the Samson parent had a larger effect on the phenotypic performance than an allele from the Impact parent (Table 5.8). SIM detected a second QTL for *d* on LG5 (qd-5.2) with a significant LOD peak near marker pps0111, but the threshold value determined for MQM for this trait was not exceeded (Table 5.7). At qTL-7, performance was largely enhanced by an allele from the Impact parent (Table 5.8).

There was only one significant QTL detected for PK (qPK-4), and this was located on LG4 (Table 5.7, Fig. 5.3). This QTL explaining 15.6% of the PVE was the sole significant QTL on LG4 (Table 5.7, Fig. 5.3). Enhanced performance at qPK-4 was largely conferred by an allele from the Impact parent (Table 5.8). Surprisingly, qPK-4 and the two QTL for *d* were mapped on separate LGs by MQM (Table 5.7, Fig. 5.3) despite the consistently high phenotypic correlation of *d* with PK (Table 5.2). However, SIM showed coincidences of QTL for *d* and PK including an overlap of their respective peaks near marker pps0660b on LG2 (Table 5.7).

There was only one significant QTL (qd/f-5) identified for *d*/*f*. This QTL was located on LG5 and away from other QTL (Fig. 5.3). This was a large effect QTL explaining 17.3% of the PVE (Table 5.7), and the effect on its performance was mainly from an allele inherited from the Samson parent (Table 5.8). Interestingly, *d*/*f* had the highest positive correlation with *d* but there was no indication by either SIM or MQM that the two traits were coupled.

Two QTL were identified for *g*, one each on LG2 (qg-7) and LG7 (qg-7) and explaining 15.3% and 14% of the PVE, respectively (Table 5.7). Both QTL were distinctly separated from other QTL for Rubisco turnover and herbage yield traits (Fig. 5.3). At both loci, increased phenotypic performance was largely enhanced in the presence of an allele inherited from the Samson parent (Table 5.8).

5.4.5.2. QTL detection for herbage yield traits

Between one and three significant QTL per trait were detected on three linkage groups (LG1, LG2, and LG5) for the five herbage yield traits evaluated in this study (Table 5.7,

Fig. 5.3). The trait PVE by individual QTL ranged from 5.3 % to 24.3% (Table 5.7). LG1 contributed one locus, while LG2 and LG5 contributed four loci each in clusters (Table 5.7, Fig. 5.3).

Two regions on LG2 and LG5 appeared to be of interest with regard to QTL for morphological traits and plant yield. On LG2, four QTL (qTN-2 (PVE = 10.1%), qLL-2 (PVE = 16.2%), qDW-2.1 (PVE = 8.9%) and qPI-2 (PVE = 7%) co-mapped near the pps0113 marker locus (Table 5.7, Fig. 5.3). The performance of all QTL at this chromosomal locus was largely enhanced in the presence of an allele from the Samson parent apart from qLL-2 for which an allele from the Impact parent had a larger positive effect (Table 5.6). On LG5, QTL for DW (qDW-5.1 (PVE = 21.3%)) and PI QTL (qPI-5.1 (PVE = 24.3%)) both peaked near marker pps1115 (Table 5.7). The support intervals of both qDW-5 and qPI-5.1 coincided with that of qTN-5.1 (marker pps0718, PVE = 12.5% (Table 5.7, Fig. 5.3)). The allelic effects were such that enhanced performance was largely due to the effect of an allele from the Impact parent for both qTN-5 and qPI-5, whereas the performance of qDW-5 was enhanced mainly by an allele from the Samson parent (Table 5.8). Besides the appearance in clusters, DW had an additional QTL (qDW-1.1) detected on LG1 near marker pps0290 (Table 5.7). This QTL explaining 12.4% of the PVE was the sole significant QTL on LG1 (Table 5.7, Fig. 5.3). Enhanced performance for qDW-1.1 was favoured largely by an allele from the Impact parent (Table 5.6).

MQM revealed one significant QTL for TW, qTW-5 (PVE = 14%), on LG5 near marker pps0036 (Table 5.7). This QTL was located very close to qd-5 (Table 5.7, Fig. 5.3). MQM indicated no support interval overlaps between qTW-5 and QTL for the other measured herbage yield traits in a cluster on LG5. The most probable position of qTW-5 fell <16cM from the probable positions of the other QTL clustered on LG5. However, SIM indicated an overlap of support intervals of QTL for TW, TN, DW, and PI on LG5 (Table 5.7)

Table 5.7: QTL identified by simple interval mapping (SIM) and multiple QTL mapping (MQM) for Rubisco turnover and herbage yield traits for 135 progeny of the Grasslands II mapping population and the parent plants (Samson and Impact), assessed in a glasshouse between May and August 2007. TW = tiller weight; LL = leaf lamina length (mm); TN = tiller number; DW = herbage dry weight (g); PI = productivity index; *d* = maximum Rubisco content (mg N/g leaf dry weight); *f* = curve width measure; *d*/*f* = *d*:*f* ratio; *g* = time of *d* (days); PK = average of the two Rubisco content determinations closest to *d* (mg N/g leaf dry weight); TL = residual Rubisco content measured as the average of the last two time points(mg N/g leaf dry weight); PVE = phenotypic variance explained.

			SIM				MQM				
Trait	QTL	Linkage group	Position (cM ± 1 LOD)	Peak LOD	PVE (%)	Nearest marker	Position (cM ± 2 LOD)	LOD threshold	Maximum LOD value	PVE (%)	Marker
d	qd-1	1	28.9 (24.2 – 43.8)	2.16	8.3	ppt0013					
	qd-3	3	31.7 (20.7 – 45.3)	2.16	7.2	pps0558					
	qd-4.1	4	65.0 (38.1 – 73.0)	2.02	7.4	pps0130					
	qd-4.1	4	97.9 (84.8 – 123.7)	2.06	10.9	pps1099					
	qd-4.2	4					50.0 (42.5 – 51.5)	3.90	3.41	8.7	lpssr006
	qd-5.1*	5	61.6 (60.5 – 61.6)	3.05	11.0	rv0340	61.6 (59.8 – 63.0)	3.90	4.37	12.5	rv0340
	qd-5.2*	5	15.5 (0.0 – 44.0)	2.64	9.7	pps0111					
	qd-7*	7	34.2 (27.6 – 49.9)	2.81	10.2	pps0065	34.2 (30.8 – 34.3)	3.90	4.30	12.3	pps0065
	Variation (%) explained by all QTL				64.7					33.5	
	Variation (%) explained by genome-wide significant QTL				30.9					24.8	
f	qf-2	2	66.1 (48.5 – 79.1)	2.07	8.4	pps0188					
	qf-7	7	19.7 (0.0 – 36.2)	2.13	10.2	pps0766					
	Variation (%) explained by all QTL				18.6						
	Variation (%) explained by genome-wide significant QTL				0.0						
d/f	qd/f-2	2	46.3 (43.7 – 59.5)	2.39	9.0	pps0153					
	qd/f-5*	5					3.1 (0.0 – 9.7)	3.95	3.95	17.3	pps0509
	Variation (%) explained by all QTL				9.0					17.3	
	Variation (%) explained by genome-wide significant QTL				0.0					17.3	

Table 5.7 (Continued)

			SIM				MQM				
Trait	QTL	Linkage group	Position (cM ± 1 LOD)	Peak LOD	PVE (%)	Nearest marker	Position (cM ± 2 LOD)	LOD threshold	Maximum LOD value	PVE (%)	Marker
g	qg-2*	2	9.9 (0.0 – 23.8)	2.77	13.7	pps0514	9.9 (0.0 – 21.6)	4.05	4.13	15.3	pps0514
	qg-3	3	1.9 (0.0 – 20.8)	2.36	12.1	pps0577					
	qg-7.1*	7					100.5 (96.7 – 101.7)	4.05	4.17	14.0	pps0002
	qg-7.2	7	98.5 (88.6 – 105.3)	2.36	9.2	rv0663					
	Variation (%) explained by all QTL				35.0					29.3	
	Variation (%) explained by genome-wide significant QTL				13.7					29.3	
PK	qPK-2*	2	60.1 (53.0 – 73.1)	2.61	9.5	pps0660	60.1 (56.5 – 61.3)	3.90	3.56	12.5	pps0660
	qPK-4*	4	65.0 (42.7 – 68.8)	2.11	7.7	pps0130	65.0 (64.6 – 70.3)	3.90	4.69	15.6	pps0130
	Variation (%) explained by all QTL				17.2					28.1	
	Variation (%) explained by genome-wide significant QTL				9.5					15.6	
TL	qTL-7.1*	7	34.4 (32.2 – 48.0)	3.37	12.0	pps0060	34.4 (31.1 – 34.8)	3.88	4.28	24.1	pps0060
	qTL-7.2	7	53.2 (49.4 – 64.9)	3.57	14.1	pps0099					
	Variation (%) explained by all QTL				26.1					24.1	
	Variation (%) explained by genome-wide significant QTL				26.1					24.1	
TW	qTW-5*	5	57.5 (50.9 – 72.5)	2.71	10.0	pps0036	57.5 (50.0 – 58.1)	3.96	4.60	14.0	pps0036
	qTW-7*	7	105.3 (93.5 – 105.3)	2.37	8.8	dlf020					
	Variation (%) explained by all QTL				18.8					14.0	
	Variation (%) explained by genome-wide significant QTL				18.8					14.0	
LL	qLL-1	1	20.6 (10.0 – 38.9)	2.29	9.2	pps0969					
	qLL-2*	2	40.1 (37.3 – 48.2)	3.60	12.8	pps0113	40.1 (38.2 – 42.0)	3.91	4.48	16.2	pps0113
	qLL-7	7	100.5 (91.3 – 105.3)	2.26	8.3	pps0002					
	Variation (%) explained by all QTL				30.3					16.2	
	Variation (%) explained by genome-wide significant QTL				12.8					16.2	
TN	qTN-1	1	20.6 (12.2 – 33.7)	2.18	9.1	pps0969					
	qTN-2.1*	2					37.6 (36.1 – 40.0)	4.00	4.26	10.1	pps0113
	qTN-2.2	2	43.7 (20.2 – 51.3)	2.62	9.9	nfa022					

Table 5.7 (Continued)

			SIM				MQM				
Trait	QTL	Linkage group	Position (cM ± 1 LOD)	Peak LOD	PVE (%)	Nearest marker	Position (cM ± 2 LOD)	LOD threshold	Maximum LOD value	PVE (%)	Marker
TN	qTN-3.1	3	31.7 (23.9 – 38.7)	2.45	10.1	pps0558					
	qTN-3.2	3	79.2 (54.3 – 106.0)	2.19	9.7	pps0164					
	qTN-4	4	30.8 (0.1 – 41.8)	2.26	8.8	pps0062					
	qTN-5.1*	5	60.6 (59.1 – 73.9)				64.6 (62.4 – 78.8)	4.00	4.34	12.1	pps0718
	qTN-5.2	5		3.10	12.0	rv1112					
	Variation (%) explained by all QTL				59.6					22.2	
	Variation (%) explained by genome-wide significant QTL				32.0					22.2	
DW	qDW-1.1*	1					32.3 (29.0 – 35.3)	4.03	4.17	12.4	pps0290
	qDW-1.2	1	28.9 (24.4 – 34.9)	2.48	9.3	ppt0013					
	qDW-2.1*	2	40.1 (36.0 – 51.4)	3.62	12.8	pps0113	40.1 (37.4 – 40.5)	4.03	5.07	8.9	
	qDW-2.2	2	89.0 (80.9 – 96.1)	2.47	9.0	rv0188					pps0113
	qDW-5.1*	5					72.1 (69.4 – 72.4)	4.03	4.29	21.3	
	qDW-5.2	5	61.6 (59.2 – 92.8)	3.09	11.1	rv0340					pps1115
	Variation (%) explained by all QTL				42.2					42.6	
	Variation (%) explained by genome-wide significant QTL				42.2					42.6	
PI	qPI-1.1	1	20.6 (13.5 – 28.9)	2.38	10.0	pps0969					
	qPI-1.2	1					32.3 (29.0 – 36.1)	3.90	3.47	5.3	rv0244
	qPI-2.1*	2					40.1 (37.2 – 41.5)	3.90	5.05	7.0	pps0113
	qPI-2.2	2	60.1 (43.6 – 66.1)	2.70	10.0	pps0660					
	qPI-2.3	2	89.0 (73.9 – 96.1)	2.26	8.3	rv0188					
	qPI-3.1	3	52.6 (40.4 – 65.1)	2.44	8.9	pps0373					
	qPI-3.2	3	101.7 (89.8 – 111.6)	2.51	9.8	pps0483					
	qPI-5.1*	5					69.6 (64.8 – 72.4)	3.90	12.32	24.3	pps1115
	qPI-5.2	5	60.6 (55.4 – 69.6)	3.08	11.2	rv1112					
	qPI-7	7	105.3 (99.0 – 105.3)	2.04	7.6	dlf020					
	Variation (%) explained by all QTL				65.8					36.6	
	Variation (%) explained by genome-wide significant QTL				49.9					31.3	

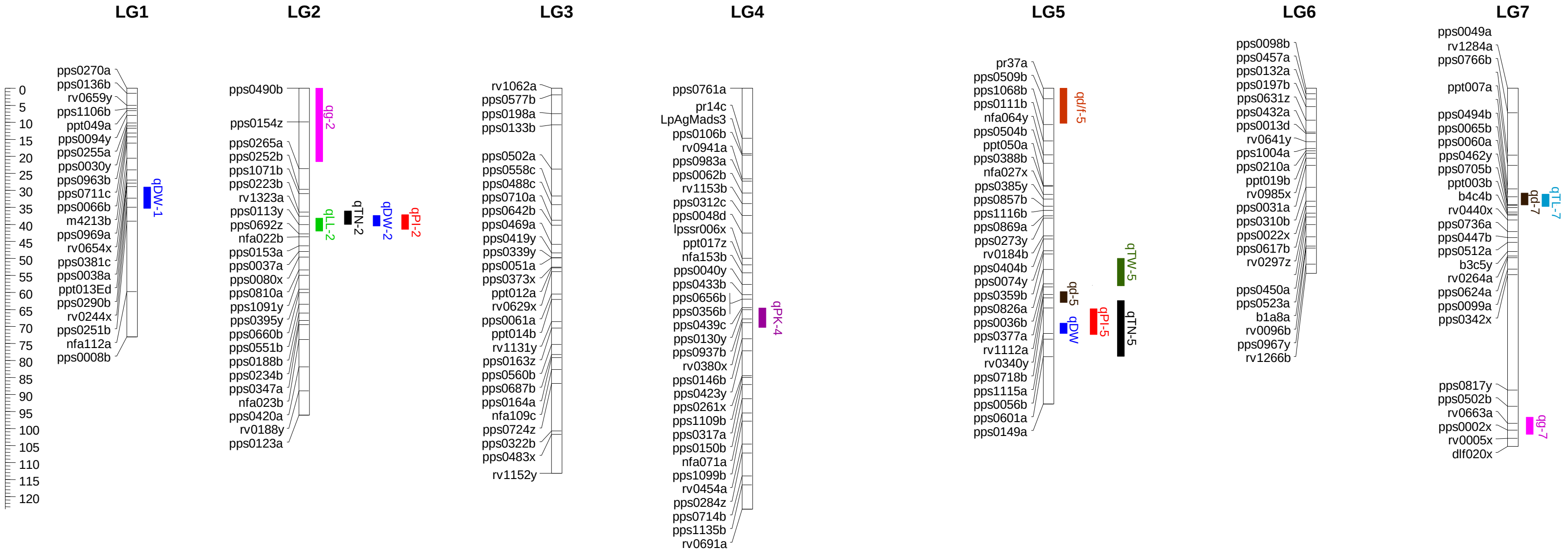


Figure 5.3: Location of QTL for Rubisco turnover and herbage yield traits on the IxS reference genetic map (mainly SSR-based) of perennial ryegrass, based on the results of multiple QTL mapping (MQM). Molecular markers are indicated on the left of each linkage group (LG), and QTL are indicated as filled bars on the right. Bar lengths indicate a LOD drop of 2.0 on either side of the maximum likelihood position. Map distances are indicated by the centimorgan scale at the left.

Table 5.8: Genotype class means and allelic effects associated with seventeen significant ($P < 0.05$) QTL for herbage yield and Rubisco turnover traits measured on 135 F_1 plants of the Grasslands II mapping population. Impact parent (I) alleles = a and b; Samson parent (S) alleles = c and d. TW = tiller weight; LL = leaf lamina length (mm); TN = tiller number; DW = herbage dry weight (g); PI = productivity index; *d* = maximum Rubisco content (mg N/g leaf dry weight); *f* = curve width measure; *d*/*f* = *d*:*f* ratio; *g* = time of *d* (days); PK = average of the two Rubisco content determinations closest to *d* (mg N/g leaf dry weight); TL = residual Rubisco content measured as the average of the last two time points(mg N/g leaf dry weight).

	Trait	Linkage	QTL Position	Genotype means				Allelic effects		
Marker	QTL	Group	(cM)	ac	ad	bc	bd	Maternal	Paternal	Parent
pps0002	qg-7	7	102.3	12.9	13.4	11.9	13.1	1.2	-1.8	S
pps0036	qTW-5	5	57.5	109.6	106.1	102.1	135.5	-22.0	-29.9	S
pps0060	qTL-7	7	34.4	17.4	26.6	33.6	38.1	-27.7	13.7	I
pps0065	qd-7	7	34.2	40.8	44.1	43.8	47.4	-6.3	-7.0	S
pps0113	qTN-2	2	37.6	69.0	49.6	69.1	64.4	-15.0	24.1	S
	qLL-2	2	40.1	326.6	372.9	242.0	172.0	285.6	23.7	I
	qDW-2	2	40.1	5.7	3.8	6.9	5.0	-2.3	3.8	S
	qPI-2	2	40.1	4.5	4.3	4.6	4.4	-0.2	0.4	S
pps0130	qPK-4	4	65.0	38.3	42.1	75.2	46.6	-37.5	28.8	I
pps0154	qg-2	2	9.9	12.9	12.0	12.6	11.3	1.0	2.2	S
pps0290	qDW-1	1	32.3	5.4	3.9	4.0	6.1	-0.8	-0.7	I
pps0509	qd/f-5	5	3.1	60.3	47.2	54.6	49.8	3.1	17.9	S
pps0660	qPK-2	2	60.1	42.3	44.6	40.8	51.0	-5.0	-12.5	S
pps0718	qTN-5	5	64.6	69.0	46.3	50.3	70.0	-5.0	2.9	I
pps1115	qDW-5	5	72.1	5.7	11.4	8.7	11.4	-2.9	-8.5	S
	qPI-5	5	69.6	4.5	3.9	5.1	5.2	-1.8	0.5	I
rv0340	qd-5	5	61.6	40.8	38.2	42.1	36.0	0.8	8.6	S

5.5. Discussion

5.5.1. Repeatability and other experimental design considerations

One point of interest in a study of this type is the repeatability of the results obtained on re-measurement or across a wider range of environments. For Rubisco turnover parameters this was not rigorously tested in the current series of experiments though the comparison between the 18 genotypes measured in both Experiments 2 and 3 (Section 5.4.2) is at least indicative, and the conclusion has to be that the cross-experiment correlation in the Rubisco turnover data is low. However, cross experiment correlation could be more rigorously examined for the 135 mapping population and two parent genotypes common to both Experiment 3 and Sartie's (Sartie, 2007) two earlier experiments. There too, cross-experiment correlation was non-significant for a number of traits (TN, DW, PI), and correlations were weak to moderate (0.19 – 0.31) where significance was obtained (Section 5.4.4). Despite the low correlation of data across experiments, it cannot be said that no 'genotypic signal' was detected, since genotype differences were highly significant within each experiment (Table 4.1, 5.1) and also significantly correlated with other traits measured (Table 4.2, 5.2). A lack of correlation in DW and TN for the same genotypes in separate experiments might be expected if one experiment measured plants under closed canopy conditions while another measured seedling plants actively expanding in size. For Experiment 3 in this study, the positive TN:TW of correlation (Table 5.2) and patterns of coefficients in PC1 (Tables 5.3 and 5.6) suggest the latter, while in Sartie's experiments and in Experiment 2 (Tables 4.2 and 4.3) there was a negative TN:TW association suggesting the former. Clearly, since Rubisco turnover parameters do not appear to correlate well between Experiments 2 and 3, yet there is a strong indication of 'genotypic signal' in the Rubisco turnover data, the reasons why data for these traits do not appear to correlate well across experiments would be an interesting area for further research.

The assumption of a 20% increase in leaf length described in Section 5.2.4 was based on knowledge that the process of coordination between successive phytomers determines that successive leaves on a grass tiller are usually larger than the previous leaf (Sartie et al., 2009; Verdenal et al., 2008). TW_{Large} as a measure of tiller weight was based on the tillers used for LL determination. The choice to use TW over TW_{Large} as an estimate of tiller weight was based on the stronger correlation of TW with the Sartie (2007) autumn data (Table 5.4). The autumn data was chosen over the spring data from the Sartie (2007) study because the relationship between correlated traits was stronger. This may possibly indicate that morphological traits reported in the Sartie (2007) spring experiment were influenced by reproductive development of plants.

5.5.2. Rubisco turnover in the mapping population

The existence of differences between genotypes seen in the mapping population sub sample and reported in Chapter 4 was reconfirmed for the larger sample of 135 genotypes from mapping population for which Rubisco turnover curve parameters were eventually obtained. The range in all three Rubisco turnover traits *d*, *f*, and *g* greatly exceeded the mean SE determined in Sigma Plot (Table 5.1). The biological significance of these genotypic differences in curve parameters was examined through simple correlation and multivariate PCA as set out in Section 5.4 and discussed below.

With respect to patterns of association between Rubisco turnover curve parameters themselves, the inverse relationship between *d* and *f* that was not significant in simple correlation analysis in Experiment 2 (Table 4.2) though apparently reflected in PCA (Table 4.3) did assume significance with the larger sample in Experiment 3 (Table 5.2) and was accompanied by a logically consistent inverse correlation between *d* and *g*. Paradoxically, however, there was also a significant inverse correlation between *f* and *g* meaning that in genotypes with wider curve width, Rubisco concentration tended to peak earlier.

With respect to the two parameters intended to quantify systematic lack of fit of the Rubisco turnover data to the log-normal model, the positive correlation of *d* with PK (Table 5.2) was to be expected, and was also consistently observed in PCA results (Table 5.3) suggesting that the two parameters captured the same information although with varied efficiency. The positive correlation observed in this study between *d* and TL was unexpected given the strong negative correlation of *d* with *f* (Table 5.2) but probably it does provide insight into the nature or mechanism of the negative correlation between *f* and *g*. This implies that different genotypes within the mapping population that attain high *d* might be broadly categorised into two groups: high-*d* and Rubisco subsequently degraded, or high-*d* and Rubisco subsequently retained. The latter case raises the question of a possible modification of the model for determination of Rubisco turnover characteristics in order to take into account this residual protein as it may have adverse effects on plant yield. A second possible explanation for the high TL values of some genotypes is that this is an evaluation artefact because synchronising leaf age was one of the most challenging and probably limiting steps in this experiment meaning that the last two sampling times may not necessarily have corresponded with the time when Rubisco concentration had levelled off in senescence. However, one point against the measurement artefact hypothesis is that TL was also significantly correlated with plant morphology traits. Both PK and TL are discussed further under model evaluation in Sections 7.2 and 7.3.

5.5.3. Rubisco turnover and herbage yield parameters

Moderate associations between Rubisco turnover and plant morphology were observed (Table 5.2, 5.3). Correlation analysis (Table 5.2) indicated that the link between Rubisco turnover and herbage yield traits was described by the amount of Rubisco (*d*) rather than Rubisco pattern of synthesis and degradation (*f* and *g*). PCA, on the other hand, identified several possible Rubisco turnover-herbage yield trait combinations, some of which were not evident in correlation analysis (Table 5.3). Since PCs are uncorrelated, this separation was possibly due to independent trait associations involving different subsets of genotypes in the mapping population in each case. This sheds light on how patterns observed among the PC coefficients (Table 5.3) can differ from those seen in the correlation analysis. Lower-order PCs could be regarded much as association of residuals from the regression of two highly correlated traits with a third trait. The third trait then explains variation around the more dominant regression relationship, with the minor association not apparent until the major source of variation is accounted for.

In PCA of Experiment 2 data, the strongest link between Rubisco turnover and yield as measured by DW was an intuitively logical pattern described in PC3 (Table 4.3) and explaining 16% of data variation, of higher DW associated with lower *d*, earlier *g* and wider *f*. An independent *d*, *f*, and *g* association pattern was also seen in PC2, but not linked to DW. By contrast in Experiment 3 it was PC1 explaining 36% of variation that was most associated with DW (Table 5.3). This PC appears to be a more general plant size descriptor than PC3 in Experiment 2, and there is a modest association of high yield with lower *d*. Meanwhile, PC2 identifies a pattern of high *d:f* ratio where *d* is, if anything, positively associated with DW (Table 5.3). Introduction of information on genotypic differences in plastochron from Sartie's (Sartie, 2007) experiment (Table 5.6) superficially appears to displace the trait association at PC2 (Table 5.3) to PC3 (Table 5.6) but the correlation of PC scores (Table 5.9) indicates that while this is true, PC2 and PC3 from the PCA analysis that includes Sartie's (2007) plastochron data are both highly correlated with the original PC2. Therefore, the addition of the plastochron data effectively allowed two independent relationships to be formed from the original PC2 (Table 5.3): long LED and low *d*/*f* (PC2, DW neutral, but indicating some evidence of co-ordination between plastochron and *f*), and long LED and high *d*/*f* (PC3, modest positive association with DW (Table 5.6)).

Table 5.9: Correlations between principal component (PC) scores without (a) or with (b) plastochron data of Sartie (2007) included in the PCA.

PC	PC1a	PC2a	PC3a
PC1b	0.949^{**}	0.115^{ns}	0.010^{ns}
PC2b	0.279^{**}	-0.704^{**}	-0.060^{ns}
PC3b	0.126^{ns}	0.700^{**}	-0.088^{ns}
PC4b	0.022^{ns}	0.016^{ns}	0.992^{**}

^{ns}, ** represent non-significant and significance at $P < 0.01$, respectively.

It is often stated that the photosynthetic inefficiency of Rubisco is compensated for, in part, by a large amount of Rubisco protein in the leaf reaching up to 50% of total leaf protein (e.g. Spreitzer and Salvucci, 2002; Stitt and Schulze, 1994). This implies that a higher leaf Rubisco concentration would increase plant productivity. It is clear from the current measurements (Table 5.2, 5.3) and backed by the result in Chapter 4 (Table 4.3) that high yield was encouraged mainly by lower rather than higher leaf Rubisco concentration. A lower *d* acted in association with a lower TL to enhance yield (PC1 (Table 5.3). A lower TL suggests that Rubisco-N was more efficiently redistributed from older leaves to new leaves in these plants leading to increased plant yield. Efficient leaf N remobilisation has been linked to increase in plant yield in numerous studies (e.g. Kichey et al., 2007; Mickelson et al., 2003; Vansanford and Mackown, 1987). From this observation, it follows that while high Rubisco concentration is favourable for increased leaf photosynthetic rate, it may not be favourable for Rubisco remobilisation and the maintenance of high whole plant photosynthetic rate. Since plant yield is a combination of the two physiological processes, it appears that these two processes are better balanced by plants with lower amounts of Rubisco in their leaves. Another notable observation in PC1(also in PC2) was that productivity of plants was a result of plant vigour since size/density compensation (Hernández Garay et al., 1999; Matthew et al., 1995) was not evident. This is possibly because plants were sufficiently spaced that they did not form a closed canopy. In these conditions induced tiller death that causes SDC would have been reduced.

The association of a higher *d* and a lower *f* with increased yield in PC2 (Table 5.3) is an example of a pattern revealed in PCA but not in the simple correlation patterns (Table 5.2) as discussed earlier in this section. In Chapter 4 (Table 4.2), a superficially similar turnover pattern was associated with low plant yield, which would be intuitively explained by the energy cost of Rubisco synthesis and leaf production that was uncompensated for in photosynthesis. While the result in the current study may appear contradictory, it is clear that the larger number of genotypes analysed in Experiment 3 has allowed detection of more complex trait association patterns than were detected in Experiment 2. Also, there is evidence that a faster decline in leaf Rubisco content if accompanied by an increase in sink capacity can be productive (e.g. Brady, 1988; Paul and Foyer, 2001; Simova-Stoilova et al., 2001). TN had a relatively large coefficient for PC2 (Table 5.3) and therefore increased sink capacity for these plants may be explained by increased tillering. TN did not correlate with Rubisco turnover (Table 5.2), but it is possible that it may correlate with other photosynthetic characteristics not evaluated in this study.

The pattern of a higher and earlier Rubisco peak identified by PC3 (Table 5.3) may represent a rapid synthesis of Rubisco during leaf expansion as described by earlier work (e.g. Mae et al., 1983; Suzuki et al., 2001). This pattern also involves a slower protein loss during senescence (as indicated by a wider *f*) and a large amount of Rubisco being retained in the senescent leaf (i.e. high TL) (Table 5.3). It is likely that under this scenario the slower protein loss was not accompanied by a slower rate of leaf senescence/ longevity (as reviewed by Masclaux-Daubresse et al., 2008). Also, the C balance could have been very high meaning that Rubisco was kept high for a long time. Degradation as predicted by the Rubisco turnover model (Irving and Robinson, 2006) may cover a large part of leaf development. In view of this, it would be important for a concurrent evaluation of leaf longevity and Rubisco degradation to identify plants with a match or mismatch in the two processes.

The tendency to high tillering exhibited by plants with a late Rubisco peak and high TL (i.e. high leaf Rubisco concentration late in the leaf's life seen in PC4 (Table 5.3), may be explained by the proximity of source (leaf) and sink (tiller bud). For example, it has been shown in previous studies that tiller buds in perennial ryegrass and tall fescue, and stolons in white clover are triggered by a good C substrate supply (Kemball and Marshall, 1995; Yang et al., 1998).

A point of consideration at this stage is the stability of the signal(s) detected in this experiment if measured in a different experiment/environment. One approach to this is to examine the relationships between the different measures of tiller weight (TW and TW_{Large}). The large correlation coefficient of TW and TW_{Large} (Table 5.4) suggests that either of the two measures could be used for estimation of tiller weight. The correlation of TW and TW_{Large} with tiller weight data from Sartie's autumn and spring experiment (Sartie, 2007) are an indication the both measures are stable across experiments. However, the strength of genotype signal appears to vary with the season (TW stronger in autumn TW_{Large} stronger in spring) (Table 5.4). Overall, TW had relatively large correlation coefficients with tiller weight data in both seasons and was therefore better than TW_{Large} for introducing information about genetic potential for tiller weight to the PCA.

Data comparison for herbage yield variables measured in this experiment and the Sartie (2007) autumn experiment showed stability of LL and TW across environments. The association between plastochron variables (Alf, Alg, and LED) and Rubisco turnover identified by multivariate analysis (Table 5.6) were biologically useful. For example, in PC2 decreased leaf appearance rate (high Alf) and an increase in LED (as reported by Bahmani et al., 2000; Robson, 1967) were associated with a wider Rubisco curve, *f*

(Table 5.6). This suggests increased Rubisco retention duration was associated with increased leaf life span, and therefore possible synchronisation between Rubisco turnover and leaf longevity. However, it was surprising that these interactions were not associated with increased DW (Table 5.6). In PC3, the pattern of Alf, Alg, LED, and LER was similar to that in PC2 but was associated with a higher *d*, a lower *f*, and a moderate increase in yield and yield components (Table 5.6). Similar counterintuitive patterns were observed in Table 5.3, and represented unique patterns exhibited by some genotypes in the mapping population. Increased LER and LL were associated with low values of *d*, PK and TL (Table 5.5). This result suggests that attainment of long leaves in these plants was achieved by increased LER (e.g. Bahmani et al., 2000; Hazard et al., 1996; Sartie, 2007). This relationship between LER and Rubisco turnover is a logical one given that in this study, size of the plant as indicated by high TW (and LL), TN and DW was mainly linked to a lower *d*, PK and TL (Table 5.3).

As described in Section 4.3.1, the pollen parent was infected with *Neotyphodium lolii* strain AR6 endophyte while the seed parent was infected with the wild-type *N. lolii*. Symbiotic plant-endophyte interactions have been known to affect plant performance parameters such as plant productivity, drought tolerance, and mineral stress tolerance. Recently, a significant interaction between endophyte infection and *L. perenne* cultivar on soluble protein accumulation has been reported (Rasmussen et al., 2007). It should be noted therefore that plant-endophyte interaction might have influenced the outcome in this study.

5.5.4. QTL discovery

QTL analysis indicated a wide dispersion of the regulation of Rubisco turnover and the herbage yield traits across the perennial ryegrass genome. Some QTL for morphological traits coincided with those discovered in a previous study (Sartie, 2007) but others were new. Some QTL were detected on LG7 where a major heading date QTL was located (Armstead et al., 2004) suggesting that these QTL may be subject to influence by time-to-flowering differences in the mapping population. Multivariate analysis showed some positive association of Rubisco turnover with herbage yield traits, but there was little evidence that the genetics of Rubisco turnover and the genetics of the herbage yield traits measured in this study were interdependent. However, some QTL identified for

Rubisco turnover were mapped in the regions previously identified as having loci for other plant growth parameters.

5.5.4.1. Regulation of Rubisco turnover

QTL for *d* were not mapped near QTL for *g* (no QTL were detected for *f*) suggesting that *d* and *g* are controlled by different genetics or by factors not well represented in the log-normal turnover model. QTL determining the leaf concentration of Rubisco were detected on LG5 and LG7 (Table 5.3, Fig.5. 3). A previous study mapped QTL for Rubisco content on rice chromosome 8 and 9 (Ishimaru et al., 2001a). Comparative analyses have shown syntenic relationships between rice chromosome 9 and perennial ryegrass LG5 (Jones et al., 2002b), and rice chromosome 8 and perennial ryegrass LG7 (Faville, 2005; Sim et al., 2005). Considering this relationship, more work will be needed to test co-location between the ryegrass and rice QTL.

The overlap of QTL for *d* and TL on LG7 reflected the correlation between these traits as demonstrated by simple correlation analysis (Table 5.2). While this overlap may indicate genetic interdependence or pleiotropic inheritance, allelic effects were opposite for these traits (Table 5.8). This suggests that there may be some complex genetic interactions between these traits that this study cannot resolve. The detection of qd/f-5 suggests that there may be some mechanisms that determine the ratio of Rubisco peak to the curve width, signifying Rubisco turnover that would have a larger cost of Rubisco synthesis and less photosynthetic contribution. The distinct position of qd/f-5 relative to the two QTL for *d*, despite the two traits having a very strong positive correlation, shows that the *d*/*f* ratio may be linked to other complex traits besides *d*. This also shows that a strong statistical correlation does not necessarily imply a common genetic basis as has been reported in other QTL studies (Ishimaru et al., 2001a; Simko et al., 1997). PK was an indicator variable for *d*, and the two traits were correlated (Table 5.2). While MQM showed no coincidence of the QTL for PK and *d*, SIM consistently co-located them on LG4 and LG7, although one or the other was suggestive. Given the strong correlation of *d* and PK (Table 5.2, 5.3), it is possible that the two traits are co-regulated but the detection accuracy by MQM may have been affected by the relatively small size of the mapping population.

LG2 and LG7 where QTL for *d* and/or *g* were identified, are known to contain a minor and a major heading date locus, respectively, as revealed by a recent QTL study (Armstead et al., 2004). In the Grasslands II mapping population, some QTL for days to heading (DH) identified by Sartie (2007) were located on these LGs. On LG2, the two QTL for DH identified in autumn and spring (Sartie, 2007) overlapped with the QTL for *g* identified in this study. On LG7, QTL for *d* and TL mapped close to the QTL for DH identified in autumn. This means that precautions must be taken as these QTL may be influenced by time-to-flowering differences. A negative correlation between heading date and N remobilisation from leaves to the grain has been reported in barley (Mickelson et al., 2003). Other studies have also reported the effect of developmental genes on physiological characteristics including the influence of heading date on grain protein concentration (Forster et al., 2000; See et al., 2002).

5.5.4.2. Co-regulation of Rubisco turnover and herbage yield parameters

Coincidence of QTL for agronomic traits and QTL for Rubisco turnover affecting herbage yield means that yield can be defined in physiological terms. In this study there was only one case representing potential genetic factors common to Rubisco turnover and herbage yield traits. This was on LG5 where QTL for TN overlapped with QTL for *d*. This interaction appears complex since the two traits involved had opposite allelic effects (Table 5.6) and correlated negatively (Table 5.2). Two logical possibilities are one gene acting to affect two traits, but in opposite directions (pleiotropy) or close genetic linkage without phenotypic relationships (as described by Tuberosa et al., 2002).

Despite the weak associations between Rubisco turnover and herbage yield traits measured in the Sartie (2007) autumn study (Table 5.5), some QTL have been mapped in common locations. Co-mapping (interval overlaps) were found for QTL for PK with Alg and LED on LG4, *d* and TL with LL on LG7. The common location of LL with *d* and TL probably reflected their strong negative correlation (Table 5.5). These coincidences did not include the near significant associations found between *g* and LED and Alf found in Chapter 4 (Table 4.2). While the effect of genotype x environment interaction is an important factor to consider, these associations mainly involving leaf development characteristics suggest that the genetics of Rubisco turnover may be related to other traits not measured in this study.

There were no coincidences between QTL for Rubisco turnover parameters and QTL for DW (Table 5.7, Fig. 5.3). A previous report (Ishimaru et al., 2001b) indicated that the structural genes controlling C metabolism including *rbcS*, did not overlap with the QTL controlling yield in rice. Given that the amount of Rubisco is partly determined by the levels of *rbcS* and *rbcL* mRNAs (Imai et al., 2005; Suzuki et al., 2001), it would be logical to deduce that yield is not limited by the amount of Rubisco. This result somewhat agrees with previous findings in different crops (Osaki et al., 1993; Simova-Stoilova et al., 2001) in which high Rubisco content was not always associated with high yield. Considering the results of PCA, particularly PC1, phenotypic performance of all measured agronomic traits was in fact enhanced by a lower Rubisco peak (Table 5.2). This finding is supported by previous work that have reported an association of higher net photosynthetic rates and NUE with lower Rubisco in rice and Australian native species (Ray et al., 2003; Warren et al., 2000). It follows therefore that although Rubisco is a key component of photosynthesis in C_3 plants, plant productivity is not always limited by the amount of Rubisco.

Rubisco peak, *d*, correlated negatively with *f* (Table 5.2). This relationship suggests an association of a lower Rubisco peak with a longer retention period and sustained photosynthesis and vice versa. Such a pattern where plants maintain a high Rubisco content and so keep photosynthetic activity high has been reported to favour high yield in a number of crops (Osaki et al., 1993). MQM did not detect any QTL for *f*, but SIM detected an indicative QTL (qf-2) on LG2 whose confidence interval overlapped with those QTL for PI, TN, and DW (Table 5.7). The confidence interval of QTL for LL mapped very close (0.3 cM) to this suggestive QTL (Table 5.7). It is also possible that the relatively low number of plant genotypes (n=135) evaluated in this study may have reduced the sensitivity of QTL detection for the parameters examined in this study.

5.5.4.3. QTL for herbage yield traits

The strong positive correlations among herbage yield traits were also reflected in support interval overlaps and/or coincidences of the peaks of their QTL on LG2 and

LG5. Such co-location of QTL for herbage yield traits is often found in perennial ryegrass (e.g. Dolstra et al., 2007; Sartie, 2007). The allelic effect was aligned for three QTL (qTN-2, qDW-2 and qPI-2) while opposite for qLL-2 at pps0113 marker locus indicating that a single gene in this region may be acting pleiotropically to increase DW and PI by increasing TN. However, the same gene seems to decrease LL. Given that LL is related to TW, this may be a reflection of the SDC phenomenon (Hernández Garay et al., 1999; Matthew et al., 1995; Sbrissia et al., 2003). Alternatively, it may be that a completely different gene is responsible for an increase in LL but it is tightly linked to the gene controlling DW, TN, and PI. In the cluster involving QTL for DW, PI, and TN on LG5, the allelic effects were similar for TN and PI and opposite for DW (Table 5.4). In this genomic region, SIM detected co-mapping of qTW-5 with qDW-5, qPI-5 and qTN-5 profiles, and it is likely MQM missed qTW-5 due the small sample size of the mapping population used in this study. If indeed the four QTL co-mapped, and taking into account that PI relates to TN and TW simultaneously (Matthew et al., 1995), then the allelic effects displayed by the four traits would have a significant biological meaning: The similar QTL effect for PI and TN and opposite to that of DW and TW indicates that a gene in this section of the genome is responsible for enhancement of PI through the increase in TN may also be responsible for a decrease in TW probably through a low leaf elongation rate (Lemaire and Chapman, 1996). Accordingly, the decrease in TW is reflected in the decrease in DW.

A recent paper (Jin et al., 2008) reported the discovery of PROG1 gene on rice chromosome 7 that controls plant architecture, with pleiotropic effects on tiller angle, tiller number and grain yield. Rice chromosome 7 is syntenic to ryegrass LG2 (Faville, Personal comm.), and the position of PROG1 gene corresponds to the interval on ryegrass LG2 containing markers pps0113 flanking TN (and also LL, DW and PI (Table 5.5)). This possible conserved syntenic relationship between the ryegrass and rice genomes provides a means for the transfer of information from well characterised crop (rice) to ryegrass for identification of markers at molecular marker level that can be of agronomic importance.

5.5.4.4. Coincidences of QTL for herbage yield traits in the studies

Co-location and possible pleiotropic/tight linkage gene effects on some QTL influencing the traits evaluated in this experiment have been reported in previous experiments. For example, in the Grasslands II mapping population (n=200), Sartie (2007) found co-locations of QTL for TN with PI and DW, DW with LL, TW with LL, and DW with TN. Co-location of DW with TN was also reported in another perennial ryegrass mapping population derived from a cross between an F_1 and a doubled haploid (Dolstra et al., 2007). There were also cases of overlaps and close location of some QTL identified in the current study and that of Sartie (2007). For example, on LG1, the support interval (29.0 – 35.3 cM) of QTL for DW (qDW-1.1) identified in this study overlapped with that found in the Sartie (2007) autumn study (qDW-03-1.2, support interval 32.5 – 40.8 cM). On LG2, the locations of QTL for DW were close in the two studies (support intervals 37.4 – 40.5 cM (in this study) and 54.0 – 56.5 cM (in the Sartie (2007) study). On LG2, QTL for TN and PI (suggestive in this study) overlapped with significant QTL found by Sartie (2007). These overlaps and close location of QTL identified in different experiments may indicate stability of the QTL across environments.

Apart from the overlaps, there were also new QTL for herbage yield traits discovered in this study that were not identified by Sartie (2007). On LG5 where Sartie (2007) discovered only one QTL for Alf (qALf-04-5), this study identified three QTL, one each for TW, TN and DW (qTW-5, qTN-5, and qDW-5, respectively) in addition to the three QTL for Rubisco turnover (Table 5.5, Fig. 5.3). For a long time, agronomists have been aware of the strong G x E component in the expression of traits in many crops. QTL analysis is now revealing that different loci may be responsible for the control of the same trait in different seasons/environments. A more systematic investigation of the way different loci contribute to specific phenotypically expressed G x E interactions would be an interesting though resource intensive follow-up project.

5.6. Conclusions

QTL were identified for Rubisco turnover and for herbage yield characteristics measured in this experiment. More QTL were detected by SIM than MQM, suggesting that the small size of the mapping population (n = 135) used in this study may have reduced the capacity for QTL detection by MQM.

The genetics of Rubisco turnover and the genetics of the morphological traits evaluated in this study were not strongly interdependent. The regulation of the primary synthesis and degradation of Rubisco could not be directly linked with the herbage yield traits evaluated in this study through QTL co-location. However, statistically significant negative ($P < 0.05$) correlations were observed between d and LL, TW, DW and PI. Some QTL discovered for Rubisco were located in chromosomal regions previously identified as having QTL for leaf development parameters, suggesting that Rubisco turnover may be related to these parameters.

Correlation analysis and PCA (PC1 explaining 36% of data variation) clarified that larger sized plants demonstrated a modest tendency to lower than average peak leaf lamina Rubisco concentration. This association did not involve any tendency for differences in f or g, but the Rubisco turnover lack of fit parameter, TL, tended to be higher in smaller plants indicating Rubisco retention at leaf senescence as another facet of this association. Multivariate analysis confirmed independent Rubisco turnover patterns (e.g. PC1 involving a lower d and a lower TL, and PC2 involving a higher d and a lower f) within the mapping population both of which had some degree of association with DW. In summary, increased leaf Rubisco concentration was linked here to decreased DW, and not the reverse as commonly assumed. It is too early to tell if this information will be able to be harnessed for plant improvement.

Some QTL positions identified by Sartie (2007) were reconfirmed in this study indicating stability across environments. Other QTL and patterns of association between traits were unique to Experiment 3 and therefore further research would be needed before selection based on them could be used in plant improvement.

The co-localisation of QTL for multiple herbage yield traits including TN, LL, DW and PI around pps0113 marker locus on LG2, DW and PI (marker pps1115) and TN (marker pps0718) on LG5, and loci on LG5 and the direction of the allelic effects at these loci provide a strong indication of potential for commercial application in relation to plant productivity enhancement.

The two QTL identified for *d* were mapped on LG5 and LG7. These LGs have syntenic relationships with rice chromosome 9 and chromosome 8 on which QTL for Rubisco content were mapped in a previous study. Similarly, QTL for TN, LL, DW, and PI on LG2 were mapped in the region likely to be syntenic to rice chromosome 7 where the gene (PROG1) controlling plant architecture (including TN, tiller angle, and grin yield) has been located. These coincidences are remarkable and may suggest conservation of gene order over evolutionary time. Further work is needed to identify the proximity of functionally associated markers and QTL in order to be useful in the implementation of MAS in perennial ryegrass.

CHAPTER 6

Rubisco turnover characteristics of long-leaved and short-leaved perennial ryegrass populations after two and four cycles of divergent selection

6.1. Introduction

Experiments in Chapter 4 and Chapter 5 evaluated genetic variation and the association of Rubisco turnover with herbage yield characteristics in the Grasslands II perennial ryegrass mapping population plants. One point of interest from these chapters is that the strongest correlative link between Rubisco turnover parameters and plant yield was that plants with higher DW exhibited on average a lower Rubisco peak either with an extended Rubisco retention period or a lower residual Rubisco content in the senescent leaf. Low leaf Rubisco concentration in larger plants may arise from an energy cost when more Rubisco than necessary is synthesised as explained in Section 5.5.3. Alternatively, it may result from a dilution effect when a leaf grows faster.

A question that arises from studies like these, therefore, is whether coupling between Rubisco turnover and leaf formation and senescence is inherent in leaf physiology and the norm, or whether there is variation in the pattern of Rubisco synthesis and degradation between ryegrass plants from differing populations with differing leaf formation strategies. An evaluation of Rubisco turnover in plants explicitly selected for different leaf length could help clarify such questions. In this chapter, the long-leaved and short-leaved INRA perennial ryegrass breeding populations derived from divergent selection for leaf length were evaluated to give some structured variation in plant morphology against which to assess Rubisco turnover.

6.2. Objectives

The objectives of this experiment were;

- To determine how morphological traits such as LER and LED varied between perennial ryegrass populations of contrasting leaf length;

- To examine differences in Rubisco turnover characteristics in perennial ryegrass breeding populations with contrasting leaf length;
- To examine the effect of divergent selection for leaf length on Rubisco turnover and on other morphogenetic characteristics.

6.3. Materials and methods

6.3.1. Plant material and growth conditions

The study was conducted at INRA, Lusignan, France, between June 2008 and October 2008. Four INRA *L. perenne* breeding populations derived from divergent selection for leaf length from a collection of French ecotypes (Hazard et al., 1996) were used in this experiment. Populations LLC2 and LLC4 were the long-leaved selections derived from the second and fourth selection cycle, respectively. SLC2 and SLC4 were the short-leaved selections derived from the second and fourth selection cycle, respectively.

Seeds of the four breeding lines were germinated on filter paper moistened with deionised water within closed Petri dishes in a temperature controlled room on January 15 2008. The Petri dishes were kept in a growth chamber at 24˚C in the dark for 3 days. The seedlings were transplanted into individual pots of approximately 3L capacity containing fine sand and transferred to a glasshouse. For each breeding population, five plants (genotypes) were selected. The seedlings were allowed to grow until they had produced several tillers. On June 30 2008, each of the five genotypes per population was sub-divided to provide thirteen clones which were transplanted into pots (one plant per pot) and cut to 4 cm height. The pots were transferred to a growth chamber and arranged on the table in a completely randomised design (Fig. 6.1). Randomisation was achieved by use of a random number generator for all plant genotypes of the four breeding populations.The growth chamber was operated with day/night temperature of 20˚C and 80% relative humidity. The photoperiod was 14 h, with quantum flux density of 500 µmol photons $m^{-2}s^{-1}$ at plant level. The tillers were cut again to 4 cm on 24 July 2008 and observed for the emergence of the third leaf.

Plants were watered automatically twice daily with the nutrient solution containing 0.02 mol m^{-3} KNO_3, 5 x 10^{-3} mol m^{-3} Ca $(NO_3)_2.4H_2O$, 0.02 mol m^{-3} NH_4NO_3, 2 x 10^{-3}

mol m^{-3} $CaCl_2.2H_2O$, 0.01 mol m^{-3} NaCl, 2 x 10^{-3} mol m^{-3} $MgSO_4.7H_2O$, 4 x 10^{-3} mol m^{-3} KH_2PO_4, 10^{-3} mol m^{-3} K_2HPO_4 (Gastal and Saugier, 1986) and 5 x 10^{-4} mol m^{-3} H_3BO_3, 5 x 10^{-5} mol m^{-} $CuSO_4.5H_2O$, 4 x 10^{-5} mol m^{-3} $MnSO_4.H_2O$, 4 x 10^{-5} mol m^{-3} $ZnSO_4.7H_2O$, 10^{-3} mol m^{-3} $Na_2MoO_4.2H_2O$, 10^{-3} mol m^{-3} $FeSO_4.H_2O$, 10^{-3} mol m^{-3} HEDTA (HEEDTA) (Maurice, 1997), dissolved in de-ionised water.

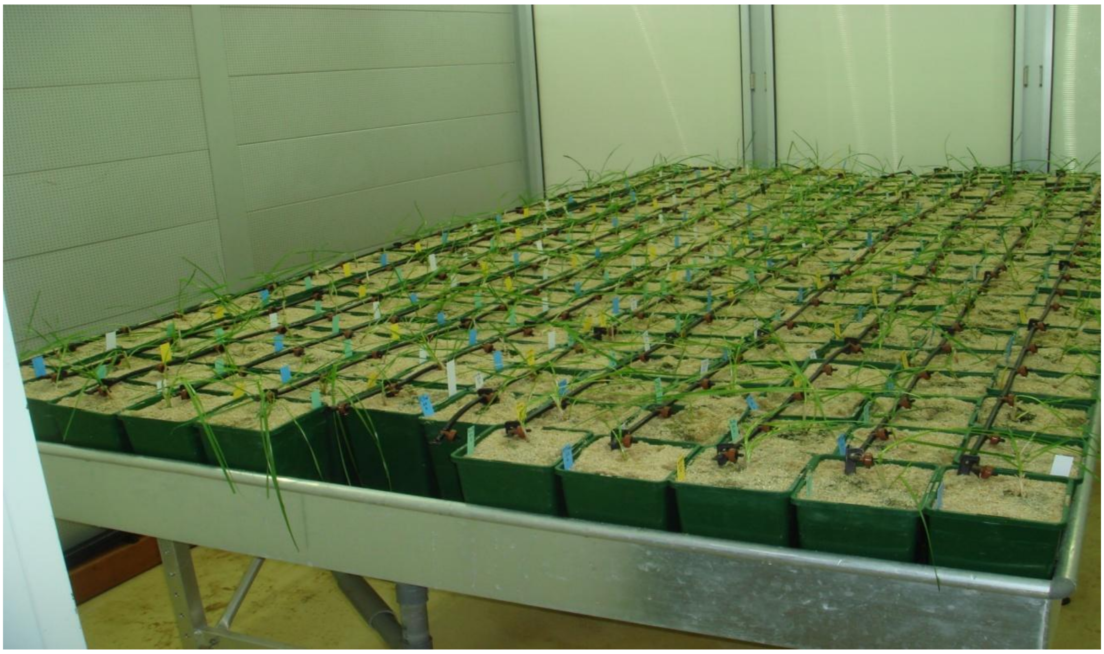


Figure 6.1: Perennial ryegrass plants of populations SLC2, SLC4, LLC2, and LLC4 in Experiment 4 growing in an environmentally controlled chamber - INRA, Lusignan, France. Light intensity was 500 µmol photons $m^{-2}s^{-1}$ at plant level. SL denotes ‘short leaf’, LL denotes ‘long leaf’, C2 indicates second selection cycle, and C4 indicates fourth selection cycle.

6.3.2. Assessment of herbage yield characteristics

Herbage yield trait measurements were taken on five genotypes (replicates) per breeding population. Leaf appearance interval (Alf) was measured as the average of the difference (days) between the appearance of the tips of five successive leaves for each genotype. Ligule appearance interval (Alg) was measured as the average of the difference between the appearances of ligules on five successive leaves per genotype. Leaf elongation duration (LED) was measured as the difference (days) between leaf tip appearance and ligule appearance of a given leaf. Leaf length (LL) was measured as the final length of the lamina. Leaf elongation (LER) was calculated as the ratio of LL:LED (cm/day). Tiller number (TN) was an average of the direct count of total number of tillers per plant and was carried out at the end of the experiment. Dry weight (DW) was measured as the average weight (grams) of the oven dried herbage above the soil level. The herbage was oven-dried at 80˚C for 48hrs. Tiller weight (TW) was derived as the ratio DW:TN (grams).

6.3.3. Sampling for Rubisco analysis

The third leaf blade was excised from the plant at the ligule 4, 7, 11, 15, 22, 29, 36 and 43 days after leaf tip emergence. In each sampling, three leaves per breeding population were harvested, had their width (LW) and length (LL) measurements taken, weighed and then immediately frozen at -80˚C until further analysis. Leaf area was estimated as LL*LW*K. Where K is the conversion constant determined for each sampling time and population by digitising the photocopied leaf blade images and determining their corresponding area using the public domain software, Image J (National Institute of Health, USA, http://rsb.info.nih.gov/ij/).

6.3.4. Determination of Rubisco content

Rubisco was determined using the procedure outlined in Chapter 3 (Section 3.2.3.2), but the calibration curve was made with reference Rubisco extracted from spinach. Leaf Rubisco concentration was expressed in three ways; (i) leaf area basis (mg Rubisco-N/cm^2), (ii) leaf fresh weight basis (mg Rubisco-N/g FW), and (iii) leaf blade basis (mg Rubisco-N/leaf blade). The concentration used for curve fitting was Rubisco-N/cm^2.

6.3.5. Photosynthesis measurements

Single leaf photosynthetic rates were determined using a portable IRGA (Horiba ASSA-1110, Horiba, Kyoto, Japan) with CO_2 control. The rate at which the gas was taken up in photosynthesis (or emitted in respiration) was measured by enclosing the leaf in a temperature-controlled chamber equipped with a fan. Measurements were made at a leaf temperature of 20˚C and a PAR of 2000 µmol photons m^{-2} s^{-1}. The ambient CO_2

concentration in the chamber was adjusted to 400 µmol CO_2 mol^{-1}. The relative humidity was maintained at 60 – 70%. Data were recorded after steady state equilibrium was achieved. Responses were determined in three replicate leaves per genotype for each time point. In all cases responses were determined on the third leaf. The first measurement was taken when the target leaves were eleven days old (i.e. eleven days after the emergence of their tips). At this age, the leaves had developed ligules and therefore were assumed to have fully expanded.

6.3.6. Statistical analyses

Rubisco turnover parameters *d*, *f* and *g* were determined in Sigma plot (version 11) using non-linear regression (data points for the log-normal curve were based on mg Rubisco-N/cm^2). These parameters together with all herbage yield parameters were subjected to analysis of variance, correlation analysis and principal component analysis (PCA), using SAS (SAS Institute Inc., Cary, NC, USA). One-way ANOVA was performed and the least significant difference (LSD, $P = 0.05$) was calculated using the Duncan New Multiple Range (DUNCAN) test. Individual values for each clonal replicate (20 plants = 5 clones x 4 populations) were the basis for the analysis of each measurement except photosynthesis. The difference in the average rate of net photosynthesis at each evaluation point was tested using Student’s t-test.

6.4. Results

6.4.1. Performance of the breeding populations

As expected, leaf length was significantly greater in the long-leaved than the short-leaved populations (Table 6.1). Increased leaf length of the long-leaved populations was associated with increase in both LER and LED, but especially LER, and also with increase in TW (Table 6.1). Differences were observed between long- and short-leaved populations for a number of herbage traits (DW, $P < 0.05$; TW, $P < 0.01$; LL, $P < 0.01$; LER, $P < 0.01$). Alf ($P = 0.096$) differed only marginally between the long- and short-leaved populations (Table 6.1). LED increased marginally ($P = 0.109$) from the 2nd to the 4th cycle populations (Table 6.1). In no case did any of the herbage traits differ significantly between 2nd and 4th selection cycle short- or long-leaved populations. However, there were at times opposing trends seen in the long- and short-leaved populations across selection cycles such that the end result was a statistically significant difference across populations. For example, Alf and DW did not differ between the LLC2 and the SLC2 plants. However, Alf was shorter and DW higher in the LLC4 than the SLC4 plants, and marginal divergence was only observed in Alf ($P = 0.072$) (Table 6.1). Among the determinants of Rubisco turnover, d differed marginally ($P = 0.109$) between the long- and short-leaved plants, but after the fourth selection cycle, a significant ($P < 0.01$) divergence had been achieved (Table 6.1). The curve width (f) did not differ between the long- and short- leaved populations or between cycles, but was wider in the LLC4 than in the LLC2 plants (Table 6.1). Even though the tendency to a narrower f through selection for short lamina length was not significant, the end result was a significant ($P < 0.05$) leaf length x interaction effect for this trait (Table 6.1). Selection for leaf size did not affect g (Table 6.1).

Table 6.1: Mean values, standard error (SEM), and effects of leaf length, cycle number, leaf length*cycle interaction of Rubisco turnover and herbage yield parameters of the four INRA perennial ryegrass breeding populations. Alf: leaf appearance interval (days/leaf), Alg: ligule appearance interval (days/ligule), LED: leaf elongation duration (days), TN: tiller number, DW: herbage dry weight (g), TW: Tiller weight (g), LL: Leaf lamina length (cm), LER: leaf elongation rate (cm/day), *d*: maximum leaf Rubisco concentration (mg/cm^2), *g*: time when d occurs (days), *f*: a measure of curve width.

						ANOVA *P* value for the effects		
Trait	LLC2	LLC4	SLC2	SLC4	SEM	Leaf length	Cycle	Leaf length*cycle
Alf	7.34^{ab}	6.66^{b}	7.28^{ab}	8.10^{a}	(0.40)	0.096	0.860	0.072
Alg	7.80^{a}	7.60^{a}	7.60^{a}	8.50^{a}	(0.54)	0.525	0.525	0.322
LED	9.30^{a}	9.70^{a}	7.80^{b}	8.70^{ab}	(0.38)	0.005	0.109	0.523
TN	47.90^{a}	72.40^{a}	72.50^{a}	53.40^{a}	(20.00)	0.890	0.894	0.291
DW	8.57^{ab}	12.48^{a}	6.82^{ab}	4.89^{b}	(1.90)	0.025	0.611	0.143
TW	0.18^{a}	0.19^{a}	0.11^{b}	0.09^{b}	(0.01)	<0.001	0.994	0.356
LL	50.00^{a}	50.80^{a}	32.90^{b}	32.90^{b}	(2.36)	<0.001	0.866	0.876
LER	5.40^{a}	5.27^{a}	4.20^{b}	3.78^{b}	(0.25)	<0.001	0.290	0.573
d	0.17^{a}	0.15^{a}	0.13^{b}	0.17^{a}	(0.01)	0.109	0.165	0.001
f	0.70^{b}	0.86^{a}	0.79^{ab}	0.71^{b}	(0.05)	0.512	0.410	0.026
g	12.00^{a}	11.80^{a}	12.20^{a}	13.00^{a}	(0.62)	0.280	0.624	0.438

Means with different letters are significantly different (LSD$_{0.05}$). See ANOVA table in Appendix 6.1.

Table 6.2: Trait correlation structure of the Rubisco turnover and herbage yield parameters of the four INRA perennial ryegrass breeding populations. Alf: leaf appearance interval (days/leaf), Alg: ligule appearance interval (days/ligule), LED: leaf elongation duration (days), TN: tiller number, DW: herbage dry weight (g), TW: Tiller weight (g), LL: Leaf lamina length (cm), LER: leaf elongation rate (cm/day), d: maximum leaf Rubisco concentration (mg/cm^2), g: time when d occurs (days), f: a measure of curve width.

Trait	Alf	Alg	LED	TN	DW	TW	LL	LER	d	f
Alg	0.84**									
LED	0.07	0.23								
TN	-0.21	-0.16	-0.24							
DW	-0.42†	-0.26	0.17	0.77**						
TW	-0.28	-0.12	0.66**	-0.24	0.31					
LL	-0.14	0.04	0.69**	-0.01	0.51*	0.76**				
LER	-0.21	-0.08	0.30	0.13	0.57**	0.59**	0.90**			
d	0.01	-0.09	0.25	-0.39†	-0.25	0.18	0.09	-0.03		
f	0.09	0.38†	0.19	-0.01	0.22	0.26	0.31	0.30	-0.32	
g	0.01	-0.04	0.02	-0.02	-0.22	-0.26	-0.23	-0.32	-0.11	-0.52*

†, *, ** Significant at $P < 0.1$, $P < 0.05$ and $P < 0.01$, respectively

6.4.2. Correlation among Rubisco turnover and herbage yield traits

Correlations between traits showed a strong relationship between LL and DW as expected (Table 6.2). LL also strongly correlated with TW, although no correlation was found between TW and DW (Table 6.2). However, DW showed a strong correlation with TN ($r = 0.77$). LL correlated positively with both LER and LED (Table 6.2). Among the curve parameters themselves, it was only the negative relationship between g and f that was significant (Table 6.2). There were also near-significant correlations between d and TN ($r = -0.39$, $P = 0.09$), and f and Alg ($r = 0.38$, $P = 0.09$) (Table 6.2).

When the analysis used Rubisco concentration expressed per unit leaf fresh weight (mg Rubisco-N/g FW) (Appendix 6.2) or per leaf blade basis (Appendix 6.3), the only statistically significant relationship was a positive correlation between f and Alg. For leaf Rubisco content calculated on a per leaf blade basis there were also positive, significant ($P < 0.01$) correlations between d and LED ($r = 0.60$), TW ($r = 0.83$), LL ($r = 0.79$) and LER ($r = 0.66$) (Appendix 6.2). These significant relationships when Rubisco was determined on a per leaf blade basis were expected because by virtue of their size, leaves of the long-leaved plants would be larger sinks that accumulate more Rubisco than the smaller-sized plants of the short-leaved populations.

6.4.3. Principal component analysis of herbage yield and Rubisco turnover traits

In PCA, the first four PCs explained 82% of the variation in the data (Table 6.3). PC1 explained 33% of the overall data variation and had large positive coefficients for LL, LER, TW, DW and LED, among the morphological traits (Table 6.3). This PC strongly discriminated between the long- and short-leaved breeding populations (Table 6.4, Appendix 6.3). Paradoxically, the coefficient for Alg was negative. There was a modest involvement of Rubisco turnover parameters f and g, with positive and negative coefficients, respectively (Table 6.3). A contrast between plants tending to have lower TN and DW on one hand and higher Alf, Alg, and d on the other hand defined the second PC explaining 22% of the data variation (Table 6.3). The scores (Appendix 6.3) as well as the ANOVA of PC scores (Table 6.4) showed that PC2 differentiated between genotypes within populations, but did not discriminate between the four population types. PC3 (17% data variation explained) had a very strong contribution from Rubisco turnover parameters, especially *d* and *f* (Table 6.3). Plants with a tendency towards lower *d*, showed a weak tendency to earlier *g* and a tendency to longer *f*. This turnover pattern was associated with higher Alf, Alg, TN, and DW, and lower TW (Table 6.3). The LLC2 and SLC2 populations were significantly separated by PC3 (Table 6.4). The separation of plants by PC4 (11% of variation explained) was closely related to the value for *g* but indicated a tendency for plants with later *g* to also have higher TN and longer Alf, Alg, LED, and LL (Table 6.3). Paradoxically, later *g* was associated with a weak tendency to reduced *f*.

Table 6.3: Principal component analysis structure of Rubisco turnover and herbage yield parameters of the four INRA perennial ryegrass breeding populations. Alf: leaf appearance interval (days/leaf), Alg: ligule appearance interval (days/ligule), LED: leaf elongation duration (days), TN: tiller number, DW: herbage dry weight (g), TW: Tiller weight (g), LL: Leaf lamina length (cm), LER: leaf elongation rate (cm/day), *d*: maximum leaf Rubisco concentration (mg/cm^2), *g*: time when *d* occurs (days), *f*: a measure of curve width, PVE (%): percent variation explained.

Variable	PC1	PC2	PC3	PC4
Alf	-0.18	0.45	0.33	0.20
Alg	-0.60	0.47	0.42	0.21
LED	0.31	0.32	-0.17	0.36
TN	0.08	-0.47	0.35	0.25
DW	0.36	-0.37	0.20	0.17
TW	0.43	0.15	-0.23	-0.03
LL	0.49	0.13	-0.05	0.18
LER	0.45	-0.02	0.04	0.03
d	0.01	0.22	-0.48	-0.18
f	0.23	0.17	0.44	-0.34
g	-0.22	-0.07	-0.21	0.71
PVE (%)	32.80	21.80	17.10	10.50

Table 6.4: Means of populations for principal component (PC) scores and statistical significance of population differences based on herbage yield traits and Rubisco turnover data of the four INRA perennial ryegrass breeding populations; LLC2: long-leaved 2nd cycle population, LLC4: long-leaved 4th cycle population, SLC2: short-leaved 2nd cycle population, SLC4: short-leaved 4th cycle population.

Trait	LLC2	LLC4	SLC2	SLC4	SEM
PC1	1.13a	2.13a	-1.28b	-1.98b	0.38
PC2	0.48a	-0.27a	-0.89a	0.69a	0.69
PC3	-0.89b	0.08ab	0.90a	-0.09ab	0.59
PC4	0.14a	-0.02a	-0.45a	0.33a	0.79

Means with different letters are significantly different ($LSD_{0.05}$)

6.4.4. Photosynthesis

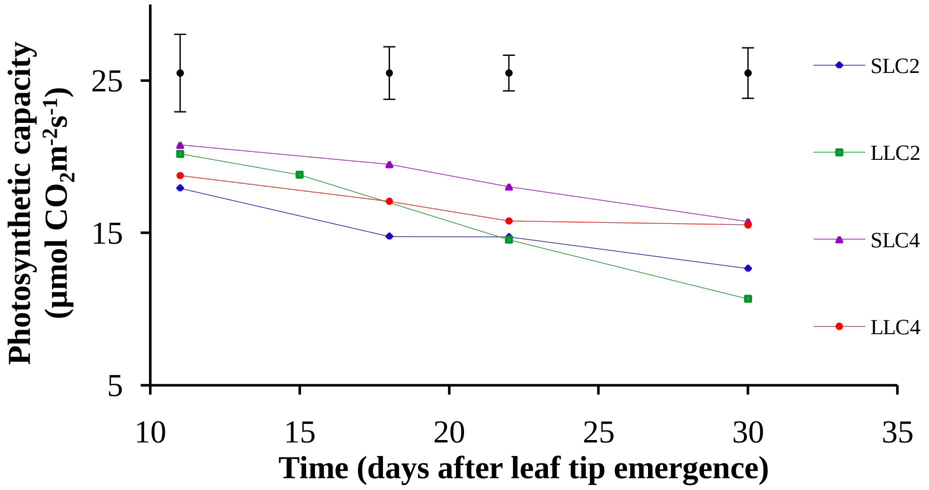


Figure 6.2: Changes in net photosynthetic rates in fully expanded 3rd leaves of the four INRA breeding populations. Each point represents a mean value (n=3); Bars above the graphs are the mean SE at each evaluation time. LLC2: long-leaved 2nd cycle population, LLC4: long-leaved 4th cycle population, SLC2: short-leaved 2nd cycle population, SLC4: short-leaved 4th cycle population.

Light saturated photosynthetic rate declined with leaf age in all four populations (Fig. 6.2). There were no significant differences in photosynthetic rate detected across populations and between the two cycles of selection (Appendix 6.4). While four data points would be considered too few for a basis for meaningful conclusions on photosynthesis, linear regression t-test of the trends depicted in Fig. 6.2 revealed a significantly ($P < 0.05$) slower decline in photosynthesis in LLC4 (slope = -0.17) than in LLC2 (slope = -0.52) plants. The slopes of SLC2 (-0.26) and SLC4 (-0.27) were not significantly different. The fact that LLC4 plants had a significantly higher f than the LLC2 plants (Table 6.1) and tended to stay green for longer (by visual observation) may warrant more investigation.

6.5. Discussion

The results indicated a marginal ($P = 0.109$) cycle effect on LED, with the rest of the evaluated variables remaining unchanged (Table 6.1). Significant differences between the long- and short-leaved population plants were mainly based on variations in LL and TW. The effect of selection for long leaves was also reflected in higher LER and to some extent, LED (Table 6.1). LER was 28.6% higher and LED was 19.2% longer in long- than short-leaved 2nd cycle plants, and a 52% increase in LL was observed. In the 4th cycle, a difference in LL (54.4%) between the long- and short-leaved plants arose from differences in LER (39.4%) and LED (11.5%). These results correlate well with other previous studies (e.g. Bahmani et al., 2000; Hazard et al., 1996; Sartie et al., 2009) which have reported achievement of long leaves in perennial ryegrass by an increase in LER and/or LED. Selection for short leaves was associated with high Alf implying low leaf appearance rate, particularly evident in the SLC4 when compared with the LLC4 plants. A low leaf appearance rate is theoretically associated with a reduction in the number of tiller buds resulting in a few large tillers (Lemaire and Chapman, 1996). Similarly, a fast leaf appearance rate implies potential for increased production with size-density compensation coming into play to reduce tiller weight (Lemaire and Chapman, 1996). While the SLC4 plants exhibited reduced tiller size, tiller density did not increase, possibly due to the low leaf appearance rate. Since plants were sufficiently spaced in this study to eliminate possible tiller death, this result suggests that four cycles of selection for short leaf lamina directly resulted in genetic depression of plant vigour.

In contrast to morphological traits and DW, Rubisco turnover parameters (*d* and *f*) showed some significant movement in response to selection for leaf lamina length. The 2nd cycle plants differed in *d* but the opposing effects from selection for short and long leaves was such that the 4th cycle plants did not differ in *d* (Table 6.1) indicating convergence, rather than divergence of this trait. Conversely, the 4th cycle plants differed in *f* even though the 2nd cycle plants were not separated (Table 6.1), indicating divergence following selection for short and long leaves. The movement in *f* between the 2nd and 4th cycle plants corresponded to the movement in DW (divergence as indicated by mean separations) even though leaf length x interaction for DW was not significant (Table 6.1). Alf, which was the only herbage yield to show some leaf length x cycle interaction ($P = 0.072$, Table 6.1), diverged after the 4th cycle of selection being longer in SLC4 than in LLC4 plants. From these results it can be argued that selection for long leaf lamina inadvertently resulted in selection for a more efficient (wider *f*) remobilisation, and presumably high yield. On the other hand, selection for short leaves was accompanied by an increase in the amount of Rubisco per unit area (higher *d*) of the leaf but Rubisco remobilisation was inefficient (narrower *f*), and Alf was remarkably increased leading to low yield in SLC4 plants.

Correlation analysis showed a link (although weak) between Alg and *f* ($P < 0.1$, Table 6.3) suggesting coupling of extended Rubisco retention with leaf longevity. Multivariate analysis partitioned populations and genotypes and unravelled more links between Rubisco turnover and the evaluated herbage yield traits. PC1, for example, linked large plant size (described by the long-leaved populations (Table 6.4, Appendix 6.3)) with an earlier Rubisco peak and an extended Rubisco retention period (Table 6.3). On average, long-leaved plants had higher LER, LED and DW (Table 6.1), and as defined by PC1 (Table 6.4), they also experienced prolonged Rubisco retention (wider *f*). This suggests that sustained photosynthesis was ensured during the life of the leaf. The high LER and LED, and sustained greenness (especially physically observed in the LLC4) of these plants likely increased both leaf area and leaf area duration leading to increased total photosynthesis (e.g. Ma and Dwyer, 1998; Richards, 2000; Tollenaar, 1991). Thus, these plants exhibited a number of productivity enhancing traits relating to leaf photosynthesis optimisation and leading to increased yield. Keeping Rubisco high for long periods of time has been associated with increased yields in a number of studies (e.g. Osaki et al., 1993; Simova-Stoilova et al., 2001). The large negative coefficient of Alg for this PC (Table 6.3) suggests that at some stage, successive leaves for the long-leaved plants may have been elongating at the same time. As seen from Table 6.1, LLC4 plants also had the highest LED:Alf ratio.

Depressed yield in plants identified by PC2 was associated with a low tiller population density, and with increased LED and Alf (i.e. decreased leaf appearance rate). Previous studies (e.g. Davies and Thomas, 1983; Robson, 1967; Skinner and Nelson, 1994) have reported a similar decrease in the number of tillers which is attributed to fewer tiller sites being available when LED is long. Plants exhibiting these characteristics also had higher peak Rubisco content per unit area of leaf but there was no evidence that Rubisco turned over rapidly. Therefore, although Rubisco turnover appeared efficient in these plants, reduced sink capacity (i.e. reduced tiller numbers) (e.g. Neales and Incoll, 1968; Paul and Foyer, 2001) effected by low leaf appearance rates (e.g. Chapman and Lemaire, 1993) was a liability to the plants. The negative relationship between *d* and DW in PC2 in the present experiment mirrors a similar *d* and DW association in PC1 for data from Grasslands II mapping population in Chapter 5 (Table 5.3). This common feature in the results of the two experiments is noteworthy because PC1 here discriminated between the long- and short-leaved populations (a source of variation not incorporated in Experiment 3) and PC2 here was the first to separate genotypes within the four populations (Table 6.4 and Appendix 6.3). Hence the trend for plants with higher *d* to have on average lower DW was common to both Experiment 3 (Chapter 5) and the present Experiment.

PC3 associated increased DW with an earlier and lower Rubisco peak (*d*) and an extended Rubisco retention period (wider *f*). A key factor in interpreting this PC is that it strongly separates the 2nd cycle populations but not the 4th selection cycle populations (Table 6.4). It is also seen on inspection of morphological trait data (Table 6.1) that DW showed the opposite trend, being similar in 2nd cycle plants and diverging in 4th cycle plants between long- and short-leaved populations. In Experiment 3 (Table 5.2) LL was correlated with DW. Therefore, a speculative hypothesis that could explain this pattern in the data is that selection of long- and short-leaved plants in early selection cycles was based on visually similar DW scores. This might have inadvertently co-selected for a less efficient Rubisco turnover pattern in long-leaved plants and a more efficient Rubisco turnover pattern in short-leaved plants, but this was lost in later selection cycles as DW was allowed to diverge. If so, PC3 may then be related to the similar conformation observed in Chapter 4 (see PC3, Table 4.3).

Considering the opposite effects of *d* and *f* displayed by this PC, the LLC2 plants with a higher Rubisco peak and a rapid Rubisco turnover also had reduced period of photosynthetic function because Rubisco content is closely related to Rubisco activity (e.g. Jiang et al., 1999; Mae et al., 1993). This is a cost to the plant because it makes a lot of Rubisco but in turn it is not efficiently utilised for biomass production. Reduced productivity with a higher Rubisco peak was observed in the earlier experiments (Chapter 4 and Chapter 5 (PC1, Table 5.3). It is argued that a high Rubisco peak which tends to result in a shorter retention Rubisco ensures a more complete Rubisco-N remobilisation but is ultimately energy-inefficient and reduces plant productivity. Such a ‘trade-off’ has been demonstrated in tall fescue (*Festuca arundinacea* Schreb.) where comparison of rhizomatous and non-rhizomatous populations found that non-rhizomatous plants had a greater leaf appearance rate and the number of leaves per tiller, and significantly higher total dry weight per tiller and per plant than rhizomatous plants (Bryant, 1997). PCA in Chapter 5 revealed that some plants that accumulate high amounts of Rubisco might also retain a lot of Rubisco in senescent leaves (positive correlation of *d* with TL (Table 5.2, 5.4)). For such plants, the cost is even higher because not only do they make incomplete use of Rubisco for photosynthesis but a substantial amount would be expected to be lost in the senescent leaf.

The tendency of plants with a later *g* to produce more tillers as shown in PC4 explaining 11% variation (Table 6.3) was also observed in Chapter 5 (PC4 explaining 10% of data variation (Table 5.3)). The fact that PC4 separated genotypes within populations (Table 6.4) implies a consistent trend among perennial ryegrass plants, explained (as in Section 5.5.3) by the proximity of the sink and the source (as reported by Kemball and Marshall, 1995; Yang et al., 1998).

As noted in Section 6.3.4, three measurements of Rubisco were taken. As addressed in Section 6.4.2, mg Rubisco-N/leaf blade had a whole host of morphological correlations but the two concentration formulations (mg Rubisco-N/cm^2, mg Rubisco-N/g FW) did not (Table 6.2, Appendices 6.2 and 6.3). This suggests a leaf size effect when Rubisco is expressed as mg Rubisco-N/leaf blade; large leaves will contain more Rubisco by virtue being larger sinks. PCA analysis using mg Rubisco-N/cm^2 indicated that PC1 extracted morphological differences between populations including LL while PC2 (Table 6.3) which differentiated genotypes within populations was like PC1 (Table 5.3) in Experiment 3 where mg Rubisco-N/g leaf dry weight concentration was used. Dry matter is an important index in pasture productivity and therefore Rubisco concentration estimate per unit mass of tissue (as in Chapter 5) and Rubisco per unit area in photosynthesis measurements (as in Chapter 6) would be important in plant breeding.

6.6. Conclusions

In relation to the objectives set out at the beginning of this chapter, the conclusions are:

- The 2nd cycle populations did not differ in herbage yield but selection for short and long leaf lamina length resulted in significantly higher herbage yield of the LLC4 than the SLC4 populations. There was no significant change in herbage yield characteristics between the 2nd and the 4th cycle long- or short-leaved populations.
- Long-leaved plants had both increased LER and LED compared to short-leaved plants. The differences were LER (28.6%), LED (19.2%) and LL (52%) for the 2nd cycle plants and LER (39.4%), LED (11.5%), LL(54.4%) for the 4th cycle plants.
- Low productivity of short-leaved plants may have been due to reduced plant vigour since selection for short leaves remarkably reduced leaf appearance rate. PC2 which separated genotypes within each population mirrored PC1 in Chapter 5 (Table 5.3) and in common modest tendency to a lower *d* and a higher DW.
- Photosynthetic capacity was statistically not affected by selection but trends in the data were consistent with measured differences in *d* and *f*.

CHAPTER 7

General discussion and conclusions

7.1. Review of thesis objectives

7.1.1. Rubisco turnover variation

One important objective of this thesis was to assess the Grasslands II mapping population for genetic variation in Rubisco turnover. Confirmation of genetic variation was an essential first step for analysis of Rubisco turnover using a quantitative genetic approach. The results of Chapter 4 (Table 4.1) involving a sub sample (16 progeny) of the mapping population and the two parent plants, showed significant ($P < 0.01$) genotypic differences in all three Rubisco turnover parameters. Comparable variation was observed in a larger sample of the mapping population (n=135) evaluated in Chapter 5 (Table 5.1), and also indicated that Rubisco turnover is quantitatively inherited (Fig. 5.2).

7.1.2. Rubisco turnover characteristics and plant yield

A second objective of this thesis was to relate specific Rubisco turnover patterns with plant morphological characteristics. In general, correlation analysis did not indicate a strong link between Rubisco turnover and herbage yield characteristics except for the association of a lower d with herbage yield and its components in Chapter 5 (Table 5.2). However, when the genotypes/populations were partitioned by PCA, more patterns emerged linking turnover parameters and plant yield. In Chapter 4, PC3 (16% of data variation) associated increased DW with an earlier and lower d and a wider f (Table 4.3). In Chapter 5, the lower d with a wider f was observed in correlation analysis (Table 5.2) and in PC2 but was not associated with increased DW (Table 5.3). Instead, PC1 (36% of data variation) associated DW with a lower d and a lower TL (Table 5.3). Chapter 6 involved an evaluation of breeding populations based on selection for short/long leaf lamina. PC2 (22% of data variation) was considered appropriate for comparison with PC1 in Chapter 5 because it involved separation of genotypes within populations and had a similar pattern of coefficients across traits (Table 6.4). This PC associated increased DW with a lower d and a shorter f (Table 6.3). Overall, these results suggest that a deficiency of Rubisco does not limit forage yield in perennial ryegrass. Therefore, even though the abundance of Rubisco protein in C_3 plants partly compensates for its catalytic inefficiency (e.g. Spreitzer and Salvucci, 2002), it appears that accumulation of 'higher than optimum' amounts of Rubisco protein may be disadvantageous to the plant. Recent studies involving transgenic rice plants over-expressing the *rbc*S gene have revealed that over-production of Rubisco does not increase photosynthetic rate or biomass; instead it causes an apparent down-regulation of the protein's active state (Suzuki et al., 2009; Suzuki et al., 2007).

The hypothesis set out in Chapter 3 (Section 3.1.1) was that Rubisco turnover that was either too fast or too slow would be inefficient and therefore reduce plant productivity. This was based on the reasoning that a fast turnover would lead to loss of photosynthetic opportunity even though potentially allowing more complete N remobilisation, while a slow turnover would compromise N remobilisation though photosynthetic opportunity should be optimised. Experiment 1 compared barley plants of similar genetic background at two contrasting N levels and there was no evidence that N level affected curve parameters *f* or *g*, though it did affect *d* calculated on a per whole leaf basis. Experiment 2 comparing a small subset of genotypes from the mapping population did appear to show evidence of lower plant DW with faster Rubisco turnover (PC3, Table 4.3) but this result was not reproduced in Experiment 3 in that PC2 (Table 5.3) that was associated with rapid Rubisco turnover in this experiment was also associated with a modest increase in DW (Table 5.3). This characteristic of perennial ryegrass may be a reflection of phenotypic plasticity, and further investigation could be carried out to determine genetic variability and heritability. In PC1 (Table 5.3), a higher *d* was associated with a higher TL (Rubisco retention in dying leaves) implying a double cost to the plant i.e. by protein synthesis (which is an energy intensive process) and by loss of the protein in senescent leaves. As the results suggest (and as expected), a higher TL was detrimental to the plant, leading to decreased yield (Table 5.3).

7.1.3. Regulation of Rubisco turnover and NUE

The third objective of this thesis was to map QTL for Rubisco turnover and herbage yield parameters in the Grasslands II mapping population in order to identify morpho-physiological genetic associations that could be used in the improvement of NUE. Four QTL (two each for *d* and *g*) were mapped for Rubisco turnover in this study (Table 5.7,

Fig. 5.3). The support interval of the QTL for *d* (qd-5) co-located with that of QTL for TN (qTN-5) on LG5, although with opposite influence (Table 5.7, Fig. 5.3). This region had multiple overlaps involving QTL for TN, DW, and PI, and was close to the QTL for TW (Fig. 5.3), and may be therefore potentially important for marker-based improvement in a perennial ryegrass breeding programme. The negative co-location suggests that selection for the increased TN via QTL on LG5 may work mechanistically to reduce *d*, hence reducing the plant's energy overload and allowing greater DW through increased tillering. As discussed in Section 7.1.2, yield was favoured in the presence of a lower *d*.

Correlation and QTL results indicated that the effect of Rubisco turnover on plant productivity was related to *d* and not *f* or *g*. There are two possibilities here: The first is that plant productivity may relate to the energy cost of Rubisco synthesis (*d* correlated negatively with plant yield) rather than photosynthetic capacity. Secondly, as laid out in Chapter 3 discussion (see Section 3.4), it is likely that the methodology was not sensitive enough to detect genotypic or treatment effects on *f* or *g* that would have been associated with yield.

7.1.3.1. Possible syntenic relationships

QTL for *d* were mapped on LGs 5 and 7 which have syntenic relationships with rice chromosomes 8 and 9, respectively (Faville, 2005; Jones et al., 2002b; Sim et al., 2005). Previously, QTL for Rubisco content have been mapped on rice chromosomes 8 and 9 by Ishimaru et al (2001a). Rice is an important model plant and its small genome size has allowed complete sequencing of its chromosomes (e.g. Matsumoto et al., 2005). The syntenic relationship of rice and perennial ryegrass allows cross-specific genetic information transfer, and identification of functionally associated molecular markers will enhance implementation of MAS in the improvement perennial ryegrass. This study did not extend to resolve the extent of synteny at the corresponding loci in the two crops, and therefore more work will be needed to clarify this. Once careful interspecific comparisons have been made, predictions of genes mapped in rice can be made in perennial ryegrass. Due to the small genome size of rice, it may be used to clone a gene of interest that is required in perennial ryegrass. Another overlap was that of QTL for *d* with QTL for TN with opposite allelic effects on LG5. A recent study (Senthilvel et al.,

2008) confirmed that QTL associated with the number of productive tillers under different N conditions corresponds to that for Rubisco content on chromosome 9 in the Ishimaru et al (2001) study. This is a demonstration of agronomic-physiological coincidence that might be important in the improvement of NUE in the two crops.

7.1.3.2. Effect of genotype x environment interactions

The variation in relationships between and among Rubisco turnover and/or the agronomic traits in experiments in Chapters 4, 5 and 6, and the Sartie (2007) study confirms the importance of genotypic, environmental, and G x E interaction effects on these traits. The correlations of Rubisco turnover traits for the same genotypes measured in Chapter 4 and Chapter 5 were numerically low and non-significant (Section 5.4.2). The G x E effect was also evident in the herbage yield traits across experiments. Of the five traits (LL, TN, TW, DW, and PI) evaluated in Chapter 5 and the Sartie (2007) the correlation was significant only for LL and TW (Section 5.4.4). These G x E effects were also observed in the differences in the strength of the relationship of the two measures of tiller weight (TW and TW_{Large}) with data from the Sartie (2007) autumn and spring experiments (Table 5.4). Furthermore, the different QTL locations for similar traits measured in this study and the Sartie (2007) study suggest different loci may be responsible for the control of the same trait in different environmental conditions. This information on repeatability of traits is important because little work has been done to verify the theoretical plant internal feedbacks between morphological traits proposed by Chapman and Lemaire (1993).

If many genes influencing a quantitative trait are specific to some environments as indicated above, detected QTL will need to be confirmed in multiple experiments (replicated over locations and/or seasons) before they are used in for breeding. This may involve independent populations constructed from the same parental genotypes or closely related genotypes used in the primary QTL study. Also, use of a larger mapping population would increase the power of detection. N treatment is an aspect that could be included in the screening of the populations to assess genotypic response to limiting N conditions and the identification of the corresponding QTL. This would be important since the initial goal of this research was the assessment of Rubisco turnover as a physiological marker that could be used for early selection of N use efficient plants.

7.1.3.3. Heritability estimates of Rubisco turnover

The results (Table 4.1) indicated that all parameters were highly heritable (97% for *d*, 68% for *f*, and 73% for *g*, respectively). A caveat should be added here that heritability estimates were based on the sub-sample of the mapping population evaluated in Chapter 4. Heritability could not be estimated for the larger sample (Chapter 5) because for each genotype, a single Rubisco turnover curve was regressed using all data points from the three replicates. *d* was the only Rubisco turnover variable that correlated (negatively) with plant morphological characteristics. High heritability of *d* in larger populations and/or in QTL validation populations may be useful for phenotypic selection at the seedling stage in forage breeding.

7.2. Rubisco turnover model performance

Evaluation of the model was an important initial step in this thesis because all determinations of Rubisco turnover were model-based. Overall, the experimental observations of Rubisco content over the life span of the leaf were consistent with the log-normal distribution pattern assumed by the model. The curve regression coefficient (R^2) for most of the mapping population plants was greater than 0.75 (the range was 0.44 to 99.3), indicating a high goodness of fit. However, there were often cases where systematic variation of points around the fitted curve was noted leading to derivation of PK and TL parameters to test the model's suitability in defining the time course of Rubisco concentration. The positive correlation of PK with *d* (Table 5.2) was somewhat expected. However, given the negative correlation of *d* with *f* in Chapter 5, and the consistency of the trend in Chapters 4 and 6, the positive correlation of *d* with TL was unexpected. There was in fact an overlap of QTL for TL and *d* on LG7 (Fig. 5.3). This shows that while high leaf Rubisco content may be associated with a rapid loss of the protein, it may not always result in a more complete remobilisation and reallocation of the protein, and the model needs modification to take into account the residual protein in a leaf in some genotypes.

In chapter 3, there was a good agreement between the model-derived and observed Rubisco peaks. In both cases, a significant effect of N supply on Rubisco peak was observed, and this was consistent with previous reports that have shown a correlation between N supply and leaf Rubisco content (Imai et al., 2005; Makino, 2003; Makino et al., 1984a). The time of the peak (*g*) and the curve width (*f*) did not differ between the N treatments. Similar results were found by Irving and Robinson (2006) for model-predicted Rubisco turnover parameters of the Makino et al. (1984b) rice data. However, numerous publications point to the fact that N is dominant in affecting senescence progress in plants, suggesting that *f* and *g* should be altered depending on the N nutrition status of the plant. For example, in Arabidopsis (Schulze et al., 1994; Thomas and deVilliers, 1996), tobacco (Terce-Laforgue et al., 2004), wheat (Crafts-Brandner et al., 1998) and grasses (Lattanzi et al., 2005) N limiting nutrition was shown to accelerate senescence. These reports collectively suggest that under reduced N supply conditions the curve width (*f*) should be reduced because Rubisco degradation is initiated upon onset of senescence. Previous reports have indicated that under reduced N supply conditions, senescence is enhanced to allow the recycling of nutrients to support the growth of new tissues (e.g. Diaz et al., 2008; Hirel and Gallais, 2006) and/or to enable the plant to complete its life cycle (e.g. Smart, 1994). Similarly, senescence progresses more slowly under high N supply (e.g. Crafts-Brandner et al., 1998; Imai et al., 2005; Ono et al., 2001) suggesting that *f* should be increased. N supply is known to increase leaf elongation rate (LER) (Gastal and Nelson, 1994; Volenec and Nelson, 1984). An increase in N supply leads to faster achievement of full leaf expansion, suggesting that time of Rubisco peak (*g*) will be earlier, although a delay in Rubisco peak with an increase in N supply has been reported in rice (Imai et al., 2005) probably because LER was also accompanied by increased leaf elongation duration (LED). Although differences in *f* may partly be explained by whether or not the plants were subjected to a steady state N nutrition or differences in the life span of the leaves, further investigation to test the sensitivity of the model to N treatments involving genetically similar experimental material would be recommended.

7.3. Physiological processes and Rubisco

Some plant physiologists presume that Rubisco degradation would be enzyme mediated and as noted in Section 2.8.3.2 some enzymes have been shown to be capable of Rubisco degradation. In this study, there was no indication of a sudden decline in leaf Rubisco concentration in the latter part of the life of the leaf. Instead, the decline was in a manner similar to that reported by other workers (e.g. Chiba et al., 2003; Friedrich and Huffaker, 1980; Mae et al., 1983; Mae et al., 1984). As discussed in the preceding section, data points of Rubisco concentration over the life span of the leaf closely followed the pattern of Rubisco turnover model (Irving and Robinson, 2006) but there were visually obvious deviations represented by PK and TL in some genotypes thus indicating shapes inconsistent with an enzyme-mediated Rubisco degradation model. Also, as reviewed by Chiba et al (2003), comparative studies have found that increase in Rubisco concentration during leaf expansion was in a like manner to LHCII and chlorophyll. However, after full expansion was achieved, Rubisco declined rapidly while LCHII and chlorophyll remained at the same level for some time (8 days) before starting to decline. This suggests differences in the degradation pattern between Rubisco and other proteins.

In this study, most log-normal curves indicated lower model-derived d than the measured d. More peak Rubisco than the model predicts could arise from synthesis having a sudden rather than a gradual ending or from an initial delay in Rubisco degradation. An interesting observation in relation to residual Rubisco-N was that in most cases Rubisco content tended to increase slightly in late leaf development resulting in more than modelled residual protein (TL). Increase in a protein that is already declining could be attributed to reverse senescence, a process that is supported by empirical results. However, reverse senescence has been found to occur under specific conditions. For example, in barley and Arabidopsis, leaves that had already started senescing exhibited reversion of senescence by later addition of N (Schildhauer et al., 2008). This reversion resulted in an increase in the efficiency of photosystem II and chlorophyll content. An earlier study in *Nicotiana rustica* L. (Zavaleta-Mancera et al., 1999b) found that decapitation of plants above a single senescent leaf induced regreening, which was promoted by cytokinin treatment. The decline in leaf protein content and increase in proteolytic activity seen in senescence were reversed on re-greening. Another study (Zavaleta-Mancera et al., 1999a) revealed that the chloroplasts in re-greening leaves arose by re-differentiation of gerontoplasts. In our study, the plants did not receive any of the reverse-senescence initiation treatments. It is therefore unclear why Rubisco protein increased late in the leaf's life. The possibility that other sources (endogenous) of N may have stimulated re-greening could be investigated.

7.4. Conclusions

This study has identified regions of the perennial ryegrass genome responsible for the control of Rubisco turnover. An important genetic link between Rubisco turnover (related to *d*) and herbage yield traits that could potentially affect plant productivity was identified on LG5. QTL overlaps in this region suggested that markers rv0340 and pps0718 may be useful for simultaneous selection of increased TN and reduced *d* with a positive effect on plant yield. On LG2 the region around marker pps0113 appears to be of interest in relation to the use of herbage yield trait associations in the improvement of yield. In this region, there were multiple overlaps involving morphological traits with aligned allelic effects. This study has provided some understanding of genotypic differences in the mode of Rubisco N management in relation to plant yield. Differences in Rubisco turnover pattern in this study were not strongly associated with plant morphology. However, the resource constraints meant that we only succeeded in evaluating 135 of the 200 genotypes of the mapping population meaning that the sensitivity of QTL detection may have been reduced. The potential use of Rubisco turnover in plant improvement is therefore open for further investigation.

7.5. Recommendations and further research

- In this study, more QTL were detected by SIM than MQM, suggesting that QTL detection power was low given the small size (n=135) of the mapping population. Therefore, further work relating to physiological and morphological trait associations evaluated in this study should use a large number of genotypes of the mapping population to increase the accuracy of detection of QTL and for a more informative genetic dissection of these traits.

- Owing to the location of QTL for *d* on LGs with syntenic relationships with rice chromosomes mapped with QTL for Rubisco content, more detailed work is required to verify collinearity of these genomic regions which can be potentially utilised for MAS in perennial ryegrass improvement programmes. However, it should be noted that genetic analysis of leaf Rubisco turnover and plant morphological characteristics was based only on one cross. Being an out-breeder, perennial ryegrass contains considerable variation for a wide range of traits. It is therefore important to evaluate these QTL effects in other marker-designed crosses, and also assess if marker selected genotypes cause a shift in phenotypic means.

- This mapping study was confined to a glasshouse experiment in only one location. As observed in the cross-experiment analysis, the magnitude QTL effects, and direction may vary depending on experimental conditions due to QTL x Environment interactions. Therefore, there is need for this mapping study to be carried out across different years/seasons and locations. This will ascertain the existence of the interactions which must be carefully considered in order to develop an effective scheme for MAS.

- One key finding in this study was the association of a lower than average Rubisco peak with plant yield common to a mapping population of the same parents (PC1 of Chapter 5) and in populations selected for contrasting leaf lamina length (PC2 of Chapter 6). A follow-up experiment to assess of performance of genotypes selected for lower leaf Rubisco concentration could help harness this effect. N treatment is an aspect that could be included in the screening of these genotypes, since the initial goal was the assessment of Rubisco turnover as a physiological marker that can be used for early selection of N use efficient plants.

Bibliography

Adam Z. (2001) Chloroplast proteases and their role in photosynthesis regulation, in: E. M. Aro and B. Andersson (Eds.), Regulation of photosynthesis, Kluwer Academic Publishers, Dordrecht, Netherlands. pp. 265-276.

Adam Z., Clarke A.K. (2002) Cutting edge of chloroplast proteolysis. Trends in Plant Science 7:451-456.

Albuquerque J.A., Esquivel M.G., Teixeira A.R., Ferreira R.B. (2001) The catabolism of ribulose bisphosphate carboxylase from higher plants. A hypothesis. Plant Science 161:55-65.

Andrews M., Lea P.J., Raven J.A., Lindsey K. (2004) Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. Annals of Applied Biology 145:25-40.

Armstead I.P., Turner L.B., King I.P., Cairns A.J., Humphreys M.O. (2002) Comparison and integration of genetic maps generated from F_2 and BC_1-type mapping populations in perennial ryegrass. Plant Breeding 121:501-507.

Armstead I.P., Turner L.B., Farrell M., Skot L., Gomez P., Montoya T., Donnison I.S., King I.P., Humphreys M.O. (2004) Synteny between a major heading-date QTL in perennial ryegrass (*Lolium perenne* L.) and the Hd3 heading-date locus in rice. Theoretical and Applied Genetics 108:822-828.

Arredondo J.T., Schnyder H. (2003) Components of leaf elongation rate and their relationship to specific leaf area in contrasting grasses. New Phytologist 158:305-314.

Asp T., Frei U.K., Didion T., Nielsen K.K., Lübberstedt T. (2007) Frequency, type, and distribution of EST-SSRs from three genotypes of *Lolium perenne*, and their conservation across orthologous sequences of *Festuca arundinacea*, *Brachypodium distachyon*, and *Oryza sativa*. BMC Plant Biology 7:36.

Bahmani I., Hazard L., Varlet-Grancher C., Betin M., Lemaire G., Matthew C., Thom E.R. (2000) Differences in tillering of long- and short-leaved perennial ryegrass genetic lines under full light and shade treatments. Crop Science 40:1095-1102.

Barcaccia G., Torricelli R., Russi L., Lucaroni B., Bozzo F., Veronesi F., Falcinelli M. (1998) Morphological and molecular characterisation of two cultivars of *Lolium perenne* L.: An example of calculating the genetic distance between a registered and a newly-constituted cultivar. Journal of Applied Seed Production 16:39-45.

Barnes D.K., Goplen B.P., Baylor J.E. (1988) Highlights in the USA and Canada in: A. A. Hanson, et al. (Eds.), Alfalfa and alfalfa improvement, American society of agronomy, Madison. pp. 1-24.

Barnes R.F., Nelson C.J. (2003) Forages and grasslands in a changing world, in: R. F. Barnes, et al. (Eds.), Forages: An introduction to grassland agriculture, Blackwell Publishing, Iowa. pp. 3-24.

Bate N.J., Rothstein S.J., Thompson J.E. (1991) Expression of nuclear and chloroplast photosynthesis-specific genes during leaf senescence. Journal of Experimental Botany 42:801-811.

Beavis W.D. (1998) QTL analyses: Power, Precision, and Accuracy CRC Press, Boca Raton, Florida.

Beckmann J.S., Soller M. (1983) Restriction fragment length polymorphisms in genetic improvement: Methodologies, mapping and costs. Theoretical and Applied Genetics 67:35-43.

Beman J.M., Arrigo K.R., Matson P.A. (2005) Agricultural runoff fuels large phytoplankton blooms in vulnerable areas of the ocean. Nature 434:211-214.

Bert P.F., Charmet G., Sourdille P., Hayward M.D., Balfourier F. (1999) A high-density molecular map for ryegrass (*Lolium perenne*) using AFLP markers. Theoretical and Applied Genetics 99:445-452.

Bertin P., Gallais A. (2001) Genetic variation for nitrogen use efficiency in a set of recombinant inbred lines II - QTL detection and coincidences. Maydica 46:53-68.

Bircham J.S., Hodgson J. (1983) The influence of sward condition on rates of herbage growth and senescence in mixed swards under continuous stocking management. Grass and Forage Science 38:323-331.

Bluett S.J. (1999) An evaluation of the nutritive value and endophyte of a new perennial ryegrass (*Lolium perenne*) cultivar (Aries HD), Institute of Natural Resources Massey University, Palmerston North.

Boschma S.P., Lodge G.M., Harden S. (2008) Herbage mass and persistence of pasture legumes and grasses at two potentially different saline and waterlogging sites in northern New South Wales. Australian Journal of Experimental Agriculture 48:553-567.

Botstein D., White R.L., Skolnick M., Davis R.W. (1980) Construction of a genetic-linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics 32:314-331.

Bowes G. (1991) Growth at elevated CO_2 - Photosynthetic responses mediated through Rubisco. Plant Cell and Environment 14:795-806.

Brady C.J. (1988) Nucleic acid and protein synthesis, in: L. D. Nooden and A. C. Leopold (Eds.), Senescence and aging in alants, Academic press, San Diego. pp. 147-171.

Bregard A., Allard G. (1999) Sink to source transition in developing leaf blades of tall fescue. New Phytologist 141:45-50.

Breseghello F., Sorrells M.E. (2006) Association mapping of kernel size and milling quality in wheat (*Triticum aestivum* L.) cultivars. Genetics 172:1165-1177.

Brown G.R., Bassoni D.L., Gill G.P., Fontana J.R., Wheeler N.C., Megraw R.A., Davis M.F., Sewell M.M., Tuskan G.A., Neale D.B. (2003) Identification of quantitative trait loci influencing wood property traits in loblolly pine *(Pinus taeda* L.). III. QTL verification and candidate gene mapping. Genetics 164:1537-1546.

Brown W.F., Moore J.E., Kunkle W.E., Chambliss C.G., Portier K.M. (1990) Forage testing using near-infrared reflectance spectroscopy. Journal of Animal Science 68:1416-1427.

Brummer E.C., Moore K.J. (2000) Persistence of perennial cool-season grass and legume cultivars under continuous grazing by beef cattle. Agronomy Journal 92:466-471.

Bryant R. (1997) Rhizome formation and response to defoliation of two tall fescue (*Festuca arundinacea* Shreb.) genotypes, Institute of Natural Resources, Massey University, Palmerston North.

Buchanan-Wollaston V. (1997) The molecular biology of leaf senescence. Journal of Experimental Botany 48:181-199.

Buchanan-Wollaston V., Earl S., Harrison E., Mathas E., Navabpour S., Page T., Pink D. (2003) The molecular analysis of leaf senescence - a genomics approach. Plant Biotechnology Journal 1:3-22.

Bushnell T.P., Bushnell D., Jagendorf A.T. (1993) A purified zinc protease of pea-chloroplasts, EP1, degrades the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase. Plant Physiology 103:585-591.

Cakmak I. (2002) Plant nutrition research: Priorities to meet human needs for food in sustainable ways. Plant and Soil 247:3-24.

Casler M.D. (2001) Breeding forage crops for increased nutritional value, Advances in Agronomy, Vol 71. pp. 51-107.

Casler M.D., Pedersen G.C., Eizenga G.C., Stratton S.D. (1996) Germplasm and Cultivar Development in: L. E. Moser, et al. (Eds.), Cool-season forage grasses, Madison. pp. 413-469.

Chapman D.F., Lemaire G. (1993) Morphogenic and structural determinants of plant growth after defoliation., in: M. J. Baker (Ed.), Grasslands for our world, SIR Publishings, Wellington. pp. 55-64.

Chiba A., Ishida H., Nishizawa N.K., Makino A., Mae T. (2003) Exclusion of ribulose-1,5-bisphosphate carboxylase/oxygenase from chloroplasts by specific bodies in naturally senescing leaves of wheat. Plant and Cell Physiology 44:914-921.

Churchill G.A., Doerge R.W. (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963-971.

Cogan N., Smith K., Yamada T., Francki M., Vecchies A., Jones E., Spangenberg G., Forster J. (2005) QTL analysis and comparative genomics of herbage quality traits in perennial ryegrass (*Lolium perenne* L.). Theoretical and Applied Genetics 110:364-380.

Collard B.C.Y., Mackill D.J. (2008) Marker-assisted selection: An approach for precision plant breeding in the twenty-first century. Philosophical Transactions of the Royal Society of Biological Sciences 363:557-572.

Collard B.C.Y., Jahufer M.Z.Z., Brouwer J.B., Pang E.C.K. (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142:169-196.

Connolly V. (2001) Breeding improved varieties of perennial ryegrass, Agriculture and Food Development Authority, Oak Park, Carlow.

Crafts-Brandner S.J., Holzer R., Feller U. (1998) Influence of nitrogen deficiency on senescence and the amounts of RNA and proteins in wheat leaves. Physiologia Plantarum 102:192-200.

Crafts-Brandner S.J., Klein R.R., Klein P., Holzer R., Feller U. (1996) Coordination of protein and mRNA abundances of stromal enzymes and mRNA abundances of the Clp protease subunits during senescence of *Phaseolus vulgaris* L. leaves. Planta 200:312-318.

Crush J.R., Easton H.S., Waller J.E., Hume D.E., Faville M.J. (2007) Genotypic variation in patterns of root distribution, nitrate interception and response to moisture stress of a perennial ryegrass (*Lolium perenne* L.) mapping population. Grass and Forage Science 62:265-273.

Daepp M., Nösberger J., Luscher A. (2001) Nitrogen fertilization and developmental stage alter the response of *Lolium perenne* to elevated CO_2. New Phytologist 150:347-358.

Darvasi A., Soller M. (1994) Optimum spacing of genetic markers for determining linkage between marker loci and quantitative trait loci. Theoretical and Applied Genetics 89:351-357.

Darvasi A., Soller M. (1995) Advanced intercross lines, an experimental population for fine genetic-mapping. Genetics 141:1199-1207.

Davies A. (1974) Leaf tissue remaining after cutting and regrowth in perennial ryegrass. Journal of Agricultural Science 82:165-172.

Davies A., Thomas H. (1983) Rates of leaf and tiller production in young spaced perennial ryegrass plants in relation to soil-temperature and solar-radiation. Annals of Botany 51:591-597.

Desimone M., Henke A., Wagner E. (1996) Oxidative stress induces partial degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. Plant Physiology 111:789-796.

Desimone M., Wagner E., Johanningmeier U. (1998) Degradation of active-oxygen-modified ribulose1-5-bisphosphate carboxylase/oxygenase by chloroplastic proteases requires ATP-hydrolysis. Planta 205:459-466.

Diaz C., Lemaitre T., Christ A., Azzopardi M., Kato Y., Sato F., Morot-Gaudry J.F., Le Dily F., Masclaux-Daubresse C. (2008) Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in Arabidopsis under low nitrogen nutrition. Plant Physiology 147:1437-1449.

Doerge R.W. (2002) Mapping and analysis of quantitative trait loci in experimental populations. Nature Reviews Genetics 3:43-52.

Doerge R.W., Churchill G.A. (1996) Permutation tests for multiple loci affecting a quantitative character. Genetics 142:285-294.

Dolstra O., Denneboom C., de Vos A.L.F., van Loo E.N. (2007) Marker-assisted selection in improvement of quantitative traits of forage crops, in: E. P. Guimaraes, et al. (Eds.), Marker Assisted Selection: Current status and future perspectives in crops, livestock, forestry and fish, Electronic Publishing, Rome. pp. 59-66.

Doveri S., Lee D., Maheswaran M., Powell W. (2008) Molecular markers: History, features and applications, in: C. Kole and A. G. Abbott (Eds.), Principles and practices of plant genomics, Science Publishers, New Hampshire. pp. 23-68.

Dumsday J.L., Smith K.F., Forster J.W., Jones E.S. (2003) SSR-based genetic linkage analysis of resistance to crown rust (*Puccinia coronata* F. sp Lolii) in perennial ryegrass (*Lolium perenne*). Plant Pathology 52:628-637.

Eckardt N.A. (2009) A new chlorophyll degradation pathway. Plant Cell 21:700-700.

Eghball B., Maranville J.W. (1991) Interactive effects of water and nitrogen stresses on nitrogen utilization efficiency, leaf water status and yield of corn genotypes. Communications in Soil Science and Plant Analysis 22:1367-1382.

Ellis R.J. (1979) Most abundant protein in the world. Trends in Biochemical Sciences 4:241-244.

Evans J.R. (1986) The relationship between carbon-dioxide-limited photosynthetic rate and ribulose-1,5-bisphosphate-carboxylase content in two nuclear-cytoplasm substitution lines of wheat, and the coordination of ribulose-bisphosphate-carboxylation and electron-transport capacities. Planta 167:351-358.

Evans J.R. (1989) Photosynthesis and nitrogen relationships in leaves of C_3 plants. Oecologia 78:9-19.

Faville M.J. (2005) An in silico DNA sequence comparison of the perennial ryegrass and rice genomes, Proceedings of the 4th International Sympossium on Molecular Breeding of Forage and Turf. pp. 181.

Faville M.J., Vecchies A.C., Schreiber M., Drayton M.C., Hughes L.J., Jones E.S., Guthridge K.M., Smith K.F., Sawbridge T., Spangenberg G.C., Bryan G.T., Forster J.W. (2004) Functionally associated molecular genetic marker map construction in perennial ryegrass (*Lolium perenne* L.). Theoretical and Applied Genetics 110:12-32.

Feller U., Fischer A. (1994) Nitrogen metabolism in senescing leaves. Critical Reviews in Plant Sciences 13:241-273.

Feller U., Anders I., Demirevska K. (2008a) Degradation of Rubisco and otherchloroplast proteins under abiotic stress. Gen. Appl. Plant Physiology 34:5-18.

Feller U., Anders I., Mae T. (2008b) Rubiscolytics: Fate of Rubisco after its enzymatic function in a cell is terminated. Experimental Botany 59:1615-1624.

Ferreira R.B., Esquivel M.G., Teixeira A.R. (2000) Catabolism of ribulose bisphosphate carboxylase from higher plants. Current Topics in Phytochemistry 3:129-162.

Fischer A., Feller U. (1994) Senescence and protein-degradation in leaf segments of young winter-wheat - influence of leaf age. Journal of Experimental Botany 45:103-109.

Fischer A.M. (2007) Nutrient remobilization during leaf senescence, in: S. Gan (Ed.), Senescence processes in plants, Annual Plant Reviews. pp. 87-107.

Forsberg J., Strom J., Kieselbach T., Larsson H., Alexciev K., Engstrom A., Akerlund H.E. (2005) Protease activities in the chloroplast capable of cleaving an LHCII N-terminal peptide. Physiologia Plantarum 123:21-29.

Forster B.P., Ellis R.P., Thomas W.T.B., Newton A.C., Tuberosa R., This D., El-Enein R.A., Bahri M.H., Ben Salem M. (2000) The development and application of molecular markers for abiotic stress tolerance in barley. Journal of Experimental Botany 51:19-27.

Forster B.P., Jones E.S., Kölliker R., Drayton M.C., Dumsday J.L., Dupal M.P., Guthridge K.M., Mahoney N.L., Van Zijll de Jong E., Smith K.F. (2001) Development and implementation of molecular markers for forage crop improvement, in: G. Spangenberg (Ed.), Molecular breeding of forage crops, Kluwer academic publishers, Dordrecht. pp. 101-134.

Fournier C., Durand J.L., Ljutovac S., Schaufele R., Gastal F., Andrieu B. (2005) A functional-structural model of elongation of the grass leaf and its relationships with the phyllochron. New Phytologist 166:881-894.

Frame J., Baker R.D., Henderson A.R. (1995) Advances in grassland technology over the past fifty years, in: G. E. Pollott (Ed.), Grassland into the 21st Century: Challenges and opportunities, British Grassland Society, Harrogate. pp. 31-63.

Friedrich J.W., Huffaker R.C. (1980) Photosynthesis, leaf resistances, and ribulose-1,5-bisphosphate carboxylase degradation in senescing barley leaves. Plant Physiology 65:1103-1107.

Gallais A., Hirel B. (2004) An approach to the genetics of nitrogen use efficiency in maize. Journal of Experimental Botany 55:295-306.

Gan S.S., Amasino R.M. (1997) Making sense of senescence - Molecular genetic regulation and manipulation of leaf senescence. Plant Physiology 113:313-319.

Gastal F., Saugier B. (1986) Nitrogen nutrition and growth of tall fescue. 2. Nitrogen uptake and partitioning among plant organs. Agronomie 6:363-370.

Gastal F., Nelson C.J. (1994) Nitrogen use within the growing leaf blade of tall fescue. Plant Physiology 105:191-197.

Giles J. (2005) Nitrogen study fertilizes fears of pollution. Nature 433:791.

Gill G.P., Wilcox P.L., Whittaker D.J., Winz R.A., Bickerstaff P., Echt C.E., Kent J., Humphreys M.O., Elborough K.M., Gardner R.C. (2006) A framework linkage map of perennial ryegrass based on SSR markers. Genome 49:354-364.

Glickman M.H., Ciechanover A. (2002) The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. Physiological Reviews 82:373-428.

Good A.G., Shrawat A.K., Muench D.G. (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends in Plant Science 9:597-605.

Grant S.A., Barthram G.T., Torvell L., King J., Smith H.K. (1983) Sward management, lamina turnover and tiller population-density in continuously stocked *Lolium perenne*-dominated swards. Grass and Forage Science 38:333-344.

Gregersen P.L., Holm P.B. (2007) Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). Plant Biotechnology Journal 5:192-206.

Grover A. (1993) How do senescing leaves lose photosynthetic activity? Current Science 64:226-234.

Guo Y., Cai Z., Gan S. (2004) Transcriptome of Arabidopsis leaf senescence. Plant Cell and Environment 27:521-549.

Guo Y.F., Gan S.S. (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant Journal 46:601-612.

Gupta P.K. (2002) Molecular markers and QTL analysis in crop plants. Current Science 83:113-114.

Gupta P.K., Rustgi S., Kulwal P.L. (2005) Linkage disequilibrium and association studies in higher plants: Present status and future prospects. Plant Molecular Biology 57:461-485.

Guthridge K.M., Dupal M.P., Kolliker R., Jones E.S., Smith K.F., Forster J.W. (2001) AFLP analysis of genetic diversity within and between populations of perennial ryegrass (*Lolium perenne* L.). Euphytica 122:191-201.

Hackett C.A. (2002) Statistical methods for QTL mapping in cereals. Plant Molecular Biology 48:585-599.

Haldane J.B.S. (1919) The combination of linkage values, and the calculation of distances between the loci of linked factors. Journal of Genetics 8:299-309.

Haley C.S., Knott S.A. (1992) A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. Heredity 69:315-324.

Hallauer A.R. (2007) History, contribution, and future of quantitative genetics in plant breeding: Lessons from maize. Crop Science 47:S4-S19.

Harrison M.A., Nemson J.A., Melis A. (1993) Assembly and composition of the chlorophyll a-b light-harvesting complex of barley (*Hordeum-vulgare* L.) - immunochemical analysis of chlorophyll b-less and chlorophyll b-deficient mutants. Photosynthesis Research 38:141-151.

Hayward M.D., Fageria N.K. (2001) The future of molecular breeding of forage crops, in: G. Spangenberg (Ed.), Developments in plant breeding. Molecular breeding of forage crops, Kluwer Academic Publishers, Netherlands. pp. 325-337.

Hayward M.D., McAdam N.J., Jones J.G., Evans C., Evans G.M., Forster J.W., Ustin A., Hossain K.G., Quader B., Stammers M., Will J.K. (1994) Genetic markers and the selection of quantitative traits in forage grasses. Euphytica 77:269-275.

Hazard L., Ghesquiere M., Barraux C. (1996) Genetic variability for leaf development in perennial ryegrass populations. Canadian Journal of Plant Science 76:113-118.

Heisler J., Glibert P.M., Burkholder J.M., Anderson D.M., Cochlan W., Dennison W.C., Dortch Q., Gobler C.J., Heil C.A., Humphries E., Lewitus A., Magnien R., Marshall H.G., Sellner K., Stockwell D.A., Stoecker D.K., Suddleson M. (2008) Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8:3-13.

Hernández Garay A., Matthew C., Hodgson J. (1999) Tiller size/density compensation in perennial ryegrass miniature swards subject to differing defoliation heights and a proposed productivity index. Grass and Forage Science 54:347-356.

Hervieu B. (2002) Multi-functionality: A conceptual framework for a new organization research and development on grasslands and livestock systems. Grassland Science in Europe 7:1-2.

Hinder B., Schellenberg M., Rodon S., Ginsburg S., Vogt E., Martinoia E., Matile P., Hörtensteiner S. (1996) How plants dispose of chlorophyll catabolites - Directly energized uptake of tetrapyrrolic breakdown products into isolated vacuoles. Journal of Biological Chemistry 271:27233-27236.

Hirel B., Gallais A. (2006) Rubisco synthesis, turnover and degradation: some new thoughts on an old problem. New Phytologist 169:445-448.

Hirel B., Bertin P., Quillere I., Bourdoncle W., Attagnant C., Dellay C., Gouy A., Cadiou S., Retailliau C., Falque M., Gallais A. (2001) Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. Plant Physiology 125:1258-1270.

Hoi-Shan K., Hai-Lou X. (2002) Construction of a genetic ma of shiitake mushroom *Lentinula edodes* strain L-54. Biochemistry and Molecular Biology 35:465-471.

Holland J.B. (2007) Genetic architecture of complex traits in plants. Current Opinion in Plant Biology 10:156-161.

Hopkins A., Holz B. (2005) Grassland for agriculture and nature conservation: Production, quality and multi-functionality. Grassland Science in Europe 10:15-29.

Hopkins A., Wilkins R.J. (2006) Temperate grassland: Key developments in the last century and future perspectives. Journal of Agricultural Science 144:503-523.

Horn R., Paulsen H. (2004) Early steps in the assembly of light-harvesting chlorophyll a/b complex - Time-resolved fluorescence measurements. Journal of Biological Chemistry 279:44400-44406.

Horst G.L., Nelson C.J., Asay K.H. (1978) Relationship of leaf elongation to forage yield of tall fescue genotypes. Crop Science 18:715-719.

Hörtensteiner S. (2006) Chlorophyll degradation during senescence. Annual Review of Plant Biology 57:55-77.

Hörtensteiner S., Feller U. (2002) Nitrogen metabolism and remobilization during senescence. Journal of Experimental Botany 53:927-937.

Hörtensteiner S., Rodoni S., Schellenberg M., Vicentini F., Nandi O.I., Qui Y.L., Matile P. (2000) Evolution of chlorophyll degradation: The significance of RCC reductase. Plant Biology 2:63-67.

Hospital F. (2003) Marker-assisted breeding, in: H. J. Newbury (Ed.), Plant molecular breeding, Oxford, Blackwell pp. 30-59.

Houtz R.L., Portis A.R. (2003) The life of ribulose 1,5-bisphosphate carboxylase/oxygenase-posttranslational facts and mysteries. Archives of Biochemistry and Biophysics 414:150-158.

Hudson G.S., Evans J.R., Voncaemmerer S., Arvidsson Y.B.C., Andrews T.J. (1992) Reduction of ribulose-1,5-bisphosphate carboxylase oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. Plant Physiology 98:294-302.

Huff D.R. (1997) RAPD characterization of heterogeneous perennial ryegrass cultivars. Crop Science 37:557-564.

Huffaker R.C. (1990) Proteolytic activity during senescence of plants. New Phytologist 116:199-231.

Humphreys M.O. (2001) Breeding methods for forage and amenity grasses, in: G. Spangenberg (Ed.), Molecular breeding of forage crops, Kluwer Academic Publishers, Netherlands pp. 41-50.

Humphreys M.O. (2005) Genetic improvement of forage crops - past, present and future. Journal of Agricultural Science 143:441-448.

Humphreys M.O., Turner L.B. (2001) Molecular markers for improving nutritive value in perennial ryegrass, Plant breeding: Sustaining the future Edinburg.

Humphreys M.W., Yadav R.S., Cairns A.J., Turner L.B., Humphreys J., Skot L. (2006) A changing climate for grassland research. New Phytologist 169:9-26.

Hyne V., Kearsey M.J., Pike D.J., Snape J.W. (1995) QTL analysis - unreliability and bias in estimation procedures. Molecular Breeding 1:273-282.

Imai K., Suzuki Y., Makino A., Mae T. (2005) Effects of nitrogen nutrition on the relationships between the levels of *rbcS* and *rbcL* mRNAs and the amount of ribulose 1.5-bisphosphate carboxylase/oxygenase synthesized in the eighth leaves of rice from emergence through senescence. Plant, Cell and Environment 28:1589-1600.

Irving L.J., Robinson D. (2006) A dynamic model of Rubisco turnover in cereal leaves. New Phytologist 169:493-504.

Ishida H., Shimizu S., Makino A., Mae T. (1998) Light-dependent fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in senescent leaves of tobacco. Planta 204:305-309.

Ishimaru K., Kosone M., Sasaki M., Kashiwagi T. (2004) Leaf contents differ depending on the position in a rice leaf sheath during sink-source transition. Plant Physiology and Biochemistry 42:855-860.

Ishimaru K., Kobayashi N., Ono K., Yano M., Ohsugi R. (2001a) Are contents of Rubisco, soluble protein and nitrogen in flag leaves of rice controlled by the same genetics? Journal of Experimental Botany 52:1827-1833.

Ishimaru K., Yano M., Aoki N., Ono K., Hirose T., Lin S.Y., Monna L., Sasaki T., Ohsugi R. (2001b) Toward the mapping of physiological and agronomic characters on a rice function map: QTL analysis and comparison between QTLs and expressed sequence tags. Theoretical and Applied Genetics 102:793-800.

Ishizuka M., Makino A., Suzuki Y., Mae T. (2004) Amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein and levels of mRNAs of rbcS and rbcL in the leaves at different positions in transgenic rice plants with decreased content of Rubisco. Soil Science and Plant Nutrition 50:233-239.

Jarvis S.C. (1996) Future trends in nitrogen research. Plant and Soil 181:47-56.

Jemison J.M., Fox R.H. (1994) Nitrate leaching from nitrogen-fertilized and manured corn measured with zero-tension pan lysimeters. Journal of Environmental Quality 23:337-343.

Jensen L.B., Andersen J.R., Frei U., Xing Y.Z., Taylor C., Holm P.B., Lubberstedt T.L. (2005) QTL mapping of vernalization response in perennial ryegrass (*Lolium perenne* L.) reveals co-location with an orthologue of wheat VRN1. Theoretical and Applied Genetics 110:527-536.

Jiang C.Z., Ishihara K., Satoh K., Katoh S. (1999) Loss of the photosynthetic capacity and proteins in senescing leaves at top positions of two cultivars of rice in relation to the source capacities of the leaves for carbon and nitrogen. Plant and Cell Physiology 40:496-503.

Jin J., Huang W., Gao J.P., Yang J., Shi M., Zhu M.Z., Luo D., Lin H.X. (2008) Genetic control of rice plant architecture under domestication. Nature Genetics 40:1365-1369.

Jones E.S., Dupal M.P., Kolliker R., Drayton M.C., Forster J.W. (2001) Development and characterisation of simple sequence repeat (SSR) markers for perennial ryegrass (*Lolium perenne* L.). Theoretical and Applied Genetics 102:405-415.

Jones E.S., Dupal M.P., Dumsday J.L., Hughes L.J., Forster J.W. (2002a) An SSR-based genetic linkage map for perennial ryegrass (*Lolium perenne* L.). Theoretical and Applied Genetics 105:577-584.

Jones E.S., Mahoney N.L., Hayward M.D., Armstead I.P., Jones J.G., Humphreys M.O., King I.P., Kishida T., Yamada T., Balfourier F., Charmet G., Forster J.W. (2002b) An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other Poaceae genomes. Genome 45:282-295.

Jones N., Ougham H., Thomas H. (1997) Markers and mapping: we are all geneticists now. New Phytologist 137:165-177.

Kao C.H., Zeng Z.B., Teasdale R.D. (1999) Multiple interval mapping for quantitative trait loci. Genetics 152:1203-1216.

Kasha K.J. (1999) Biotechnology and world food supply. Genome 42:642-645.

Kato Y., Yamamoto Y., Murakami S., Sato F. (2005) Post-translational regulation of CND41 protease activity in senescent tobacco leaves. Planta 222:643-651.

Kato Y., Murakami S., Yamamoto Y., Chatani H., Kondo Y., Nakano T., Yokota A., Sato F. (2004) The DNA-binding protease, CND41, and the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in senescent leaves of tobacco. Planta 220:97-104.

Kearsey M.J. (1998) The principles of QTL analysis (a minimal mathematics approach). Journal of Experimental Botany 49:1619-1623.

Kearsey M.J., Farquhar A.G.L. (1998) QTL analysis in plants; where are we now? Heredity 80:137-142.

Kemball W.D., Marshall C. (1995) Clonal integration between parent and branch stolons in white clover - A developmental study. New Phytologist 129:513-521.

Kemp D.R., Michalk D.L. (2007) Towards sustainable grassland and livestock management. Journal of Agricultural Science 145:543-564.

Kichey T., Hirel B., Heumez E., Dubois F., Le Gouis J. (2007) In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. Field Crops Research 102:22-32.

Kim T.H., Ourry A., Boucaud J., Lemaire G. (1991) Changes in source sink relationship for nitrogen during regrowth of lucerne (*Medicago-sativa* L.) following removal of shoots. Australian Journal of Plant Physiology 18:593-602.

King J., Thorogood D., Edwards K.J., Armstead I.P., Roberts L., Skot K., Hanley Z., King I.P. (2008) Development of a genomic microsatellite library in perennial ryegrass (*Lolium perenne*) and its use in trait mapping. Annals of Botany 101:845-853.

King J., Roberts L.A., Kearsey M.J., Thomas H.M., Jones R.N., Huang L., Armstead I.P., Morgan W.G., King I.P. (2002) A demonstration of a 1 : 1 correspondence between chiasma frequency and recombination using a *Lolium perenne*/*Festuca pratensis* substitution. Genetics 161:307-314.

Knapp S.J., Bridges W.C. (1990) Programs for mapping quantitative trait loci using flanking molecular markers. Journal of Heredity 81:234-235.

Knott S.A., Neale D.B., Sewell M.M., Haley C.S. (1997) Multiple marker mapping of quantitative trait loci in an outbred pedigree of loblolly pine. Theoretical and Applied Genetics 94:810-820.

Kokubun N., Ishida H., Makino A., Mae T. (2002) The degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase into the 44-kDa fragment in the lysates of chloroplasts incubated in darkness. Plant and Cell Physiology 43:1390-1395.

Kölliker R., Boller B., Widmer F. (2005) Marker assisted polycross breeding to increase diversity and yield in perennial ryegrass (*Lolium perenne* L.). Euphytica 146:55-65.

Kölliker R., Stadelmann F.J., Reidy B., Nösberger J. (1999) Genetic variability of forage grass cultivars: A comparison of *Festuca pratensis* Huds., *Lolium perenne* L., and *Dactylis glomerata* L. Euphytica 106:261-270.

Kosambi D.D. (1943) The estimation of map distances from recombination values. Annals of Eugenics 12:172-175.

Kraft E., Stone S.L., Ma L.G., Su N., Gao Y., Lau O.S., Deng X.W., Callis J. (2005) Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. Plant Physiology 139:1597-1611.

Kumar L.S. (1999) DNA markers in plant improvement: An overview. Biotechnology Advances 17:143-182.

Lander E.S., Botstein D. (1989) Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185-199.

Landi P., Sanguineti M.C., Salvi S., Giuliani S., Bellotti M., Maccaferri M., Conti S., Tuberosa R. (2005) Validation and characterization of a major QTL affecting leaf ABA concentration in maize. Molecular Breeding 15:291-303.

Langridge P., Lagudah E.S., Holton T.A., Appels R., Sharp P.J., Chalmers K.J. (2001) Trends in genetic and genome analyses in wheat: a review. Australian Journal of Agricultural Research 52:1043-1077.

Lattanzi F.A., Schnyder H., Thornton B. (2004) Defoliation effects on carbon and nitrogen substrate import and tissue-bound efflux in leaf growth zones of grasses. Plant Cell and Environment 27:347-356.

Lattanzi F.A., Schnyder H., Thornton B. (2005) The sources of carbon and nitrogen supplying leaf growth. Assessment of the role of stores with compartmental models. Plant Physiology 137:383-395.

Lea P.J., Ireland R.J. (1999) Nitrogen metabolism in higher plants, in: B. K. Singh (Ed.), Plant amino acids: Biochemistry and biotechnology, Marcel Dekker, New York. pp. 49-109.

Lecker S.H., Goldberg A.L., Mitch W.E. (2006) Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. Journal of the American Society of Nephrology 17:1807-1819.

Ledgard S.F., Crush J.R., Penno J.W. (1998) Environmental impacts of different nitrogen inputs on dairy farms and implications for the Resource Management Act of New Zealand, in: K. W. VanderHoek, et al. (Eds.), 1st International Nitrogen Conference 1998, Elsevier Science Bv, Noordwijkerhout, Netherlands. pp. 515-519.

Lemaire G., Chapman D. (1996) Tissue flows in grazed plant communities, in: J. Hodgson and A. W. Llius (Eds.), The ecology and management of grazing systems CAB International, Wallingford. pp. 3-36.

Lincoln S., Daly M., Lander E. (1993) Mapping genes controlling quantitative ttraits using MAPMAKER/QTL. Version 1.1 Whitehead institute of Biomedical Research technical report.

Liu B. (1998) Statistical genomics: Linkage, mapping and QTL analysis, CRC Press, Boca Raton.

Liu B.H., Knapp S.J. (1990) Gmendel - a program for Mendelian segregation and linkage analysis of individual or multiple progeny populations using log-likelihood ratios. Journal of Heredity 81:407-407.

Liu J., Wu Y.H., Yang J.J., Liu Y.D., Shen F.F. (2008) Protein degradation and nutrient remobilization during senescence. Plant Biology 51:11-19.

Lonsdale W.M., Watkinson A.R. (1982) Light and self-thinning. New Phytologist 90:431-445.

Louahlia S., Macduff J.H., Ourry A., Humphreys M., Boucaud J. (1999) Nitrogen reserve status affects the dynamics of nitrogen remobilization and mineral nitrogen uptake during recovery of contrasting cultivars of *Lolium perenne* from defoliation. New Phytologist 142:451-462.

Louahlia S., Laine P., Thornton B., Ourry A., Boucaud J. (2000) The role of N-remobilisation and the uptake of NH_4^+ and NO_3^- by *Lolium perenne* L. in laminae growth following defoliation under field conditions. Plant and Soil 220:175-187.

Lozatavera H., Martinezbarajas E., Sanchezdejimenez E. (1990) Regulation of ribulose-1,5-bisphosphate carboxylase expression in 2nd leaves of maize seedlings from low and high-yield populations. Plant Physiology 93:541-548.

Lübberstedt T. (2002) QTL mapping of forage traits, in: S. Mohan, et al. (Eds.), Molecular techniques in crop improvement, Kluwer Academic Publishers, Dordrecht. pp. 585-600.

Ma B.L., Dwyer L.M. (1998) Nitrogen uptake and use of two contrasting maize hybrids differing in leaf senescence. Plant and Soil 199:283-291.

Macadam J.W., Volenec J.J., Nelson C.J. (1989) Effects of nitrogen on mesophyll cell-division and epidermal-cell elongation in tall fescue leaf blades. Plant Physiology 89:549-556.

Macadam J.W., Nelson C.J., Sharp R.E. (1992a) Peroxidase-activity in the leaf elongation zone of tall fescue .1. Spatial-distribution of ionically bound peroxidase-activity in genotypes differing in length of the elongation zone. Plant Physiology 99:872-878.

Macadam J.W., Sharp R.E., Nelson C.J. (1992b) Peroxidase-activity in the leaf elongation zone of tall fescue .2. Spatial-distribution of apoplastic peroxidase-activity in genotypes differing in length of the elongation zone. Plant Physiology 99:879-885.

Mach J.M., Castillo A.R., Hoogstraten R., Greenberg J.T. (2001) The Arabidopsis-accelerated cell death gene ACD2 encodes red chlorophyll catabolite reductase and suppresses the spread of disease symptoms. Proceedings of the National Academy of Sciences of the United States of America 98:771-776.

Mackay T.F. (2001) The genetic architecture of quantitative traits. Annual Review of Genetics 35:303-339.

Mae T. (1997) Physiological nitrogen efficiency in rice: Nitrogen utilization, photosynthesis, and yield potential. Plant and Soil 196:201-210.

Mae T., Ohira K. (1981) The remobilization of nitrogen related to leaf growth and senescence in rice plants (*Oryza sativa* L). Plant and Cell Physiology 22:1067-1074.

Mae T., Makino A., Ohira K. (1983) Changes in the amounts of ribulose bisphosphate carboxylase synthesized and degraded during the life-span of rice leaf (*Oryza-sativa*-L). Plant and Cell Physiology 24:1079-1086.

Mae T., Makino A., Ohira K. (1987) Plant Senescence: Its biochemistry and physiology, in: W. W. Thomson, et al. (Eds.), The American Society of Plant Physiologists, Rockville. pp. 123-131.

Mae T., Kai N., Makino A., Ohira K. (1984) Relation between ribulose bisphosphate carboxylase content and chloroplast number in naturally senescing primary leaves of wheat. Plant and Cell Physiology 25:333-336.

Mae T., Thomas H., Gay A.P., Makino A., Hidema J. (1993) Leaf development in *Lolium-temulentum* - photosynthesis and photosynthetic proteins in leaves senescing under different irradiances. Plant and Cell Physiology 34:391-399.

Mae T., Kamei C., Funaki K., Miyadai K., Makino A., Ohira K., Ojima K. (1989) Degradation of Ribulose-1,5-bisphosphate carboxylase oxygenase in the lysates of the chloroplasts isolated mechanically from wheat (*Triticum aestivum* L) leaves. Plant and Cell Physiology 30:193-200.

Makino A. (2003) Rubisco and nitrogen relationships in rice: Leaf photosynthesis and plant growth. Soil Science and Plant Nutrition 49:319-327.

Makino A., Osmond B. (1991) Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. Plant Physiology 96:355-362.

Makino A., Mae T., Ohira K. (1984a) Relation between nitrogen and ribulose-1,5-bisphosphate carboxylase in rice leaves from emergence through senescence. Plant and Cell Physiology 25:429-437.

Makino A., Mae T., Ohira K. (1984b) Changes in photosynthetic capacity in rice leaves from emergence through senescence - analysis from ribulose-1,5-bisphosphate carboxylase and leaf conductance. Plant and Cell Physiology 25:511-521.

Makino A., Mae T., Ohira K. (1985) Enzymic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase purified from rice leaves. Plant Physiology 79:57-61.

Makino A., Mae T., Ohira K. (1986) Colorimetric measurement of protein stained with coomassie brilliant blue-R on sodium dodecyl sulfate-polyacrylamide gel-electrophoresis by eluting with formamide. Agricultural and Biological Chemistry 50:1911-1912.

Makino A., Sakashita H., Hidema J., Mae T., Ojima K., Osmond B. (1992) Distinctive responses of ribulose-1,5-bisphosphate carboxylase and carbonic-anhydrase in wheat leaves to nitrogen nutrition and their possible relationships to CO_2-transfer resistance. Plant Physiology 100:1737-1743.

Makino A., Shimada T., Takumi S., Kaneko K., Matsuoka M., Shimamoto K., Nakano H., MiyaoTokutomi M., Mae T., Yamamoto N. (1997) Does decrease in ribulose-1,5-bisphosphate carboxylase by antisense *rbcS* lead to a higher N-use efficiency of photosynthesis under conditions of saturating CO_2 and light in rice plants? Plant Physiology 114:483-491.

Manly K.F., Cudmore R.H., Meer J.M. (2001) Map manager QTX, cross-platform software for genetic mapping. Mammalian Genome 12:930-932.

Martin A., Lee J., Kichey T., Gerentes D., Zivy M., Tatout C., Dubois F., Balliau T., Valot B., Davanture M., Terce-Laforgue T., Quillere I., Coque M., Gallais A., Gonzalez-Moro M.B., Bethencourt L., Habash D.Z., Lea P.J., Charcosset A., Perez P., Murigneux A., Sakakibara H., Edwards K.J., Hirel B. (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. Plant Cell 18:3252-3274.

Martinez D.E., Costa M.L., Guiamet J.J. (2008) Senescence-associated degradation of chloroplast proteins inside and outside the organelle. Plant Biology 10:15-22.

Masclaux-Daubresse C., Reisdorf-Cren M., Orsel M. (2008) Leaf nitrogen remobilisation for plant development and grain filling. Plant Biology 10 23-36.

Masclaux C., Quillere I., Gallais A., Hirel B. (2001) The challenge of remobilisation in plant nitrogen economy. A survey of physio-agronomic and molecular approaches. Annals of Applied Biology 138:69-81.

Masclaux C., Valadier M.H., Brugiere N., Morot-Gaudry J.F., Hirel B. (2000) Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. Planta 211:510-518.

Matsumoto T., Wu J.Z., Kanamori H., Katayose Y., Fujisawa M., Namiki N., Mizuno H., Yamamoto K., Antonio B.A., Baba T., Sakata K., Nagamura Y., Aoki H., Arikawa K., Arita K., Bito T., Chiden Y., Fujitsuka N., Fukunaka R., Hamada M., Harada C., Hayashi A., Hijishita S., Honda M., Hosokawa S., Ichikawa Y., Idonuma A., Iijima M., Ikeda M., Ikeno M., Ito K., Ito S., Ito T., Ito Y., Iwabuchi A., Kamiya K., Karasawa W., Kurita K., Katagiri S., Kikuta A., Kobayashi H., Kobayashi N., Machita K., Maehara T., Masukawa M., Mizubayashi T., Mukai Y., Nagasaki H., Nagata Y., Naito S., Nakashima M., Nakama Y., Nakamichi Y., Nakamura M., Meguro A., Negishi M., Ohta I., Ohta T., Okamoto M., Ono N., Saji S., Sakaguchi M., Sakai K., Shibata M., Shimokawa T., Song J.Y., Takazaki Y., Terasawa K., Tsugane M., Tsuji K., Ueda S., Waki K., Yamagata H., Yamamoto M., Yamamoto S., Yamane H., Yoshiki S., Yoshihara R., Yukawa K., Zhong H.S., Yano M., Sasaki T., Yuan Q.P., Shu O.T., Liu J., Jones K.M., Gansberger K., Moffat K., Hill J., Bera J., Fadrosh D., Jin S.H., Johri S., Kim M., Overton L., Reardon M., Tsitrin T., Vuong H., Weaver B., Ciecko A., *et al.* (2005) The map-based sequence of the rice genome. Nature 436:793-800.

Matthew C., Lemaire G., Hamilton N.R.S., Hernández Garay A. (1995) A modified self-thinning equation to describe size/density relationships for defoliated swards. Annals of Botany 76:579-587.

Maurice I. (1997) Mise en place du volume et de´poˆ t de compose´s carbone´s et azote´s au cours de la croissance de la feuille de fe´tuque e´leve´e (*Festuca arundinacea* Schreb.), Institut National Polytechnique de Lorraine, Nancy-Met, France.

Mauricio R. (2001) Mapping quantitative trait loci in plants: Uses and caveats for evolutionary biology. Nature Reviews Genetics 2:370-381.

Mazzanti A., Lemaire G. (1994) Effect of nitrogen-fertilization on herbage production of tall fescue swards continuously grazed by sheep. 2. Consumption and efficiency of herbage utilization. Grass and Forage Science 49:352-359.

McGrath J.M., Penno J.W., Macdonald K.A., Carter W.A. (1998) Using nitrogen fertiliser to increase dairy farm profitability, New Zealand Society of Animal Production. pp. 117-120.

McKersie B.D. (1997) Improving forage production systems using biotechnology, in: B. D. McKersie and D. C. W. Brown (Eds.), Biotechnology and the improvement of forage legumes, CAB International, Wallingford. pp. 444.

Melchinger A.E., Utz H.F., Schon C.C. (1998) Quantitative trait locus (QTL) mapping using different testers and independent population samples in maize reveals low power of QTL detection and large bias in estimates of QTL effects. Genetics 149:383-403.

Messeguer R., Ganal M., Devicente M.C., Young N.D., Bolkan H., Tanksley S.D. (1991) High-resolution RFLP map around the root-knot nematode resistance gene (*Mi*) in tomato. Theoretical and Applied Genetics 82:529-536.

Michelmore R., Paran I., Kesseli R.V. (1991) Identification of marker linked to disease resistance gene by bulk segregant analysis: A rapid method to detect markers in specific genomic regions using segregating populations. Proc. Natl Acad Sci USA 88:9828-9832.

Mickelson S., See D., Meyer F.D., Garner J.P., Foster C.R., Blake T.K., Fischer A.M. (2003) Mapping of QTL associated with nitrogen storage and remobilization in barley (*Hordeum vulgare* L.) leaves. Journal of Experimental Botany 54:801-812.

Millard P. (1988) The accumulation and storage of nitrogen by herbaceous plants. Plant Cell and Environment 11:1-8.

Miller B.L., Huffaker R.C. (1985) Differential induction of endoproteinases during senescence of attached and detached barley leaves. Plant Physiology 78:442-446.

Mitsuhashi W., Craftsbrandner S.J., Feller U. (1992) Ribulose-1,5-bis-phosphate carboxylase oxygenase degradation in isolated pea-chloroplasts incubated in the light or in the dark. Journal of Plant Physiology 139:653-658.

Mollaretti G., Bosio M., Gentinetta E., Motto M. (1987) Genotypic variability for N-related traits in maize: Identification of inbred lines with high or low-levels of NO_3-N in the stalks. Maydica 32:309-323.

Monaghan R.M., Paton R.J., Smith L.C., Drewry J.J., Littlejohn R.P. (2005) The impacts of nitrogen fertilisation and increased stocking rate on pasture yield, soil physical condition and nutrient losses in drainage from a cattle-grazed pasture. New Zealand Journal of Agricultural Research 48:227-240.

Mühlecker W., Kräutler B. (1996) Breakdown of chlorophyll: Constitution of nonfluorescing chlorophyll-catabolites from senescent cotyledons of the dicot rape. Plant Physiology and Biochemistry 34:61-75.

Muylle H., Baert J., Van Bockstaele E., Pertijs J., Roldan-Ruiz I. (2005) Four QTLs determine crown rust *(Puccinia coronata* f. sp lolii) resistance in a perennial ryegrass (*Lolium perenne*) population. Heredity 95:348-357.

Myles S., Peiffer J., Brown P.J., Ersoz E.S., Zhang Z.W., Costich D.E., Buckler E.S. (2009) Association mapping: Critical considerations shift from genotyping to experimental design. Plant Cell 21:2194-2202.

Neales T.F., Incoll L.D. (1968) Control of leaf photosynthesis rate by level of assimilate concentration in leaf - A review of hypothesis. Botanical Review 34:107.

Nelson J.C. (1997) QGENE: Software for marker-based genomic analysis and breeding. Molecular Breeding 3:239-245.

Nikolau B.J., Klessig D.F. (1987) Coordinate, organ-specific and developmental regulation of Ribulose 1,5-bisphosphate carboxylase gene-expression in amaranthus-hypochondriacus. Plant Physiology 85:167-173.

Nösberger J., Staszewski Z. (2002) Overview of the changes in research on grasslands in Europe: Multifunctional grasslands, quality forages, animal products, and landscapes, Proceedings of the 19th General Meeting of the European Grassland Federation, La Rochelle, France. pp. 17-27.

Obara M., Kajiura M., Fukuta Y., Yano M., Hayashi M., Yamaya T., Sato T. (2001) Mapping of QTLs associated with cytosolic glutamine synthetase and NADH-glutamate synthase in rice (*Oryza sativa* L.). Journal of Experimental Botany 52:1209-1217.

Obara M., Sato T., Sasaki S., Kashiba K., Nagano A., Nakamura I., Ebitani T., Yano M., Yamaya T. (2004) Identification and characterization of a QTL on chromosome 2 for cytosolic glutamine synthetase content and panicle number in rice. Theoretical and Applied Genetics 110:1-11.

Ono K., Nishi Y., Watanabe A., Terashima I. (2001) Possible mechanisms of adaptive leaf senescence. Plant Biology 3:234-243.

Ookawa T., Naruoka Y., Yamazaki T., Suga J., Hirasawa T. (2003) A comparison of the accumulation and partitioning of nitrogen in plants between two rice cultivars, Akenohoshi and Nipponbare, at the ripening stage. Plant Production Science 6:172-178.

Osaki M., Morikawa K., Matsumoto M., Shinano T., Iyoda M., Tadano T. (1993) Productivity of high-yielding crops III. Accumulation of ribulose-1,5-bisphosphate carboxylase/oxygenase and chlorophyll in relation to productivity of high-yielding crops. Soil Science and Plant Nutrition 39:399-408.

Otegui M.S., Noh Y.S., Martinez D.E., Vila Petroff M.G., Andrew Staehelin L., Amasino R.M., Guiamet J.J. (2005) Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean. Plant Journal 41:831-844.

Pan L., Ober C., Abney M. (2007) Heritability estimation of sex-specific effects on human quantitative traits. Genetic Epidemiology 31:338-347.

Papakosta D.K., Gagianas A.A. (1991) Nitrogen and dry-matter accumulation, remobilization, and losses for Mediterranean wheat during grain filling. Agronomy Journal 83:864-870.

Paterson A.H. (1995) Molecular dissection of quantitative traits - Progress and prospects. Genome Research 5:321-333.

Paterson A.H., Lander E.S., Hewitt J.D., Peterson S., Lincoln S.E., Tanksley S.D. (1988) Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335:721-726.

Paterson A.H., Damon S., Hewitt J.D., Zamir D., Rabinowitch H.D., Lincoln S.E., Lander E.S., Tanksley S.D. (1991) Mendelian factors underlying quantitative traits in tomato - Comparison across species, generations, and environments. Genetics 127:181-197.

Paul M.J., Foyer C.H. (2001) Sink regulation of photosynthesis. Journal of Experimental Botany 52:1383-1400.

Peoples M.B., Dalling M.J. (1988) The interplay between proteolysisand amino acid metabolism during senescence and nitrogen reallocation, in: L. D. Nooden and A. C. Leopold (Eds.), Senescence and aging in plants, Academic Press, San Diego. pp. 181-217.

Pickart C.M. (2004) Back to the future with ubiquitin. Cell 116:181-190.

Piepho H.P., Mohring J. (2007) Computing heritability and selection response from unbalanced plant breeding trials. Genetics 177:1881-1888.

Pollock C.J., Humphreys M.O., Morris P., Abberton M.T., W. H.M. (2005) Biotechnological approaches to temperate forage improvement, in: S. G. Reynolds and J. Frame (Eds.), Grasslands: Developments, opportunities, perspectives, Science publishers, Plymouth. pp. 281-301.

Powell W., Machray G.C., Provan J. (1996) Polymorphism revealed by simple sequence repeats. Trends in Plant Science 1:215-222.

Prins A., Van Heerden P.D.R., Olmos E., Kunert K.J., Foyer C.H. (2008) Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. Journal of Experimental Botany 59:1935-1950.

Prins W.H. (1984) Limits to nitrogen-fertilizer on grassland. Netherlands Journal of Agricultural Science 32:319-321.

Pružinská A., Tanner G., Anders I., Roca M., Hörtensteiner S. (2003) Chlorophyll breakdown: Pheophorbide a oxygenase is a Rieske-type iron-sulfur protein, encoded by the accelerated cell death 1 gene. Proceedings of the National Academy of Sciences of the United States of America 100:15259-15264.

Racusen D., Foote M. (1965) Protein synthesis in dark-grown bean leaves. Canadian Journal of Botany 43:817-824.

Rae A.N. (1999) Livestock production and the environment: An initial assessment of some impacts of trade liberalisation, NZ Consortium, New Zealand.

Rafalski J.A. (2002) Novel genetic mapping tools in plants: SNPs and LD-based approaches. Plant Science 162:329-333.

Rasmussen S., Parsons A.J., Bassett S., Christensen M.J., Hume D.E., Johnson L.J., Johnson R.D., Simpson W.R., Stacke C., Voisey C.R., Xue H., Newman J.A. (2007) High nitrogen supply and carbohydrate content reduce endophyte and alkaloid concentration in *Lolium perenne*. New Phytologist 173:787-797.

Raven J.A., Wollenweber B., Handley L.L. (1992) Ammonia and ammonium fluxes between photolithotrophs and the environment in relation to the global nitrogen-cycle. New Phytologist 121:5-18.

Ray D., Sheshshayee M.S., Mukhopadhyay K., Bindumadhava H., Prasad T.G., Kumar M.U. (2003) High nitrogen use efficiency in rice genotypes is associated with higher net photosynthetic rate at lower Rubisco content. Biologia Plantarum 46:251-256.

Richards R.A. (2000) Selectable traits to increase crop photosynthesis and yield of grain crops. Journal of Experimental Botany 51:447-458.

Risch N. (1992) Genetic-linkage - interpreting LOD scores. Science 255:803-804.

Risch N.J. (2000) Searching for genetic determinants in the new millennium. Nature 405:847-856.

Roberts I.N., Murray P.F., Caputo C.P., Passeron S., Barneix A.J. (2003) Purification and characterization of a subtilisin-like serine protease induced during the senescence of wheat leaves. Physiologia Plantarum 118:483-490.

Robson M.J. (1967) A comparison of British and North African varieties of tall fescue (*Festuca arundinacea*). I. Leaf growth during winter and the effects on temperature and daylength. Journal of Applied Ecology 4:475-484.

Rodermel S. (1999) Subunit control of Rubisco biosynthesis - A relic of an endosymbiotic past? Photosynthesis Research 59:105-123.

Rodoni S., Mühlecker W., Anderl M., Kräutler B., Moser D., Thomas H., Matile P., Hörtensteiner S. (1997) Chlorophyll breakdown in senescent chloroplasts - Cleavage of pheophorbide a in two enzymic steps. Plant Physiology 115:669-676.

Roulin S., Feller U. (1997) Light-induced proteolysis of stromal proteins in pea (*Pisum sativum* L.) chloroplasts: Requirement for intact organelles. Plant Science 128:31-41.

Ruane J., Colleau J.J. (1995) Marker assisted selection for genetic improvement of animal population when a single QTL is marked. Genetical Research 66:71-83.

Rumball W. (1983) Pasture plant breeding in New Zealand, in: G. S. Wratt and H. C. Smith (Eds.), Plant breeding in New Zealand, Butterworths & DSIR, Wellington. pp. 215-220.

Ryden J.C., Ball P.R., Garwood E.A. (1984) Nitrate leaching from grassland. Nature 311:50-53.

Sakamoto W. (2006) Protein degradation machineries in plastids. Annual Review of Plant Biology 57:599-621.

Sane P.V., Amla D.V. (1990) Genetics of photosynthetic components in relation to productivity, in: K. R. Khanna (Ed.), Biochemical aspects of crop improvement, CRC Press. pp. 109-152.

Sartie A.M. (2007) Phenotypic assessment and quantitative trait locus (QTL) analysis of herbage and seed production traits in perennial ryegrass (*Lolium perenne* L.), Institute of Natural Resources, Massey University, Palmerston North.

Sartie A.M., Easton H.S., Matthew C. (2009) Plant morphology differences in two perennial ryegrass cultivars. New Zealand Journal of Agricultural Research 52:391-398.

Sawbridge T., Ong E.K., Binnion C., Emmerling M., McInnes R., Meath K., Nguyen N., Nunan K., O'Neill M., O'Toole F., Rhodes C., Simmonds J., Tian P., Wearne K., Webster T., Winkworth A., Spangenberg G. (2003) Generation and analysis of expressed sequence tags in perennial ryegrass (*Lolium perenne* L.). Plant Science 165:1089-1100.

Sayed H., Kayyal H., Ramsey L., Ceccarelli S., Baum M. (2002) Segregation distortion in doubled haploid lines of barley (*Hordeum vulgare* L.) detected by simple sequence repeat (SSR) markers. Euphytica 125:265-272.

Sbrissia A.F., da Silva S.C., Matthew C., de Carvalho C.A.B., Carnevalli R.A., Pinto L.F.D., Fagundes J.L., Pedreira C.G.S. (2003) Tiller size/density compensation in grazed Tifton 85 bermudagrass swards. Pesquisa Agropecuaria Brasileira 38:1459-1468.

Schaufele R., Schnyder H. (2001) Carbon and nitrogen deposition in expanding tissue elements of perennial ryegrass (*Lolium perenne* L.) leaves during non-steady growth after defoliation. Plant Cell and Environment 24:407-417.

Schejbel B., Jensen L.B., Xing Y., Lübberstedt T. (2007) QTL analysis of crown rust resistance in perennial ryegrass under conditions of natural and artificial infection. Plant Breeding 126:347-352.

Schejbel B., Jensen L.B., Asp T., Xing Y., Lübberstedt T. (2008) Mapping of QTL for resistance to powdery mildew and resistance gene analogues in perennial ryegrass. Plant Breeding 127:368-375.

Schildhauer J., Wiedemuth K., Humbeck K. (2008) Supply of nitrogen can reverse senescence processes and affect expression of genes coding plastid glutamine synthetase and lysine-ketoglutarate reductase/saccharopine dehydrogenase. Plant Biology 10:76-84.

Schnyder H., de Visser R. (1999) Fluxes of reserve-derived and currently assimilated carbon and nitrogen in perennial ryegrass recovering from defoliation. The regrowing tiller and its component functionally distinct zones. Plant Physiology 119:1423-1435.

Scholefield D., Tyson K.C., Garwood E.A., Armstrong A.C., Hawkins J., Stone A.C. (1993) Nitrate leaching from grazed grassland lysimeters: Effects of fertilizer input, field drainage, age of sward and patterns of weather. Journal of Soil Science 44:601-613.

Schulze W., Schulze E.D., Stadler J., Heilmeier H., Stitt M., Mooney H.A. (1994) Growth and reproduction of Arabidopsis-thaliana in relation to storage of starch and nitrate in the wild-type and in starch-deficient and nitrate-uptake-deficient mutants. Plant Cell and Environment 17:795-809.

See D., Kanazin V., Kephart K., Blake T. (2002) Mapping genes controlling variation in barley grain protein concentration. Crop Science 42:680-685.

Senthilvel S., Vinod K.K., Malarvizhi P., Maheswaran M. (2008) QTL and QTL x environment effects on agronomic and nitrogen acquisition traits in rice. Journal of Integrative Plant Biology 50:1108-1117.

Sewell M.M., Bassoni D.L., Megraw R.A., Wheeler N.C., Neale D.B. (2000) Identification of QTLs influencing wood property traits in loblolly pine (*Pinus taeda* L.). I. Physical wood properties. Theoretical and Applied Genetics 101:1273-1281.

Sewell M.M., Davis M.F., Tuskan G.A., Wheeler N.C., Elam C.C., Bassoni D.L., Neale D.B. (2002) Identification of QTLs influencing wood property traits in loblolly pine (*Pinus taeda* L.). II. Chemical wood properties. Theoretical and Applied Genetics 104:214-222.

Shaikh N.U., Quick W.P., Rolfe S.A. (2000) The base of the leaf acts as a localized sink for photosynthate in mature barley leaves. New Phytologist 148:257-266.

Sim S., Chang T., Curley J., Warnke S.E., Barker R.E., Jung G. (2005) Chromosomal rearrangements differentiating the ryegrass genome from the triticeae, oat, and rice genomes using common heterologous RFLP probes. Theoretical and Applied Genetics 110:1011-1019.

Simko I., McMurry S., Yang H.M., Manschot A., Davies P.J., Ewing E.E. (1997) Evidence from polygene mapping for a causal relationship between potato tuber dormancy and abscisic acid content. Plant Physiology 115:1453-1459.

Simova-Stoilova L., Stoyanova Z., Demirevska-Kepova K. (2001) Ontogenic changes in leaf pigments, total soluble protein and Rubisco in two barley varieties in relation to yield. Bulgarian Journal of Plant Physiology 27:15-24.

Simpson E. (1978) Biochemical and genetic studies of the synthesis and degradation of RuBP carboxylase, in: H. W. Siegelman and G. Hind (Eds.), Photosynthetic carbon assimilation, Plenum Press, London. pp. 113-125.

Simpson E., Cooke R.J., Davies D.D. (1981) Measurement of protein-degradation in leaves of *Zea-mays* using [H^{-3}]-labeled acetic-anhydride and tritiated-water. Plant Physiology 67:1214-1219.

Singh U., Ladha J.K., Castillo E.G., Punzalan G., Tirol-Padre A., Duqueza M. (1998) Genotypic variation in nitrogen use efficiency in medium- and long-duration rice. Field Crops Research 58:35-53.

Skinner R.H., Nelson C.J. (1994) Effect of tiller trimming on phyllochron and tillering regulation during tall fescue development. Crop Science 34:1267-1273.

Skinner R.H., Nelson C.J. (1995) Elongation of the grass leaf and its relationship to the phyllochron. Crop Science 35:4-10.

Smalle J., Vierstra R.D. (2004) The ubiquitin 26S proteasome proteolytic pathway. Annual Review of Plant Biology 55:555-590.

Smart C.M. (1994) Gene-expression during leaf senescence. New Phytologist 126:419-448.

Sodin S. (1991) Achievements in fodder crops breeding in nordic Europe, in: A. P. M. D. Nijs and A. Elgersma (Eds.), Proceedings of the 16th Meeting of the fodder crops section of Eucarpia, Pudoc, Wagenigen. pp. 7-12.

Sohl M. (2005) Grasslands: developments, opportunities, perspectives, in: S. G. Reynolds and J. Frame (Eds.), Food and Agriculture Organization of the United Nations, Science Publishers, Enfield, Plymouth. pp. v - ix.

Soler J.M.P., Pereira A.C., Torres C.H., Krieger J.E. (2006) Gene by environment QTL mapping through multiple trait analyses in blood pressure salt-sensitivity: identification of a novel QTL in rat chromosome 5. BMC Medical Genetics 7.

Southern E. (1979) Gel electrophoresis of restriction fragments. Method Enzymol 68:152-176.

Spreitzer R.J. (1999) Questions about the complexity of chloroplast ribulose1,5-bisphosphate carboxylase/oxygenase. Photosynthesis Research 60:29-41.

Spreitzer R.J., Salvucci M.E. (2002) RUBISCO: Structure, regulatory interactions, and possibilities for a better enzyme. Annual Review of Plant Biology 53:449-475.

Stam P. (1993) Construction of integrated genetic-linkage maps by means of a new computer package - JOINMAP. Plant Journal 3:739-744.

Stammers M., Harris J., Evans G.M., Hayward M.D., Forster J.W. (1995) Use of random PCR (RAPD) technology to analyze phylogenetic-relationships in the *Lolium-Festuca* complex. Heredity 74:19-27.

Stitt M., Schulze D. (1994) Does Rubisco control the rate of photosynthesis and plant-growth - An exercise in molecular ecophysiology. Plant Cell and Environment 17:465-487.

Studer B., Asp T., Frei U., Hentrup S., Meally H., Guillard A., Barth S., Muylle H., Roldan-Ruiz I., Barre P., Koning-Boucoiran C., Uenk-Stunnenberg G., Dolstra O., Skot L., Skot K.P., Turner L.B., Humphreys M.O., Kölliker R., Roulund N., Nielsen K.K., Lübberstedt T. (2008) Expressed sequence tag-derived microsatellite markers of perennial ryegrass (*Lolium perenne* L.). Molecular Breeding 21:533-548.

Sullivan J.A., Shirasu K., Deng X.W. (2003) The diverse roles of ubiquitin and the 26S proteasome in the life of plants. Nature Reviews Genetics 4:948-958.

Suzuki Y., Makino A., Mae T. (2001) Changes in the turnover of Rubisco and levels of mRNAs of *rbc*L and *rbc*S in rice leaves from emergence to senescence. Plant, Cell and Environment 24:1353-1360.

Suzuki Y., Miyamoto T., Yoshizawa R., Mae T., Makino A. (2009) Rubisco content and photosynthesis of leaves at different positions in transgenic rice with an overexpression of rbcS. Plant Cell and Environment 32:417-427.

Suzuki Y., Ohkubo M., Hatakeyama H., Ohashi K., Yoshizawa R., Kojima S., Hayakawa T., Yamaya T., Mae T., Makino A. (2007) Increased Rubisco content in transgenic rice transformed with the 'sense' *rbc*S gene. Plant and Cell Physiology 48:626-637.

Tabuchi M., Sugiyama K., Ishiyama K., Inoue E., Sato T., Takahashi H., Yamaya T. (2005) Severe reduction in growth rate and grain filling of rice mutants lacking OsGS1;1, a cytosolic glutamine synthetase1;1. Plant Journal 42:641-651.

Takeuchi A., Yamaguchi T., Hidema J., Strid A., Kumagai T. (2002) Changes in synthesis and degradation of Rubisco and LHCII with leaf age in rice (*Oryza sativa* L.) growing under supplementary UV-B radiation. Plant Cell and Environment 25:695-706.

Tanksley S.D. (1993) Mapping polygenes. Annual Review of Genetics 27:205-233.

Tas B. (2006) Nitrogen utilization of perennial ryegrass in dairy cows, in: A. Elgersma, et al. (Eds.), Fresh herbage for dairy cattle Springer, Netherlands. pp. 125-140.

Terce-Laforgue T., Mack G., Hirel B. (2004) New insights towards the function of glutamate dehydrogenase revealed during source-sink transition of tobacco (*Nicotiana tabacum*) plants grown under different nitrogen regimes. Physiologia Plantarum 120:220-228.

Teyker R.H., Moll R.H., Jackson W.A. (1989) Divergent selection among maize seedlings for nitrate uptake. Crop Science 29:879-884.

Thomas H., deVilliers L. (1996) Gene expression in leaves of Arabidopsis thaliana induced to senesce by nutrient deprivation. Journal of Experimental Botany 47:1845-1852.

Thorogood D., Kaiser W.J., Jones J.G., Armstead I. (2002) Self-incompatibility in ryegrass 12. Genotyping and mapping the S and Z loci of *Lolium perenne* L. Heredity 88:385-390.

Tollenaar M. (1991) Physiological-basis of genetic-improvement of maize hybrids in Ontario from 1959 to 1988. Crop Science 31:119-124.

Tuberosa R., Salvi S., Sanguineti M.C., Landi P., MacCaferri M., Conti S. (2002) Mapping QTLs regulating morpho-physiological traits and yield: Case studies, shortcomings and perspectives in drought-stressed maize. Annals of Botany 89:941-963.

Turner L.B., Cairns A.J., Armstead I.P., Ashton J., Skot K., Whittaker D., Humphreys M.O. (2006) Dissecting the regulation of fructan metabolism in perennial ryegrass (*Lolium perenne*) with quantitative trait locus mapping. New Phytologist 169:45-57.

Tziveleka L.A., Argyroudi-Akoyunoglou J.H. (1998) Implications of a developmental-stage-dependent thylakoid-bound protease in the stabilization of the light-harvesting pigment-protein complex serving photosystem II during thylakoid biogenesis in red kidney bean. Plant Physiology 117:961-970.

Van Loo E.N. (1992) Tillering, leaf expansion and growth of plants of 2 cultivars of perennial ryegrass grown using hydroponics at 2 water potentials. Annals of Botany 70:511-518.

Van Loo E.N., Dolstra O., Humphreys M.O., Wolters L., Luessink K., De Riek J., Bark N. (2003) Lower nitrogen losses through marker assisted selection for nitrogen use efficiency and feeding value in ryegrass (NIMGRASS). Vortrage für Pflanzenzuchtung 59:270-279.

Van Ooijen J.W. (1992) Accuracy of mapping quantitative trait loci in autogamous species. Theoretical and Applied Genetics 84:803-811.

Van Ooijen J.W., Maliepaard C. (1996) MapQTL version 3.0: Software for the calculation of QTL position on genetic linkage map. Plant Genome 4:105.

Van Ooijen J.W., Boer M.P., Jansen R.C., Maliepaard C. (2002) MapQTL® 4.0, Software for the calculation of QTL positions on genetic maps. Plant Research International, Wageningen, Netherlands.

Vansanford D.A., Mackown C.T. (1987) Cultivar differences in nitrogen remobilization during grain fill in soft red winter-wheat. Crop Science 27:295-300.

Varshney A., Mohapatra T., Sharma R.P. (2004) Molecular mapping and marker assisted selection of traits for crop improvement, in: P. S. Srivastava, et al. (Eds.), Plant biotechnology and molecular markers, Kluwer Academic Publishers, New Delhi. pp. 289-330.

Varshney R.K., Graner A., Sorrells M.E. (2005) Genic microsatellite markers in plants: features and applications. Trends in Biotechnology 23:48-55.

Verdenal A., Combes D., Escobar-Gutierrez A.J. (2008) A study of ryegrass architecture as a self-regulated system, using functional-structural plant modelling. Functional Plant Biology 35:911-924.

Vicentini F., Hortensteiner S., Schellenberg M., Thomas H., Matile P. (1995) Chlorophyll breakdown in senescent leaves - identification of the biochemical lesion in a stay-green genotype of *Festuca pratensis* huds. New Phytologist 129:247-252.

Vierstra R.D. (1987) Ubiquitin, a key component in the degradation of plant-proteins. Physiologia Plantarum 70:103-106.

Vierstra R.D. (1993) Protein-degradation in plants. Annual Review of Plant Physiology and Plant Molecular Biology 44:385-410.

Vierstra R.D. (2003) The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends in Plant Science 8:135-142.

Vinod K.K. (2007) Marker assisted selection for nitrogen use efficiency in crop plants, Genetics and plant breeding training programme on "Innovative tools in crop improvement, Coimbatore.

Vitousek P.M., Mooney H.A., Lubchenco J., Melillo J.M. (1997) Human domination of Earth's ecosystems. Science 277:494-499.

Volenec J.J., Nelson C.J. (1983) Responses of tall fescue leaf meristems to N-fertilization and harvest frequency. Crop Science 23:720-724.

Volenec J.J., Nelson C.J. (1984) Carbohydrate-metabolism in leaf meristems of tall fescue. 1. Relationship to genetically altered leaf elongation rates. Plant Physiology 74:590-594.

Wada S., Ishida H., Izumi M., Yoshimoto K., Ohsumi Y., Mae T., Makino A. (2009) Autophagy plays a role in chloroplast degradation during senescence in individually darkened leaves. Plant Physiology 149:885-893.

Warren C.R., Adams M.A., Chen Z.L. (2000) Is photosynthesis related to concentrations of nitrogen and Rubisco in leaves of Australian native plants? Australian Journal of Plant Physiology 27:407-416.

Warren C.R., Dreyer E., Adams M.A. (2003) Photosynthesis-Rubisco relationships in foliage of Pinus sylvestris in response to nitrogen supply and the proposed role of Rubisco and amino acids as nitrogen stores. Trees-Structure and Function 17:359-366.

Watson C.J., Jordon C., Taggart P.J., Laidlaw A.S., Gatrrett M.K., Steen R.W.J. (1992) The leaky cycle on grazed grassland. Aspects of Applied Biology 30:215-222.

White R., Murray W.R., Rohweder M. (2000) Pilot analysis of global ecosystems (PAGE), Grasslands Ecosystems, World Resource Institute (WRI). pp. 100.

Wilkins P.W. (1991) Breeding perennial ryegrass for agriculture. Euphytica 52:201-214.

Wilkins P.W., Humphreys M.O. (2003) Progress in breeding perennial forage grasses for temperate agriculture. Journal of Agricultural Science 140:129-150.

Wilkins P.W., Lovatt J.A. (2004) Recent gains from forage grass breeding. IGER Innovations 8: 18-21.

Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic-markers Nucleic Acids Research 18:6531-6535.

Williams W.M., Easton H.S., Jones C.S. (2007) Future options and targets for pasture plant breeding in New Zealand. New Zealand Journal of Agricultural Research 50:223-248.

Winter P., Kahl G. (1995) Molecular marker technologies for plant improvement. World Journal of Microbiology and Biotechnology 11:438-448.

Wittenbach V.A. (1979) Ribulose bisphosphate carboxylase and proteolytic activity in wheat leaves from anthesis through senescence. Plant Physiology 64:884-887.

Wright I.A. (2005) Future prospects for meat and milk from grass-based systems in: S. G. Reynolds and J. Frame (Eds.), Grasslands: Developments, opportunities, perspectives, Science Publishers, Enfield USA; Plymouth, UK. pp. 161-179.

Wu K.K., Burnquist W., Sorrells M.E., Tew T.L., Moore P.H., Tanksley S.D. (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theoretical and Applied Genetics 83:294-300.

Xu Y., Zhu L., Xiao J., Huang N., McCouch S.R. (1997) Chromosomal regions associated with segregation distortion of molecular markers in F_2, backcross, doubled haploid, and recombinant inbred populations in rice (*Oryza sativa* L.). Molecular & General Genetics 253:535-545.

Xu Z.L., Zou F., Vision T.J. (2005) Improving quantitative trait loci mapping resolution in experimental crosses by the use of genotypically selected samples. Genetics 170:401-408.

Yamada T., Forster J.W. (2005) QTL analysis and trait dissection in ryegrasses (*Lolium spp*.), in: M. O. Humphreys (Ed.), Molecular breeding for the genetic improvement of forage crops and turf. Proceedings of the 4th international symposium on molecular breeding of forage and turf, a satellite workshop of the XXth International Grassland Congress, July 2005, Wagenigen Academic Publishers, Aberystwyth, Wales. pp. 43-53.

Yamada T., Jones E.S., Cogan N.O.I., Vecchies A.C., Nomura T., Hisano H., Shimamoto Y., Smith K.F., Hayward M.D., Forster J.W. (2004) QTL analysis of morphological, developmental, and winter hardiness-associated traits in perennial ryegrass. Crop Science 44:925-935.

Yamaya T., Obara M., Nakajima H., Sasaki S., Hayakawa T., Sato T. (2002) Genetic manipulation and quantitative-trait loci mapping for nitrogen recycling in rice. Experimental Botany 53:917-925.

Yang J.Z., Matthew C., Rowland R.E. (1998) Tiller axis observations for perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*): Number of active phytomers, probability of tiller appearance, and frequency of root appearance per phytomer for three cutting heights. New Zealand Journal of Agricultural Research 41:11-17.

Yashitola J., Thirumurugan T., Sundaram R.M., Naseerullah M.K., Ramesha M.S., Sarma N.P., Sonti R.V. (2002) Assessment of purity of rice hybrids using microsatellite and STS markers. Crop Science 42:1369-1373.

Yoneyama T., Sano C. (1978) Nitrogen nutrition and growth of rice plant. 2. Considerations concerning dynamics of nitrogen in rice seedlings. Soil Science and Plant Nutrition 24:191-198.

Young N.D. (1994) Constructing a plant genetic map with DNA markers, in: R. L. Phillips and I. K. Vasil (Eds.), DNA-based markers in plants, Kluwer Academic Publishers, London. pp. 39-57.

Young N.D. (1996) QTL mapping and quantitative disease resistance in plants. Annual Review of Phytopathology 34:479-501.

Young N.D. (1999) A cautiously optimistic vision for marker-assisted breeding. Molecular Breeding 5:505-510.

Youngquist J.B., Maranville J.W. (1992) Patterns of nitrogen mobilization in grain-sorghum hybrids and the relationship to grain and dry-matter production. Journal of Plant Nutrition 15:445-455.

Yu J.M., Holland J.B., McMullen M.D., Buckler E.S. (2008) Genetic design and statistical power of nested association mapping in maize. Genetics 178:539-551.

Zavaleta-Mancera H.A., Thomas B.J., Thomas H., Scott I.M. (1999a) Regreening of senescent Nicotiana leaves II. Redifferentiation of plastids. Experimental Botany 50:1683-1689.

Zavaleta-Mancera H.A., Franklin K.A., Ougham H.J., Thomas H., Scott I.M. (1999b) Regreening of Nicotiana leaves I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll a/b-binding protein. Experimental Botany 50:1677-1682.

Zelisko A., Jackowski G. (2004) Senescence-dependent degradation of Lhcb3 is mediated by a thylakoid membrane-bound protease. Journal of Plant Physiology 161:1157-1170.

Zemenchik R.A., Albrecht K.A. (2002) Nitrogen use efficiency and apparent nitrogen recovery of Kentucky bluegrass, smooth bromegrass, and orchardgrass. Agronomy Journal 94:421-428.

Zeng Y., Li J., Wang C., Chang M.M., Yang R., Wu R. (2008) Genetic mapping of quantitative trait loci, in: C. Kole and A. G. Abbott (Eds.), Principles and practices of plant genomics, Science Publishers, New Hampshire. pp. 175-204.

Zeng Z.B. (1994) Precision mapping of quantitative trait loci. Genetics 136:1457-1468.

Zheng B., Halperin T., Hruskova-Heidingsfeldova O., Adam Z., Clarke A.K. (2002) Characterization of chloroplast Clp proteins in Arabidopsis: Localization, tissue specificity and stress responses. Physiologia Plantarum 114:92-101.

Zhu C., Gore M., Buckler E.S., Yu J. (2008) Status and prospects of association mapping in plants. The Plant Genome 1:5-20.

Appendices

Appendix 3.1: Changes in fresh weight (g), total N (mg/leaf) and Rubisco content (mg N/leaf) in leaves at different positions of barley plants growing under low N (N-) and high N (N+) concentrations. Values are means (n=3) except for Rubisco in the 4th and 6th leaves which were based on single leaf measurements. N loss (%) represents the proportion of the maximum amount of total N/Rubisco-N lost at the leaf age indicated by the asterisk (*).

		Leaf age (days)									
4th leaf	N level	4	7	11	13	17	20	24	27	31*	N loss (%)
Fresh weight	N-	0.25	0.33	0.35	0.33	0.30	0.31	0.27	0.24	0.13	
	N+	0.26	0.30	0.39	0.36	0.36	0.44	0.35	0.31	0.19	
Total N	N-	1.30	2.18	3.74	2.81	-	-	0.94	1.17	0.70	81.3
	N+	1.55	1.65	3.69	2.99	-	-	2.52	2.17	1.76	52.3
Rubisco	N-	0.19	0.48	0.57	-	-	0.12	0.21	0.30	0.10	82.5
	N+	0.36	0.55	-	0.73	-	0.23	0.27	0.19	-	-
Rubisco/total N	N-	0.15	0.22	0.15	-	-	-	0.22	0.26	0.14	
	N+	0.23	0.33	-	0.24	-	-	0.13	0.11	-	

		Leaf age (days)								
6th leaf	N level	4	8	11	15	18	24	30	37*	N loss (%)
Fresh weight	N-	0.35	0.67	0.68	0.78	0.80	0.72	0.60	0.42	
	N+	0.57	0.81	0.96	0.88	0.88	0.71	0.68	0.49	
Total N	N-	2.66	4.46	4.11	4.50	4.81	2.89	2.13	1.63	66.1
	N+	4.22	6.17	7.59	6.93	6.02	3.99	2.85	2.16	71.5
Rubisco	N-	0.45	0.73	0.90	0.99	0.53	0.59	0.23	0.24	75.7
	N+	0.69	0.15	1.64	0.95	0.91	0.70	0.32	0.42	74.3
Rubisco/total N	N-	0.17	0.16	0.22	0.22	0.11	0.20	0.11	0.15	
	N+	0.16	0.19	0.22	0.14	0.15	0.18	0.11	0.20	

Appendix 3.1 (Continued)

		Leaf age (days)								
8th leaf	N level	4	7	11	15	18	26	33*	40	N loss (%)
Fresh weight	N-	0.62	0.72	0.68	0.71	0.67	0.68	0.52	0.38	
	N+	0.85	0.89	0.93	0.85	0.92	0.93	0.80	0.48	
Total N	N-	3.03	3.33	3.37	3.17	3.24	3.12	2.12	1.60	37.1
	N+	4.19	6.47	5.71	6.10	6.51	5.51	3.82	2.29	41.3
Rubisco	N-	0.55	0.82	0.63	0.60	0.62	0.55	0.36	-	56.1
	N+	0.78	1.38	1.31	0.80	1.00	0.90	0.68	-	50.1
Rubisco/total N	N-	0.18	0.25	0.19	0.19	0.19	0.18	0.17	-	
	N+	0.19	0.21	0.23	0.13	0.15	0.16	0.18	-	

		Leaf age (days)								
10th leaf	N level	4	7	10	13	16	23	29*	36	N loss (%)
Fresh weight	N-	0.51	0.42	0.59	0.38	0.42	0.41	0.50	0.38	
	N+	0.79	0.82	0.89	0.94	0.96	0.82	0.70	0.34	
Total N	N-	4.81	4.51	5.80	3.07	2.56	2.21	3.44	2.11	66.1
	N+	5.93	6.87	8.99	7.75	6.29	5.36	5.43	1.99	71.5
Rubisco	N-	0.41	0.54	0.67	0.61	0.54	0.58	0.40	0.36	75.7
	N+	0.66	0.86	0.95	1.13	0.86	0.64	0.57	-	74.3
Rubisco/total N	N-	0.08	0.12	0.11	0.20	0.21	0.26	0.12	0.17	
	N+	0.11	0.13	0.11	0.15	0.14	0.12	0.11	-	

Appendix 4.1: Principal component structure of the first five PCs for ten variables measured in 200 genotypes of the Grasslands II mapping population in autumn 2003 (Sartie, 2007). LER, leaf elongation rate (cm/day); LED, leaf elongation duration (days); Alg, ligule appearance interval (days/leaf); LL, leaf lamina length (cm); Alf, leaf appearance interval (days/leaf); TN, tiller number; TW, tiller weight (g); PI, productivity index; CP, crude protein content; DW, herbage dry weight (g).

Trait	PC1	PC2	PC3	PC4	PC5
LER	-0.297	0.132	0.450	-0.176	0.404
LED	0.377	0.318	-0.145	0.033	-0.035
Alg	0.364	0.342	-0.209	-0.059	0.195
LL	-0.037	0.497	0.302	-0.182	0.387
Alf	0.390	0.365	-0.189	-0.032	0.033
TN	0.420	-0.285	0.309	-0.042	0.050
TW	-0.322	0.383	0.095	0.278	-0.192
PI	0.415	-0.256	0.360	-0.001	0.027
CP	-0.028	-0.190	-0.218	-0.045	0.479
DW	0.173	0.112	0.557	0.324	-0.169
Variation (%)	29.800	19.600	14.700	9.400	8.700

Appendix 4.2: Principal component scores of the first five PCs for ten variables measured in 200 genotypes of the Grasslands II mapping population in autumn 2003 (Sartie, 2007). Variation explained: PC1 (29.8%), PC2 (19.6%), PC3 (14.7%), PC4 (9.4%), and PC5 (8.7%).

Genotype	PC1	PC2	PC3	PC4	PC5
1	2.375	-1.607	3.282	1.147	-1.056
2	3.106	0.986	4.573	0.578	0.918
3	1.729	-2.295	-0.102	-0.967	-0.785
4	-0.848	0.636	1.614	1.197	0.544
5	0.462	-1.715	0.258	2.019	0.298
6	-2.185	-3.783	-0.894	-0.769	0.081
7	0.064	-1.586	2.670	-2.758	-1.015
8	0.147	-0.439	-1.576	-1.966	0.085
9	2.113	-3.737	0.443	-1.493	0.388
10	2.934	0.493	0.270	-1.806	-0.942
11	2.112	0.869	0.313	-2.758	1.568
12	-0.862	1.522	-1.396	-0.374	-0.928
13	-0.217	0.670	-2.017	1.944	-0.176
14	-2.349	1.148	-1.543	-0.129	0.825
15	1.526	3.584	-2.270	-1.467	0.129
16	0.735	-3.004	-0.149	0.858	-0.023
17	-0.187	0.706	-0.032	0.393	-0.786
18	-2.331	-1.173	0.588	-0.891	-1.159
19	1.973	-0.634	0.641	-1.069	1.458
20	1.377	-2.028	1.120	-0.340	-0.259
21	1.359	2.307	-0.510	-0.806	-0.364
22	3.118	-0.751	-1.252	0.207	-1.767
23	2.215	-0.035	-0.832	-0.353	-0.234
24	-0.235	-0.273	-2.321	0.726	0.360
25	-1.340	-2.093	-1.143	-0.720	0.616
26	-0.671	0.428	0.288	0.142	-1.115
27	-0.773	0.150	0.440	-0.128	1.074
28	0.887	-0.841	-1.553	0.315	1.089
29	2.357	0.199	-2.411	1.005	1.660
30	-1.543	1.482	-0.523	1.375	0.510
31	4.159	-1.164	1.501	1.684	0.534
32	0.740	-3.685	-0.434	1.855	0.492
33	1.764	-0.829	0.260	0.629	1.284
34	1.586	2.599	-0.775	0.893	0.647
35	0.838	0.118	1.240	-0.682	0.975
36	-0.287	-2.230	2.104	1.684	0.453
37	-1.438	-1.915	0.650	0.212	1.044
38	1.711	-0.533	-0.162	1.561	-0.961

Appendix 4.2 (Continued)

39	-0.848	-0.931	0.896	0.078	-0.505
40	0.830	-0.961	0.948	-1.273	-0.654
41	0.285	-0.837	-1.041	-0.244	-0.592
42	-0.568	-2.080	-1.931	0.526	-0.060
43	-2.036	1.279	1.552	0.691	-0.627
44	0.088	1.245	-2.291	0.132	0.284
45	-0.504	-1.210	-0.052	-0.561	-0.335
46	3.453	-0.640	2.790	1.633	-1.684
47	3.892	-1.752	0.596	-1.081	1.030
48	2.738	0.216	0.098	-0.446	-0.856
49	0.845	0.137	-0.347	0.890	-0.719
50	3.132	0.862	-0.981	-0.105	-1.296
51	0.245	-1.994	0.276	-1.824	-2.153
52	1.304	-0.896	-0.147	-0.680	0.390
53	1.018	1.652	-0.432	0.407	0.779
54	-1.211	1.245	1.096	0.706	0.808
55	0.211	-2.044	-0.738	-1.564	-1.182
56	0.800	1.213	-0.812	0.752	0.087
57	2.365	0.680	0.509	0.739	0.321
58	-1.773	-0.883	-0.832	-2.352	-1.927
59	2.613	-1.756	-0.322	-2.044	0.030
60	-3.089	-1.370	-2.855	0.409	1.615
61	6.003	-1.724	0.310	0.686	0.349
62	4.635	-1.076	-1.324	0.775	-1.992
63	3.382	0.847	-1.275	-0.928	0.793
64	2.679	2.083	2.242	-1.785	2.782
65	0.218	-1.049	-0.310	1.784	1.689
66	0.747	-0.293	0.770	-0.395	-1.923
67	-1.250	3.978	-2.084	0.035	0.376
68	-3.469	-2.360	-1.560	-0.663	1.071
69	1.424	1.694	-0.361	-1.223	0.479
70	0.595	-1.514	0.510	-1.496	-0.643
71	1.564	-0.359	0.571	1.882	0.157
72	-0.167	-2.030	-0.594	0.213	-1.104
73	-2.817	0.837	0.381	-1.168	-0.212
74	0.203	1.820	0.665	-0.498	0.475
75	0.759	1.646	-2.157	0.319	0.515
76	1.643	-1.262	-0.823	-0.422	0.230
77	3.335	1.914	-1.566	1.197	0.075
78	0.683	-1.363	-0.383	0.110	-0.479
79	0.077	0.227	-0.121	-0.310	-2.457
80	2.007	0.915	-0.036	-1.725	-1.025
81	-1.794	-0.012	0.177	-0.427	-1.708
82	0.424	3.268	1.911	0.559	0.450

Appendix 4.2 (Continued)

83	-1.311	2.976	0.619	-1.672	0.447
84	0.140	1.681	0.213	-1.130	-0.936
85	-2.088	-0.143	-0.406	-0.778	0.359
86	2.248	-1.415	0.515	-1.634	-0.290
87	-1.245	-1.082	0.505	-0.181	-0.564
88	-0.707	-0.458	-0.116	1.543	-0.027
89	-0.133	-1.380	-2.846	0.095	0.744
90	-1.353	-0.372	0.691	0.452	0.554
91	-1.221	0.467	-0.362	-0.348	-0.228
92	2.302	-1.292	-2.197	0.598	-2.816
93	0.374	-0.188	-0.189	-0.666	1.929
94	-1.149	-2.152	-2.121	-0.869	0.946
95	-1.359	-2.241	-1.569	-1.389	2.364
96	0.498	-0.088	0.178	0.294	0.016
97	1.330	1.891	-0.686	0.125	-0.281
98	-1.148	0.107	-2.597	-1.090	-0.191
99	-1.184	0.798	-0.339	-0.874	-0.214
100	1.520	0.765	-0.346	0.049	-1.280
101	0.780	-0.079	-0.711	0.160	-0.887
102	0.562	0.737	-1.288	-0.418	2.142
103	-2.649	2.147	3.832	-1.196	0.096
104	1.275	3.045	-0.806	-0.431	0.235
105	0.618	-0.880	-0.125	-1.017	-0.486
106	0.971	-3.066	2.176	-1.415	0.936
107	3.398	-0.793	-0.926	1.138	0.629
108	0.010	0.973	-1.143	-0.082	1.754
109	-0.261	1.535	1.305	-0.902	-0.057
110	-2.710	1.219	-2.163	-0.158	0.054
111	-2.797	-1.438	1.538	-1.614	1.235
112	0.568	-2.291	-1.299	0.039	-0.306
113	-1.366	0.094	-0.426	-0.599	-1.491
114	-1.295	1.775	-1.103	-1.651	-0.337
115	-0.530	0.091	-1.435	-0.096	1.045
116	-3.154	-0.421	0.853	2.493	-1.191
117	-1.524	-1.465	0.391	-0.057	0.525
118	-0.193	-2.850	-1.886	-0.155	-0.098
119	-2.936	-0.093	2.172	-0.137	2.113
120	0.161	-0.184	-1.180	1.072	0.059
121	1.307	-1.786	1.780	0.765	-0.225
122	0.476	0.217	-1.002	-1.799	0.642
123	1.603	2.180	1.433	-0.415	-1.118
124	2.277	0.767	-0.581	-0.475	0.768
125	-0.590	0.014	-0.513	-1.592	1.970

Appendix 4.2 (Continued)

126	0.740	-0.503	-1.860	-0.145	0.839
127	-3.113	2.727	-1.982	-0.857	-0.040
128	1.013	3.144	-1.963	1.629	-0.336
129	0.488	0.661	-0.011	0.202	-0.788
130	0.048	1.250	0.285	1.576	-0.535
131	1.251	-1.084	1.196	0.333	1.460
132	0.430	1.187	1.659	1.607	0.596
133	-1.017	2.088	-0.337	1.391	-0.125
134	-4.733	0.339	0.228	-0.032	-4.242
135	-3.179	-0.866	-0.838	-0.857	-0.234
136	3.292	2.205	2.693	1.044	1.446
137	-1.081	0.700	0.240	1.065	-0.173
138	1.276	1.322	-0.007	1.158	-1.456
139	0.393	-1.138	1.198	-0.385	-0.596
140	-1.822	0.061	-0.062	-0.477	-1.089
141	0.798	2.384	-1.190	0.439	0.942
142	-1.326	-0.441	-0.455	0.527	-0.176
143	-3.846	0.206	-1.209	0.956	-0.565
144	-2.796	-0.862	-0.639	0.440	-0.748
145	-0.460	-1.630	-1.528	-0.769	-0.939
146	2.676	1.016	-1.170	0.835	0.615
147	-1.115	1.847	2.905	-1.296	0.364
148	-2.203	-0.540	0.138	0.763	-1.211
149	-1.548	1.796	0.974	0.243	2.629
150	0.446	-1.040	1.123	1.584	0.854
151	1.324	1.248	0.352	-0.271	-1.120
152	-2.847	-0.148	0.383	1.125	-0.299
153	-0.602	1.346	3.134	-1.098	0.225
154	0.467	1.288	0.923	0.566	-0.383
155	-0.005	0.267	0.912	0.043	0.231
156	-0.833	-0.050	2.413	0.440	-1.642
157	-1.388	-0.986	-1.053	1.287	0.407
158	-2.430	-0.128	0.764	-0.137	0.576
159	-1.116	-2.049	1.122	1.517	0.040
160	-1.014	0.784	1.682	-0.166	-0.186
161	-1.330	0.691	-0.656	0.229	0.071
162	-3.138	1.424	0.555	-1.043	-0.394
163	-2.009	-0.510	0.217	1.196	0.868
164	-1.137	-0.271	-1.092	-0.028	-0.886
165	-2.877	1.421	-1.045	0.655	0.691
166	-0.387	-1.385	2.032	1.103	0.354
167	-2.679	-1.134	0.520	0.549	0.519
168	-1.612	0.103	2.414	-0.436	-0.999

Appendix 4.2 (Continued)

169	-1.404	-1.215	-0.756	1.339	0.145
170	-1.172	1.323	0.557	-0.402	0.779
171	-0.733	-0.940	-0.211	1.510	1.205
172	-0.115	0.401	0.643	-0.319	-0.949
173	-1.803	1.408	1.927	-0.552	2.328
174	-1.738	0.853	0.679	0.882	0.241
175	-0.297	-1.016	0.108	-1.467	-1.475
176	-1.486	0.809	2.262	-0.489	0.123
177	-2.273	1.839	0.310	-0.631	-0.574
178	0.054	-1.419	-1.014	0.272	-0.442
179	2.423	1.856	-1.506	0.816	0.074
180	1.000	0.171	-2.143	2.566	0.059
181	4.192	2.163	-0.636	-1.014	-0.070
182	1.962	0.883	1.322	0.961	-0.349
183	-2.826	0.548	1.216	1.568	-0.031
184	-1.151	-1.671	0.244	0.645	0.651
185	0.824	1.784	1.311	0.771	-1.791
186	1.299	2.632	-0.479	0.547	-0.093
187	1.724	1.708	0.573	-1.569	-0.739
188	0.092	1.515	0.152	-0.732	-0.239
189	-2.037	2.394	-0.776	0.491	-0.633
190	-1.835	-0.199	0.837	1.874	-0.712
191	-1.113	0.983	0.335	-0.672	-0.257
192	-3.462	0.475	0.024	0.672	-0.621
193	-0.891	-0.479	-0.226	0.009	0.017
194	0.087	-2.650	1.456	-0.218	0.666
195	0.444	1.029	-0.636	-1.760	-0.077
196	0.927	0.192	0.487	1.792	-0.379
197	0.657	-2.318	0.871	0.848	-0.174
198	-0.483	-0.802	0.748	1.044	0.535
Impact	2.637	-3.441	-0.803	-1.448	1.564
Samson	-4.809	-1.788	1.039	-0.082	2.003

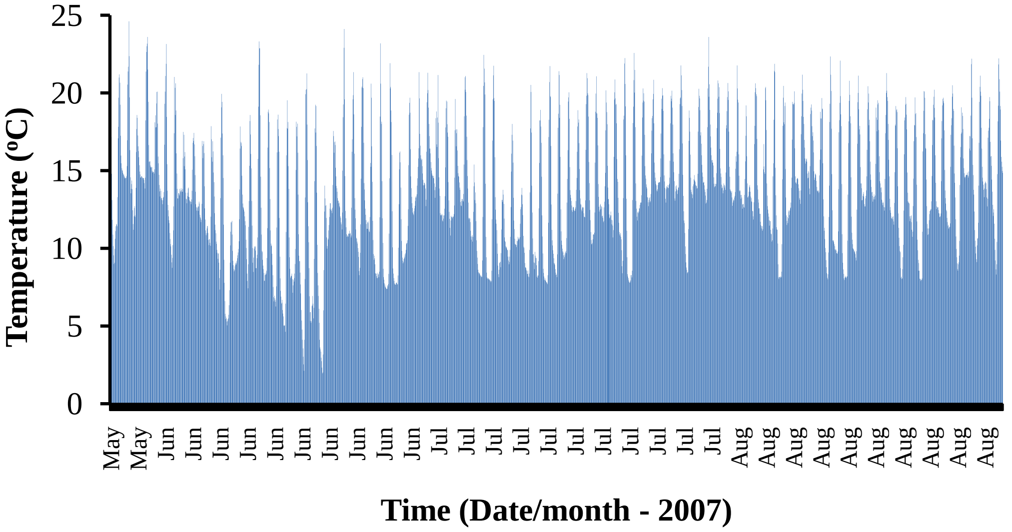
Appendix 4.3: ANOVA of Rubisco turnover parameters measured in the 16 genotypes of the Grasslands II mapping population. d = curve peak (mg Rubisco-N/leaf); f = measure of curve width; g = time of curve peak (days).

Variable	Source	DF	SS	MS	F	*P*
d	Genotype	15	0.2592	0.0173	78.86	0.0001
	Rep	1	0.0011	0.0011	4.88	0.0430
	Error	15	0.0033	0.0002		
	Total	31	0.2636			
f	Genotype	15	0.7451	0.0497	5.19	0.0014
	Rep	1	0.0069	0.0069	0.73	0.4079
	Error	15	0.1435	0.0096		
	Total	31	0.8956			
g	Genotype	15	126.6418	8.4428	6.40	0.0004
	Rep	1	0.2343	0.2343	0.18	0.6795
	Error	15	19.7999	1.3200		
	Total	31	146.6760			

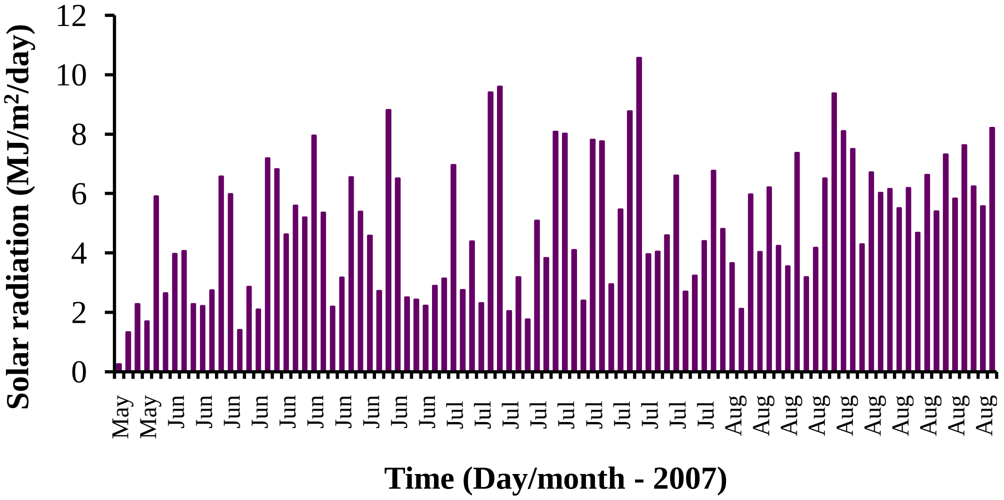
Appendix 4.4: Principal component scores of the first five PCs for the 16 genotypes of the Grasslands II mapping population and the two parent plants. Variation explained by each PC; PC1 (35.6%), PC2 (22.2%), PC3 (15.9%), PC4 (10.1%), and PC5 (7.4%).

Genotype	PC1	PC2	PC3	PC4	PC5
1	0.407	-0.577	-2.756	-0.023	1.662
7	-0.710	-0.793	-0.150	-0.444	1.511
29	1.722	0.326	0.290	0.380	-0.637
51	-0.190	-2.067	-0.006	0.350	-0.501
59	1.744	-1.776	2.181	-0.291	0.396
62	3.339	-0.447	-2.281	-0.963	-1.173
63	2.213	1.386	-0.069	-0.502	0.345
67	-0.360	2.613	1.730	0.703	-0.724
68	-2.292	-0.979	1.403	-1.028	-0.782
70	0.171	-0.957	0.021	-0.219	0.111
111	-2.192	-0.582	0.707	0.056	0.876
134	-2.913	-0.258	-1.781	0.221	-1.672
141	0.694	1.465	0.394	1.285	-0.436
149	-1.315	1.859	0.365	0.856	1.195
165	-1.316	3.014	-0.244	-2.789	0.049
181	2.592	1.033	-0.361	1.802	0.111
Impact	1.756	-2.180	1.442	-0.673	-0.121
Samson	-3.350	-1.079	-0.886	1.277	-0.209

Appendix 5.1: Mean daily temperature record in the glasshouse during the period of assessment of the Grasslands II perennial ryegrass mapping population for Rubisco turnover and herbage yield traits in 2007. Recording time was May 28 to August 31 2007.



Appendix 5.2: Solar radiation measurements in the glasshouse during the period of assessment of the Grasslands II perennial ryegrass mapping population for Rubisco turnover and herbage yield traits in 2007



Appendix 5.3: The final data of herbage yield and Rubisco turnover measurements of the 135 F_1 and the two parent plants of Grasslands II perennial ryegrass mapping population plants. TW = tiller weight (mg); TW_{Lg} = average weight of the three largest tillers (mg); LL = leaf lamina length (mm); TN = tiller number; DW = herbage dry weight (g); PI = productivity index; *d* = maximum Rubisco content (mg N/g leaf dry weight) ; *f* = curve width measure; *d*/*f* = *d*:*f* ratio; *g* = time of *d* (days); PK = average of the two Rubisco content determinations closest to *d* (mg N/g leaf dry weight); TL = residual Rubisco content measured as the average of the last two time points (mg N/g leaf dry weight).

Genotype	TW	TW_{Lg}	LL	TN	DW	PI	*d*	*f*	*d/f*	*g*	PK	TL
1	125	218	316	47	3.48	4.53	59.5	0.57	104.3	13.6	48.6	16.4
2	159	250	317	40	4.21	4.53	45.1	0.77	58.6	13.0	47.5	15.0
3	95	221	365	68	5.64	4.64	52.1	0.91	57.0	10.4	46.9	20.7
4	118	225	357	68	5.85	4.74	49.2	0.91	53.9	11.7	54.7	25.3
5	59	140	238	42	1.80	4.13	53.7	0.91	59.1	12.0	52.4	25.2
6	71	115	237	41	0.76	4.18	61.9	0.80	77.3	10.8	55.4	18.4
7	132	225	346	89	9.93	4.97	52.3	0.78	66.9	11.8	47.7	17.2
8	109	265	340	104	8.63	4.98	54.5	0.73	74.6	11.5	51.3	15.5
9	102	177	403	77	5.72	4.75	40.0	0.89	44.8	11.4	41.4	24.6
10	100	204	257	79	7.19	4.77	40.6	0.86	47.0	12.5	40.5	19.6
12	167	294	306	54	7.69	4.75	51.2	0.81	63.0	10.6	49.2	14.5
13	86	180	306	74	5.01	4.66	46.4	1.03	44.9	11.9	47.0	27.4
14	100	209	349	55	3.26	4.53	50.3	0.74	68.2	12.2	47.4	14.3
15	46	151	318	53	2.75	4.16	38.8	0.96	40.4	13.7	31.2	16.3
17	68	179	291	70	3.18	4.52	45.9	0.92	50.1	14.0	48.5	25.1
18	85	158	204	50	1.03	4.40	45.3	0.87	51.9	11.1	47.7	28.0
19	157	420	424	75	9.38	4.92	40.7	0.90	45.3	13.1	38.1	23.2
20	94	212	296	62	5.64	4.58	49.3	0.78	63.2	12.0	46.3	18.8
21	82	136	311	26	1.30	3.95	38.3	0.75	50.9	14.6	41.4	24.6
22	55	97	206	23	0.54	3.70	40.4	0.93	43.3	16.6	32.7	15.6
23	72	102	204	25	1.08	3.87	46.6	0.81	57.8	13.7	39.3	21.3
24	87	200	302	58	3.23	4.50	53.3	0.71	75.3	13.8	44.2	20.0
25	106	232	341	75	6.27	4.76	38.8	0.86	45.0	15.2	40.6	22.8
26	85	217	327	58	4.58	4.49	36.3	0.78	46.7	12.9	34.6	11.2
28	69	129	255	46	2.84	4.25	49.0	0.80	61.2	11.2	50.4	12.8
30	110	206	278	72	5.35	4.74	35.6	0.93	38.4	12.4	42.2	19.3
31	81	141	295	51	2.52	4.39	44.2	0.79	56.2	11.0	33.9	18.4
32	91	128	317	40	1.53	4.28	46.3	1.03	45.0	12.6	44.6	24.2
34	66	141	290	95	5.54	4.70	50.0	0.90	55.8	11.6	57.1	18.0
35	76	305	278	76	6.64	4.62	41.2	0.81	51.1	12.5	39.4	14.7
36	107	209	266	57	6.09	4.58	51.9	0.81	64.1	11.9	46.6	18.1
37	95	250	340	87	7.78	4.81	37.7	0.78	48.1	11.7	32.2	11.5
40	128	311	314	76	8.94	4.85	40.9	0.82	49.7	14.3	29.1	21.2
41	95	178	250	26	1.51	4.02	44.9	0.87	51.4	13.6	46.1	25.0
42	51	109	217	56	2.21	4.25	58.3	1.00	58.4	14.6	63.4	38.7
43	145	309	348	79	9.12	4.92	40.1	0.85	47.0	13.8	31.8	17.8
44	166	372	336	66	8.95	4.87	48.5	0.82	58.8	12.3	48.4	18.9

Appendix 5.3 (Continued)

45	97	256	352	103	8.78	4.92	56.7	0.90	63.0	10.2	38.9	19.5
46	71	131	234	64	2.32	4.48	45.8	0.66	69.2	14.3	40.1	24.4
47	59	98	216	17	0.60	3.55	48.5	0.84	58.0	9.8	43.9	22.9
48	57	129	263	42	0.79	4.11	41.5	0.92	45.3	12.2	38.5	17.8
50	58	132	230	50	1.89	4.23	45.3	0.90	50.2	12.0	39.0	23.7
51	59	109	187	34	1.17	3.99	43.0	0.69	62.8	11.0	38.2	16.7
53	76	170	262	38	1.85	4.16	46.4	0.82	56.5	10.5	39.1	18.2
55	62	138	248	57	1.80	4.35	55.5	0.68	82.0	17.6	56.1	28.7
56	71	94	213	19	0.46	3.70	46.2	0.77	60.0	16.5	45.8	25.2
57	75	186	319	45	2.69	4.28	43.4	0.88	49.4	10.9	40.0	21.1
59	103	207	263	56	5.10	4.56	39.4	0.97	40.8	13.0	39.8	22.8
61	62	127	245	69	3.66	4.47	44.4	0.79	56.0	13.5	42.5	17.0
62	70	176	244	92	6.09	4.71	40.3	0.86	46.8	14.9	38.5	19.8
63	100	220	299	46	2.69	4.42	44.7	0.89	50.1	12.6	37.1	21.1
64	112	216	347	55	4.96	4.58	38.0	0.65	58.6	13.4	35.6	16.1
65	64	159	271	41	1.78	4.14	42.4	1.08	39.3	9.0	44.3	13.6
66	96	250	360	55	3.65	4.51	49.9	0.68	73.8	14.0	41.7	18.9
67	73	143	252	30	1.37	3.99	45.4	1.15	39.4	13.3	42.6	21.7
68	88	305	295	100	7.46	4.86	41.7	0.82	50.7	14.5	41.4	19.4
69	88	236	265	55	2.72	4.47	37.1	0.68	54.5	14.2	31.2	13.2
70	61	138	240	34	1.07	4.01	49.3	0.95	52.1	13.4	46.9	27.6
71	80	173	233	44	2.08	4.29	49.0	0.86	57.0	12.4	45.4	22.3
73	143	233	336	77	7.17	4.90	43.0	0.81	52.7	12.8	41.8	15.6
76	65	137	268	45	2.59	4.22	53.4	0.82	64.9	12.1	60.4	27.0
78	59	117	178	35	1.11	4.01	45.6	0.82	55.8	14.9	45.6	23.9
79	121	384	416	106	9.85	5.04	44.7	0.81	55.0	13.8	47.0	19.2
80	68	174	343	88	5.02	4.67	36.6	0.88	41.8	14.1	34.1	20.0
81	94	168	303	72	4.95	4.67	54.6	0.70	77.5	11.3	39.6	15.4
82	109	285	427	50	3.88	4.51	44.2	0.83	53.1	12.2	33.3	15.4
84	225	205	377	92	6.30	5.21	31.6	1.23	25.8	9.9	33.3	18.0
85	105	258	338	41	3.89	4.36	38.2	0.97	39.4	10.9	36.2	16.7
86	8	123	349	49	2.33	3.36	45.3	0.89	50.8	10.9	41.5	13.2
87	77	194	311	71	4.84	4.58	38.8	0.94	41.2	13.0	35.8	19.7
89	55	120	216	56	2.85	4.28	54.5	0.89	61.2	12.2	47.4	25.5
91	91	198	324	69	7.00	4.64	50.3	0.86	58.7	12.8	51.5	27.3
92	107	146	231	29	1.48	4.15	64.3	0.71	90.2	10.7	77.1	18.5
93	85	221	335	82	5.09	4.72	40.6	0.93	43.5	12.6	36.2	17.5
95	102	201	323	54	4.45	4.52	39.4	0.82	48.0	12.3	38.7	15.0
98	142	229	298	47	4.33	4.58	48.7	0.97	50.0	11.9	52.3	26.6
99	104	200	285	75	6.53	4.75	39.7	0.76	52.2	12.0	45.7	16.8
100	114	240	378	70	6.12	4.74	40.1	0.84	47.7	14.9	40.5	22.9
101	77	116	223	42	1.62	4.25	51.8	0.74	70.1	13.6	38.6	23.0
102	125	268	326	72	7.13	4.80	36.4	0.87	42.0	14.6	33.4	18.3
104	111	198	319	75	6.35	4.77	35.1	0.78	45.2	16.1	31.6	19.9
110	86	128	324	77	5.95	4.68	42.4	0.77	55.3	12.6	40.0	21.0
111	50	161	276	75	5.61	4.43	44.2	0.76	58.5	12.2	44.1	14.7
115	97	150	281	39	1.61	4.29	54.1	0.77	70.5	12.0	49.7	18.1
116	125	182	297	36	3.19	4.35	43.2	0.68	63.6	15.9	31.4	12.7
117	109	181	278	89	7.90	4.88	46.6	0.85	54.7	12.2	43.5	19.8
118	78	186	284	41	3.02	4.23	49.1	0.80	61.1	14.6	52.4	16.5
120	89	127	233	30	1.82	4.09	47.6	0.84	56.7	13.8	33.4	13.9

Appendix 5.3 (Continued)

121	119	188	320	94	11.23	4.95	46.7	0.78	60.1	13.8	47.6	21.7
123	155	287	370	85	12.56	5.00	41.0	0.59	69.7	14.5	41.5	13.0
125	141	270	358	103	12.13	5.09	35.1	0.72	48.9	14.8	27.8	13.0
130	136	221	256	46	3.68	4.55	46.8	0.99	47.1	10.6	46.3	20.6
131	135	191	307	62	6.36	4.74	34.3	0.90	38.3	14.1	34.3	17.9
134	154	317	346	75	10.11	4.92	41.2	0.84	49.0	12.6	40.6	15.8
141	77	166	227	52	4.14	4.37	48.6	0.84	57.5	11.6	47.4	18.9
142	99	189	314	74	5.16	4.72	48.2	0.93	52.0	11.3	46.0	20.0
143	121	221	217	79	8.80	4.85	42.4	0.86	49.3	10.4	34.1	15.1
144	114	262	357	80	7.52	4.83	61.5	0.77	80.0	12.1	67.2	19.1
145	86	120	231	83	4.69	4.73	52.1	0.72	72.1	12.5	51.7	15.0
147	90	168	256	87	8.33	4.79	46.3	0.69	67.3	14.3	44.2	16.9
149	145	341	368	51	5.04	4.64	54.3	0.83	65.3	11.7	52.7	18.9
152	138	224	300	75	8.43	4.87	51.4	0.68	76.2	13.9	46.3	18.5
154	134	322	350	63	5.98	4.75	39.2	0.79	49.5	14.2	36.7	17.5
155	41	49	235	14	0.20	3.24	49.0	0.81	60.4	13.1	36.7	21.7
157	88	119	197	31	0.75	4.10	51.2	0.73	69.8	13.3	59.6	21.6
158	115	190	314	74	7.82	4.78	46.9	0.77	61.3	11.5	48.4	17.3
159	79	73	197	13	0.28	3.49	34.6	0.89	38.9	13.1	34.8	15.7
160	169	344	341	83	11.22	5.03	38.0	0.81	46.7	13.0	36.8	13.6
161	131	256	380	64	6.20	4.75	38.2	0.73	52.5	13.5	35.8	14.2
163	87	139	303	89	5.89	4.78	44.0	0.68	64.5	13.4	37.7	13.2
164	76	134	222	36	1.02	4.13	46.9	0.83	56.4	13.8	44.0	26.9
165	73	236	266	36	4.37	4.13	53.2	0.87	61.0	11.8	57.1	25.7
166	144	299	351	80	9.23	4.93	37.1	0.77	48.1	13.5	37.5	15.9
167	92	166	317	52	3.51	4.46	44.9	0.62	72.2	15.8	38.8	18.1
168	141	281	381	65	9.88	4.78	40.3	0.72	56.2	14.2	40.9	20.6
170	104	140	332	101	6.88	4.94	40.6	0.74	55.0	14.1	41.5	17.0
171	89	193	246	78	5.62	4.71	42.9	0.84	51.1	14.0	39.8	22.4
172	71	113	260	71	5.58	4.54	46.4	0.74	63.1	13.9	41.4	20.6
173	98	134	241	53	3.63	4.49	45.9	0.72	64.0	13.7	45.0	19.4
174	78	195	292	76	7.17	4.63	48.8	0.75	65.1	12.5	51.0	14.3
175	59	77	222	40	1.48	4.10	48.3	0.78	61.6	11.4	58.7	15.7
176	118	196	274	74	7.07	4.80	38.7	0.75	51.5	13.7	37.5	16.4
178	79	185	283	83	5.78	4.70	48.8	0.64	76.5	14.1	52.7	16.5
180	92	179	312	77	6.08	4.71	57.0	0.76	74.6	11.7	54.6	18.7
182	79	139	312	56	3.10	4.43	32.8	0.89	36.8	10.8	27.1	17.5
185	78	123	198	71	1.73	4.59	46.1	0.58	79.9	17.9	39.2	22.0
186	56	129	213	106	3.91	4.71	41.2	0.87	47.4	12.1	41.8	25.1
187	141	282	433	61	7.54	4.75	45.5	0.73	62.0	14.8	33.5	18.6
192	92	163	275	74	6.33	4.68	34.5	1.02	33.8	12.4	31.9	14.7
194	69	160	289	96	5.63	4.73	53.1	0.73	72.4	15.8	44.5	24.1
195	112	266	268	91	7.56	4.91	60.0	0.69	87.2	13.5	52.9	22.7
196	78	156	234	40	1.69	4.21	57.2	0.67	85.1	12.0	51.5	22.6
197	87	178	323	68	4.83	4.61	51.2	0.89	57.4	10.5	41.7	17.4
199	76	133	284	59	2.98	4.45	39.9	0.76	52.3	14.8	40.7	22.0
200	96	158	289	76	5.04	4.72	41.7	0.87	48.2	13.0	47.8	22.1
Samson	94	209	321	52	4.83	4.47	42.8	0.84	50.7	13.5	44.9	18.7
Impact	80	197	336	104	6.74	4.85	49.4	0.77	64.0	12.2	47.6	13.6

Appendix 5.4: ANOVA of the herbage yield traits measured in the 135 genotypes of the Grasslands II mapping population in 2007. TW = tiller weight (mg); TW_{Lg} = average weight of the three largest tillers (mg); TN = tiller number; PI = productivity index; DW = herbage dry weight (g); LL = leaf lamina length (mm).

Variable	Source	DF	SS	MS	F	*P*
TW	Genotype	134	232157.212	1732.516	1.99	<0.001
	Rep	1	916.004	916.004	1.05	0.307
	Error	114	99178.496	869.987		
	Total	249	332514.400			
TW_{Lg}	Genotype	134	1134136.789	8463.707	3.79	<0.001
	Rep	1	19628.856	19628.856	8.78	0.004
	Error	119	266070.475	2235.886		
	Total	254	1425197.962			
TN	Genotype	134	109628.684	818.1245	2.31	<0.001
	Rep	1	2687.817	2687.8166	7.59	0.007
	Error	115	40727.545	354.1526		
	Total	250	153535.297			
PI	Genotype	134	28.600	0.213	2.92	<0.001
	Rep	1	0.566	0.566	7.74	0.006
	Error	111	8.117	0.073		
	Total	246	37.448			
DW	Genotype	134	2203.336	16.443	3.28	<0.001
	Rep	1	47.701	47.701	9.53	0.003
	Error	116	580.729	5.006		
	Total	251	2831.985			
LL	Genotype	134	756627.425	5646.473	2.64	<0.001
	Rep	1	248.974	248.974	0.12	0.7336
	Error	117	250222.471	2138.654		
	Total	252	1008204.968			

Appendix 5.5: PC scores of the first the first four PCs for herbage yield and Rubisco turnover measurements on the 135 F_1 and the two parent plants of the Grasslands II perennial ryegrass mapping population. Variation explained by each PC: PC1 (35.9%), PC2 (23.3%), PC3 (13.9%), PC4 (10.2%).

Genotypes	PC1	PC2	PC3	PC4
1	-1.690	4.106	-1.603	-1.351
2	0.864	0.339	-0.254	-1.162
3	0.112	0.608	2.154	-0.468
4	0.373	0.772	2.288	0.898
5	-3.467	-0.195	1.313	0.511
6	-4.295	1.791	1.069	-1.454
7	1.699	2.607	1.062	-0.410
8	1.151	3.382	0.879	-0.731
9	1.491	-0.564	1.577	0.693
10	1.408	-0.606	0.353	0.478
12	1.181	1.540	1.322	-1.724
13	0.132	-0.963	2.407	1.663
14	-0.103	1.151	-0.186	-1.522
15	0.236	-3.027	-0.637	-0.265
17	-1.052	-0.469	0.704	1.713
18	-2.172	-1.029	1.301	0.889
19	3.707	-0.157	1.066	1.023
20	-0.186	0.886	0.313	-0.452
21	-1.221	-1.828	-1.446	0.725
22	-2.087	-3.496	-2.331	0.144
23	-3.030	-1.394	-1.134	-0.083
24	-1.104	1.525	-0.917	-0.031
25	1.893	-0.744	-0.375	1.765
26	1.475	-1.389	-1.265	-1.469
28	-1.809	0.147	0.197	-1.953
30	1.493	-1.590	0.744	0.419
31	-0.851	-0.760	-0.082	-1.178
32	-0.853	-1.983	1.479	0.636
34	-0.286	1.041	1.748	0.072
35	1.336	-0.527	-0.440	-0.590
36	-0.239	0.943	0.536	-0.624
37	2.896	-0.495	-0.442	-1.385
40	2.711	-0.419	-0.709	1.058
41	-1.993	-1.573	-0.026	0.715
42	-4.119	0.285	2.215	4.184
43	3.425	-0.317	-0.250	0.391
44	2.045	1.439	1.089	-0.152
45	1.460	1.681	2.119	-0.326
46	-1.397	0.525	-1.654	1.029
47	-4.135	-1.613	0.717	-1.332
48	-1.257	-2.499	-0.030	-0.713
50	-1.625	-1.787	0.396	0.373

Appendix 5.5 (Continued)

51	-2.344	-1.095	-1.270	-1.828
55	-3.839	2.479	-1.719	2.793
56	-4.263	-0.897	-1.921	1.379
57	-0.491	-1.554	0.811	-0.645
59	0.734	-1.975	0.702	0.951
61	-0.810	-0.243	-0.773	-0.052
63	-0.096	-1.400	0.209	0.007
64	1.293	0.027	-1.746	-0.829
65	-0.634	-3.045	2.177	-2.166
66	-0.120	1.366	-1.285	-0.301
67	-2.122	-3.161	1.379	0.577
68	1.867	0.200	-0.432	1.299
69	0.858	-1.214	-2.599	-0.958
70	-3.475	-1.341	0.732	1.333
71	-1.846	-0.662	0.354	0.150
73	2.475	0.476	0.199	-0.414
76	-2.881	0.752	1.269	0.800
78	-2.644	-1.434	-1.185	1.104
79	3.178	1.670	0.617	0.950
80	1.752	-1.512	-0.487	1.049
81	-0.269	2.020	-0.210	-1.509
82	1.732	-0.808	-0.106	-1.365
84	5.142	-2.542	3.695	-0.120
85	0.862	-2.475	1.016	-1.226
86	-2.167	-2.104	0.135	-2.280
87	1.119	-1.879	0.258	0.509
89	-3.415	0.246	1.056	0.844
91	-0.152	0.947	1.367	1.538
92	-4.880	3.762	1.563	-1.658
93	1.574	-1.224	0.520	0.083
95	1.246	-1.175	-0.307	-1.028
98	-0.294	-0.287	2.308	0.963
99	1.372	0.137	0.017	-0.440
100	1.927	-0.420	-0.282	1.481
101	-2.292	0.128	-1.136	0.251
102	2.754	-1.215	-0.656	0.756
104	2.482	-1.047	-1.877	1.420
110	0.890	0.137	-0.126	0.325
111	-0.069	0.097	-0.506	-0.901
115	-2.260	0.994	0.155	-1.152
116	-0.377	0.054	-2.968	-0.895
117	1.294	0.706	0.862	0.460
118	-1.715	0.290	-0.696	-0.248
120	-1.447	-1.396	-1.384	-1.238
121	2.304	1.832	0.312	1.299
123	3.852	2.652	-1.767	-0.530

Appendix 5.5 (Continued)

125	4.998	0.472	-1.695	0.130
130	-0.267	-1.031	2.150	-0.435
131	2.518	-1.794	-0.405	0.460
134	3.279	0.348	0.499	-0.345
141	-1.435	-0.185	0.592	-0.551
142	0.276	0.077	1.716	-0.058
143	1.617	-0.324	0.742	-1.019
144	-0.768	4.339	1.852	-0.167
145	-0.723	2.084	-0.183	-0.669
147	0.935	1.681	-1.239	0.343
149	0.301	1.586	1.427	-0.783
152	0.949	2.818	-0.657	0.099
154	2.295	-0.445	-0.817	0.116
155	-3.854	-2.217	-1.275	-0.746
157	-3.660	0.835	-0.300	-0.072
158	1.165	1.331	0.656	-0.647
159	-2.155	-4.050	-1.352	-1.252
160	4.297	0.293	-0.036	-0.458
161	2.581	-0.072	-1.104	-0.835
163	1.283	1.081	-1.413	-0.757
164	-2.315	-1.187	-0.362	1.169
165	-2.796	0.366	1.518	0.447
166	3.442	0.134	-0.499	-0.051
167	-0.279	0.944	-2.676	0.117
168	2.774	0.818	-0.708	0.596
170	2.381	0.795	-0.774	0.526
171	0.195	-0.300	-0.250	1.338
172	-0.963	0.797	-0.893	0.588
173	-0.815	0.458	-1.027	0.025
174	-0.129	1.660	-0.020	-0.792
175	-3.623	0.404	0.402	-1.401
176	2.002	-0.171	-1.038	0.037
178	-0.389	2.597	-1.183	-0.033
180	-0.797	2.736	1.016	-0.463
182	1.069	-3.062	0.033	-0.996
185	-2.202	1.743	-3.684	1.843
186	-0.092	-0.750	0.712	1.617
187	2.409	0.983	-1.136	0.131
192	2.192	-2.713	0.490	-0.263
194	-0.865	2.255	-0.974	2.074
195	-0.451	4.056	0.148	0.899
196	-4.101	2.243	-0.207	-0.566
197	0.318	0.097	1.359	-1.035
199	-0.121	-0.889	-1.331	1.020
200	0.748	-0.396	0.630	0.969
Impact	1.115	1.859	0.336	-0.723
Samson	0.252	-0.584	-0.059	0.049

Appendix 6.1: ANOVA of the herbage yield traits and Rubisco turnover parameters measured in the four INRA perennial ryegrass mapping populations. Alf: leaf appearance interval (days/leaf), Alg: ligule appearance interval (days/ligule), LED: leaf elongation duration (days), TN: tiller number, DW: herbage dry weight (g), TW: Tiller weight (g), LL: Leaf lamina length (cm), LER: leaf elongation rate (cm/day), *d*: maximum Rubisco content (mg/cm^2), *f*: a measure of curve width, *g*: time when *d* occurs (days).

Variable	Source	DF	SS	MS	F	*P*
Alf	Lamina length	1	2.381	2.381	3.13	0.0957
	Cycle	1	0.025	0.025	0.03	0.8597
	Lamina length*cycle	1	2.813	2.813	3.70	0.0723
	Error	16	12.152	0.760		
	Total	19	17.370			
Alg	Lamina length	1	0.613	0.613	0.42	0.5250
	Cycle	1	0.613	0.613	0.42	0.5250
	Lamina length*cycle	1	1.513	1.513	1.04	0.3223
	Error	16	23.200	1.450		
	Total	19	25.938			
LED	Lamina length	1	7.813	7.813	10.68	0.0048
	Cycle	1	2.113	2.113	2.89	0.1085
	Lamina length*cycle	1	0.313	0.313	0.43	0.5226
	Error	16	11.700	0.731		
	Total	19	21.938			
TN	Lamina length	1	39.200	39.200	0.02	0.8901
	Cycle	1	36.450	36.450	0.02	0.8940
	Lamina length*cycle	1	2376.200	2376.200	1.19	0.2907
	Error	16	31840.100	1990.010		
	Total	19	34291.950			
DW	Lamina length	1	109.295	109.295	6.08	0.0254
	Cycle	1	4.852	4.852	0.27	0.6105
	Lamina length*cycle	1	42.631	42.631	2.37	0.1431
	Error	16	287.596	17.975		
	Total	19	444.373			
TW	Lamina length	1	0.033	0.033	35.63	<0.001
	Cycle	1	0.000	0.000	0.00	0.9944
	Lamina length*cycle	1	0.001	0.001	0.90	0.3558
	Error	16	0.015	0.001		
	Total	19	0.049			

Appendix 6.1 (Continued)

Variable	Source	DF	SS	MS	F	*P*
LL	Lamina length	1	1530.375	1530.375	54.83	<.0001
	Cycle	1	0.820	0.820	0.03	0.8661
	Lamina length*cycle	1	0.703	0.703	0.03	0.8759
	Error	16	446.614	27.913		
	Total	19	1978.512			
LER	Lamina length	1	9.079	9.079	28.48	<0.001
	Cycle	1	0.383	0.383	1.20	0.2896
	Lamina length*cycle	1	0.106	0.106	0.33	0.5725
	Error	16	5.101	0.319		
	Total	19	14.668			
d	Lamina length	1	0.001	0.001	2.89	0.1087
	Cycle	1	0.001	0.001	2.12	0.1650
	Lamina length*cycle	1	0.004	0.004	18.35	0.0006
	Error	16	0.004	0.000		
	Total	19	0.009			
f	Lamina length	1	0.005	0.005	0.45	0.5117
	Cycle	1	0.009	0.009	0.72	0.4095
	Lamina length*cycle	1	0.071	0.071	6.00	0.0262
	Error	16	0.191	0.012		
	Total	19	0.276			
g	Lamina length	1	2.369	2.369	1.25	0.2800
	Cycle	1	0.473	0.473	0.25	0.6241
	Lamina length*cycle	1	1.197	1.197	0.63	0.4384
	Error	16	30.315	1.895		
	Total	19	34.353			

Appendix 6.2: Correlation analysis of the parameters of four INRA perennial ryegrass breeding populations Alg: ligule appearance interval (days/ligule), LED: leaf elongation duration (days), TN: tiller number, DW: herbage dry weight (g), TW: Tiller weight (g), LL: Leaf lamina length (cm), LER: leaf elongation rate (cm/day), *d*: maximum leaf Rubisco concentration (mg N/g leaf fresh weight), *f*: a measure of curve width, *g*: time of *d* (days).

Trait	Alf	Alg	LED	TN	DW	TW	LL	LER	*d*	*f*
Alg	0.84**									
LED	0.07	0.23								
TN	-0.21	-0.16	-0.24							
DW	-0.42	-0.26	0.17	0.77**						
TW	-0.28	-0.12	0.66**	-0.24	0.31					
LL	-0.14	0.04	0.70*	-0.01	0.51*	0.76**				
LER	-0.21	-0.08	0.30	0.13	0.57**	0.59**	0.90**			
d	-0.20	-0.23	-0.17	-0.20	-0.10	0.04	0.07	0.20		
f	0.20	0.54*	0.30	-0.09	0.11	0.17	0.23	0.11	-0.09	
g	0.21	0.21	-0.08	-0.12	-0.41	-0.37	-0.36	-0.42	-0.30	-0.29

*, ** Significant at $P < 0.05$ and $P < 0.01$, respectively

Appendix 6.3: Correlation analysis of the parameters of four INRA perennial ryegrass breeding populations Alg: ligule appearance interval (days/ligule), LED: leaf elongation duration (days), TN: tiller number, DW: herbage dry weight (g), TW: Tiller weight (g), LL: Leaf lamina length (cm), LER: leaf elongation rate (cm/day), *d*: maximum leaf Rubisco concentration (mg N/g leaf), *f*: a measure of curve width, *g*: time of *d* (days).

Trait	Alf	Alg	LED	TN	DW	TW	LL	LER	*d*	*f*
Alg	0.84**									
LED	0.07	0.23								
TN	-0.21	-0.16	-0.24							
DW	-0.42	-0.26	0.17	0.77**						
TW	-0.28	-0.12	0.66**	-0.24	0.31					
LL	-0.14	0.04	0.70*	-0.01	0.51*	0.76**				
LER	-0.21	-0.08	0.30	0.13	0.57**	0.59**	0.90**			
d	-0.40	-0.20	0.60**	-0.12	0.43	0.83**	0.79**	0.66**		
f	0.29	0.50*	0.07	-0.20	-0.10	-0.06	0.05	0.03	-0.12	
g	0.23	0.37	0.09	-0.05	-0.17	-0.22	-0.15	-0.26	-0.31	-0.12

*, ** Significant at $P < 0.05$ and $P < 0.01$, respectively

Appendix 6.4: PC scores for the first four PCs for herbage yield and Rubisco turnover measurements taken on four INRA perennial ryegrass populations. Variation explained by each PC: PC1 (32.8 %), PC2 (21.8%), PC3 (17.1%), PC4 (10.5%).

Population	PC1	PC2	PC3	PC4
SLC2	-2.538	-1.307	-0.378	-1.858
SLC2	-0.199	0.277	1.103	-0.528
SLC2	-1.965	-0.939	0.798	-0.743
SLC2	-1.569	-3.177	2.249	0.539
SLC2	-0.122	0.676	0.750	0.341
LLC2	1.438	-1.000	-1.070	-0.453
LLC2	0.656	0.124	-1.293	1.495
LLC2	1.490	2.180	1.414	0.413
LLC2	-0.025	0.287	-2.464	0.354
LLC2	2.093	0.784	-1.043	-1.126
SLC4	-1.556	0.512	-1.026	-0.883
SLC4	-1.288	-1.697	-0.117	1.556
SLC4	-1.622	3.247	2.341	-1.210
SLC4	-2.291	1.621	0.785	2.482
SLC4	-3.139	-0.256	-2.450	-0.285
LLC4	1.765	1.365	-1.013	0.324
LLC4	2.244	-0.698	0.176	1.132
LLC4	1.102	1.295	-0.546	-0.466
LLC4	2.946	-1.400	1.095	-0.861
LLC4	2.581	-1.896	0.690	-0.222

Appendix 6.5: Mean values of light-saturated net photosynthesis (µmol CO_2 $m^{-2}s^{-1}$) of the third leaves of the four INRA perennial ryegrass breeding populations. SLC2: short-leaved 2nd cycle population, SLC4: short-leaved 4th cycle population, LLC2: long-leaved 2nd cycle population, LLC4: long-leaved 4th cycle population.

A. Population differences between cycles

	Leaf age (days)			
Population	11	18	22	30
SLC2	17.94	14.77	14.55	12.66
SLC4	20.78	19.51	15.79	15.57
SE	2.84	2.00	1.30	1.91
P value	***0.50***	***0.13***	***0.11***	***0.28***
LLC2	20.18	17.00	14.55	10.67
LLC4	18.78	17.07	15.79	15.52
SE	2.84	2.00	1.30	1.91
P value	***0.73***	***0.98***	***0.52***	***0.11***

B. Differences between short- and long-leaved populations

	Leaf age (days)			
Population	11	18	22	30
Short-leaved	19.36	17.14	16.38	14.20
Long-leaved	19.48	17.04	15.17	13.10
SE	1.87	1.47	1.00	1.51
P value	***0.96***	***0.96***	***0.41***	***0.62***