



**UNIVERSITY OF ADELAIDE, AUSTRALIA**

**CHARACTERISATION OF GLUTAMINE SYNTHETASE  
TO MAP NEW REGULATORY LOCI MODULATING  
NITROGEN USE EFFICIENCY IN HEXAPLOID WHEAT**

**Jonathan Castel Djietror**

Thesis submitted in fulfillment of the requirements for  
the degree of Doctorate of Philosophy in the Faculty of  
Sciences at the University of Adelaide, Australia

**The Australian Centre for Plant Functional  
Genomics (ACPFG), Adelaide**

September 2, 2016

*This thesis is dedicated to Catherine and the Kids,  
my mum Dora Akleh Djietror and to loving memory of  
Samuel Ayiku Djietror*

# **Characterisation of Glutamine synthetase to Map New Regulatory Loci Modulating Nitrogen Use Efficiency in Hexaploid Wheat**

**Jonathan Castel DJIETROR**

## **Supervisors:**

### **Associate Prof. Ken Chalmers**

Senior Research Fellow, Lecturer - Plant Breeding and Genetics  
University of Adelaide-Waite Campus  
PMB1, Glen Osmond, SA5064, Australia  
ken.chalmers@adelaide.edu.au

### **Prof. Diane Mather**

Professor - Plant Breeding and Genetics  
JAT Mortlock Chair - Crop Improvement and Deputy Head (Research)  
University of Adelaide-Waite Campus  
PMB1, Glen Osmond, SA5064, Australia  
diane.mather@adelaide.edu.au

### **Associate Prof. Sigrid Heuer**

Science Coordinator and Program Leader  
Australian Centre for Plant Functional Genomics  
University of Adelaide-Waite Campus  
PMB1, Glen Osmond, SA5064, Australia  
sigrid.heuer@acpfg.com.au

### **Dr. Darren Plett**

Research Fellow  
Australian Centre for Plant Functional Genomics  
University of Adelaide-Waite Campus  
PMB1, Glen Osmond, SA5064, Australia  
darren.plett@acpfg.com.au

**Dr. Adam Croxford**

Postdoctoral Researcher  
University of Adelaide-Waite Campus  
PMB1, Glen Osmond, SA5064, Australia  
adam.croxford@adelaide.edu.au

**Dr. Julian Taylor**

Research Scientist  
University of Adelaide-Waite Campus  
PMB1, Glen Osmond, SA5064, Australia  
julian.taylor@adelaide.edu.au

Thesis submitted in fulfilment of the requirements for the degree of **Doctor of Philosophy, Science**

School of Agriculture, Food and Wine

Faculty of Science

The University of Adelaide

Waite Research Institute, Glen Osmond, SA 5064

**Jonathan Castel DJIETROR**

Email:

[jonathan.djietror@acpfg.com.au](mailto:jonathan.djietror@acpfg.com.au)

[jonathan.djietror@adelaide.edu.au](mailto:jonathan.djietror@adelaide.edu.au)

## DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

.....

02/ 09 /2016

(Signed)

(Date)

**Jonathan Castel DJIETROR**

(1637082)

## **ACKNOWLEDGEMENTS**

The contribution of the following persons at the Australian Centre for Plant Functional Genomics and University of Adelaide, Waite Campus is duly acknowledged and deeply appreciated. Ute Baumann is acknowledged for providing technical support in identifying reference gene sequences from polyploid species. Timo Tiirika served as a consultant on bioinformatics and phylogenetic assessment of glutamine synthetase and ammonium transporter genes. Trevor Garnett provided materials for cultivating plants used in the hydroponic experiments to evaluate enzyme activity and expression analysis. Yuan Li provided technical and advisory support on optimising qPCR experiment. Radovslav Sucheski served as advisor and consultant for the technical aspects of the POTAGE programme. Adam Croxford provided technical and advisory support on the sequencing of GS genes and the optimisation of PCR experiments. Beata Sznajder and Julian Taylor gave valuable technical assistance on the statistical analysis of enzyme activity, gene expression and QTL analysis. Members of the Molecular Marker Laboratory provided technical and advisory support in various ways and their contribution is very much appreciated.

## **LIST OF KEY TERMS AND ABBREVIATIONS**

**AMT:** Ammonium transporter

**cDNA:** complimentary DNA

**DNA:** Deoxyribonucleic acid

**FL:** Flag leaf

**FL-1:** Fully extended leaf next the flag leaf

**GDH:** Glutamate dehydrogenase

**GOGAT:** Glutamate 2-oxoglutarate transaminase

**GS:** Glutamine synthetase

**HN:** High nitrogen treatment (5.0 mM  $\text{NO}_3^- + \text{NH}_4^+$ )

**KASP:** Kompetitive allele specific primer

**LN:** Low nitrogen treatment (0.5 mM  $\text{NO}_3^- + \text{NH}_4^+$ )

**N:** Nitrogen

**$\text{NH}_4^+$ :** Ammonium

**$\text{NO}_3^-$ :** Nitrate

**NUE:** Nitrogen use efficiency

**NRT:** Nitrate transporter

**OFB:** Older fully extended leaf

**PCA:** Principal component analysis

**PCR:** Polymerase chain reaction

**PTM:** Post translational modification

**POTAGE:** PopSeq ordered *Triticum aestivum* gene expression



**qPCR:** Quantitative polymerase chain reaction

**SNP:** Single nucleotide polymorphism

**YEB:** Young fully extended leaf

**Zadoks stages:** distinct phases of cereal growth and development

# TABLE OF CONTENTS

|   |           |
|---|-----------|
| DECLARATION.....  | vi        |
| ACKNOWLEDGEMENTS.....   | vii       |
| LIST OF KEY TERMS AND ABBREVIATIONS.....                                    | viii      |
| TABLE OF CONTENTS.....  | 1         |
| GENERAL INTRODUCTION.....   | 6         |
| <b>CHAPTER ONE: Introduction and Literature Review.....</b>                 | <b>10</b> |
| 1. Introduction.....  | 10        |
| 1.1 Importance of Nitrogen in Crop Production.....                          | 12        |
| 1.2 Glutamine synthases in N-linked and Metabolic pathways.....             | 16        |
| 1.3 Quantifying Nitrogen Use Efficiency (NUE).....                          | 20        |
| 1.4 Genetic Control of Nitrogen Use Efficiency.....                         | 21        |
| 1.4.1 Evidence for Heritable Genetic Variation for NUE Traits.....          | 21        |
| 1.4.2 Forward Genetic Studies Involving QTL Mapping.....                    | 22        |
| 1.4.3 Candidate Genes and Productivity QTL Collocation.....                 | 24        |
| 1.4.4 Gene Families in Nitrogen Assimilation and Metabolism .....           | 26        |
| 1.5 Glutamine synthase Functional Traits and Sub-cellular Localisation..... | 28        |
| 1.6 Quantitative Expression of <i>GS1</i> and <i>GS2</i> .....              | 34        |
| 1.7 Post-translational Modification of <i>GS1</i> and <i>GS2</i> .....      | 40        |
| 1.8 Characterisation of Enzyme Activity .....                               | 42        |

## **CHAPTER TWO: Gene Identification, SNP Genotyping and Locus**

### **Mapping of *TaGS1.1* and *TaGS2* Conserved Domains .....45**

2. Abstract .....45

2.1 Introduction.....46

2.2 Materials and methods .....50

2.2.1 Overview of Experimental Activity.....50

2.2.2 Germplasm Material.....51

2.2.3 In-silico Gene Sequence Identification.....53

2.2.4 Phylogenetic Analysis of *TaGS1* and *TaGS2* Homologues.....57

2.2.5 Primer Design and Gene-Genome Specificity Confirmation.....57

2.2.6 Polymerase Chain Reaction and Optimisation.....59

2.2.7 SNP Marker Assay and Fine Mapping of GS Homologues.....62

2.2.8 Marker-based Analysis of Genetic Structure.....64

2.3 Results.....67

2.4 Discussion.....85

2.5 Conclusion.....89

### **CHAPTER THREE: Full-length Gene Sequencing to Capture Phylogeny and Allelic Variation in Wheat Glutamine synthetases .....90**

3. Abstract.....90

3.1 Introduction... .....91

3.2 Materials and methods.....97

|   |            |
|---|------------|
| 3.2.1 Sequence Assembly and Identification of GS Genes.....                                   | 97         |
| 3.2.2 Primer Design and Gene Sequence Amplification.....                                      | 99         |
| 3.2.3 Next Generation Sequencing with MiSeq Sequencing Tools.....                             | 101        |
| 3.2.4 Polypeptide Function Detection Using Web-Based Tools.....                               | 103        |
| 3.2.5 Phylogenetic Analysis.....  | 104        |
| 3.3 Results and Discussion.....   | 105        |
| 3.3.1 Phylogeny of GS Sequences from Different Plant Species.....                             | 105        |
| 3.3.2 Polymorphism between Wheat GS Alleles.....  | 112        |
| 3.4 Conclusion.....   | 121        |
| <b>CHAPTER FOUR: Regulation of Glutamine synthetases in Wheat.....</b>                        | <b>122</b> |
| 4. Abstract .....   | 122        |
| 4.1 Introduction.....   | 123        |
| 4.2 Materials and methods .....   | 129        |
| 4.2.1 Experiment 1 - Gene Expression Analysis Using a Novel Tool.....                         | 129        |
| 4.2.2. Experiment 2 - Confirmation of the POTAGE Analysis of GS Transcript<br>Abundance ..... | 130        |
| 4.2.2.1 Primer Design, PCR Amplification and PCR Product Sequencing.....                      | 132        |
| 4.2.3 Experiment 3 - Hydroponic Assessment of GS Expression in Plants.....                    | 135        |
| 4.2.4 cDNA Synthesis from RNA Samples.....  | 138        |
| 4.2.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR) .....                           | 138        |
| 4.2.6 Gene Expression Analysis.....   | 139        |
| 4.3 Results.....  | 141        |

|  |            |
|--|------------|
| 4.4 Discussion.....  | 150        |
| 4.4.1 Transcript Abundance of <i>TaGS1.1</i> .....   | 150        |
| 4.4.2 Transcript Abundance of <i>TaGS2</i> .....   | 151        |
| 4.4.3 <i>GS1.1</i> and <i>GS2</i> Transcript Abundance in Wheat Tissues.....                         | 152        |
| 4.4.4 Nitrogen Effect on GS Expression.....  | 154        |
| 4.5 Conclusion.....  | 157        |
| <b>CHAPTER FIVE: Glutamine synthetase Activity in Wheat at High and Low Nitrogen Treatments.....</b> | <b>159</b> |
| 5. Abstract.....   | 159        |
| 5.1 Introduction.....  | 160        |
| 5.2 Materials and methods.....   | 166        |
| 5.2.1 Experiment 1: GS Activity at Four Growth Stages.....   | 166        |
| 5.2.2 Experiment 2: GS activity based on Zadoks Growth Stages.....                                   | 167        |
| 5.2.2.1 Germplasm Material for Experiment 2.....   | 167        |
| 5.2.2.2 Experiment 2 Activities.....   | 168        |
| 5.2.2.3 Hydroponic System Set-up and Growth Conditions.....  | 169        |
| 5.2.2.4 Buffer Preparation and Biochemical GS Assays.....  | 170        |
| 5.2.2.5 GS Activity Determination.....   | 172        |
| 5.2.2.6 Genetic Analysis of GS Activity.....   | 173        |
| 5.2.3 Experiment 3: GS Activity in Plants Grown in Soil.....   | 174        |
| 5.3. Results.....  | 175        |
| 5.4 Discussion.....  | 193        |

|   |            |
|---|------------|
| 5.5 Conclusion.....   | 200        |
| <b>CHAPTER SIX: General Overview and Future Perspectives for GS<br/>Characterisation.....</b> | <b>201</b> |
| 6. Introduction.....  | 201        |
| 6.1 Advances in Knowledge from the Present Study.....   | 203        |
| 6.2 Future Directions for GS Characterization in Wheat.....                                   | 210        |
| 6.3 Concluding Remarks.....   | 214        |
| <b>Appendix.....</b>  | <b>216</b> |
| <b>List of References.....</b>  | <b>238</b> |

## GENERAL INTRODUCTION

Each year, cereal crops such as wheat, rice, maize and barley are cultivated and harvested to serve as staple foods that contain calories, dietary fibre, vitamins and minerals in the diets of over 70% of the world population. Wheat is among the most cultivated cereal crops. The major wheat production areas include the Mediterranean production areas of the Middle East (Fertile Crescent Region), Europe, North America, Asia (India and China) and Australia (Fig. G.1). Wheat cultivation involves considerable application of nitrogenous fertilisers to the plants in order to maximise yield. Annually, the global nitrogen (N) fertiliser application in crop production estimates at 85 – 90 mMt; of which 53.3 mMt is applied to cereals. Nitrogen fertilizer is vital for crops as the plants utilise nitrogenous compounds ( $\text{N}_2\text{O}$ ,  $\text{NO}$ ,  $\text{N}_2$  and  $\text{NH}_3^+$ ) to synthesise amino acids essential for grain development.

Nitrogen is added to soils in the form of inorganic fertiliser and processes such as precipitation, atmospheric nitrogen fixation (lightning and thunderstorms), and the activity of soil micro-organisms in root nodules of leguminous plants. The movements of N out of agricultural soils is by gaseous losses (volatilisation) to atmosphere, leaching from topsoil and uptake in crop plants for growth and physiological development (conversion of N to biomass). Concerns over excess  $\text{NH}_3^+$  on the atmosphere and climate, environmental impact of excess nitrogenous fertilisers in croplands and the damaging effects in aquatic

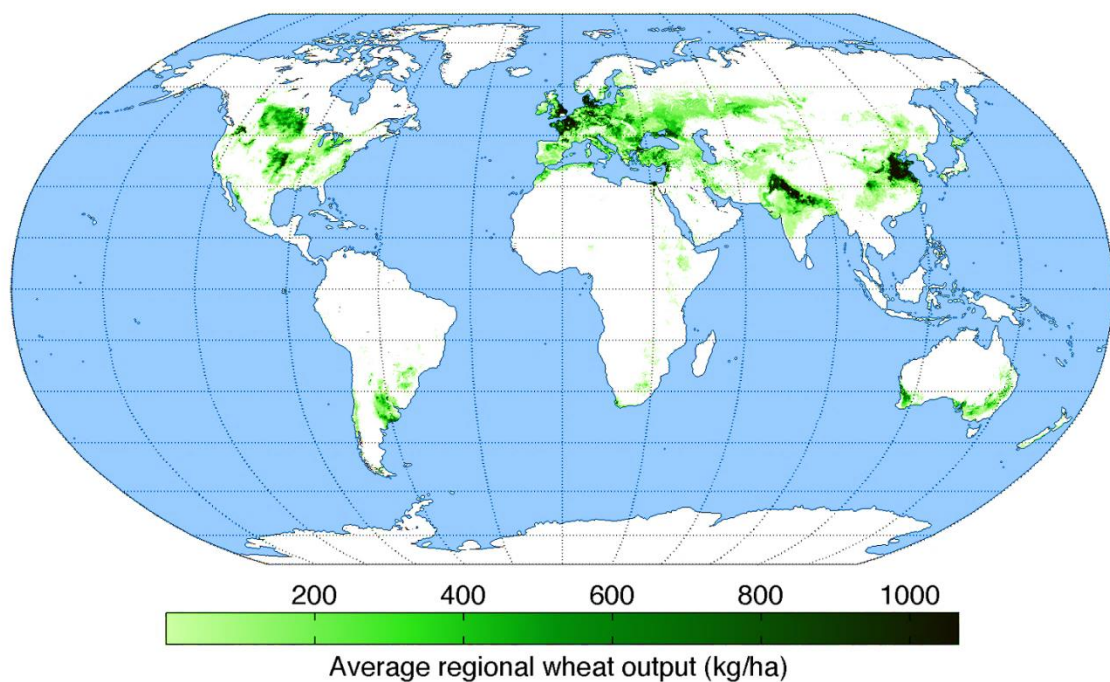
ecosystems have highlighted the need for the introduction of more N-efficient crop varieties into cropping systems.

Nitrogen response in plants can be assessed in terms of grain yield per N supplied per area and in terms of physiological variables including plant height, biomass, chlorophyll content, leaf area (Asplund et al. 2016, Barutçular et al. 2016; Elazab et al. 2016; Singh et al. 2016). These traits are controlled by genetic factors including specific enzymes such as Glutamine synthetase (GS) that affect N uptake in plants and may determine positive correlation between N-uptake genes and plant (grain and stem) N content (Habash et al. 2007). Various isoforms of GS have been shown to catalyse metabolic processes in N uptake and biosynthetic pathways within cereal crops including hexaploid wheat (Sukanya, et al. 1994; Singh & Ghosh, 2013; Urriola & Rathore, 2015; Basuchaudhuri, 2016).

Currently, there are few studies of GS in wheat. Glutamine synthetase studies in related cereal species have, revealed however, crucial links between the GS enzyme and N- related traits. For example, quantitative trait analysis has revealed genetic loci for GLN1 cytosolic GS isoform, whose activity relates to grain production in maize (Hirel et al, 2007; Galais and Hirel 2004) and rice where there is correlation between cytosolic GS protein content and grain number/size (Yamaya et al. 2002, Obara et al. 2004).



This current study attempts to decipher the genetic characteristic of GS in N metabolism principally in hexaploid wheat, but may be applicable to related cereal crop species and non-related species more generally. The objective of this study is to characterise the different isoforms of GS enzymes that are actively involved in nitrogen metabolism in hexaploid wheat.



**Fig. G.1** Global map of wheat production (mean percentage of cultivation land x mean yield in each grid cell) by the University of Minnesota, Institute of Environment. Source: [https://en.wikipedia.org/wiki/International\\_wheat\\_production\\_statistics#/media/File:WheatYield.png](https://en.wikipedia.org/wiki/International_wheat_production_statistics#/media/File:WheatYield.png).

## **Specific Aims and Objectives of the Present Study**

The main aim of this research project is to identify and characterise genetic variation within a diverse collection of wheat germplasm for key enzymes linked with N metabolism in wheat and related cereal species. Moreover, the study will attempt to address the following research objectives:

- Identity and confirm the genetic loci of GS homologues in hexaploid wheat genome.
- Characterise sequence diversity among different accessions of wheat through phylogenetic analysis.
- Develop molecular markers within the GS conserved domain sequences.
- Assess GS expression by quantifying the transcript abundance under high and low N treatment of wheat plants.
- Evaluate effects of genetic variation on GS activity under high and low N.

The germplasm for this project is sourced from a genetically diverse pool of wheat, adapted for the growth in the major cultivation zones around the world, and represent a unique resource for use in this study.

# CHAPTER ONE

## INTRODUCTION AND LITERATURE REVIEW

### 1. INTRODUCTION

The effective uptake of mineral nitrogen (N) by crop plants is an important agronomic target for increased crop yields and sustainable food production. Central to N nutrition research are several attempts (Masclaux-Daubresse et al. 2008; Castaings et al. 2011; Krapp et al. 2011; Hsu & Tsay, 2013; Avice & Etienne, 2014; Betti et al. 2014; Gelli et al. 2016) to characterise the N-linked characteristics of nuclear genes and the enzymes that these genes encode. These include glutamine synthetase (GS, EC 6.3.1.2), glutamine-2-oxoglutarate aminotransferase (GOGAT), nitrate transporter genes (NRTs) and ammonium transporters (AMTs). These enzymes act as important catalysts in metabolic reactions that contribute N to support the development of physiological traits in plants as they progress through the life cycle.

Researchers have observed a number of physiological trait changes in response to differential N supply in different accessions of crop plants. In attempts to quantify N uptake and to evaluate genetic and physiological differences among accessions, researchers conducted tests to evaluate the response of the different accessions to varied N levels under field and laboratory conditions (Neilson et al. 2015; Mahjourimajd et al. 2015; Melino et al. 2015,

Büchi et al. 2016; Mon et al. 2016). Plant responses to N treatment have been measured in terms of increased grain yield (Kaur et al. 2016; Salo et al. 2016; Zhen et al. 2016), grain protein content (Gelli et al. 2016; Rial-Lovera et al. 2016; Taulemesse et al. 2016; Li et al. 2016) and thousand grain weight (Valkama et al. 2013). Both grain protein and grain N content are very important for grain metabolic activity involving enzymes and storage in the endosperm is a vital source of N needed for rapid growth after the seed germinates. Grain proteins such as gliadins and glutenins are responsible for some desired rheological properties including dough extensibility and elasticity (Shewry & Tatham, 2000; Kuktaite et al. 2004) for bread making. Increases in thousand-grain weight in response to N application serve as indicators of the grain biomass and a measure of the potential flour yield. A major challenge for achieving higher grain protein content is that under most N regimes, varieties that produced higher grain yield simultaneously yield relatively low grain protein (Clarke et al. 1990; Peltonen-Sainio et al. 2012). Increasing N application can increase grain protein content (Kienzler et al. 2011; Kaur et al. 2016; Liu et al. 2016; Otie et al. 2016). However, the N increase may not positively affect the nitrogen use efficiency (NUE) in specific genotypes that lack the capacity to; absorb ammonium compounds and nitrates from the available N, store the N for biosynthesis and reduce the amount of alluvial N loss (Hirel et al. 2007).

The GS enzymes are the primary catalysts for ammonium assimilation in plants. Two GS enzymes (*GS1* and *GS2*) catalyse the synthesis of glutamate from

glutamine and ammonia. These enzymes are involved in primary ammonia assimilation (plastid GS) and ammonium remobilisation (cytosolic) and GS mediated N metabolism is complemented by the N-related biosynthetic activities of AMTs and NRTs (Xu et al. 2012; Luo et al. 2013). Many studies of GS genes have been used to explain N uptake in *Arabidopsis thaliana* (Li et al. 2016) and maize (Bailey & Leegood, 2016, Wei et al. 2016). Such studies have confirmed the sub-cellular localisation of these N-linked enzymes and provided useful information on how GS genes regulate N uptake in these species. In wheat, there has been relatively little investigation of GS enzymes and their effects on N response (Bernard et al. 2009; Wang et al. 2014). It is not clear whether differences among GS alleles affect gene expression or enzyme activity. It is not clear how different N regimes affect metabolic activity. Evaluation of the genetic basis for the effect of N treatment on N metabolism will advance current understanding of how N treatments might affect the regulation of the GS alleles in wheat and other cereal crops.

## **1.1 IMPORTANCE OF NITROGEN IN CROP PRODUCTION**

Nitrogen is the most important nutrient for crop plants, as it is a primary chemical constituent of proteins and nucleic acids, which are required for sustained growth. The amount of N uptake is crucial to the level of grain proteins in cereal crops (Valkama et al. 2013; Otie et al. 2016; Tsukaguchi et al. 2016). The main source of mineral N in agricultural soils is nitrate. Typically, plants absorb ammonium (the reduced form of nitrate) which is synthesised from nitrate

by the catalytic action of N-related enzymes (Xu et al. 2012; Thomsen et al. 2014). Balkos et al. (2010) reported that ammonium tolerant plant species, such as rice, express GS and have high GS activity.

Due to the important status of N in attaining high yields in crop production, modern agricultural systems have become increasingly reliant on the use of commercially produced inorganic N fertilisers to attain the desired high yields. The advent of the Green Revolution accelerated this process. During the Green Revolution, commercial scale level of cereal crop production improved from high inorganic fertiliser inputs. Increased application of inorganic N to rice and wheat crops was complemented by the breeding and cultivation of semi-dwarf varieties, which were resistant to root and stem lodging (an agronomic bottleneck that resulted in large quantities of grain loss). The increased demand for high crop yields has also contributed to research activities using genetic approaches targeted at breeding high yielding crops with improved NUE. For instance, genetic approaches aimed at improving NUE have been used to target yield improvement in wheat (An et al. 2006; Cormier et al. 2016; Kong et al. 2016). This also in rice (Shrawat et al. 2008; Araus et al. 2016), maize (Bertin & Gallais, 2000; Hirel et al. 2001; Otie et al. 2016), barley (Bingham et al. 2012; Kindu et al. 2014) and sorghum (Urriola & Rathore, 2015; Gelli et al. 2016). The genetic characterisation of crop NUE can contribute to the identification of genetic tools that are applicable to breeding high-NUE crop varieties and there are some

agronomic advantages for sustainable crop production (Hirel et al. 2007; Masclaux-Daubresse et al. 2010).

Generally, cropping systems around the world depend on the use of large quantities (85 - 90 million tonnes) of inorganic N fertilisers annually (Good et al. 2004; FAO, 2011; Nazir et al. 2016). This has highly significant economic implications for farmers of field crops such as wheat. In recent years, high crop yields resulted from increased N fertiliser application in cropping systems (Bouwman & Goldewijk, 2013; Chen et al. 2014; Grant et al. 2016) and current estimates predict 100 - 110% increment in global crop production. This will require a 60% increase (ca. 225 Mt per annum) in global N inputs by the mid-21<sup>st</sup> century (Tilman et al. 2011; Fowler et al. 2013; Ladha et al. 2016). However, the application of large quantities of inorganic N fertilisers have also been characterised by logistic, energy, economic and environmental challenges, access and cost issues.

Increased N application has not always yielded the desired objectives due to unintended side effects. Residual N in soils and water run-off can create acute ecological risks to soil, air and water systems through pollution (Kondo et al. 2003; Gu et al. 2015). Accumulation and leaching of N in aquatic systems may cause eutrophication. N accumulation also promotes growth of microbial populations and disrupt delicate biochemical processes of flora-fauna interactions in natural systems. Beside the crucial environmental issues, there are sensitive economic issues with N fertiliser use that have wider implications for food

production and sustainability of farming systems in the developing world. There is a widening gap between developed and developing countries with respect to the amount of N fertiliser available for crop production (Mueller et al. 2014). The production of N efficient varieties (crop varieties with the capacity and potential for increased yields consistent with the level of N applied) may help to address the economic imbalance in global N distribution and fertiliser application in farming systems. This will also ease the pressure on natural systems and cycles impacted by the ground water contamination from excess applied N (Sheldrick, 1987; Vitousek et al. 1997; Novotny et al. 2007).

Selection and breeding programmes have traditionally aided the development of high yield varieties, but cereal crop breeding is trending towards the development of varieties with improved NUE, requiring reduced N fertiliser input (Hirel et al. 2007; Sylvester-Bradley & Kindred, 2009; Butterly et al. 2015; Gioia et al. 2015; Melino et al. 2015). In addition, the use of new high-throughput genomic approaches has provided an opportunity to characterise genetic differences between many of the candidate genes believed to be involved in NUE (Le Gouis et al. 2000; Presterl et al. 2003; Anbessa et al. 2009; Namai et al. 2009).

Phenotyping or genotyping methods (Dawson et al. 2008; Winfield et al. 2015) can serve as selection tools and phenotypic traits can be selection criteria (Kaur et al. 2016) which can be effectively utilised to characterise desired accessions due to the differential responses to different stress inducements. N research has produced findings that seem to indicate genetic variation in the



response to varying levels of N treatment (Tian et al 2015; Xu et al. 2016). Therefore, the future of sustainable N use in cereal crop production may partly depend on successful breeding programmes. Modern genomic approaches and molecular breeding strategies can be utilised to take advantage of the existing genetic variability between accessions towards the main goal of developing highly N efficient plant cultivars. This will have broad positive implications for sustainable food production. This review includes some of the latest trends in NUE research, and follows the N metabolic pathway in wheat and related cereal crop species. It also examines role of gene expression in relation to N metabolism and the functional role of N metabolic genes in plant growth and development.

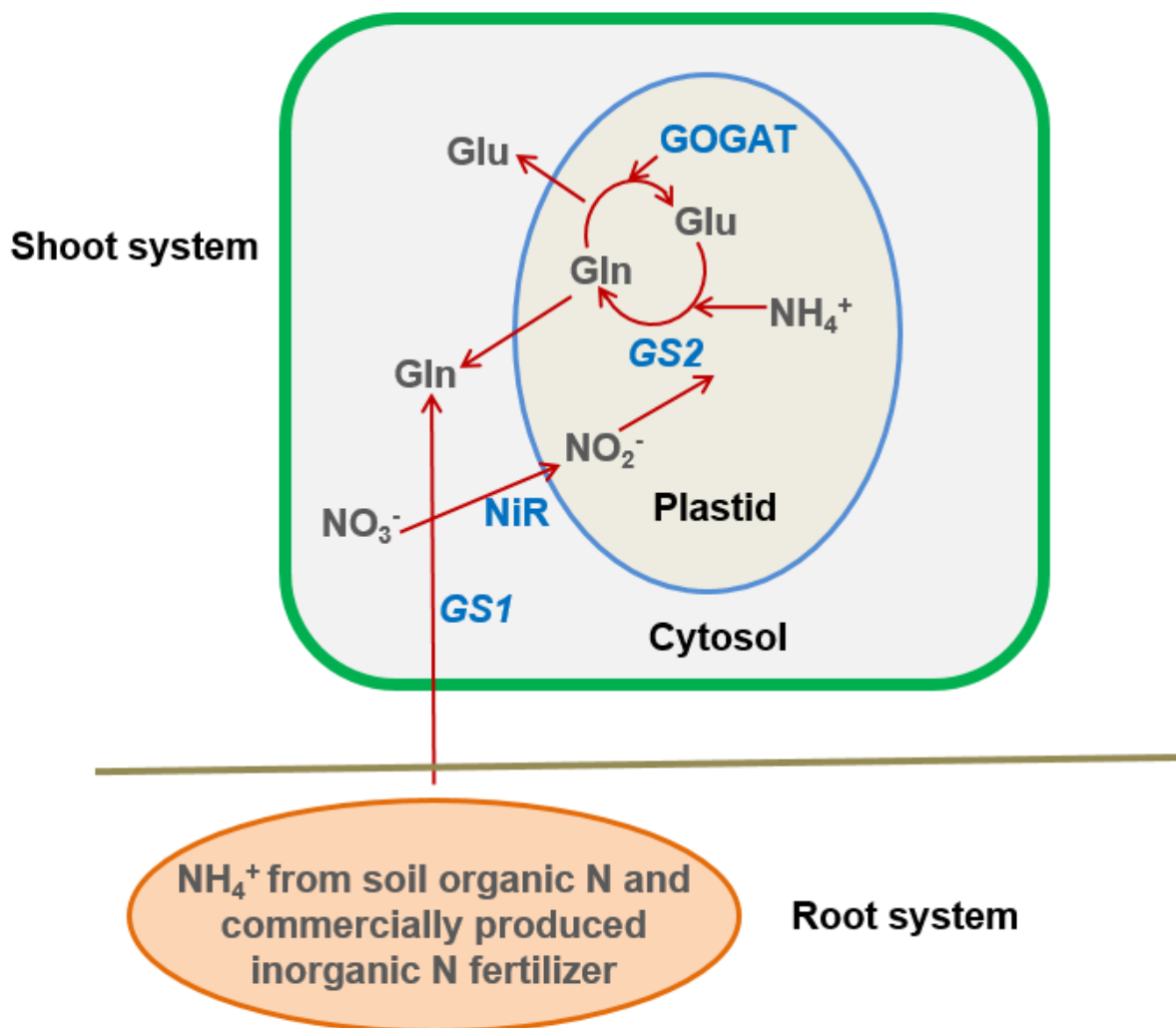
## **1.2 GLUTAMINE SYNTHASES IN NITROGEN-LINKED BIOSYNTHESIS AND METABOLIC PATHWAYS**

Plants assimilate N via four major transport mechanisms involving ammonium, nitrate, amino acids and sugars (Xu et al. 2012). In the ammonium pathway, soil organic N in the form of ammonia or ammonium is absorbed into primary and lateral roots by ammonium transporters. The ammonium is catalysed in the GS/GOGAT cycle and transferred into the stem and young leaves for vegetative growth. In the nitrate transport system, nitrate produced from the nitrification of ammonium from urea is converted to nitrite by the catalytic action of nitrate transporters in the roots. The nitrate is transported to stems, young leaves and developing grains. Nitrate can also be recycled from senescing leaves (Kong et al. 2016) by the coordinated action of *GS1* enzymes. The third transport

system involves amino acids (aspartic acid, asparagine, glutamic acid and glutamine) transport from primary and lateral roots to grains principally and to other developing tissues in the shoot system. Finally, the sugar transport system carries the complex carbohydrates produced from the photo-respiratory activity of plastid GS genes in leaves to roots and grains. Sugars from senescing leaves are also mobilised and transferred into the stem, roots stem and developing grains.

Generally, plants exhibit a complex series of biochemical reactions involving N-linked enzymes including GS (*GS1*, *GS2*) and GOGAT (*Fd-GOGAT*, *NADH-GOGAT*). GS enzymes play important biosynthetic roles in the GS/GOGAT cycle in which ammonium catalysed by *GS1* in the cytosol is utilised in converting glutamate to glutamine. The decomposition of organic material releases N in the form of nitrate and ammonium in agricultural soils. Nitrate is reduced to form ammonium. Both ammonium and nitrate assimilation pathways constitute two important biochemical processes through which N is transferred from roots to shoots. The ammonium taken up by the roots is largely assimilated in the cytoplasm using the GS-GOGAT metabolic cycle (Miflin & Habash, 2002; Lea & Miflin, 2003). In this pathway, *GS1* catalyses the assimilation of ammonium in the cytosol and *GS2* catalyses the assimilation of photo-respiratory ammonium in the plastids. Other enzymes involved in N assimilation include nitrate reductase (NR) that catalyses the reduction of nitrate to nitrite that is further absorbed into the chloroplasts in leaves. In the stems, nitrate generally undergoes a two-phase conversion reaction in which nitrate

reductase converts nitrate to nitrite in the cytoplasm and nitrite reductase (NiR) catalyses the conversion of the nitrite to ammonium within the plastid. This catabolic reaction produces ammonium. The ammonium ion is then absorbed into the GS-GOGAT cycle to produce glutamine and glutamate. Besides ammonium directly assimilated by AMTs, ammonium generated from the two-step nitrate catalysis, is utilised in the GS/GOGAT cycle to form amino acids including asparagine, asparagine synthetase, aspartate, glutamine and glutamate (Prins et al. 2016; Zhou et al. 2016). The GS/GOGAT cycle (Fig. 1) is an integral component of the biosynthetic processes utilised to incorporate inorganic N into plant tissues (Xu et al. 2012; Shat et al. 2016). The AMTs play key roles in making ammonia available from soil for onward transfer to the amino acid pathway leading to the shoot system via the GS/GOGAT cycle. In the nitrate transport system, nitrate may be directly absorbed into primary and lateral roots or transported to the stem, leaves and grains (Xu et al. 2012; Basuchaudhuri, 2016). When compared to the shoot organs (leaves, stem grains, inflorescence), roots assimilate relatively high quantity of N in the form of nitrate (Carvalho et al. 2000). Plants assimilate N via four major transport mechanisms involving ammonium, nitrate, amino acids and sugars (Xu et al. 2012).



**Fig. 1.1** Schematic representation of the GS/GOGAT cycle shows the catalytic reactions of *GS1* and *GS2* enzymes in the cytoplasm and the plastid. Abbreviations: Gln - glutamine; Glu - glutamate; GOGAT - glutamine-2-oxoglutarate aminotransferase; GS - glutamine synthetase and NiR - nitrite reductase.

### **1.3 QUANTIFYING NITROGEN USE EFFICIENCY (NUE)**

Nitrogen use efficiency is usually considered a ratio of nitrogen output to input. The output has been calculated as the total plant N, grain N, biomass or grain yield and the input has been measured as the total N, soil N or N supplied from inorganic sources (Masclaux-Daubresse et al. 2010). No single definition of NUE has been generally accepted. For example, NUE has been explained as total grain yield produced per unit of applied fertiliser N (Dhugga & Waines, 1989; Giambalvo et al. 2010; Chen et al. 2013; Kaur et al. 2015; Mahjourimajd et al. 2015). Alternatively, NUE has been defined as biomass produced per unit of applied fertiliser N (Xu et al. 2012) and in terms of dry matter output versus the total plant N (Good et al. 2004; McAllister et al. 2012). NUE has also been categorised into two distinct biological processes as the product of (1). N uptake efficiency that describes the efficiency of N assimilation from N sources such as soil or nutrient solution and (2), the N utilisation efficiency that is the effectiveness of N remobilisation (Kaur et al. 2015). Generally, the central dogma implicit to NUE definitions currently remain the biological and chemical processes involved in N uptake efficiency. These biological activities include N absorption and translocation, N utilisation (assimilation) efficiency, agronomy efficiency of N inorganic fertiliser usage, physiological use, transport, remobilisation and remobilisation rate by means of recovery and recycling (Guohua et al. 2012). For the current study, NUE is defined in a conceptual framework as the metabolic efficiency (capacity for uptake, assimilation and

remobilisation) and physiological performance of plants in extracting and converting N from organic and inorganic sources and retaining for use in source (root) and sink organs (leaves, stem, ear, inflorescence and grains).

## **1.4 GENETIC CONTROL OF NITROGEN USE EFFICIENCY**

### **1.4.1 Evidence for Heritable Genetic Variation for NUE Traits**

There have been studies using measurements of physiological variables to quantify NUE. Studies of Arabidopsis (Kant et al. 2008), barley (Kjaer & Jensen, 1995), wheat (Laperch et al. 2006) and maize (Agrama et al. 1999; Bertin & Gallais 2001; Coque et al. 2006) have yielded vital information on the signaling aspects of glutamine mechanisms in plants. Among wheat lines however, there are limitations on investigations to detect genetic variation between different accessions due to the complexity of the wheat genome (Tanksey & McCouch, 1997) and currently, most studies focus on few accessions (Nigro et al. 2016). Consequently, it has become necessary to identify and characterise allelic variations that control or mediate N related metabolic traits. Hirel et al. (2007) recognised the necessity to design populations derived from exotic strains adaptable to low N growing conditions. Some studies have investigated the efficiency of metabolic processes in the N assimilation pathway by determining flag leaf and grain biomass change in response to high and low N treatments in a diverse set of wheat accessions (Gaju et al. 2014; Kaur et al. 2015). In this analysis, the grain yields and shoot biomass of the N-efficient variety was higher

than that of the N-inefficient variety at maturity stages in the low N treatment and in the high N treatment. However, genetic mapping of N regulating genes has largely concentrated on maize and rice (Hirel et al. 2001; Gallais & Hirel, 2004; Lv et al. 2016).

The genetic mapping of these genes in wheat has been limited (Nowak, 2014; Molnar et al. 2015) and fewer number of wheat accessions have been studied (Bernard et al. 2008; Kichey et al. 2005) to characterise the metabolically important glutamine synthetase gene family. Another limitation is that the wheat accessions covered in N focused genetic studies are not representative of the wide diversity observed in wheat accessions cultivated in markedly different geographical regions. This aspect of NUE research is important as the prospect of high yield wheat production partly depends on N-efficient high yielding cultivars that are adaptable to several cultivation ecologies and with adaptation to local environment and stress factors such as N limitation.

#### **1.4.2 Forward Genetic Studies Involving QTL Mapping**

Many studies of GS genes have aimed to clone, characterise and utilise the genes that control the expression of traits related to NUE among a number of cereal crop species. Some GS loci have been extensively investigated for NUE in maize, rice, barley and to lesser extent in wheat. According to Bernard et al. (2008), the genotypic differences in GS abundance and activity can be exploited by using quantitative trait loci (QTL) analysis to decipher the role of GS genes in improving NUE and yield output.

A QTL is a region of the genome that is associated with variation in a quantitative trait expressed in the phenotype of the plant. QTL analysis can be used to confirm that the variation in NUE parameters (grain yield, grain protein content) can be associated with genetic differences within the DNA sequences.

In order to characterise yield related phenotypes in maize, Hirel et al. (2001) searched for and identified some QTL on the genetic map. There were coincidences of QTL for grain yield parameters with the genes encoding cytosolic GS and associated with GS activity. The study of higher wheat grain N content was confirmed through QTL mapping to identify correlations between grain protein content and GS activity (Habash et al. 2007). Quantifying tissue-specific enzyme activity is an aspect of NUE research in cereal crops that have utilised the advantages of forward genetics through QTL mapping. The flag leaf has been of particular interest due to its importance in N assimilation and translocation to grains during both early and late reproductive development. Undertaking QTL analysis for flag leaf GS activity in wheat, Habash et al. (2007) associated enzyme activity with the cytosolic *GSI* gene and confirmed that it co-localises with a QTL for grain N content.



### 1.4.3 Candidate Genes and Productivity QTL Collocation

Researchers have studied the GS activity variation and consequently profiled the expression patterns of *GS1* and *GS2* genes in a number of plant species. QTL for GS activity, at the cytosolic GS locus (*GLN1*) have been associated with maize grain yield (Hirel et al. 2001, 2007; Galais & Hirel, 2004). Similarly, GS activity (*GS2* and *GS1*) was positively correlated with wheat grain yield and stem N concentration (Habash et al. 2007). The investigation of a number of maize orthologues has shown that there is close homology between four GS genes (*GSI-GRMSM2G050514*, *GSII-GRMSM2G115646*, *GOGAT-GRMSM2G077054*, *GOG-GRMSM5G813007*) as active catalysts within the ammonium assimilation, glutamine synthesis and other key N metabolic pathways in maize (Brancourt-Hulmel et al. 2005; Hirel et al. 2005).

The cytosolic *GS1* is highly expressed in senescing leaves of wheat (Bernard et al. 2008). Kichey et al. (2005) reported that, during leaf senescence, *GS1* protein accumulation shifts from phloem companion cells to mesophyll cells. This change in protein level may be responsible for N remobilisation to apical meristem and growing tissues. The flag leaf has been of particular interest due to its importance in N assimilation and translocation to grains during both early and late reproductive development. Gadaleta et al. (2011) linked plastid GS with grain protein content on polymorphic regions of the *GS2* genes on Chromosome 2A and 2B of two wheat varieties.

Some studies demonstrated correlation between GS activity and grain yield for the *GS1* gene in maize (Hirel et al. 2001, 2007; Galais & Hirel, 2004). These studies confirmed co-localisation of the *GS1* protein with QTL linked to some grain characteristics. For example, some QTL of grain yield and thousand grain weight were found to co-localise (Galais & Hirel, 2004) with the *GS1* allele *ZmGln4* (*SmGln1.3*). In wheat, metabolic activity involving the cytosolic *GS1* family and the plastid *GS2* isoenzyme was proved to positively correlate with grain and shoot N content (Habash et al. 2007). In rice, there is a significant correlation between grain number/size and a grain protein content modulating locus for cytosolic *GS1* protein (Yamaya et al. 2002; Obara et al. 2004). Tabuchi et al. (2005) suggested that due to the strong effect of *GS1* in generating glutamine during N remobilisation the *GS1* protein could be responsible for severe decrease in grain filling in knockout mutant variants of rice. In a study of rice *GS1* gene was found to co-localise with a QTL for panicle weight and spikelet number (Obara et al. 2001). In wheat *GS1* *TaGSe* (*GS1.3*) have been linked to QTL for increased grain protein content (Gadaleta et al. 2014). Moreover, Habash et al. (2007) characterised a QTL for leaf enzyme activity that was mapped to *TaGSr*. It was demonstrated that the *TaGSr* locus co-localized with another locus associated with grain N content.

#### 1.4.4 Gene Families in Nitrogen Assimilation and Metabolism

Generally, the complex regulatory mechanisms that underlie NUE in plants are mediated and controlled by specific genes that encode enzymes including, nitrate transporters (NRTs) nitrate reductases (NRs), nitrite reductases (NiRs) and ammonium transporters (AMTs) in particular tissues (Le Gouis et al. 2000; Sonoda et al. 2003). A number of other genes are active in the N metabolic pathway include nicotinamide adenine dinucleotide hydrate (NADH) dependent glutamine synthase, glutamine dehydrogenase and transaminases of aspartate aminotransferases which are the reactants of glutamate synthesis (Lutts et al. 1999).

The glutamine synthetases (GS) are a gene family that play key roles in N metabolism. Two distinct gene groups within the GS family (Goodall et al. 2013; Yamaya & Kusano, 2014; Wang et al. 2015) relate to enzymes that control normal growth and development - *GS1* and *GS2*. Researchers have identified between 3-5 GS genes in different species and characterised these as members of the cytosolic group (*GS1*). Carvalho et al. (2000) identified two functional *GS1* types in *Medicago truncatula* as *MtGSa* and *MtGSb* and labelled the plastid GS as *GSr* in an expression analysis using *M. truncatula*, *Glycine max* and *Zea mays* (Torreira et al. 2014; Masalkar & Roberts, 2015) as the focus species. In addition, studies of GS genes have explained gene expression, enzyme activity, protein level differences and ammonia assimilation in cereal crop species, principally maize. In rice, Tabuchi et al. (2005) identified three isoforms of *GS1* (*OsGS1.1*,

*OsGS1.2*, and *OsGS1.3*) and there are a number of genes in the GS subfamily that encode *NADH-GOGAT*. In addition, Tamura et al. (2011) classified the rice NADH-glutamate synthases such as *OsNADH-GOGAT1* and *OsNADH-GOGAT2*. The knockout mutants of these genes have been considerably analysed for characterisation of GS function in rice (Yamaya & Kusano, 2014).

The nature of tissue expression suggests that only one main GS enzyme occurs in the plastid (*GS2*). The abundance of the *GS2* enzyme is highest in leaf mesophyll cells and catalyses ammonia assimilation using the ammonia released from photorespiration (Yamaya, 2001). For example, studies of barley mutants have shown that plastid *GS2* controls photo-respiratory ammonia assimilation (Wallsgrave et al. 1987; Reddy et al. 2015).

In wheat, Wang et al. (2015) demonstrated that three subunits of cytosolic GS isoforms (*GS1*, *GSr1*, and *GSr2*) were detectable during leaf emergence and maintained active metabolic activity as the plant matured. Moreover, the plastid GS was abundant in photosynthetic tissues and was associated with increased GS activity. The characterisation of GS functions in wheat (Bernard et al. 2008) showed that the *GS* enzyme catalyses the production of glutamine and *GOGAT* functions to produce glutamate towards grain filling. This study showed that the cytosolic *GS1.1* was upregulated during leaf senescence suggesting that the *GS1.1* plays an active role in N remobilisation during this stage of plant development. The GS isoforms identified in other species have not been characterised in wheat targeted GS analysis is very limited.

## 1.5 GLUTAMINE SYNTHETASE FUNCTIONAL TRAITS AND SUB-CELLULAR LOCALISATION

The GS enzyme has been one of the key enzymes of plant nutrition research as it regulates essential biochemical processes in the assimilation of inorganic N into plant tissues. GS has been identified as a principal enzyme in the biochemical pathways of N assimilation and remobilisation (Lea & Ford, 1994; Susuki & Knaff, 2005). The biochemical processes involving two classes of GS enzymes are essential to the fixation of inorganic N from ammonium sources into glutamine, amino acids and proteins transferred to tissues (Plett et al. 2016) and organs (Fig. 1.1). GS2 is the principal isoform associated with  $\text{NH}_4^+$  assimilation for vegetative growth of foliar mesophyll cells. The plastid GS enzyme is known to be actively involved assimilating ammonium produced in photorespiration (Pessaraki, 2005; Delgado, 2015; Döring, 2016). The photo-respiratory flux of ammonium is combined with inorganic N assimilation from sink organs such as roots and leaves (Novitskaya et al. 2002). These may be key catalysts to the release of amino acid compounds for protein synthesis and N metabolism (Malek et al. 1984; Gallais et al. 2006).

In *Arabidopsis*, three genes encode the cytosolic GS isoenzymes and five encode *GS1* isoforms including *GLN1.2* involved in N remobilisation (Berhard & Matile, 1994; Lothier et al. 2011). In this species, different isoforms of the GS complex (*GS1* and *GS2*) have been identified (Rochat & Boutin, 1991; Lam et al. 2003). A variable number of *GS1* genes in different plant species (Swarbreck et

al. 2011) encodes the cytosolic isoform. In addition, the *GS1* enzyme is encoded by a unit of five different genes differentially localised and differentially expressed in separate in the tissues (Guo et al. 2004; Ishiyama et al. 2004; Li et al. 2006; Dragicevic et al. 2014). *GLNI.2* is mostly expressed in leaf parenchyma and is over-expressed in roots where it is used for N assimilation in optimum nitrate supply conditions. However, the *GS2* proteins are highly expressed in young leaves of Arabidopsis. A comparative analysis of nucleotide and protein unit sequences shows that wheat GS sequences can be placed into four GS groupings (Habash et al. 2007) designated as *GS2* (a, b and c), *GS1* (a, b and c), *GSr* (1 and 2) and *GSe* (1 and 2). The *GS2*, *GS1*, *GSr*, and *GSe* group have been mapped to four different chromosomal loci (Habash et al. 2007). Phylogenetic analysis of wheat DNA and proteins sequences of different GS enzymes depict convergent clustering with GS sequences from maize, rice and barley, however, these sequences did not cluster with GS sequences from non-cereal species such as Arabidopsis (Thomsen et al. 2014).

In rice, three GS genes encode cytosolic *GS1* isoforms (*GS1.1*, *GS1.2* and *GS1.3*) and one gene encodes chloroplastic *GS2* in rice. *GS1* proteins are encoded by a small but complex gene family. This includes three rice genes namely *GS1.1*, *GS1.2* and *GS1.3* (Tabuchi et al. 2005). *OsGS1.2* plays a principal role in ammonium assimilation in roots and towards ammonium build-up for stem elongation (Funayama et al. 2013). *In-vivo* analysis (Hirel & Gadai, 1980; Grabowska et al. 2012; Wang et al. 2016) and the use of abundance prediction

tools have shown that *GS1* genes are largely expressed in roots and *GS2* in leaves (Wallsgrave et al. 1987). *GS2* predominantly assimilates ammonium in the leaf tissues during the vegetative growth phase, especially from transient photorespiratory mechanisms (Wallsgrave et al. 1987; Tobin & Yamaya, 2001).

In hexaploid wheat, multigene families may encode a variety of isoforms (Xing-Peng-et al. 2011). A family of genetically diverse glutamine synthetase isoenzymes, are localised in the cytosol (*GS1*) and the chloroplast (*GS2*) and there is evidence suggesting that GS generally exists as multiple isoforms. Similar to the multiple GS enzymes in *Arabidopsis* and rice, a set of seven GS enzymes were identified in wheat (Bernard et al. 2008). In maize, there are five cytosolic GS enzymes which are integral to N remobilisation (Martin et al. 2006). The *GS2* enzyme has been located in the chloroplast or plastid in a number of other species including *Glycine max* and *Medicago truncatula* (Seabra et al. 2010) and plays a crucial role in photorespiration. Therefore, within plant cells, sites of N-metabolic activity are the cytosol (cytosolic *GS1*), and chloroplast. Martin et al. (2006) identified cytosolic GS as occurring in the vascular bundles of  $C_3$  and  $C_4$  species. Expression of these isoforms has been found to vary between organs. In wheat ( $C_3$ ), cytosolic GS has a reduced occurrence in the leaves compared to roots. Further GS transcript analysis will highlight the expression patterns within different tissues and may clarify if sub-cellular localization of the GS enzymes could be linked to specific metabolic functions of the GS enzymes in a diversity of species.

Experimental analysis of GS genes and N metabolism has shown that GS enzymes play crucial roles in growth, organ development and yield in cereal crop species. For example, GS has been demonstrated to regulate grain development and grain filling in rice, maize and wheat (Tabuchi et al. 2005; Martin et al. 2006; Bernard et al. 2008). GS genes are also involved in vegetative development in barley and rice (Goodall et al. 2013). In addition to N assimilation, GS is required for reassimilation of ammonium produced via protein synthesis, protein formation and photorespiration (Guan & Schjoerring, 2014; Li et al. 2014). The chloroplast is believed to directly reassimilate ammonium by the action of chloroplastic *GS2* that is a product of photorespiration. For example, studies involving barley (*Hordeum vulgare*) mutants lacking plastid *GS2* show the loss of photo-respiratory ammonium (Oliveira et al. 2002). This confirms that reactions of plastid *GS2* are major enzymatic requirements for leaf localised ammonium reassimilation.

GS isoforms catalyse amino acid reactions for protein synthesis and N metabolism (Gallais et al. 2006; Cánovas et al. 2007). Prior to wheat anthesis the plants tend to transfer amino acids from leaves towards the developing grains for the synthesis of grain proteins. Matsumoto et al. (2000) and Xu et al. (2004) reported that other enzymes contribute to this process. For example, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase play key roles in increasing the protein concentration of the endosperm of developing grains.



The leaf to grain N translocation and remobilisation from the senescing leaves to the developing grain facilitates the increase in the grain protein concentration. It has been suggested that the accumulation of proteins (Martre et al. 2003; Yang et al. 2016; Moore et al. 2016) in the developing grain is regulated by glutamine based enzymatic activities. In addition, during periods of N starvation, glutamine and other amino acids are translocated from source organs such as senesced leaves (Masclaux-Daubresse et al. 2008). Senescence rate and N remobilisation in the leaf are driven by the N balance between the sources (root) and sink (leaf) organs (Masclaux et al. 2000). In a study of maize, *GDH1* and *GS1.1* gene transcripts were downregulated under high N treatment. Moreover, studies of maize have revealed crucial metabolic functions for cytosolic *GS1.1* during N depleted phases of the life cycle. For example, this enzyme plays a key role during leaf senescence and periods of significant N deficit by balancing N levels through remobilisation and amino acid recycling in leaves (Masclaux-Daubresse et al. 2010; Lothier et al. 2011). This suggests that *GDH* and *GS1.1* may contribute to reduce N remobilisation and that sufficient N supply might delay N remobilisation by reducing *GDH* and *GS1* gene expression and their corresponding enzyme activities.

In a study of gene function, Unno et al. (2006) characterised the protein structure of maize cytosolic GS to explain the functional roles of *GS2* polypeptides. Cytosolic GS plays an active role in N assimilation and recycling and is vital for grain development and biomass accumulation (Bernard & Habash,

2009). *GLN* and *ASN* (amido group of glutamine and aspartate - asparagine) groups are identified with nitrite to ammonium conversion and the accumulation of nitrate in the vacuole. Other associated functional traits include the metabolism of ammonium leading to the production of glutamine and proteins required for normal growth and reproductive development (Ishiyamma et al. 2003; Tabuchi et al. 2007). Habash et al. (2007) discovered GS related enzymatic activities corresponding to QTL for N assimilation, leaf protein and yield characteristics in wheat.

Another isoform of GS (*GSI*) has been shown to activate the catalysis of other distinct and important functions in the N pathway. Chloroplast-localised *GS2* isoforms react with ferredox independent isoenzyme GS needed to absorb remobilised ammonia in foliar cells and tissues (Masclaux- Daubresse et al. 2010; Hirel et al. 2011). Studies involving rice have been used to identify QTL corresponding to leaf protein level, spikelet weight and other grain characteristics (Obara et al. 2004). Some of these QTL have been associated with efficient grain filling at maturity (*OsGSI*) under optimum N regimes (Tabuchi et al. 2005). Hirel et al. (2001) and Hayashi and Chino (1990) showed that glutamine serves as the main N transport product in rice. *OsGln1.2* transcripts have been shown to correspond to the sites of ammonium uptake (Sonoda et al. 2003, Tabuchi et al. 2007). In maize *Gln1.3* and *Gln1.4* are essential for seed germination (Limami et al. 2002). These isoforms also regulate ear development and contribute to 1000 grain weight and grain size (Cañas et al. 2010). Further, cytosolic *GSI* is active

in assimilating  $\text{NH}_4^+$  in sink organs such as in the prenatal maize tassels, cobs and husks. There have also been several efforts to characterise the functional roles of cytosolic GS in regulating ammonium uptake, assimilation and remobilisation in maize, rice and Arabidopsis. However, the process of N uptake and assimilation have not been linked specifically to GS allelic variation in hexaploid wheat.

## **1.6 QUANTITATIVE EXPRESSION OF *GS1* AND *GS2***

To quantify GS expression in real-time, quantitative reverse transcription polymerase chain reaction (qRT-PCR) procedures have been used extensively to test the expression of GS genes in response to supplied inorganic N. For example, Tian et al. (2014) tested two GS isoforms (*TaGS1c*; *TaGS2a*) involved in N metabolism in two wheat accessions (N-efficient and N-inefficient) with high and low N uptake efficiency at low N (no N application) and high N (0.3 g N kg<sup>-1</sup>) treatments. Leaf tissues were sampled at the tillering, jointing, heading and grain filling stages. The N-efficient genotype produced significantly higher gene expression and grain N compared to the N-inefficient genotype and it is proposed that this was related to its higher N remobilisation efficiency. In maize plants cultivated under contrasting N conditions, showed elevated N use efficiency in response to high N treatment (Guo et al. 2015). This was believed to be the effect of the high levels of GS transcripts that were constitutively expressed.

Generally, plastid *GS2* is more highly expressed in leaf mesophyll cells than the cytosolic *GS1.1*. Recent biochemical, genetic and molecular studies have highlighted a number of genetically important biosynthetic mechanisms,

including the high-level of *GS2* expression that can be explained as a consequential ammonium recycling effect (Lea & Forde, 1994; Kozaki & Takeba, 1996; Migge et al. 2000). Bernard et al. (2008) investigated the expression profile of GS genes in wheat. High levels of *GS2* mRNA was detected in mature leaf, peduncle tissues and photosynthetic glumes, but with roots showing low expression levels.

A number of studies quantifying NUE variables have utilised constitutive promoters to evaluate the effect of *GSI* overexpression on N assimilation and physiological development (Oliveira et al. 2002; Fei et al. 2006; Brauer & Shelp, 2010; Urriola & Rathore, 2015; Castro-Rodríguez et al. 2016). Moreover, some studies that investigated enzyme activity associated elevated GS activity with overexpression of *GSI* genes (Oliveira et al. 2002; Lima et al. 2006a; Thomsen et al. 2014). In a related experiment involving *Lotus corniculatus*, N remobilisation was synthetically induced by overexpression of the *GSI* isoform (Vincent et al. 1997). Transgenic wheat plants overexpressing the *GSI* gene have shown increases in grain number and grain weight per grain (Tian et al. 2014).

Though *GSI* overexpression in *Nicotiana tabacum* was linked with increased growth and high shoot biomass turnover (Fuentes et al. 2001; Oliveira et al. 2002), the overall effect on grain yield has been neutral or even, negative. It has been suggested that a number of factors including critical environmental stress and metabolic imbalances that may downregulate GS1 activity (Thomsen et al. 2014) may cause this inconsistency. According to Bao et al. (2014), imbalance in

C-N metabolism in plants with *GSI.1*, *GSI.2* overexpression may contribute to reduced growth rate and reproductive development resulting in reduced grain yield (Rhodes et al. 1978). Sufficient amounts of N and C skeletons are required to activate GS protein (Rhodes et al. 1978) and the two elemental tissue constituents are interactively coordinated in metabolic reactions to offset energy loss and C-N imbalances under reduced N conditions.

In addition, it has been shown that physiological changes can induce the expression of certain genes, for example, a study of *Brassica napus*, species isolated distinct *GSI* genes that exhibited variable expression patterns during leaf senescence when the plants were cultured under different N treatments (Orsel et al. 2014). The transgenic plants overexpressing *BnGSI.2* produced high biomass and increased plant height. In a study of rice, Martin, et al. (2006) showed that the expression of certain isoforms could vary considerably between species. The same study showed that expression of *GSI.1* and *GSI.2* mRNA in shoots along with significant levels of *GSI.2* mRNA in roots. In rice, the *GSI.1* expression level is normally high under optimum N conditions. However, the expression level drops significantly following ammonium application (Ishiyama et al. 2004, Tabuchi et al. 2007). In wheat, a study of high and low N responsive accessions was used to demonstrate that the magnitude of *TaGSIc* gene expression in the N-efficient genotype (YM18) was significantly higher than that produced from the N-inefficient (XY6) during reproductive development in both low and high N treatments (Bernard et al. 2008). It was proposed that this difference in expression

pattern might contribute significantly to the high N remobilisation efficiency of the N-efficient genotype.

In direct contrast, the rapid up-regulation of rice *GS1.2* gene suggests that independent regulatory mechanisms may control the expression of rice *GS1.1* and *GS1.2* genes in the roots. These regulatory mechanisms appear to be mediated and driven by the mutation of *GS1.1* that reduced or eliminated *GS1* function and this affected the accumulation of metabolites. For example, rice *GS1.1* mutants produced imbalanced metabolite levels (sugars, amino acids) highlighting the potential effect of GS mutation (Kusano et al. 2011). Unlike *GS1.1*, *GS2* expression was consistent with leaf *GS2* levels in other cereal species. In wheat, expression of *GS1.1* was upregulated in response to low ammonium content (Caputo et al. 2009). However, high ammonium rates specifically induced the expression of *GS1.3* in barley and sorghum (Fuentes, 2001; Habash et al. 2001). Goodall et al. (2013) demonstrated in barley that there was a direct correlation between the expression of *GS1* with grain protein and GS activity, and this suggested that *GS1* regulation influences GS activity. Contrary to this, Bao et al. (2014) recently demonstrated that *GS1.2* overexpressing plants produce reduced grain yield and amino acid concentrations in response to low N treatment.

These contrasting findings on GS overexpression are consistent with the dynamic and fluid nature of the NUE trait, GS gene expression and irregular results of enzyme activity experiments. Enzyme activity and phenotypic data from field studies of GS gene expression in response to N treatment does not

indicate any clear pattern either (Bishop et al. 1976; Smith et al. 1985; Gigova & Ivanova, 2015). A major flaw of evaluation is that current N treatment assessments exploring overexpression-yield linkages are unguided by any known consensus on what levels of N concentration is acceptable as high or low N treatment. Since different accessions respond differently to N availability, the current process of determining how gene expression affects yield, measured by the multiply different N amounts reported in published research, is confounded and subjective.

In other studies of the GS gene family, researchers confirmed that the pattern of gene expression in roots and leaves was dependent on the developmental stage of the plant (Peterman & Goodman, 1991). The above results reported by Bao et al. (2014) regarding *GSI.2* overexpressing plants seem to contradict studies in transgenic cereal plants that demonstrated that grain yield and protein content were increased by *GSI* overexpression (Temple et al. 1993; Fuentes et al. 2001). It is clear that further expression analysis is required to clarify the exact effects and regulatory mechanisms underlying gene expression in cereal species. It is expected that analysis of gene expression could provide a clear molecular benchmark for predicting and measuring the N content of nutrient deficient plants. This could guide the decisions necessary for applying N fertilisers to optimise grain yield in commercial production systems (Yang et al. 2011). Over-expression analysis of the cytosolic *GSI* gene provides a robust analytical tool for evaluating NUE and understanding the genetic basis of nutrient

use dynamics in cereal crops. However, due to the complexity and diversity observed in *GSI* alleles, the results of *GSI* overexpression analysis in many plant species so far show considerable inconsistencies in the NUE and yield effect.

To clarify these inconsistencies, a combination of approaches is required to study *GSI* post-transcriptional regulatory factors at gene, transcript and protein levels (Ortega et al. 2006). For example, tests of wheat accessions demonstrated that the flag leaf produced high levels of *GS2* proteins at anthesis (Tian et al. 2014). However, anthesis triggers several changes in leaf enzyme metabolic activity because of regulatory changes that alter protein and chlorophyll, and it is not clear how these changes are mediated by N treatment differences at other growth stages. It is also not clear how *GSI* isoforms and proteins are regulated during the upsurge in levels of *GS2* flag leaf-protein. However, it is possible that gene and protein regulatory changes will be mediated by post-translational modifications that alter cellular metabolism (Ghosh & Adams, 2011).

Furthermore, it is critical to investigate the direct effect of elevated *GSI.1*, *GS2* mRNA transcriptional levels on the expression pattern of other N related genes that control ammonium assimilation and remobilisation. Studies of GS expression and enzyme activity seem to suggest that increased *GSI* activity contributes to accelerated vegetative growth (Bernard et al. 2008; Tian et al. 2014). A proposal is that high N uptake (during vegetative growth) and remobilisation from senescing leaves drive this up-regulation of metabolic activity. An analysis of physiological plant variables is required to assess



phenotypic changes that may occur in response to the expression of GS isoforms. Such analysis would include plant root length, plant height, biomass (root and shoot dry weight) leaf area and SPAD value of vegetative tissues at specific growth stages in the life cycle, such as specified in Zadoks scale that is a standardised quantitative tool for assessment of cereal plant growth (<https://www.agric.wa.gov.au/grains/zadoks-growth-scal>). Moreover, *GS1* and *GS2* overexpressing plants and wild type plants can be compared in different growth environments (soil or hydroponics) and N treatments. Some of the yield components to be measured at the mature stage include seed setting rate panicles/plant, filled grains/panicle, 1000 grain weight, kernel size and grain protein content.

## **1.7 POST-TRANSLATIONAL MODIFICATION OF *GS1* AND *GS2***

A number of post-translational modification (PTM) mechanisms such as phosphorylation drive the modification of post-translational regulation GS proteins. It was observed in the GS protein of *M. truncatula* (dicotyledonous species with GS protein almost identical to wheat GS alleles) that the phosphorylated *GS2* interacts with specific proteins that induce selective proteolysis (Lima, 2006a) that renders the chloroplastic isoform inactive. A similar inactivation was recorded for the post-translational modification (adenylation) of the bacteria *Escherichia coli* *GS2* isoform (Liaw et al. 1993). However, a similar test of soybean GS indicated a lack of *GS2* phosphorylation (Ortega et al. 1999). A further study of bacteria species by Stadtman (2001)

confirmed that GS proteins undergo adenylation/deadenylation. Finnemann & Schjoerring (2000) demonstrated that *GS1* is post-translationally modified by the action of 14-3-3 protein in the root nodule of *B. napus*. Lima et al. (2006b) improved the understanding of this PTM through studies of *M. truncatula* that demonstrated that the phosphorylation of *GS1A* and *GS1B* was contrasted to that of GS2 enzymes, as the phosphorylation of the plastid GS involved the catalytic reaction of calcium-dependent kinases that were not involved in the phosphorylation of the cytosolic enzymes. However, despite these advances in GS PTM studies no conclusive evidence explaining definitely the plant post-translational regulatory mechanisms that modulate GS enzymes has yet been published.

## 1.8 CHARACTERISATION OF ENZYME ACTIVITY

Throughout the history of crop nutrition research, several studies aimed at identifying N linked QTL and other NUE variables in crop plants have examined enzyme activity (Kaiser & Lewis 1983; Lea & Miflin 2003; Masalkar & Roberts, 2015). This has become a useful benchmark for measuring the effect of GS isoforms in many crop species in terms of N uptake, assimilation and remobilisation (Silveira et al. 2003; Wang et al. 2006; Grabowska et al. 2012). The metabolic activity of important N-related enzymes is essential for N assimilation including the Asparagine synthetase family (*AspAT-GRMSM2G400604*, *AlaAT-GRMSM2G088028*) and *NRT* group (*NRT2.1-GRMSM2G010280*, *NRT2.2-GRMSM2G010251*, *NRT2.5-GRMSM2G455124*, *NRT-3.1A-GRMSM2G179294*). Additionally, the metabolic activity involving nicotinamide adenine dinucleotide hydrate (NADH) dependent glutamine synthase, glutamine dehydrogenase and transaminases of aspartate aminotransferases are the important for glutamate synthesis (Lutts et al. 1999; ).

A critical aspect of NUE research is to enquire how GS enzyme activity contributes to increased or reduced N assimilation. Another important focus of GS activity analysis is to characterise the N-induced rate of metabolic activity in the tissues at particular growth and time points between vegetative growth and maturity. This will inform decisions on N-fertiliser application at specific growth stages of plants. A variety of laboratory techniques and strategies have been used to quantify GS activity in plant tissues with the objective of identifying GS

isoforms and determining GS function in particular tissues (Bernard & Gadai, 1982; Avila-Ospina et al. 2015; Wang et al. 2015; Guan et al. 2016). Some of the strategies have used tissue assays to characterise GS structural units and grain protein (Nigro et al. 2016), subcellular localisation (Taira et al. 2004; Chao et al. 2016) and potential function linked to N assimilation (Guan et al. 2016; Plett et al. 2016). A notable approach to evaluating GS activity has been the use of tissue analysis to measure the level of absorbance of GS isoform reaction with  $\gamma$ -glutamyl monohydroxamate used as the standard (Rhodes et al. 1975; Fraser & Ridley, 1984). There have been useful insights in studying GS activity with respect to phenotyping physiological responsiveness of accessions to stress such as drought and salinity. For example, studies of drought in maize by Li et al. (2016) have shown that level of N remobilisation in the ear was elevated under post-silking drought and this influenced *GS1* and asparagine synthetase activity in the leaves surrounding the ears. Reguera et al. (2013), demonstrated under stress conditions cytokinin could increase drought tolerance through induced N assimilation. Similarly, an investigation into GS activity patterns in salinity stress induced tobacco transgenic plants suggested that GS activity contributes significantly to proline accumulation (Brugiere et al. 1999).

The rate of *GS2* metabolic activity has been shown to be significantly higher than *GS1* activity, however, the discrepancy in metabolic activity depreciates with the onset of leaf senescence (McNally et al. 1983; Bernard et al. 2008; Thomsen et al. 2014). Considerable amount of work exists on GS activity

in other accessions but in wheat accessions, the exact effect of N treatment on enzyme activity and GS protein content is not clear between cytosolic and plastidic GS isoforms and at high or low metabolic rates. The result of GS activity assays so far have shown that GS activity varies significantly between wheat accessions (Tabuchi et al. 2007). Due to this considerable GS activity, variation between wheat accessions (Tabuchi et al. 2007); additional characteristics of GS activity can be identified through assays of several different tissue samples across different organs in diverse accessions. This can be addressed by analysing a larger set of accessions from genetically diverse backgrounds. Such an approach will be important for understanding the genetic and molecular processes that link efficient N metabolism with yield improvement, abiotic stress tolerance and other agronomic traits of interest for high yield wheat production.

## CHAPTER TWO

### GENE IDENTIFICATION, SNP GENOTYPING AND LOCUS MAPPING OF *TaGS1.1* AND *TaGS2* CONSERVED DOMAINS

#### 2. ABSTRACT

The conserved domains of two highly expressed glutamine synthase (GS) isoforms *TaGS1.1* and *TaGS2* were identified and genotyped using *in-silico* sequence analysis tools. Using standard PCR-based amplification protocols, six GS homologues were isolated and sequenced from a diverse panel of 100 hexaploid wheat accessions (Diversity Panel) that were representative of spring wheat accessions from widely different genetic backgrounds (ACPFPG unpublished). Consensus alignment of *TaGS1* and *TaGS2* sequences from *Arabidopsis thaliana*, *Oryza sativa*, *Brachypodium distachyon* and *Zea mays* derived from standard wheat genome databases and subsequent sequencing of the wheat amplicons, revealed a high level of conservation of *TaGS1* and *TaGS2* localised to *Chr.6* (*GS1.1A*, *GS1.1B*, *GS1.1D*) and *Chr.2* (*GS2A*, *GS2B*, *GS2D*) respectively. Phylogenetic analysis of the reference GS transcripts show a clear dichotomy of the cytosolic and plastidic GS isoforms. Both *GS1.1* and *GS2* homologues are anchored to the *GS1.1A* transcript *6AL\_5748909* which appear to be the major ancestral genetic root of the other five GS homologues. SNP genotyping with unique gene specific markers (developed from the conserved domains) accurately mapped these homologues to their respective chromosome

loci within three mapping populations. Principal component analysis demonstrated a distinct segregation of *TaGS1.1* and *TaGS2* genes along clearly defined nitrogen response clusters. This analysis may provide data relevant for the selective breeding of elite N - efficient spring wheat lines.

## **2.1 INTRODUCTION**

Important physiological processes such as nutrient assimilation and other traits that regulate plant growth and development are under close genetic control (Lopes et al. 2006). The conserved domains of these genes constitute important recurring and inheritable structural units that modulate gene and protein function in eukaryotic organisms including wheat. Single nucleotide polymorphisms (SNPs) occur frequently in plant gene sequences (Ching et al. 2002), though differentially abundant between different genetic clades. The density and genetic locus of SNPs vary between species largely because of bi-allelic nucleotide change in the targeted DNA sequences (Deulvot et al. 2010). Conserved domain SNPs are crucial genetic sign posts that flag the sites of allelic variation, and amino acid substitution that may confer functional variation in trait linked characteristics of the genes (Tian et al. 2004; Van Nguyen et al. 2016). Identification of SNP loci in conserved domains is therefore, an important step towards genotyping genetically diverse varieties of wheat and towards gene-wide sequencing and successful molecular characterisation (Causse et al. 1995).

Generally, SNP genotyping is hampered by the quantity of allelic variants captured (Deulvot et al. 2010). This constraint results from the misalignment of

gene sequences leading to false SNP identification and pseudo-location of polymorphic regions. This challenge is compounded in paralogous gene sequences alignments, which are misread as polymorphic. Erroneous sequence assemblage and inconsistent alignment may result in ignoring actual SNPs. The current trend of marker detection is gearing towards high-throughput SNP genotyping which quicker and simpler than the previously popular microsatellite marker (Loridon et al. 2005) genotyping approaches and superseded restriction fragments length polymorphism (RFLP) mapping.

Despite advancement in cloning technology and molecular characterisation approaches, there is scarcity of molecular tools (Rodríguez-Lázaro et al. 2007) for breeding high yielding wheat cultivars, especially for NUE targeted molecular breeding. Currently, there is a need to broaden the scope of genetic research targeting GS genes and nitrogen use efficiency (NUE). For example, many published works typically explore just one analytical aspect of GS genes such as gene expression, protein function characterisation or enzyme activity. The focus of discussions based on such studies often treat the experimentation of these experimental units as separate entities which are isolated from each other rather than inter-related complementary units. In wheat, few studies have targeted the allelic variation representative of the three wheat genomes (A, B and D). Studies of maize Glutamine (*GLN*) mutants have produced vital genetic data on the effect of knock-out mutation of *GLN1.3* and *GLN1.4* on grain yield and number (Hirel et al. 2001; Martin et al. 2006). Hanley et al. (2000) showed that *GSI* isogene



mutants might lose some functionality resulting from sequence insertion events observable in some of the maize mutants profiled. A study of wild type maize accessions revealed different cellular localisation of *GLN1.2* and *GLN1.3* transcripts (Sakakibara et al. 1996). In addition, a *GLN1.4* loci for grain yield was proposed for NUE in maize (Hirel, 2001).

Unlike maize however, molecular tools and gene sequences are not available for most wheat varieties for sequence comparison, molecular characterisation and phylogenetic assessment. The large size of the wheat genome and gene duplication events compound this problem (Scofield et al. 2005). Crucially, sequences information for the accessions tested in this study has previously not been isolated for members of the GS gene family. Such gene sequence information is important in reconstructing the genetic history of the distinct isoforms within the GS gene complex.

Molecular markers are obviously robust tools for genotyping to detecting allelic variation. However, due to the lack of information on genetic diversity among orthologues, phylogenetic analysis can provide the relevant background information needed to gain an in-depth understanding of the allelic variation among GS genes. Such analysis is useful for accurately interpreting patterns of genetic diversity and similarity between multiple alleles. Generally, phylogenetic analysis also provides the basis for the prediction of the kind of allelic variation that will possibly emerge in the future (Wheeler, 2000; Sullivan et al. 2012; Lutz et al. 2016). The combined application of genetic markers and phylogenetic

assessment is not only useful in profiling genetic relationships (Raina et al. 2001; Wang et al. 2009; Garcia-Lor, 2013), but also in determining whether specific accessions are linked by a common ancestral genotype.

A considerable number of articles have been published examining GS genes in maize, rice and Arabidopsis. However, wheat GS sequence analysis still very limited and until the complete wheat genome becomes available, efforts to characterise these genes among wheat accessions will continue with a high degree of difficulty. In the meantime, conserved domain analysis of *TaGS* genes will remain one of the most effective strategies for NUE molecular characterisation. The sequencing information presented in this study is a novel attempt to characterise these GS homologues in spring wheat accessions. This study seeks to clarify the genetic relationships among a diverse set of genetic material represented by spring wheat varieties and potentially exploit the available genetic variation for molecular breeding. The varieties investigated constitute a pool of diverse accessions used in wheat genomic studies and serves as an important resource for NUE related genomic and physiological analyses. The analysis also aims to provide useful insights on sequence divergence among accessions, which can be related to N-linked genes of wheat and closely related species.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Overview of Experimental Activity

Six GS genes (*TaGS1.1A*, *TaGS1.1B*, *TaGS1.1D*, *TaGS2A*, *TaGS2B* and *TaGS2D*) are collectively representative of the three wheat genomes (A, B and D). The genes were isolated through sequencing of the genes in nine spring wheat accessions. Among these wheat accessions were accessions that have been used as parents of a double haploid mapping populations and recombinant inbred lines. A total of 25 DNA markers was designed to map the SNP loci identified from within the conserved regions of the GS genomic sequences. The SNP markers were mapped to four different mapping populations comprising the double haploid population (DH) of RAC875 x Kukri, Excalibur x Kukri, Yallaroi x AUS24152\_Janz and recombinant in-bred line population of Gladius x Drysdale. Sequence analysis of amplicons was also undertaken in the accessions Gladius, Drysdale, Excalibur, RAC875, Kukri, Espada, Mace and Chinese spring. These lines were selected from in-house DNA stocks at the Australian Centre for Plant Functional Genomics (ACPFPG, <http://www.acpfg.com.au/index>).

### **2.2.2 Germplasm Material**

The germplasm that was examined comprised 100 wheat accessions with considerable genetic variation. These spring-wheat accessions were selected for adaptation to a Mediterranean type climate representing different geographically and diverse wheat cultivation areas. The list of accessions included Gladius, Drysdale, Excalibur, RAC875, Kukri, Chinese Spring, Mace, Scout and Espada. The complete list of varieties is presented in Table 2.1. Some of these varieties are widely cultivated in South Australia owing to their high yield and improved levels of resistance and tolerance to boron toxicity, and resistance to black stem rust and yellow dwarf virus diseases. Gladius for example, is a mid-season maturing variety that maintains a steady height and grain yield under drought. Gladius was bred from crosses involving RAC875, Excalibur, Kukri, Krichauff and a derivative of another variety Trident ([http://modraseeds.com.au/pdf/AGT\\_Gladius.pdf](http://modraseeds.com.au/pdf/AGT_Gladius.pdf)). Another accession Drysdale is a semi-dwarf variety bred from a cross between Hartog 3 and Quarrion. The seeds of all the wheat accessions were planted in pots in a controlled chamber under moderate conditions of nitrogen and soil moisture until the seedling were 4 weeks old. The leaves were harvested and DNA samples were extracted using the freeze-dried method. These DNA samples were used as the template for sequencing and developing SNP markers and conduct SNP genotyping.

**Table 2.1** List of 100 wheat accessions included in the Diversity Panel used in SNP marker assays. Unique accession numbers (AUS) or Diversity Panel codes (DPG) designates accessions.

| Serial Code | Wheat Accession        | Accession Number | Country of Origin | Serial Code | Wheat Accession | Accession Number | Country of Origin |
|-------------|------------------------|------------------|-------------------|-------------|-----------------|------------------|-------------------|
| 1           | AUS28080_4             | AUS28080         | India             | 51          | Granarolo       | DPG_479          | Italy             |
| 2           | AUS5186_6              | AUS5186          | Iran              | 52          | S975_A4_A1      | Unknown          | Zimbabwe          |
| 3           | AUS19123_5             | AUS19123         | Iran              | 53          | Andes 56        | AUS1786          | Colombia          |
| 4           | AUS17838_4             | AUS17838         | Pakistan          | 54          | LW336           | Unknown          | Morocco           |
| 5           | AUS14828_6             | AUS14828         | Australia         | 55          | Menflo          | DPG_704          | Chile             |
| 6           | AUS4885_6              | AUS28080         | Australia         | 56          | Buck-Atlantico  | DPG_175          | Argentina         |
| 7           | PBW 343_6              | Unknown          | Unknown           | 57          | Mace            | AUS36462         | USA               |
| 8           | Opata 85_5             | AUS23399         | Mexico            | 58          | Gladius         | AUS167576        | Australia         |
| 9           | Jupateco 73_4          | AUS39225         | Mexico            | 59          | Excalibur       | AUS39210         | Australia         |
| 10          | Inia 66_4              | AUS359           | Australia         | 60          | Espada          | AUS36391         | Australia         |
| 11          | Genaro F81_4           | AUS90831         | Mexico            | 61          | Merlin          | AU17148          | Germany           |
| 12          | Ciano79_6              | AUS100122        | Mexico            | 62          | Kukri           | AUS29472         | Australia         |
| 13          | Suzuki_12_6            | Unknown          | Mexico            | 63          | Drysdale        | AUS30498         | Australia         |
| 14          | Suzuki_3_4             | Unknown          | Mexico            | 64          | Spitfire        | AUS39574         | Australia         |
| 15          | Sokoll_4               | DPG_863          | Mexico            | 65          | RAC875          | AUS34632         | Australia         |
| 16          | Siete Cerros_6         | AUS10684         | Mexico            | 66          | Halberd         | AUS11612         | Australia         |
| 17          | Roelfs F2007_5         | DPG_834          | Unknown           | 67          | Wyalkatchem     | AUS30784         | Australia         |
| 18          | Pitic 62_6             | AUS801           | Mexico            | 68          | Janz            | AU39211          | Australia         |
| 19          | Tacupeto_5             | AUS29169         | Mexico            | 69          | Stylet          | AUS30405         | Australia         |
| 20          | Synthetic W7985_5      | AUS24280         | Australia         | 70          | Young (AGT)     | AUS35917         | Australia         |
| 21          | Suzuki 9_6             | Unknown          | Mexico            | 71          | Scout           | AUS10681         | USA               |
| 22          | Suzuki 24_4            | Unknown          | Mexico            | 72          | Trident         | AUS25924         | Australia         |
| 23          | Suzuki 17_5            | Unknown          | Mexico            | 73          | Yipti           | AUS30492         | Australia         |
| 24          | Suzuki 23_4            | Unknown          | Mexico            | 74          | Magenta         | AUS16501         | Italy             |
| 25          | Yecora 70_6            | AUS15920         | Mexico            | 75          | Suntop          | AUS39653         | Australia         |
| 26          | Vorobey_5              | AUS177749        | Mexico            | 76          | Krichauff       | AUS27203         | Australia         |
| 27          | Odessa Ex. St. 17413   | DPG_743          | Syria             | 77          | Wallup          | AUS39655         | Australia         |
| 28          | Odessa ES19565         | DPG_744          | Ethiopia          | 78          | Estoc           | AUS39492         | Australia         |
| 29          | Taferstat              | Unknown          | France            | 79          | Frame           | AUS25601         | Australia         |
| 30          | Thori 212-Var. 8/1     | DPG_904          | Pakistan          | 80          | Kord CL Plus    | AUS39552         | Australia         |
| 31          | Buck-Buck              | AU20044          | Mexico            | 81          | Axe             | AUS36389         | Australia         |
| 32          | Frontana 3671          | AUS2452          | Brazil            | 82          | EGA Gregory     | AUS34283         | Australia         |
| 33          | G72300                 | Unknown          | Unknown           | 83          | N46             | DPG_725          | Israel            |
| 34          | Meira                  | DPG_701          | Spain             | 84          | LW335           | Unknown          | Unknown           |
| 35          | Mocho De Espiga Blanca | AUS39331         | Portugal          | 85          | Coppadra        | DPG_316          | Unknown           |
| 36          | Palestinskaya          | DPG_758          | Palestine         | 86          | M70811G25/N163  | Unknown          | Israel            |
| 37          | Pamukale               | DPG_760          | Turkey            | 87          | Zilve           | DPG_975          | Turkey            |
| 38          | U-Man-Syao-Mai         | DPG_913          | China             | 88          | LW337           | Unknown          | Ethiopia          |
| 39          | Emu                    | AUS17224         | Mexico            | 89          | BT2277          | Unknown          | Tunisia           |
| 40          | Estanzuela Dorado      | AUS2832          | Uruguay           | 90          | LW334           | Unknown          | Turkey            |
| 41          | LW349                  | Unknown          | Unknown           | 91          | 117 var. 12/564 | Unknown          | Pakistan          |
| 42          | Daeraad                | AUS149           | South Africa      | 92          | N67 M2          | Unknown          | Unknown           |
| 43          | LW369                  | Unknown          | Unknown           | 93          | AUS14810        | AUS14810         | Australia         |
| 44          | Bahatane 87            | AUS12908         | Algeria           | 94          | AUS14681        | AUS14681         | Australia         |
| 45          | LW333                  | Unknown          | Unknown           | 95          | AUS5190         | AUS5190          | Australia         |
| 46          | Academie De Pekin      | DPG_097          | China             | 96          | AUS5207         | AUS5207          | Australia         |
| 47          | 6_1_3                  | Unknown          | Egypt             | 97          | AUS28093        | AUS28093         | Australia         |
| 48          | Africa Mayo            | AUS2435          | Kenya             | 98          | AUS14616        | AUS14616         | Australia         |
| 49          | H742                   | AU13492          | Afghanistan       | 99          | AUS27832        | AUS27832         | Australia         |
| 50          | BT2281                 | DPG_174          | Tunisia           | 100         | Chinese Spring  | AUS90539         | China             |

### 2.2.3 In-silico Gene Sequence Identification

Reference gene sequences of the GS family were selected for analysis because of their previously reported links with N uptake, assimilation and remobilisation in maize and other genetically related species. The reference sequences used included *TaGS1.1A*, *TaGS1.1B* and *TaGS1.1D* genes on *Chr.6* (>lcl|6AL\_5820788 reverse complementary; >lcl|6BL\_227297 reverse complementary incomplete sequence at 5'; >lcl|6DL\_2494823 3419 46201) and *GS2A*, *GS2B* and *GS2D* genes on *Chr.2*. (>lcl|2AL\_6391136; >lcl|2BL\_7944286; >lcl >2DL\_9842556). The genomic sequences, coding DNA sequences (CDS) and translated protein sequences of these GS transcripts were extracted from the ACPFG databases.

The characteristics of the genes of interest including conserved domains, were examined across multiple databases to confirm the accuracy and degree of genomic and mRNA sequence conservation. Sequence data of genomic DNA of GS homologues from maize, rice, Brachypodium and barley were retrieved from in-house ACPFG DNA sequence assemblies. This included RAC875-ACPF 454 + Solexa Assembly, Kukri -ACPF 454 Assembly, Excalibur - ACPFG 454 Assembly, Drysdale - ACPFG 454 Solexa Assembly, Gladius - ACPFG 454 Solexa Assembly, Havard TC Rice 19, Havard TC Barley Release 12 and Havard TC Maize 19. Additionally, other standard databases including the GenBank of National Center for Biotechnology Information (NCBI) and European

Bioinformatics Institute (EMBL) were utilised. To resolve quality issues within each database, there was a search of GS sequences in the databases by using the contig identifiers or gene codes of the GS transcripts (the sequences are presented in the Appendix). A review of the sequences and another round of searching the databases to refine the sequences followed this.

In addition, a number of homologues from diploid grasses were obtained from *Phytosome* (<https://phytozome.jgi.doe.gov/pz/portal.html>). A search of all genomic and cDNA based wheat databases was used to identify all known homologous sequences. A comparison of these sequences was aligned with the gene sequences from reference lines retrieved from “The Arabidopsis Information Resource” (TAIR-<https://www.arabidopsis.org>). The retrieved sequences were consolidated through consensus alignment with predicted gene sequences from wheat (Chinese Spring), maize, rice, barley and sorghum.

The accuracy of the resultant alignment was checked by using the candidate orthologue sequences and Basic Local Alignment Search Tool (BLAST) algorithm to search against the species of sequence origin. The results were carefully checked and the hit sequences (longest homologues with the least nucleotide mismatches) were downloaded to do multiple sequence alignments using different programmes *MAFFT* ([www.ebi.ac.uk/Tools/msa/mafft/](http://www.ebi.ac.uk/Tools/msa/mafft/)), *ClustalW* ([www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)), and the *Geneious* programme (<http://www.geneious.com>, Kearse et al. 2012). The sequences were then consolidated using the *Geneious* sequence alignment tools. The alignment was

checked to remove any irregular sequences (sequences wrongly assembled or interrupted by suspiciously high number of indels and abnormal deletions that were not consistent with sequences from other databases) and then realigned for accuracy before designing primers to flank the conserved domains. Based on sequence comparison, the primers were placed within or flanking the conserved domains. Primer location was dependent on the variation and position of conserved domains identified within the gene sequences. Moreover, the primers were confirmed to be gene specific (conserved domain primers specific to *TaGS1.1* on *Chr.6* and *TaGS2* on *TaChr.2*). Purified DNA sample were sequenced using Sanger sequencing and these sequences were compared with the reference sequences in a multiple alignment to check if they correctly aligned to the conserved regions of the reference sequence (Fig. 2.1).





**Fig. 2.1** Sequence alignment of GS1B wheat conserved domains showing consensus alignment and the positioning of KASP markers at SNP locus. Forward-oriented markers are represented by dark green and reverse markers by light/summer green colour.

#### **2.2.4 Phylogenetic Analysis of *TaGS1* and *TaGS2* Homologues**

Phylogenetic analysis was used to evaluate and infer the ancestral relationships between gene sequences of the GS family. A phylogenetic tree was constructed using the *Geneious R9* programme and evaluated to determine if the extracted reference sequences from different but related species (orthologous sequences) and predicted DNA sequences share homology and common phylogenetic history. The analysis also confirmed the accuracy and specificity of the sequence origin. The evaluation was used as a basis to determine whether sequence variability within the targeted wheat sample set contained enough genetic diversity detectable within the conserved domains.

#### **2.2.5 Primer Design and Gene-Genome Specificity Confirmation**

GS gene sequences were consolidated using the *Geneious R9* alignment tools and conserved domain primers specific to both A, B and D genomes, and homologues designed (Fig. 2.1). The genes investigated included; *TaGS1.1* (11 kb), *TaGS1.1* (5.4 kb), *TaGS1.1* (4 kb), *TaGS2* (10.6 kb), *TaGS2* (10.4 kb) and *TaGS2* (10.7kb). Homologue-specific primers (Appendix Table A2.1 and Table A2.2) were designed using *Geneious R9* and the annealing temperature of each primer set (sense and anti-sense) was standardised by calculations of the *NEB Tm Calculator* ([tcalculator.neb.com](http://tcalculator.neb.com)). Genome specificity of the desired amplicons was verified with aneuploid wheat DNA samples.

The aneuploids are accessions resulting from errors in cell division and chromosome mutation that produces an additional or missing a chromosome (Law et al. 1987; Griffiths et al. 2000; Endo, 2015). Therefore, the aneuploid the chromosome number is different from the wild type and is unequal to a multiple of the haploid number for the wild type that particular species. In the Chinese spring aneuploids has two pairs of chromosomes representing two different genomes of either A, B or D and lack the third chromosome pair needed to complete the hexaploid. For example, the nullitetrasonic N2AT2B represents nullisomic for A ( $2n-2$ ) and tetrasomic for B or D on chromosome 2. Hexaploid wheat is resistant to the mutative effects of nullisomy as it exhibits meiosis similar to a diploid (Plaschke et al. 1996; Griffiths, 2000; Wang et al. 2014). The tetrasomic pairs compensate for the missing nullisomic chromosome pairs.

The primers used were found to be specific to Chinese Spring aneuploid lines and were used in standard PCR protocols to generate PCR amplification products that matched the conserved domains of A, B and D genome-specific GS genes (Table 2.1 and 2.2). The Chinese spring aneuploid DNA stocks were assayed along with the reference line Chinese Spring and nine additional lines listed above (Table 2.1). The variety was Scout added as a parent of an additional mapping population.

**Table 2.2** Composition of the reaction mix representing six different protocols (P1, P2, P3, P4, P5 and P6) used to optimize PCR amplification of GS conserved domains.

|  | P1   | P2   | P3   | P4   | P5   | P6   |
|--|------|------|------|------|------|------|
| Template DNA 50 (ng/ $\mu$ L)                    | 3.0  | 3.0  | 3.0  | 3.0  | 3.0  | 3.0  |
| Immolase polymerase (5U/ $\mu$ L)                | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 |
| dNTPs 1.25 (mM)                                  | 1.6  | 1.6  | 1.6  | 1.6  | 1.6  | 1.6  |
| Immolase buffer without (MgCl <sub>2</sub> 10 x) | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  |
| MgCl <sub>2</sub> (50 $\mu$ M)                   | 0.5  | 0.8  | 1    | 0.5  | 1.5  | 1.6  |
| Forward primer each (10 $\mu$ M)                 | 0.5  | 0.5  | 0.5  | 1.0  | 0.5  | 0.4  |
| Reverse primer each (10 $\mu$ M)                 | 0.5  | 0.5  | 0.5  | 1.0  | 0.5  | 0.4  |
| Water  | 2.87 | 2.57 | 2.37 | 1.87 | 1.87 | 1.97 |
| Total ( $\mu$ L)                                 | 10   | 10   | 10   | 10   | 10   | 10   |

## 2.2.6 Polymerase Chain Reaction and Optimisation

Gene-specific primers were used in PCR optimisation experiments to generate the desired amplicons. DNA polymerase Immolase was used for its high level of specificity of amplification, its capacity to remove primer-dimers and generally achieving thermo-stability (<http://www.bioline.com/au/immolase-dna-polymerase.html>). 0.03  $\mu$ L of Immolase was used to formulate a 10ul reaction mix containing template DNA (3.0 $\mu$ L), MgCl<sub>2</sub> (0.5 $\mu$ L), dNTP (1.6 $\mu$ L), Immolase Buffer (0.03 $\mu$ L), Forward and Reverse Primers (1.0 $\mu$ L) and Water (2.87 $\mu$ L) as recommended by Bioline (<http://www.bioline.com/au/immolase-dna-polymerase.html>). The reaction mix was prepared for thermo-cycling (Temperature control mode constant at 100°C, incubation at 95°C – 10mins,

incubation at 94 – 30s, incubation at 60°C – 30s, incubate at 72°C – 60s, cycles to step 2 for 34 more times, incubate at 72°C – 5min, incubate at 20 – forever). This protocol operated on MJ Research PTC-200 PCR Peltier Thermal Cycler 60-well PCR machine ([www.thermofisher.com/](http://www.thermofisher.com/)). Amplification issues were resolved through PCR optimisation. Different primers sets were combined to amplify short sequence amplicons (0.5 - 1kb) from *TaGS1* and *TaGS2* homologs. Primers were used to amplify across longer sequences to generate larger fragments that were previously not covered with the shorter amplicons.

Different polymerases including reduced stock concentration of the Phusion High-Fidelity DNA Polymerase ([www.thermofisher.com/](http://www.thermofisher.com/)) were used in different PCR protocols. In these protocols, the annealing temperature was programmed in a temperature gradient (55.8°C – 65.0°C). The PCR reaction mix was constituted by slight variations of the volume of MgCl<sub>2</sub> (0.5µL, 0.8µL, 1.0µL, 1.5µL and 1.6µL). This altered the final concentration of MgCl<sub>2</sub> in the reaction mix to optimise the PCR conditions (Table 2.1). The PCR run produced a high yield of PCR products that were suitable for purification and sequencing. The PCR experiments were utilised to isolate *TaGS1.1* and *TaGS2* amplicons (500 - 1000 bp) from Gladius, Drysdale, Chinese spring, Excalibur, RAC875, Kukri, Mace and Espada. Several PCR tests were conducted to optimise the amplification conditions for each primer set corresponding to each of the *TaGS1.1* and *TaGS2* genes. Agarose gel electrophoresis was used to confirm the size of each conserved

domain amplicon. The band representing each amplicon was measured with a 1kb DNA ladder to verify if the band corresponded to the expected amplicon size.

The PCR products were purified using *Invitrogen Pure Link Quick PCR Purification* (<https://www.thermofisher.com/order/catalog/product/K310001>) and 3 replicates of each DNA sample were sequenced to produce 3 replicate gene amplicons which were compared by alignment with the reference sequences for accuracy. Using the *Geneious* programme the PCR amplified sequences were consolidated in a sequence alignment of *TaGS1.1A* (Chr.6A), *TaGS1.1B* (Chr.6B), *TaGS1.1D* (Chr.6D) and *TaGS2A* (Chr.2A), *TaGS2* (Chr.2B), *TaGS2* (Chr.2D). Automated Sanger DNA sequencing involving DNA fragment analysis and capillary electrophoresis (Mitchelson, 2001; 2007; Kricka & Fortina, 2016) sequenced sixty amplicons corresponding to the conserved domains of the GS genes across the selected lines.

Prior to sequencing the DNA, the template is subjected to heat to denature the double helix DNA into single strands. A primer is then annealed to a single DNA strand which is fluorescently labelled for easy detection on a gel (Marras et al. 2002). To build an extended strand, nucleotides are continually added as DNA polymerase copies the DNA templates and forms an extended strand to the primer at the 3' end. Automated Sanger sequencing is a chain termination that uses dideoxynucleotides (ddNTPs) to terminate addition of more nucleotides when incorporated into a DNA sequence as this process prevents the formation of a phosphodiester bond between the ddNTPs and further addition of the next

nucleotide, and therefore causes the chain of DNA to be terminated. After the attachment of the primer to the DNA, reagents (four dNTPs, ddGTP, ddATP, ddTTP, ddCTP and DNA polymerase) are added the reaction mix containing four ddNTPs which are marked with different fluorescent dyes for detection of "G", "A", "T" and "C" nucleotides at different wavelengths using capillary electrophoresis. Capillary electrophoresis is a high resolution and high throughput method that detects fluorescently labelled nucleic acid templates as they travel on arrays of microcapillaries (Mitchelson, 2001; Greenough et al. 2016). Twenty amplicons corresponding to the conserved domains of six *TaGS* genes across the lines were sequenced by using AGRF Sanger sequencing services ([www.agrf.org.au/services/sanger-sequencing](http://www.agrf.org.au/services/sanger-sequencing)).

### **2.2.7 SNP Marker Assay and Fine Mapping of GS Homologues**

A number of KASP (<http://www.lgcgroup.com/products/kasp-genotyping-chemistry/>) markers were designed for SNPs that were identified within the extracted conserved domain sequences of the six GS homologs (*TaGS1.1A*, *TaGS1.1B*, *TaGS1.1D*, *TaGS2A*, *TaGS2B*, *TaGS2D*). These markers were genotyped and mapped onto three different wheat mapping populations for which genotype information has been previously assembled in related studies. The goal of this study was to map all six GS genes in mapping populations for which there exists considerable genomic characterisation. In all six genes, number of SNP events including 40 SNP loci on the conserved domains of the *TaGS* genes was identified and for these a set of 300 SNP markers were designed using the *Kraken*

programme. The markers designed were used to genotype Yallaroi x AUS24152\_Janz, RAC875 x Kukri, Excalibur x Kukri double haploid Gladius x Drysdale recombinant inbred lines.

To identify the SNP marker, three different primers were designed. These comprised two allele-specific primers consisting of sense and antisense primers and a third primer termed the Common primer was positioned to overlap the targeted SNP. For each marker, the last 3' nucleotide of the two allele-specific primers was positioned on the SNP being investigated (Fig. 2.1). The Common primer length and allele-specific primers without the FAM/VIC tag were 21-25 bp and annealing temperature was set at 60°C - 61°C. Allele specific KASP markers were developed for SNP genotyping of identified polymorphic regions.

The protocol used included DNA (5ng/ $\mu$ L = 2 $\mu$ L), sense primer (24.0 $\mu$ L), antisense primer (24.0 $\mu$ L), Common (60.0 $\mu$ L) and H<sub>2</sub>O (92.0 $\mu$ L) added to a final volume of 200.0 $\mu$ L. DNA was stamped into 384-Black PCR plates on a robotic *Oktopure SNP Line* (<https://www.youtube.com/watch?v=lQxMZ3U0IJQ>) and oven dried at 65°C for 60min. An amount of 2 $\mu$ L of KASP Master Mix was added to each well and 384-Black PCR plates were sealed with optically clear plate seal on utilising an *LGC Fusion3 Plate Sealer* KBS-0007-001 (<http://www.lgcgroup.com/products/genotyping-instruments/>). This was set on KASP 61-55 PCR programme that comprised an initialising step: 94.0°C - 15min, denaturing: 94.0 °C - 20s, annealing 60.0°C - 60s, extension: 94.0°C - 20s cycling steps: decrease by 0.6 °C every cycle, cycle to step 2 - 9 more times,



and final extension: 55.0°C - 60s on a *HydroCycler16* (<http://www.gcbiotech.com/liquidhandlingautomation?pid=56&sid=116:HydroCycler-16>). The discriminant fluorescence data of contrasting alleles was obtained by spectral measurement of *TaGS* tissue assays (extracted in phenolic reagents) using the PHERAstar Plus (BMG Labtech-[www.bmglabtech.com/en/products/.../pherastar-plus-obj-42-742.html](http://www.bmglabtech.com/en/products/.../pherastar-plus-obj-42-742.html)).

### **2.2.8 Marker-based Analysis of Genetic Structure**

The Kraken programme (<http://www.lgcgroup.com/products>) was used to distinguish the segregation of the accessions. *Kraken* was utilised with a barcoding system to generate master DNA layout graphics and to ensure fully automated tracking of the barcoded plates stamped with DNA on the SNP line. The Kraken Meridian Interface (Octopure-05) was used to stamp the DNA samples into the plates and the KBiosciences Dispenser Engine (Meridian) was used to aspirate and dispense 2.0µL of the KASPA mix (KASP 1X) and 0.028 µL primer mix into each well of the 384-well plates. The Kraken-Pheraster Global functions (SNP Viewer) was utilised automatically to cluster heterozygous alleles between two accessions in a mapping population. Consequently, the marker based genotyping data was segregated into two segregating clusters that represented the two alleles of the mapping population that was investigated. This discriminant data set was mapped to the corresponding chromosomal loci using *Map Manager* (Manly et al. 2001) was utilised in calculating linkage map distances for the SNP markers based on single-locus association and interval

mapping. Subsequently, a genetic linkage map was generated using *Map Chart* (2.3 RE Vorrips 2015) - (<https://www.wageningenur.nl/en/show/Mapchart-2.30.htm>).

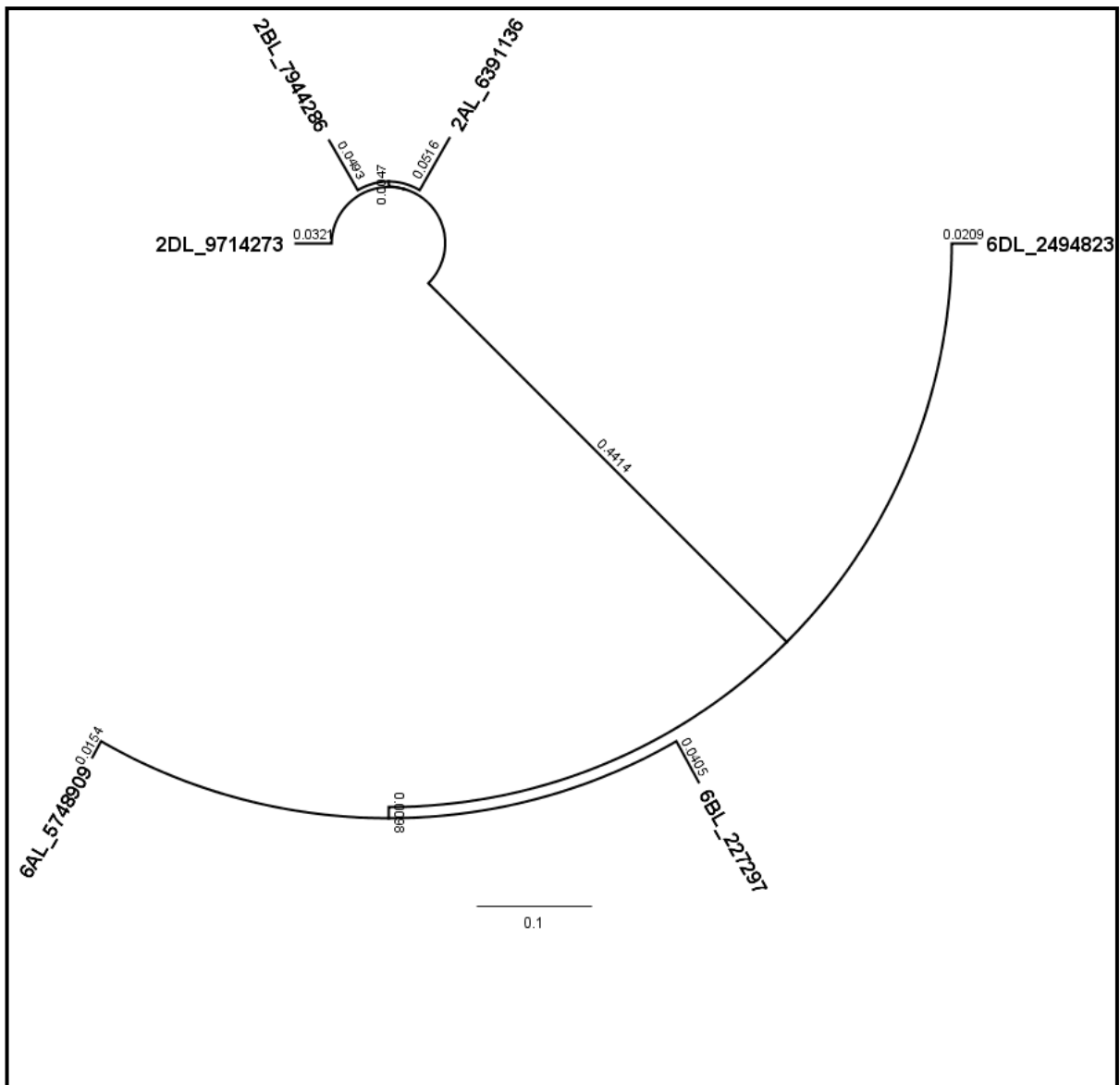
A diversity panel set of 100 wheat accession (Table 1) was genotyped for 25 SNP markers across *GS1.1A*, *GS1.1B*, *GS2A* and *GS2B*. To evaluate genetic diversity inherent in the diversity panel, two different approaches were used to assess the genetic structure: Hierarchical agglomerative clustering (R package hclust) and Principal component analysis (PCA, R function prcomp). The Hierarchical agglomerative clustering method was modelled on three different approaches used to assess the inter-genotype genetic structure (Griffiths et al. 1984; de la Vega et al. 2002; Crossa & Franco, 2004; Team R Core, 2013). This involved using pairwise dissimilarity measures first calculated all the lines (Hamming distance or number of inconsistent allele calls). This was then clustered the lines using two different hierarchical clustering methods (Crossa & Franco, 2004; Verbyla et al. 2012; Bonneau et al. 2013). The methods were “Complete” (“the furthest neighbour” method that groups data points by maximising the distance among the clusters and disregarding the homogeneity within the clusters), and “Ward” (“minimum variance” method that cluster data points by minimizing within-cluster variance and maximising between-cluster variance).

Principal component analysis and model-based high-dimensional (HD) clustering (R package HDclassif) were done using data recoded to numeric format (within marker: recoded to “1” if allele 1 and recoded to “2” if allele 2) and imputed with most common allele variant (missing values not allowed in HDclassif). PCA served to transform and visualise the data points of the individual lines in a reduced number of dimensions, for example along the first 3 principal components. Subsequently, the cluster assignments obtained with hierarchical clustering method described above were superimposed on the plot of the PCA and are presented as hierarchical clustering method (Ward minimum variance) and hierarchical method (Complete).

Most of the 100 wheat accessions were already covered in a related study that evaluated the N response of Australian wheat accessions under different N levels (Mahjourimajd et al. 2016). In that study, a total of five field experiments was conducted in the 2010 and 2011 growing seasons in South Australia with three replicates of stratified 18 - 87kgNha<sup>-1</sup> (with a single urea supply at planting). N response parameters were measured by Grain yield (kgha<sup>-1</sup>) and NUE which was quantified as the grain yield per unit of N supplied (kgGYkg<sup>-1</sup> N). The cluster assignments of 100 accessions in the current study was therefore, compared with the N response patterns recorded in the previous study. Based on this comparative assessment, the 100 wheat accessions were classified as *C1*= high, *C2*= low, *C3*= moderate and *C4*= variable N responsive (Fig. 12 & 13).

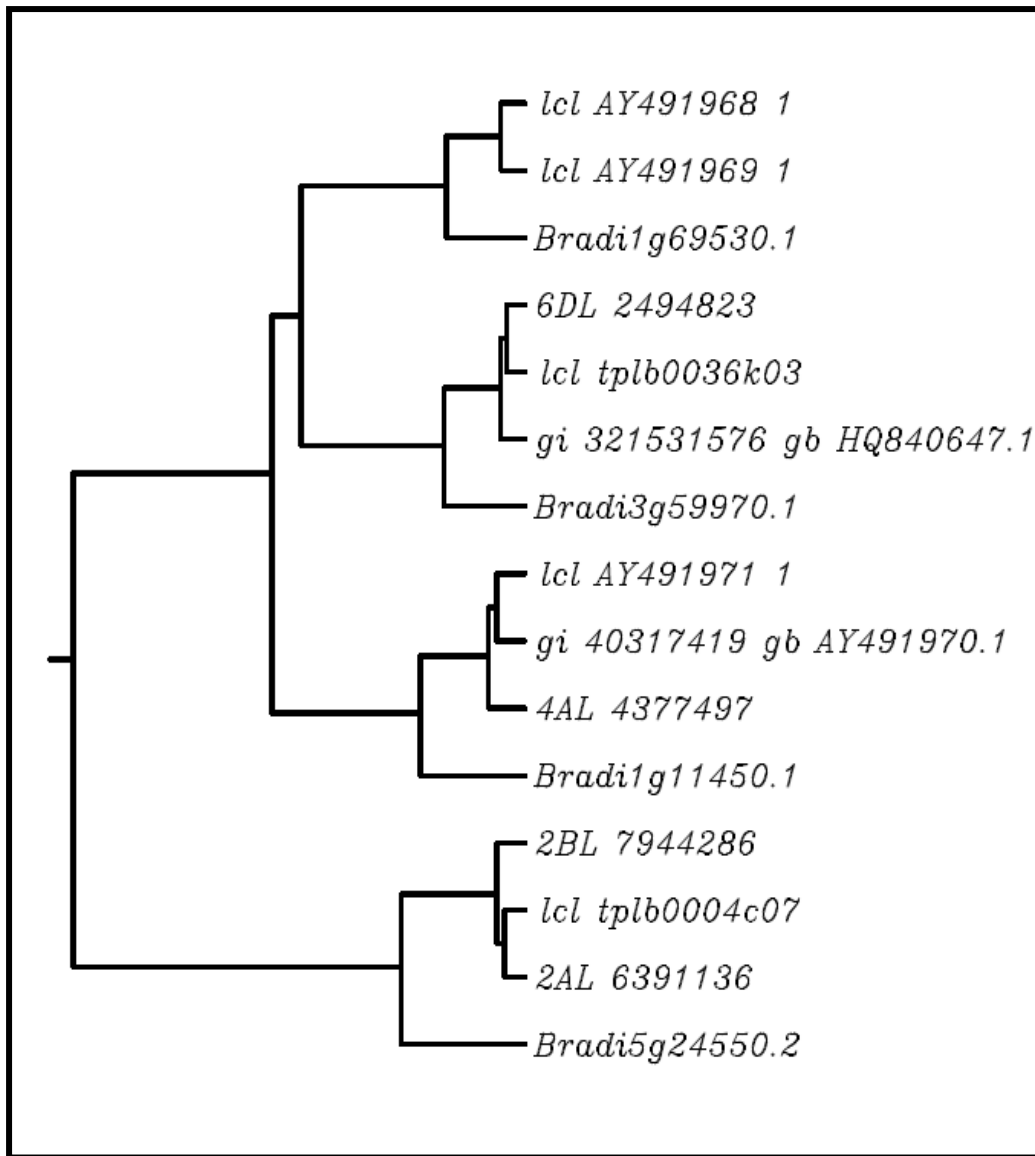
## 2.3 RESULTS

The sequence studies identified three chromosomes (*Chr.*) where GS genes are located: *Chr. 6 (GS1.1)*, *Chr.2 (GS2)* and *Chr.4 (GS1.4)*. Sequence analysis revealed clear diversity between the *TaGS1.1* and *TaGS2* reference gene sequences (Fig. 2.2) extracted from standard database archives. A search through standard gene databases showed considerable variation of the number of GS genes in different plant species. For example, in the *Phytozome* database there are 4 GS genes in Sorghum, 5 genes in maize, 4 genes in rice and *Brachypodium dystachyon*. A search in the National Center for Biotechnology Information (NCBI) database, revealed that there are 5 genes; *GLN1.1*, *GLN1.2*, *GLN1.3*, *GLN1.4* and *GLN1.5* in *Arabidopsis* and one *GLN2* gene. Similarly, maize *GLN1.4*, the wheat *GS1.4* genes are located on *Chr.4BS*, *Chr.4AL* and are considered as homologues (there has been a large translocation onto *Chr.4BS*).



**Fig. 2.2** Phylogeny of reference *GS1.1* and *GS2* sequences extracted from standard gene databases. Phylogeny reveals clear dichotomy between the two *GS* isoforms across all three wheat genomes.

There was evidence of gene duplication as was expressed to maize *GLN1.2*; there are three wheat *GS1.2* alleles also on chromosomes 4BS, 4DS and 4AL. On *Chr. 6*, the three alleles are maize *GLN2* types: (plastid-targeted) 2AL, 2BL, 2DL. From the comparison of the sequences with those of *Brachypodium* CDS, the genes identified are orthologous to the *Brachypodium* alleles (Fig. 2.3). A comparison of GS mRNA sequences was evaluated between species. For example, in maize it was found that for the GS2 reference transcript *>lcl/2AL\_6391136* (reverse complement), the deduced CDS was 90% identical to *>gi|71362454|gb|DQ124212.1| T. aestivum* plastid glutamine synthetase isoform *GS2A* mRNA (complete CDS) and a nuclear gene for the plastid product.



**Fig. 2.3** Phylogenetic profile of orthologous GS sequences extracted from standard gene databases.

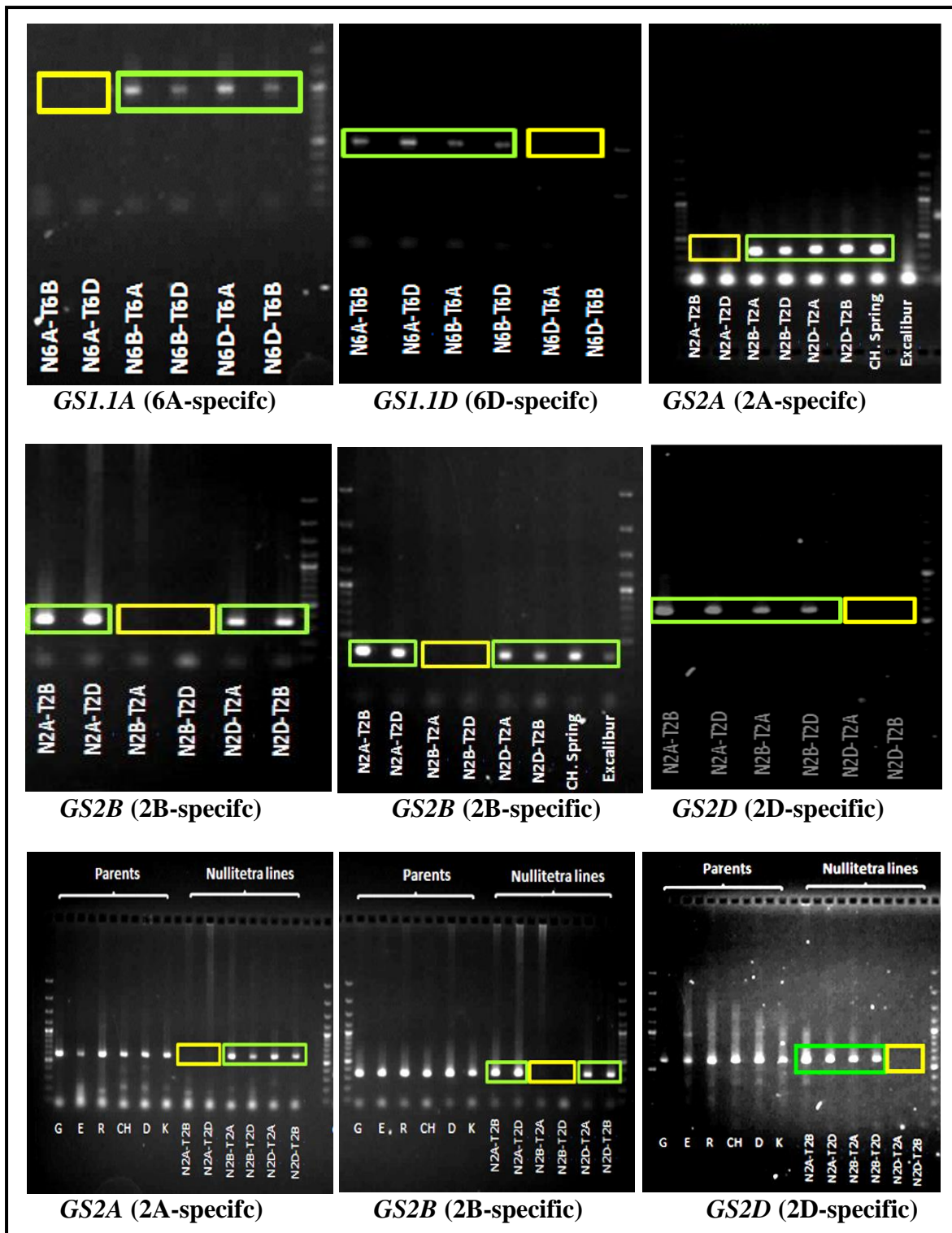
In the SNP marker genotyping analysis, four genes in the A and B genomes (*GS1.1A*, *GS1.1B*, *GS2A*, *GS2B*) were directly mapped to Yallaroi x AUS24152\_Janz population, one to the Gladius x Drysdale mapping population (*GS1.1A*) and one to the Excalibur x Kukri double haploid (DH) population (*TaGS2A*). Analysis of in-house genotypic data available for the Yallaroi x AUS24152\_Janz may produce important genetic information linked the SNP data

of the four GS genes that accurately mapped to genome-specific chromosomes, for this population. Each gene was specific to the respective genome and those genes of the A- genome were the longer (*GS1.1A*- 7981bp: *GS2A*- 8540bp). There were 10 exons in *TaGS1.1A*, 5 exons in *TaGS1.1B* and 11 exons in *TaGS1.1D*. All three *GS2* genes consisted of 14 exons. DNA data showed that the *TaGS1.1* and *TaGS2* conserved domain sequences extracted from the wheat accessions were genome specific.

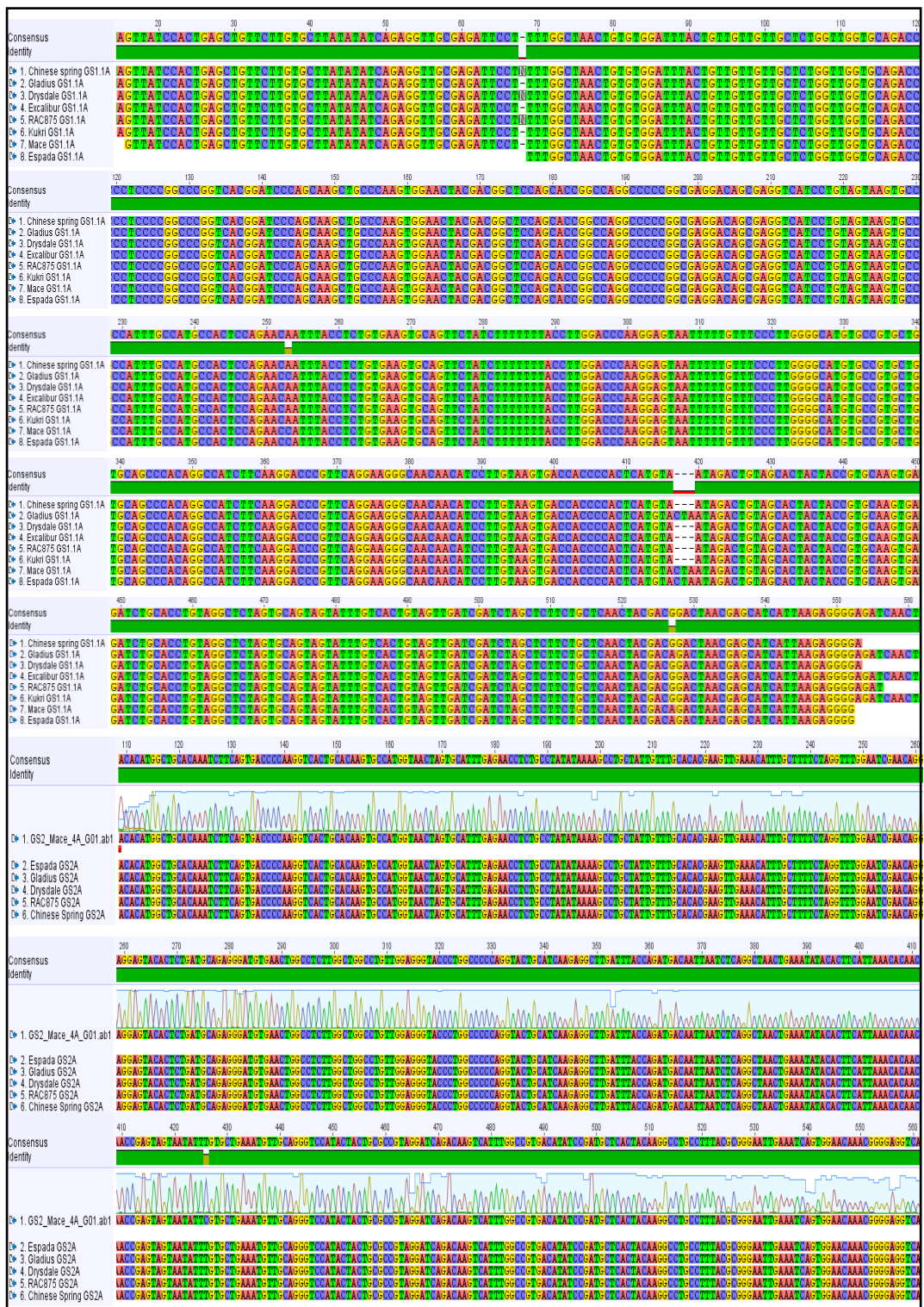
The PCR analysis completed with Chinese spring aneuploid lines produced no amplification of *GS1.1D* on N6DT6A aneuploid (Fig. 2.4), but showed clear bands for *TaGS1.1A* (A-genome specific) anchored to *Chr. 6A* and *GS1.1B* (B-genome specific) on *Chr. 6B*. Similarly, there was no amplification of the *GS1.1D* specific primers on the B-genome specific aneuploid as shown in Fig. 2.4, on *Chr. 6B*. While both *TaGS1.1A* and *TaGS1.1B* specific primers produced clear bands that confirmed amplification of the A and B genomes on *Chr.6 (TaGS1.1A)* and *Chr.2 (TaGS2A, TaGS2B)*. These results were consistent with the amplicon verification recorded for all *GS1.1* homologues. It was observed however, that all the *TaGS1.1* homologues were amplified in the *Chr. 2* localised aneuploid lines including N2BT2A (A-genome specific), N2BT2D (D-genome specific). Moreover, Agarose gel electrophoresis of the PCR products of nullisomic *TaGS2* aneuploids expectedly showed no amplicons for *TaGS2B* on N2BT2A and N2BT2D (Fig. 2.4). Moreover, the sequences from the eight lines revealed a high level of homology (98% identity) between *TaGS2A*, *TaGS2B* and *TaGS2D* and



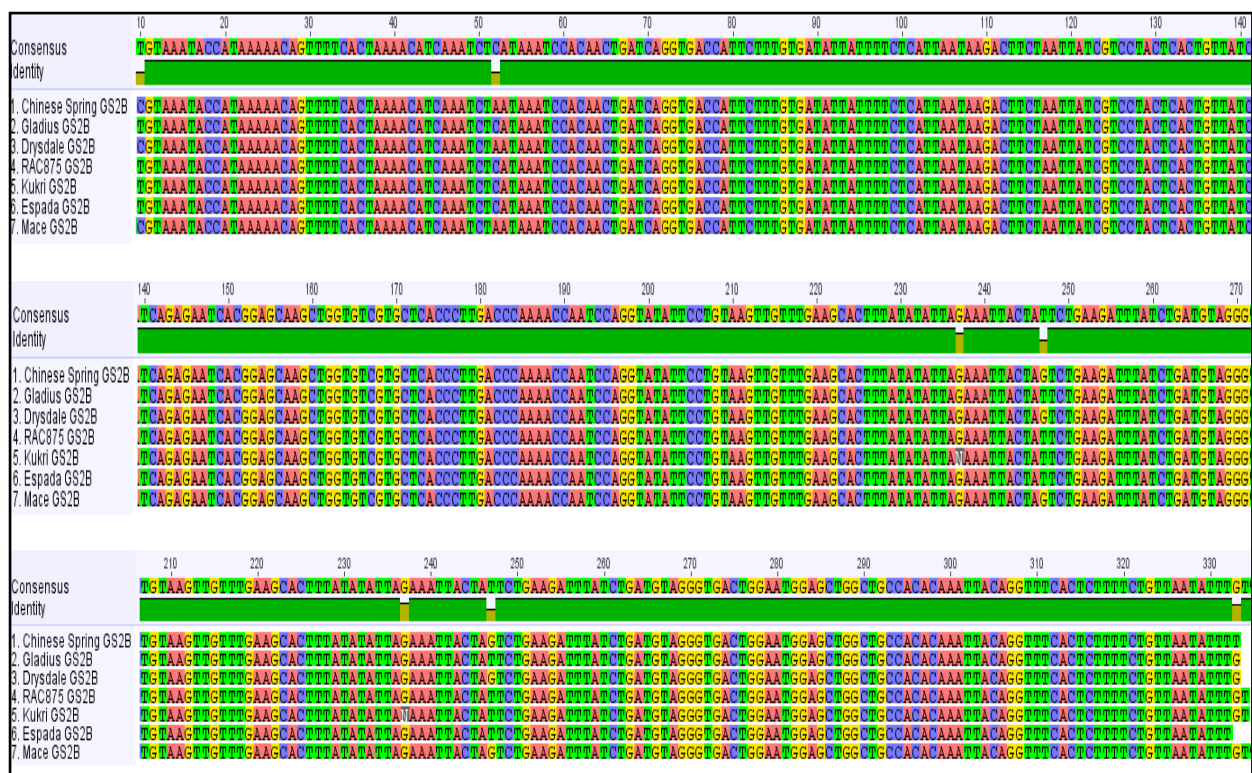
about 70% sequence homology between the *TaGS1.1* homologues (Fig. 2.5 & 2.6).



**Fig. 2.4** Gel electrophoresis image showing *TaGS1.1* and *TaGS2* homologue specific amplification (green box) of aneuploid tetrasomic lines and specific non-amplification (yellow box) of nullisomic lines using Chinese spring aneuploid lines used as control parameters: G - Gladius; E - Excalibur, R - RAC875; CH - Chinese spring; D - Drysdale; K - Kukri).

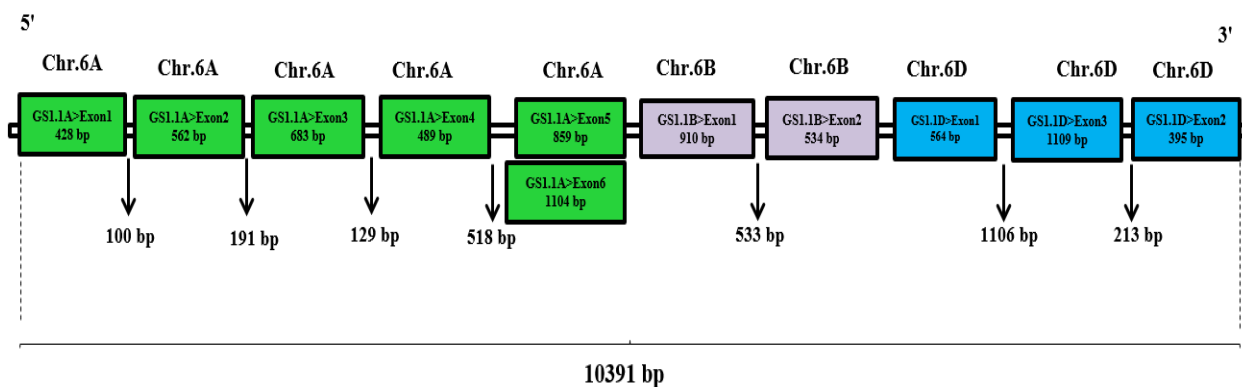


**Fig. 2.5** Sequence alignment of *TaGS1.1A* and *TaGS2A* spring wheat coding sequences shows high level of homology in the conserved domains.

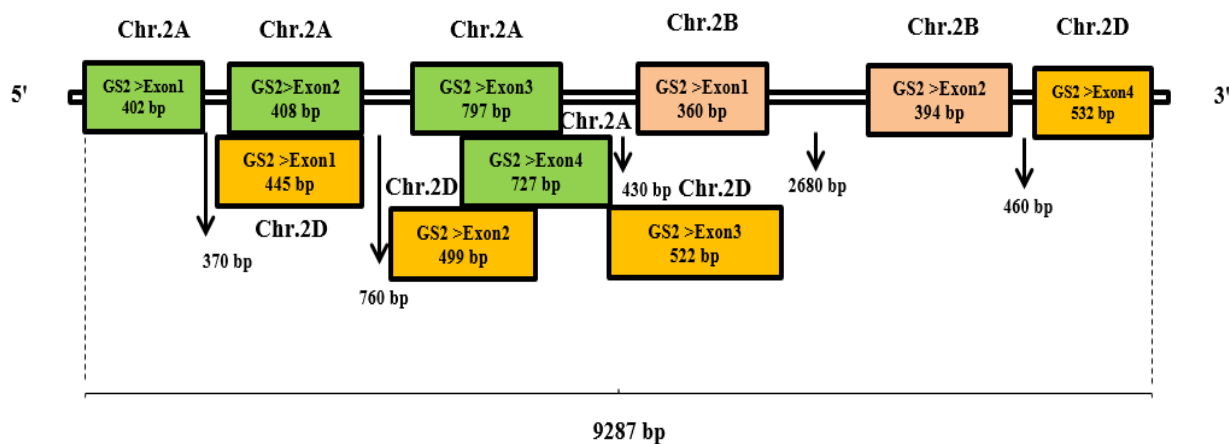


**Fig. 2.6** Sequence alignment of *TaGS1.1* and *TaGS2* spring wheat coding sequences shows high level of homology in the conserved domains.

The sequence comparison of *TaGS1.1* and *TaGS2* homologues among the lines confirmed high level of sequence similarity between lines and the reference GS sequences. An alignment of the conserved domains of *TaGS1.1* and *TaGS2* show up to 98% similarity between adjacent exons. There was considerable homology in *TaGS1.1A* between Exons 5 and 6, but not *TaGS1.1B* and *TaGS1.1D* (Fig. 2.7). Homology is shared between *TaGS2A* Exon 2 and *TaGS2D* Exon 1. There is also sequence similarity between of *TaGS2A* Exons 2 with *TaGS2D* exon 2. A similar level of homology was observed between *TaGS2B* and *TaGS2D* Exon 3 (Fig. 2.8).



**Fig. 2.7** Consensus map of wheat *GS1.1* homologue (reference sequences: (>lcl|6AL\_5820788, >lcl|6BL\_227297 and >lcl|6DL\_2494823 3419 46201) showing homology of the reference conserved domains (color boxes) and intervening non-conserved regions arrowed. Similar colour assignments represent exons of the same gene.

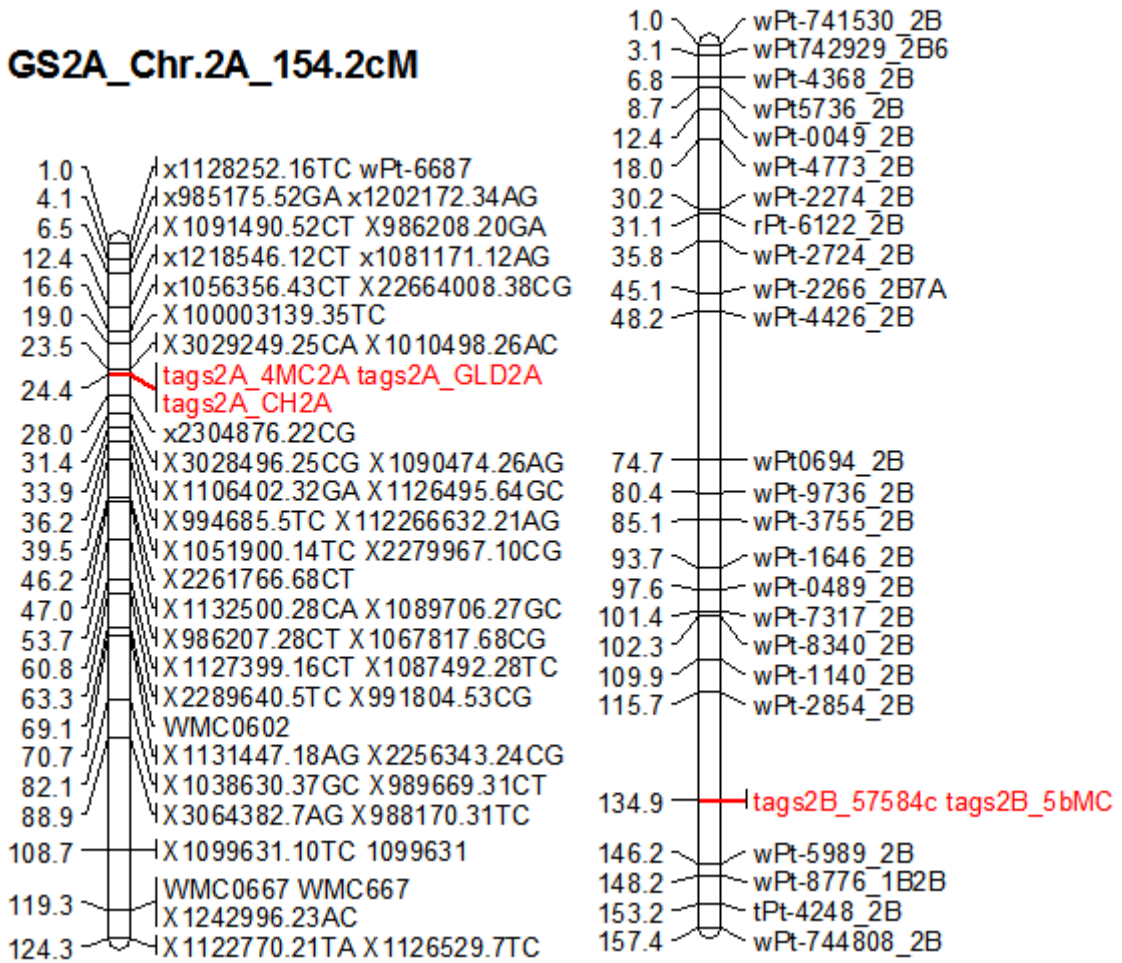


**Fig. 2.8** Consensus map of wheat *GS2* homologue (reference sequences: >lcl|2AL\_6391136; >lcl|2BL\_7944286 and >lcl|2DL\_9842556) showing homology of the reference conserved domains (color boxes) and intervening non-conserved regions arrowed. Similar colour assignments represent exons of the same gene.

The marker-based SNP genotyping of *TaGS1.1* and *TaGS2* genes showed clear bi-allelic variation with 20 of the 300 SNP markers genotyped in the diversity panel. There was clear and consistent segregation of allelic variants from the genotyping of two DH populations including RAC875 x Kukri, Excalibur x Kukri and recombinant inbred line (RIL) for the Gladius x Drysdale RIL population. The SNP markers specific to *TaGS1.1A*, *TaGS1.1B*, *TaGS2A* and *TaGS2B* were mapped to the expected chromosomal loci. For example, five *GS2A* markers including *tags2A\_42862*, *tags2A\_MC* or *tags2A\_GLD\_ek2471392* mapped to *Chr.2A* *tags2A\_42862*, *tags2A\_MC* or *tags2A\_GLD\_ek2471392* at 17.1 cM and 18.1 cM (Fig. 2.9). In addition, three *TaGS2B* markers (*tags2B\_574584*, *tags2B\_5bMC* and *tags2B\_Es\_2471396*) correctly mapped to the long-arm of *Chr. 2B* at 134.9 cM in the Excalibur x Kukri DH (Fig. 2.9). The same *TaGS2A* markers were mapped to *Chr.2A* in the Yallaroi x AUS24152 \_Janz mapping population (Fig. 2.10). Further, one *GS1.1A* SNP marker *tags1A\_13CH* (*tags1A \_sn1952823*) mapped at 74.1 cm in the Gladius x Drysdale recombinant inbred line population (Fig. 2.11).

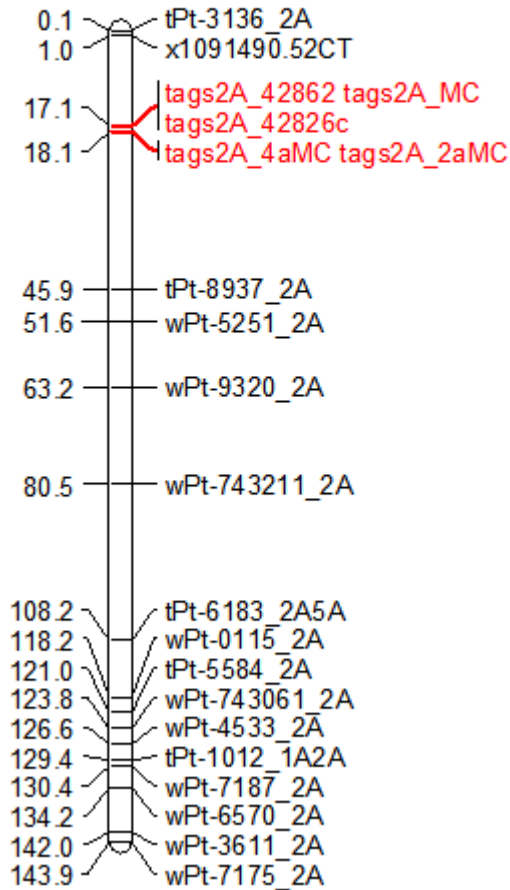
**GS2B\_Chr.2B\_157cM**

**GS2A\_Chr.2A\_154.2cM**

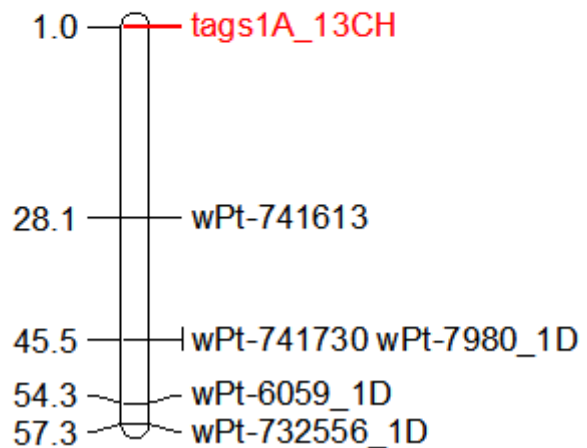


**Fig. 2.9** Linkage map showing the locus of conserved domain *GS2A* SNP markers on mapped to *Chr. 2A* in Excalibur x Kukri double haploid population and *GS2B* markers *tags2B\_574584*, *tags2B\_5bMC* and *tags2B\_Es\_2471396* (not shown) mapped to *Chr. 2B*. The locus and annotation of KASP markers are highlighted in red letters.

## GS2A\_Chr.2A\_145.9cM



## GS1.1A\_Chr.6D\_59.2cM

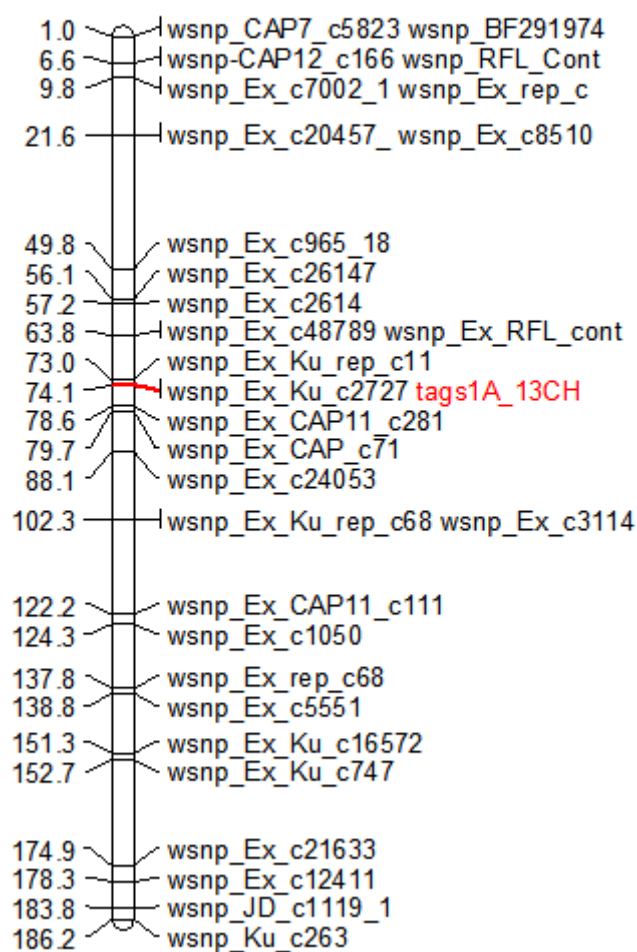


Janz x Yallaroi mapping population      Excalibur x Kukri double haploid population

**Fig. 2.10** Linkage map showing the locus of conserved domain *GS2A* SNP markers *tags2A\_42862*, *tags2A\_MC* (*tags2A\_GLD\_ek2471392*) on *Chr.2A* Janz x Yallaroi mapping population and *GS1.1A* *tags1A\_13CH* (*tags1A\_sn1952823*) gene on *Chr.6D* in the Excalibur x Kukri double haploid population. The locus and annotation of KASP markers are highlighted in red letters.



## GS1.1A\_Ch6A\_192.1cM

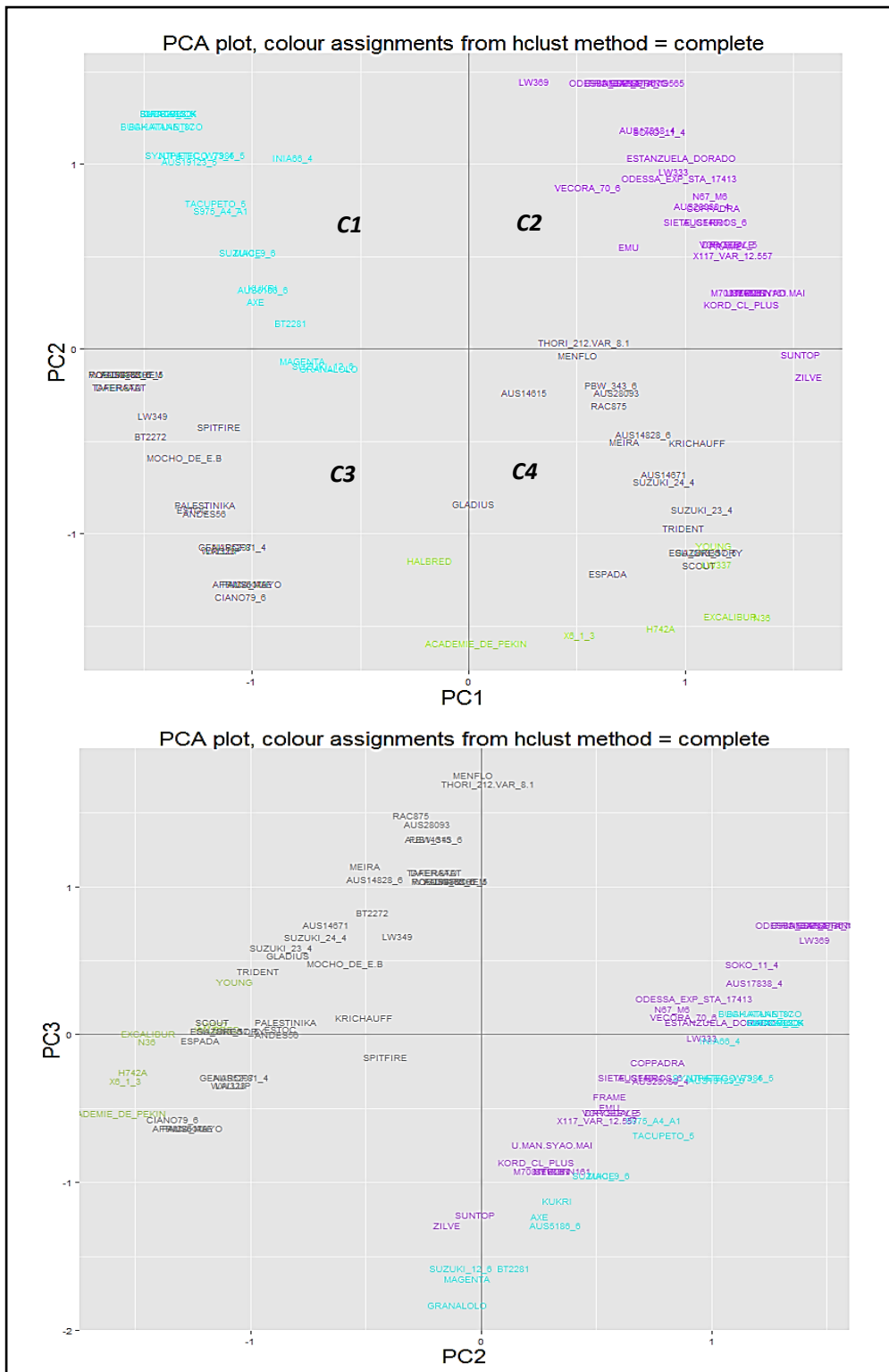


### Gladius x Drysdale RIL population

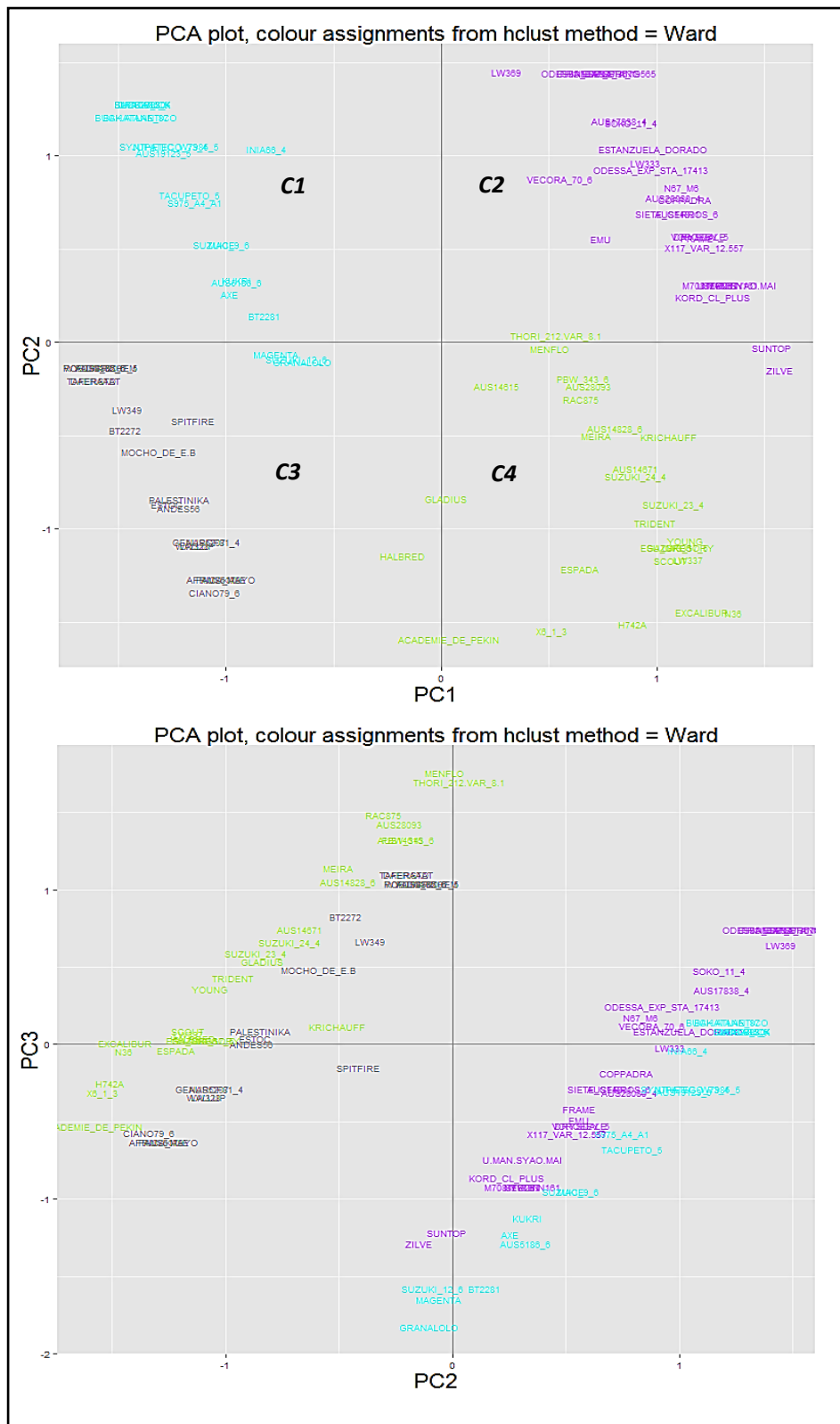
**Fig. 2.11** Linkage map showing the locus of conserved domain *GS1.1A* SNP marker *tags1A\_13CH* (*tags1A \_sn1952823*) on *Chr. 6A* among Gladius x Drysdale recombinant in-bred lines. The locus and annotation of KASP markers are highlighted in red letters.

Principal component analysis of the diversity panel accessions that the wheat accessions were clustered per N response and communal breeding background. The marker-trait linkage assessment of the first three principal components (PC1, PC2, PC3) of the genetic structure assessment were identified and presented in colour assignments that indicate cluster assignments from different methods (refer to Fig. 2.12 & 2.13 titles). Overall, the results of PCA of KASP data indicate a sub-structure (sub-populations), as most of the variation (90%) is explained by seven first principal components (Fig. 2.12 & 2.13). The results of model-based HD clustering indicated that the best model has four clusters. The classification of the four clusters (C1-high N response, C2-low N response, C3- moderate N response and C4-variable N response) are presented in the second principal component (PC2). The cluster groups are consistent with N response data (recorded in separate experiments) from field trials involving four different wheat cultivation areas across Australia. The C2 cluster mostly contain CIMMYT (International Maize and Wheat Improvement Centre) wheat accessions. The PCA algorithm served to transform and visualize the data points of the individual lines in a reduced number of dimensions, for example along the first three principal components, as shown in the cluster assignment of 100 accessions.

The cluster assignments obtained with hierarchical clustering described above were superimposed on the plot of PCA and are presented below for method *Complete* (Fig. 2.12) and method *Ward* (Fig. 2.13). The hierarchical agglomerative clustering analysis revealed four high-level major groupings of lines, of cluster assignments mostly consistent between the methods “complete” and “Ward” (minimum variance)”. However, the dendrogram of hierarchical clustering based on KASP data strongly suggested that there is a lot of sub-structure (large number of sub-populations) within the major groupings. The cluster data of PC1 and PC2 in both *Ward* and *Complete* models showed 4 sub-populations which differentially corresponded to results of clearly distinct N response clusters (high, medium, low and variable) which were reflective of the observed patterns produced in the study of Australian wheat accessions undertaken by (Mahjourimajd et al. 2016).



**Fig. 2.12** Cluster assignment of four GS genes (*GS1.1A*, *GS2A*, *GS2B* and *GS2D*) linked with contrasting N response in wheat. The plots of the first 3 principal components of the analysis based on KASP marker data. The colour code indicates the cluster assignments due to hierarchical clustering method “complete”. Cluster assignments are defined in terms of N response: *C1*= high, *C2*= low, *C3*= moderate and *C4*= variable.



**Fig. 2.13** Plots of the first three principal components of the analysis based on KASP marker data for four GS genes (*GS1.1A*, *GS1.1B*, *GS2A*, *GS2B*). The colour code indicates the cluster assignments due to hierarchical clustering method “Ward”. Cluster assignments are defined in terms of N response: *C1*= high, *C2*= low, *C3*= moderate and *C4*= variable.

## 2.4 DISCUSSION

The phylogenetic assessment of closely related cereal species and *Arabidopsis* exposed that the different allelic variants of GS genes are orthologous and there are multiple gene copies across the three genomes. It is suspected therefore that, there was extensive genetic divergence from the ancestral gene pool of these GS isoforms. Gene flow analysis suggests that there may be additional gene copies in wheat similar to the *Arabidopsis* genes that arose after speciation (Xu & Messing, 2006; Rieseberg & Blackman, 2010; Feder et al. 2012). Homology studies of the conserved domains shows also that considerable genetic differences may be present within intronic regions that may account for the generally observable structural and possibly functional variation in GS genes. Consequently, intronic domain polymorphism need not be classified as “junk” and disregarded in any attempt at GS allele molecular characterisation. Altogether, 300 KASP markers were targeted at SNPs in the conserved domains of all the *Chr.6* and *Chr.2 TaGS* gene sequences extracted *in vivo*.

However, alignment of the gene sequences from *TaGS1.1D* and *TaGS2D* (representing genes from the D genome) showed that the majority of the markers lacked polymorphism (at SNPs linked to *D-genome* genes (*TaGS1.1D* and *TaGS2D*) for all accessions within the mapping populations investigated. The absence of polymorphism at the targeted SNP loci in the D genome genes is attributable to the high level of synteny in the conserved domains. Concatenation studies of long history genes in rice and *Arabidopsis* produced equally high level

of synteny on chromosomes on Groups 1 and 2 (Salichos & Rokas, 2013). This is indicative of the high level of sequence conservation and reduced number of allelic variation existing at the SNPs of the GS genes. Generally, selective breeding over a long period is expected to reduce the level of genetic diversity (Smith & Cooper, 2004) between the GS genes. This may imply that despite a long history of speciation and genetic divergence from the original hybridisation of the diploid *Aegilops tauschii* and tetraploid *Triticum turgidum* (Jia et al. 2013; Wang et al. 2014), there is continuing genetic conservation among homologues of the different GS genes in the hexaploid *T. aestivum* as has been concluded for another species. Alternatively, this could be explained because of the increased selection for desirable accessions. This process effectively reduces genetic diversity and creates a convergent domestication bottleneck of the wheat species (Shavrukov et al. 2016).

A crucial finding that emerges from the review of the SNP genotyping results is the specific mapping of markers to the expected loci on genome-specific chromosomes within mapping populations. The fine mapping of four (*TaGS1.1A*, *TaGS1.1B*, *TaGS2A* and *TaGS2B*) different genes across different mapping populations make these SNP markers ideal candidates for allelic segregation across a larger number of wheat populations. This opens the potential of exploring the markers that map directly or proximately to the GS based SNP markers for N-linked traits. The variation in the number of GS genes accurately mapped to the different mapping populations explains the context of allelic variation

between the parents of the respective populations. Despite differences in the number of genes accurately mapped, there were observable consistencies when comparing the bi-allelic cluster patterns of the SNP markers mapped to different mapping populations (Wang et al. 2014). Such consistent segregation pattern was obvious between the Gladius x Drysdale and Diversity panel that showed highly consistent distribution of the segregate accessions (refer to Appendix Fig. A2.1, A2.2 and A.2.3).

An assessment of the accessions within the diversity panel based on the comparative N response clusters indicated strong similarity of the accessions within the clusters. Within KASP dataset assessed, the cluster assignments of individual lines conformed to clearly distinct genotype groups that originated from the same breeding programme. The marker-trait and linkage assessment was effective in distinguishing genetic variance (Leonforte et al. 2013; Gill et al. 1991) within the diversity panel dataset and assess the cluster assignments of individual lines within the population. Within KASP dataset assess the robustness of cluster assignment to linkage group was sustained by using two different clustering methods (*Ward* and *Complete*). When comparing cluster assigns derived from KASP data, it was evident that there was no clear distance-separation between different clusters (Crossa & Franco, 2004; Verbyla et al. 2012; Bonneau et al. 2013). Secondly, the groupings due to different methods roughly corresponded between *Ward* minimum variance method and model-based HD clustering, while the *Complete* method tended to conservatively put



most lines into two major clusters. Finally, in the PCA the first three principal components combined explained only 18% of the variance. To account for most of variance, (example, 75% of variance) one need to look at the first 40 principal components. However, there were clear genetic clusters for the SNP genotyping based on the *TaGS* gene based KASP markers.

It was evident that accessions related by pedigree tended to cluster together. For example, the *C2* cluster are generally known to be International Maize and Wheat Improvement Center (CIMMYT) bred lines, while the *C4* cluster were largely bred as Australian Grain Technologies (AGT) lines. From the field study of wheat accessions and the response to varied N treatments, undertaken in South Australia (Mahjourimajd et al. 2016) these accessions are generally identifiable with clearly contrasting N responses (GY, kg ha<sup>-1</sup>, harvest at 15 % moisture content). It appears therefore that, the wheat accessions that originated from a common breeding background inherited similar genetic marker traits because of pedigree selection; in this case, SNP-marker based genetic classification that tended to generate clear patterns consistent with clearly distinct N response patterns.

## 2.6 CONCLUSION

The conserved domain analysis provides evidence of clear genetic and allelic variation between wheat *GS 1.1* and *GS2* genes. There is also consistent sequence similarity between homologues of the two genes. The amplification tests of aneuploidy lines with genome-specific DNA markers confirmed that the cytosolic *TaGS1.1A*, *TaGS1.1B* and *TaGS1.1D* were sequentially anchored to *Chr.6A*, *Chr.6B* and *Chr.6D*. Moreover, the plastid *TaGS2A*, *TaGS2B* and *TaGS2D* were anchored to *Chr.2A*, *Chr.2B* and *Chr.2D*. This confirms the unique genome identity of each gene in the GS complex. The linkage-trait analysis affirmed the presence of sizeable genetic sub-structure within the four major clusters confirming that the diversity panel lines are genetically quite diverse.

This information will be a useful to identify allelic variation among the GS genes and can provide the genetic basis for developing molecular tools needed to annotate potential GS functional diversity within a large wheat genotype set. This is expected to contribute to genetic diversity data and molecular information that could be a reliable reference for breeding N-efficient hexaploid wheat lines. Analysis of the genotypic data available for the specific mapping populations may produce information on QTL and possible functional annotation of the SNP loci of the four GS genes characterised and accurately mapped to genome-specific chromosomes.

## CHAPTER THREE

### FULL-LENGTH GENE SEQUENCING TO CAPTURE PHYLOGENY AND ALLELIC VARIATION IN WHEAT GLUTAMINE SYNTHETASES (GS)

#### 3. ABSTRACT

Forty-eight full-length DNA sequences representing six *TaGS1.1* and *TaGS2* alleles identified by sequence comparison with maize and barley Glutamine synthetase (GS) sequences, were amplified from 8 hexaploid wheat accessions on an *Illumina-MiSeq* next generation sequencing platform. The sequences were consolidated through multiple gene *de novo* sequence assembly and alignment with the correspondent genome-specific reference sequences. Phylogenetic analysis revealed a clear divergence at molecular level between cytosolic *TaGS1.1* and plastid *TaGS2* and an even wider genetic divergence among the plastid GS alleles.

A modelling system based on a translation of the full length *TaGS1.1* and *TaGS2* mRNA sequences showed that the dominant amino acid residues in the active sites were aspartic acid, glutamic acid and arginine (*TaGS1.1*, *TaGS2*); threonine, tyryptophan and leucine (*TaGS2*). In addition, a comparative assessment of GS orthologue sequences between the isolated wheat sequences and other plant species revealed high-level sequence homology between the wheat and barley *GS2* sequences. Kukri *GS2B* was homologous with *Glycine max* *GS2*.

The allelic variation among GS genes highlighted in this study will be crucial in determining whether functional variation exists between the GS alleles at the molecular level which might be explored further for the potential to enhance nitrogen use efficiency (NUE) in hexaploid wheat.

### **3.1 INTRODUCTION**

The uptake of N in higher plants is a biosynthetic process controlled by complex mechanisms that are regulated by a number of gene classes. Cytosolic glutamine synthetase (*GS1*) and plastid glutamine synthetase (*GS2*) are alleles in the multigene GS family that synthesizes glutamine. The cytosolic GS isoform (*GS1*) has largely been associated with root enzyme activity, ammonia translocation. Conversely, the plastid form (*GS2*) has been shown to recycle photo-respiratory ammonia and its activity is regulated by light and N stress (Habash et al. 2001; Hirel et al. 2001; Avila-Ospina et al. 2015; Wang et al. 2016). Generally, metabolic processes directed by GS alleles are finely regulated at the molecular level. In order to explore the correlation between sequence differences in functional domains and N metabolism, it is necessary to investigate biochemical reactions across accessions. Key aspects of N metabolism in plants may well be better understood by studying the allelic variation at individual loci, through comparative assessment of molecular functions (Yamaya & Kusano, 2014; Zheng et al. 2015) of the genes (protein domain functions). This may provide information relating to the genetic control of N metabolism.

Generally, gene function is a product of the genetic conformation (Seabra & Carvalho, 2015; Pantoja-Uceda et al. 2016) of molecular constituents at gene conserved domains (CD). CDs are regions within DNA sequences; RNA or protein sequences existing across orthologous or paralogous sequences and may indicate conservation within a gene. Due to the link to N metabolic activity, the CDs of GS genes could be crucial genetic diversity indicators containing polymorphic loci in the coding sites fundamental to gene function (Goodall et al. 2013; Gupta et al. 2013; Nigro et al. 2013). Functionally, GS enzymes are known to catalyse the assimilation of inorganic N in the form of ammonia and subsequently mediate N remobilisation during reproductive development. The level and localisation of enzyme activity may be markedly different between accessions because of post-translational modification (PTMs).

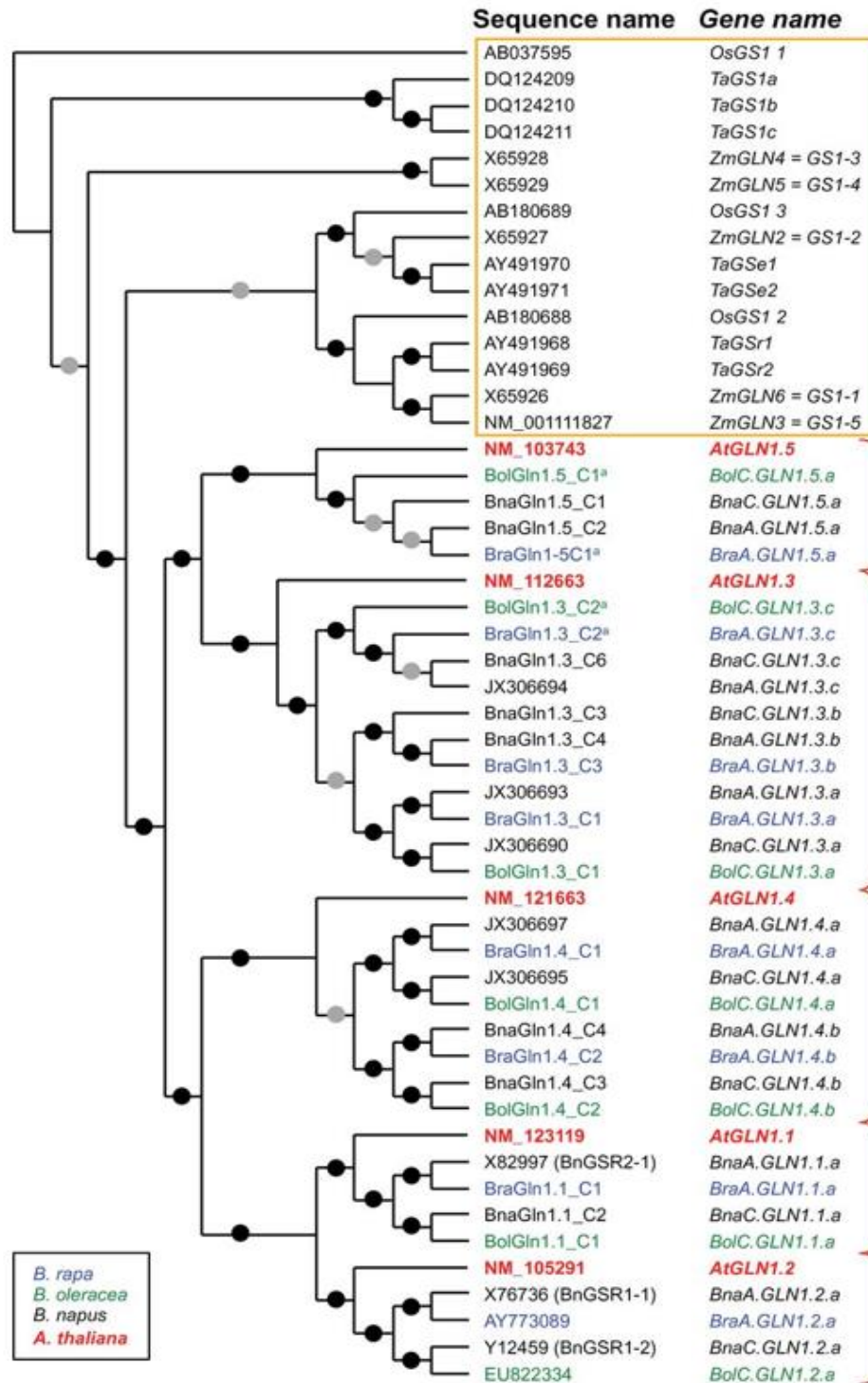
Several PTMs have been shown to significantly alter gene function. Liaw et al. (1993) demonstrated that adenylation could interrupt the functional roles of GS genes. A common form of PTM is phosphorylation that may involve (the addition of phosphate functional groups). Phosphorylation is a key mechanism for regulating enzyme activity in plants. For example, studies by Finnemann & Schjoerring (2000) has shown that phosphorylation of *GS2* is higher than that of *GS1* and that this pattern is dependent on the age and incidence of leaf senescence. Based on the evaluation of detailed studies of GS activity and active sites it has been suggested that a number of GS alleles may actually be altered by post-translation modifications that change gene biosynthetic functions (Moorhead et

al. 1999; Riedel et al. 2001; Ortega, et al. 2012). For example, PTM studies of *Medicago truncatula*, a species that has 98 % identity to maize GS (GLN), have shown a high level of variation between GS alleles (Lima et al. 2006).

Additionally, mutations in GS genes have been profiled and sometimes associated with yield effects in model species like maize (Martin et al. 2006; Weissmann et al. 2016), wheat (Lan et al. 2013) and rice (Tian et al. 2015) and *Arabidopsis thaliana* (Somerville & Ogren, 1980; Guan et al. 2015). GS genes play a principal role in converting ammonium into amino acids (Lea, 2014). Therefore, by comparing amino acid variation in different GS alleles, it may be possible to detect functional changes to GS activity of *GS1.1* and *GS2* genes and associate amino acid variation with NUE traits in wheat. Ideally amino acids at polymorphic regions should be indicators of possible allelic variation connected to GS function, as they constitute fundamental units (monomers) of proteins and they contain amine (-NH<sub>2</sub>) and carboxylic acid (-COOH) functional groups which determine protein functions.

Another approach to analyse allelic variation in wheat is through phylogenetic studies of the various homologues. These can possibly be isolated across a diverse panel of accessions (Ortega, et al. 2012; Pineda-Hidalgo et al. 2013). Phylogenetic analysis is an effective method to understand differences of heritable traits such as NUE variables in different accessions. Such analysis can serve as an effective analytical tool to infer the genetic relatedness of a large number of genes and accessions by comparing homology and polymorphism in the sequences. Phylogeny is therefore, generally important for explaining both the ancestral background homology and genetic distance between different accessions (Ahn et al. 1993; Bortiri et al. 2002; Benett & Mathews, 2006; Baker et al. 2011). Orsel et al. 2014 conducted a comprehensive study of cytosolic GS (GS1) (Fig. 3.1). Cytosolic GS isoforms including *BnaGLN1.1*, *BnaGLN1.2*, *BnaGLN1.3*, *BnaGLN1.4*, and *BnaGLN1.5* were sequenced and phylogenetically compared with mRNA contigs from *Brassica napus*, *B Brassica rapa*, and *B Brassica oleracea*. The comparison revealed high sequence identity with 5 *GLN1* sequences from *A. thaliana*. The phylogeny of the *BnaGLN1* sequences clustered separately into the cereal phylogenetic groups and non-cereal species groups. That phylogenetic study identified homology (Hampson et al. 2003) of *A. thaliana* glutamine synthetase *AtGLN1* mRNA in *Brassica napus*, *Brassica rapa*, and *Brassica oleracea*. The analysis also confirmed that *GLN1* mRNA sequences for wheat, maize, and rice were distinct from and *B. napus*, *B. rapa*, and *B.*

*oleracea* showing a clear dichotomy between monocotyledonous and dicotyledonous species.



**Fig. 3.1** Cytosolic glutamine synthetase (*GSI*) phylogenetic clustering. Source: Orsel et al. 2014. Journal of Experimental Botany; 65:3927-3947.

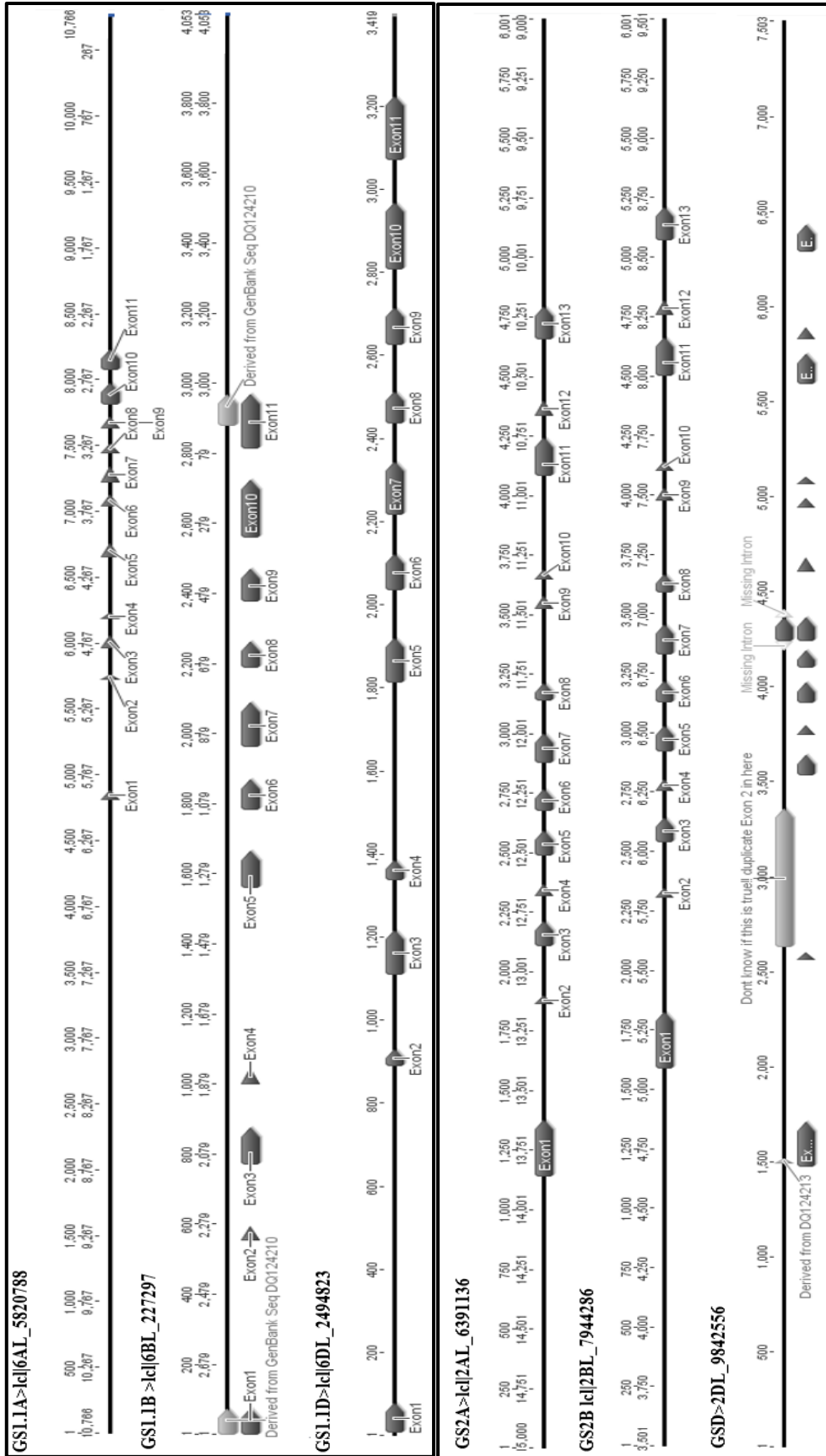


Despite the availability of polymorphic domain gene function studies in barley (Goodall et al. 2013), maize (Dell'Acqua et al. 2015; Weissmann et al. 2016) and rice (Funayama et al. 2013; Ishimaru et al. 2013; Yamaya et al. 2014; Zheng et al. 2015). There is currently very little additional published material available on full-length GS alleles in wheat (Li et al. 2011). Notwithstanding the considerably large number of accessions investigated and accuracy of the information provided on exon and intron profiles, only three genes (plastid *TaGS2-A1*, *TaGS2-B1* and *TaGS2-D1*) were examined in Li et al. (2011); the cytosolic *TaGS1* genes were not studied making it difficult to make any meaningful genetic comparisons between cytosolic and plastid forms of the GS gene. The current study aims to present a comprehensive assessment of both cytosolic and plastid GS. This will be important for clarifying the functional roles and allelic diversity of N metabolism genes (Gupta & Dhugga, 2013; Nigro et al. 2013). This investigation aims to characterise GS allelic variation in a range of Australian wheat accessions.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Sequence Assembly and Identification of GS Genes

GS reference gene sequences from *Hordeum vulgare*, *S. bicolor*, *Oryza sativa* and *Zea mays* were extracted from the standard gene sequence assembly NCBI (<http://www.ncbi.nlm.nih.gov/gene>). Full-length sequencing of six GS genes were based on this information using high throughput sequencing of the genes in nine selected wheat accessions. The reference sequences for each GS allele were aligned with corresponding alleles of different species. This was consolidated across different genomes from different species, with separate consensus alignments between the homologues of *GS1.1* and *GS2* genes. The GS alleles investigated included *GS1.1A* (>lcl/6AL\_5820788 rev.com - 11kb), *GS1.1B* (>lcl/6BL\_227297 reverse complementary 5.4kb), *GS1.1D* (>lcl/6DL\_2494823 - 4kb), *GS2A* (>lcl/2AL\_6391136; - 10.6kb), *GS2B* (>lcl/2BL\_7944286 -10.4kb) and *GS2D* (>2DL\_9842556-10.7kb). The gene map of the reference sequences is displayed in Fig. 3.2.



**Fig. 3.2** Complete reference and sequence map of *TaGS1.1* and *TaGS2* gene sequences extracted from NCBI and other standard databases.

### 3.2.2 Primer Design and Gene Sequence Amplification

DNA samples were extracted by phenol method and purified from 8 DNA samples representative of the 9 wheat accessions (Gladius, Drysdale, Excalibur, Chinese Spring, RAC875, Kukri, Mace, Scout and Espada). DNA concentration and purity was checked by Nanodrop on a Thermo Scientific NanoDrop 2000 and 2000c (<https://www.thermofisher.com/order/catalog/product/ND-2000>) in a ratio of absorbance 260 - 280 nm. The full-length gene sequences were isolated in polymerase chain reaction (PCR). The primer pairs used in the PCR, targeted full coverage of the corresponding gene sequences. Genome-specific primer sets were designed using the *Geneious* software package (<http://www.geneious.com>, Kearse et al. 2012) and the annealing temperatures standardised by using the *New England BioLabs Tm Calculator* (<http://tmcalculator.neb.com/#/>). Primers were designed 100 bp upstream from both 5' and 3' terminus of the genome specific *GS1.1* and *GS2* alleles (Glass & Donaldson, 1995). The primers were used in a standard PCR experiment to generate amplicons (4 – 10kb) from DNA samples. The amplicons consisted both exons and introns representative of each gene across the eight wheat accessions. The fragments representing the GS alleles were amplified when Immolase polymerase (<http://www.bioline.com/au/immolase-dna-polymerase.html>) was used in the PCR reaction mix in the protocol. The reaction included Immolase polymerase 0.03 $\mu$ L (5U/ $\mu$ L), DNA – 3.0 (50ng/ $\mu$ L), dNTPs (0.2mM) – 1.6 $\mu$ L, Immolase Buffer – 1.0 $\mu$ L, MgCl<sub>2</sub> – 0.5 $\mu$ L, Forward Primer – 0.5 $\mu$ L (10 $\mu$ M), Reverse Primer – 0.5 $\mu$ L (10 $\mu$ M), Water 2.87 $\mu$ L.

However, when the DNA fragments representing the larger GS alleles could not be amplified, the PCR protocol was modified to use Phusion taq and the PCR protocol further optimised. For PCR protocol optimisation, the reaction volume was altered in a reaction mix of 10 $\mu$ L as follows: DNA - 3.0 $\mu$ L (1.5mM), water - 1.2 $\mu$ L, Phusion HF Buffer – 2.0 $\mu$ L, DNTPs (0.2mM) – 1.6 $\mu$ L (1.25mM), forward and reverse primers – 2.0 $\mu$ L, Phusion Polymerase - 0.2 $\mu$ L). Phusion High-Fidelity DNA Polymerase was used due to the suitability for maximal success with minimal optimisation, high fidelity (>50 times greater than *Taq* polymerase and reduced extension times (10 times faster than *Pfu*) and high yield output in terms of increased product yield using minimal amount of enzyme (Chester & Marshak, 1993; Frey & Suppman, 1995). To verify the molecular size of each fragment and detect the presence of primer dimers, 3.0 $\mu$ L of SYBR Safe DNA Gel Stain (<https://www.thermofisher.com/order/catalog/product/S33102>) was added to an aliquot (7.0 $\mu$ L) of the PCR product. This product was centrifuged and run using 1.0% Agarose gel electrophoresis for 80min after which the gel image was visualised using Bio-Rad Gel Imaging System (<http://www.bio-rad.com/en-au/category/gel-imaging-systems>). The optimum conditions (details of the Phusion-based PCR protocol as described above) for the primer sets were confirmed to yield the expected amplicon sizes at 60.0°C. This was without addition of MgCl<sub>2</sub>. The PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) – Macherey-Nagel Purification

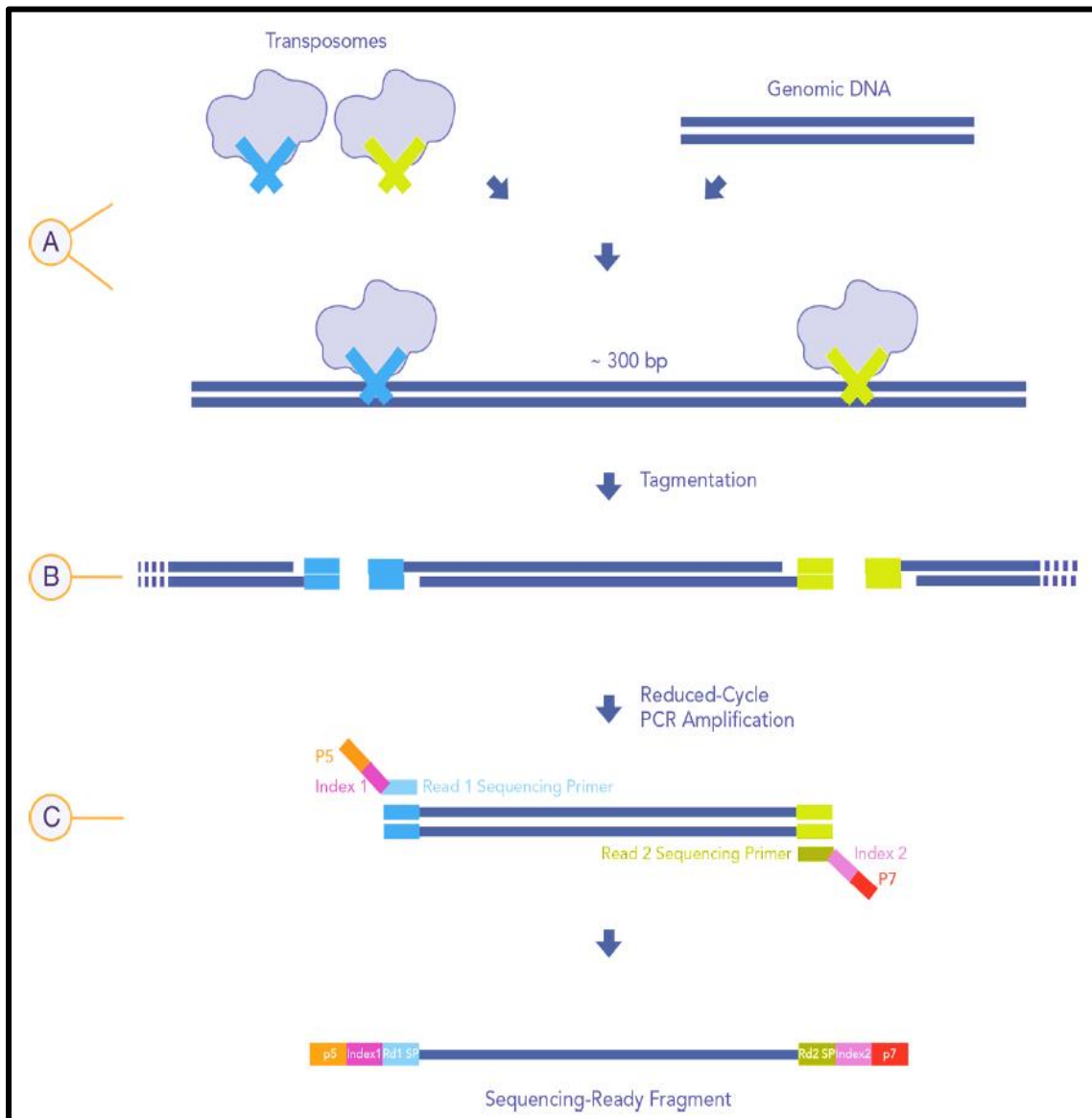
Kit prior to purification and preparation for sequencing on the Illumina *MiSeq* platform MO2262 (Quail et al. 2012).

### 3.2.3 Next Generation Sequencing with MiSeq Sequencing Tools

Nextera DNA Sample Preparation Kit (Illumina) was used to generate multiplexed paired end sequencing library of the wheat accessions ([http://www.illumina.com/products/nextera\\_dna\\_library\\_prep\\_kit.html](http://www.illumina.com/products/nextera_dna_library_prep_kit.html)). This kit applies an engineered transposome to simultaneously fragment and tag the DNA template and adds a unique adapter to the respective sequence. The adapter is then employed to amplify the DNA template in a modified PCR reaction that indexes DNA sequences on both terminuses (Fig. 3.3). There were 3 main steps prior to sequencing the amplified genes on the Illumina *MiSeq* MO2262: Tagmentation, Purification and Indexing (refer to Appendix for sequencing cycle and index data – Fig. A3.1 and Fig. A3.2).

The quantification of each step was prepared as follows: Tagmentation - TD Buffer = 5.0 $\mu$ L, TD1 = 1.0 $\mu$ L, DNA (2.5ng/ $\mu$ L) = 4.0 $\mu$ L, 10 $\mu$ L total, 55°C for 5 mins (thermocycler). Purification with Ampure Beads (0.8 x beads to product) added to 15.0 $\mu$ L water and eluted to 10.0  $\mu$ L. Indexing: NPM = 7.5 $\mu$ L, PPC = 2.5 $\mu$ L, Index I = 2.5 $\mu$ L, Index II = 2.5 $\mu$ L, DNA = 10 $\mu$ L, 25.0 $\mu$ L total; PCR (72°C – 3min, 98°C – 1min, 6 cycles of: 98°C – 10s, 63°C – 30 s, 72°C – 3min. There was another purification of Ampure beads (0.6 x beads to product) qPCR on *Rotorgene* ([www.qiagen.com/Rotor-Gene-Q](http://www.qiagen.com/Rotor-Gene-Q)), water = 3.8 $\mu$ L, Kapa Sybr Fast = 5.1 $\mu$ L, Forward primer (10 $\mu$ M) = 0.1 $\mu$ L, Reverse primer (10 $\mu$ M) =

0.1 $\mu$ L, DNA (1/50 dilution) = 1.0 $\mu$ L, 10.0 $\mu$ L total. The final template was loaded on the MiSeq at 12pM DNA with 10% Phi control library. There were 618 cycles (% PF: 87.96) and average %Q30 of 66.22% that yielded 15.69Gbp sequence data (Appendix Fig. A3.1 & Table. A3.1).



**Fig. 3.3** MiSeq flow chart showing A: Nextera XT transposome with adapters is combined with template DNA, B: Tagmentation to fragment and add adapters and C: Limited cycle PCR to add sequencing primer sequences and indices. Source: Nextera DNA Sample Preparation Guide, Illumina, Incorporated (2012).

### 3.2.4 Polypeptide Function Detection Using Web-Based Tools

The GS sequences extracted from the eight wheat accessions were translated into protein sequences using *GENCSAN* (Burge & Karlin, 1997) and aligned to compare amino acid substitution. *PredictProtein* (Rost et al. 2004) was used to identify the proteins aligned to the complete coding regions of the sequences. The coding regions were determined by identifying annotated exons between (start-AUG and stop codons - UGA, UAA, UAG) in the reference mRNA and aligning them with the exons of the wheat mRNA sequences translated from the genomic DNA sequences.

To predict gene ontology terms for protein sequences through homology, a BLAST (Basic Local Alignment Search Tool) query was matched to a given target against a custom BLAST database containing sequences and GO terms of previously annotated proteins of *Zea mays* and *M. truncatula*. Finally, an algorithm meta-classifier converts the information into one dataset. If there was no similar sequence in the alignment search database, there was no prediction made. In addition to structure modelling, the protein sequences were consolidated in a cluster alignment. Single nucleotide polymorphism (SNP) loci were identified and the corresponding amino acids codons specific to the SNPs between those sequences were annotated.



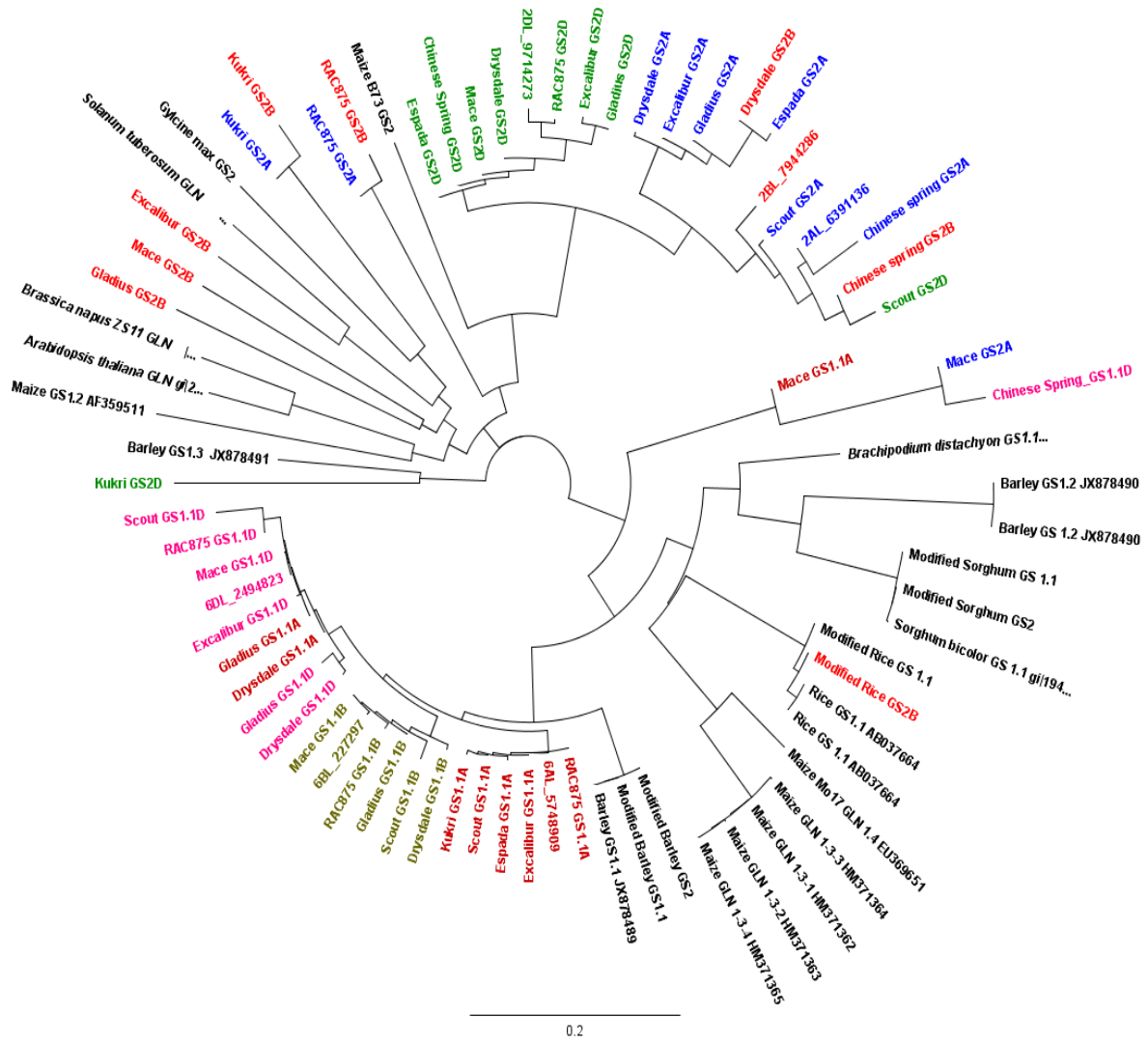
### 3.2.5 Phylogenetic Analysis

For phylogenetic analysis, the alignment algorithm in *Geneious 9.0.5* (<http://www.geneious.com>, Kearse et al. 2012) was utilised. The settings used for the analysis included alignment type (global with free ends); 65% similarity (5.0 / 4.0); genetic distance mode (Tamura-Nei) and tree building method (Neighbouring-joining) with no outgoing group. The programme was used to construct a phylogenetic tree for *GS1.1* and *GS2* alleles between wheat and alleles in related species through comparative alignment of the nucleotide sequences. The interactive distance matrix viewer was used to calculate statistically significant genetic distances between neighbouring genomic DNA sequences. A total of 77 sequences comprising 26 sequences from of other plant species, 45 wheat sequences (extracted from 8 accessions) and 6 reference sequences (based on the multiple alignment of *GS1* and *GS2* sequences from barley, rice, maize, *S. bicolor* and *A. thaliana*) were used in the phylogenetic analysis.

### **3.3 RESULTS AND DISCUSSION**

#### **3.3.1 Phylogeny of GS Sequences from Different Plant Species**

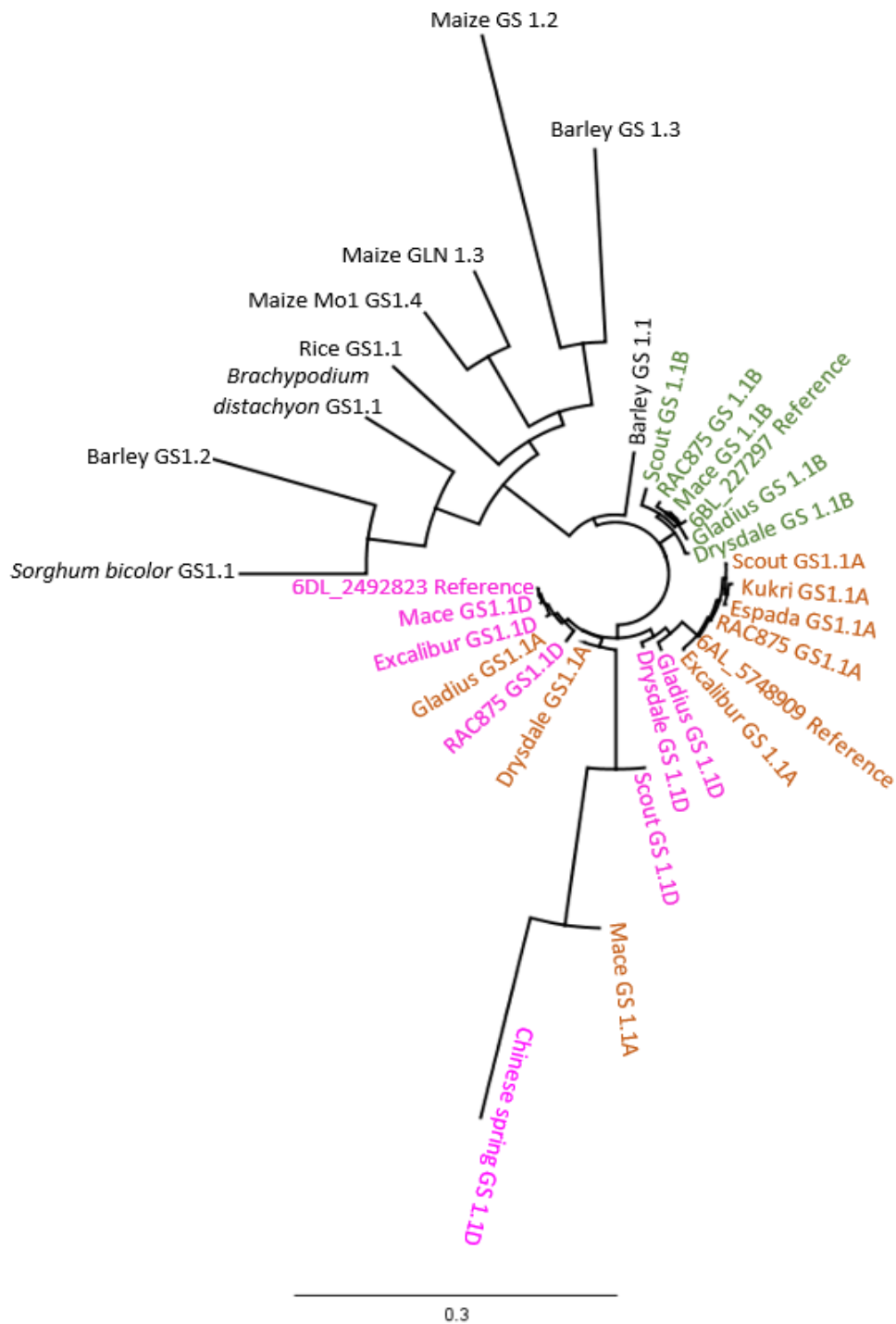
Phylogenetic analysis is useful to explore natural variation among allelic variants that control quantitative traits such as NUE variables among cereal crop species including wheat (Lu et al. 2011; Meihls et al. 2011). The phylogenetic analysis in the current study included *GS1* sequences of 5 species (*Brachipodium distachyon*, *Zea mays*, *S. bicolor* *Hordeum vulgare* and *Oryza sativa*) in addition to *GS2* and *GLN* sequences from 7 different species (*Z. mays*, *H. vulgare*, *O. sativa*, *S. tuberosum*, *A. thaliana*, *B. napus* and *G. max*). The phylogenetic analysis of 77 sequences of two GS isoforms showed that the *GS2* homologues were divergent genetically from *GS1.1* sequences (Fig. 3.4).



**Fig. 3.4** Phylogeny of all *TaGS1.1* and *TaGS2* genes extracted *in-vivo* from spring wheat accessions (coloured) and from additional plant species (black). Genes of the same genome are similarly coloured.

Among the *GSI.1* sequences of the cereal species of the mean genetic distance of the wheat *GSI.1* sequences were closer to barley *GSI.1* (0.13) than to Brachipodium *GSI.1* (0.26), rice *GSI.1* (0.27), maize (*GLN1.2* – 0.52; *GLN1.3* – 0.3; *GLN1.4* – 0.29) and sorghum (0.32) showing that barley and wheat were genetically related to each other than to either Brachipodium or rice. This result marks the wheat *GSI.1* sequences (Fig. 3.5) as being genetically divergent from the other cereal species examined (Castro-Rodríguez et al. 2011; Torreira, 2014), especially as *GSI* sequences of barley, maize, rice and *B. distachyon* were all anchored to a node on the phylogenetic tree that is shared with the wheat *GSI.1* sequences.

This also suggests that the *GSI.1* sequences of wheat, barley, Brachipodium and rice are orthologous to a common ancestor *GSI.1* sequence (Martin et al. 2006; Chen et al. 2007). Swarbreck et al. (2011) reported that the genetic relationship between six *GSI.1* alleles including the cereal species investigated in the present study was not clear; however, the current study shows that the *GSI.1* sequences of Brachipodium (0.14) and rice (0.15) and sorghum (0.16) were evenly distanced from barley. However, the barley *GSI.1* sequences (*GSI.3*, *GSI.2*) were situated at a genetic distance of 0.05 on the same node as was 0.01 to the wheat *GSI.1B* (Scout, RAC875, Mace, Gladius and Drysdale) pointing possibly to a history of co-speciation (Gaut & Doebley, 1997; Salse & Feuillet, 2007).

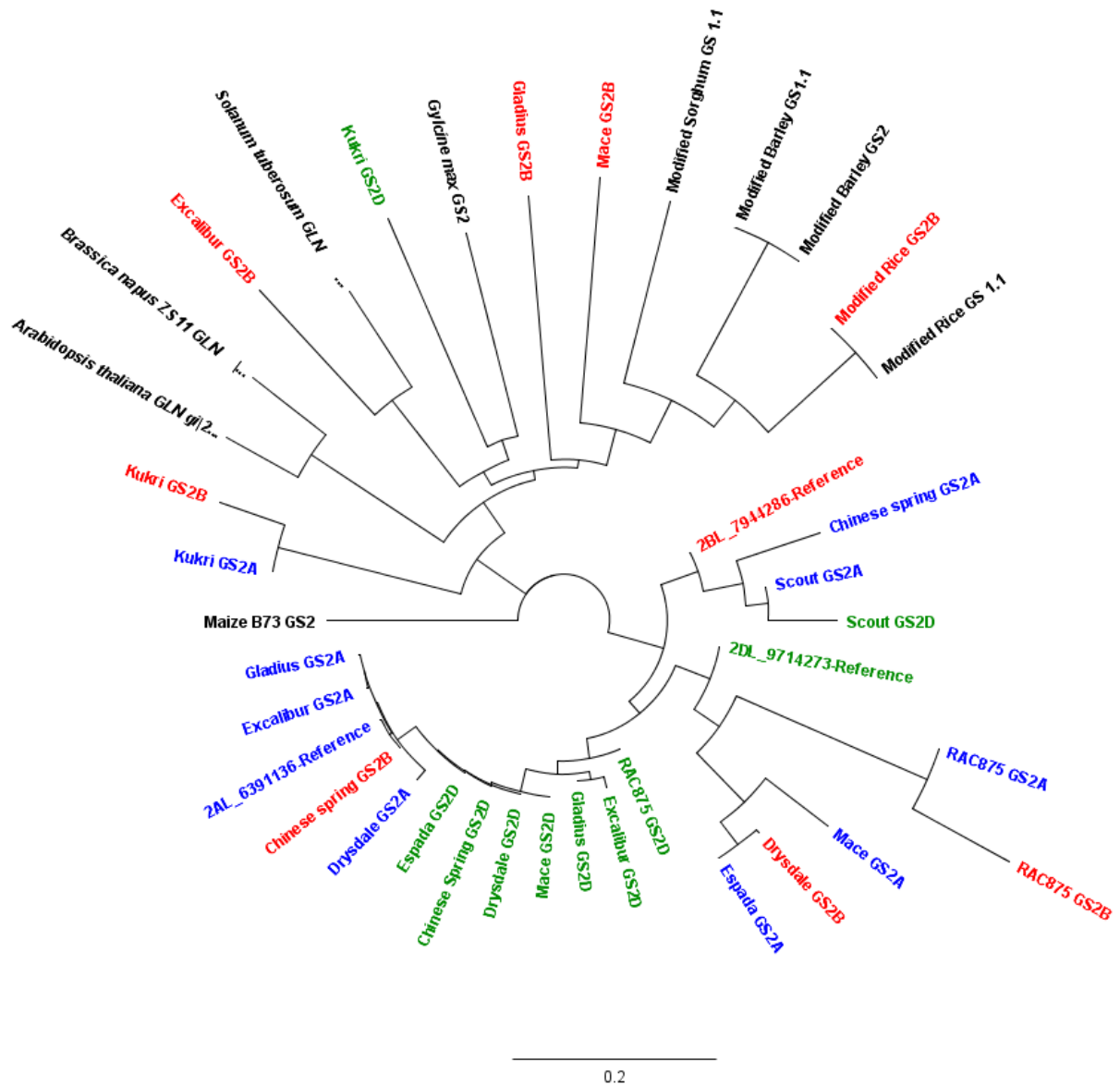


**Fig. 3.5.** Dichotomy of *TaGS1.1* genes extracted in-vivo from spring wheat accessions (coloured) and from additional plant species (black). Genes of the same genome are similarly coloured.

The maize *GSLN1.4* sequence also diverged into two branches from the same ancestral root as was *S. bicolor* (also two branches from the root), though they were clustered into separate nodes. This is a key finding confirmed by Swigonová et al. (2004) who studied the genomic progenitors of the two species and concluded that genetic divergence between maize and *S. bicolor* occurred contemporaneously. The finding of Swigonová et al. (2004) is confirmed in this study, as the maize genetic distance of the maize genotype Mo17(EU369651) *GLN1.4* sequence was found to be the same genetic distance (0.23) as the sorghum *GS1.1* sequence was from the base root.

In addition, the rice *GS1.1* (0.12) and Brachipodium *GS1.1* (0.13) were clustered closely and situated at an even distance between barley *GS1.1*, sorghum *GS1.2* and maize *GS1.4* and *GLN1.3*. Of the six GS2 genes studied, Kukri *GS2B* was the most genetically diverse (Fig. 3.6). This gene shared close sequence identity to *G. max* *GS2*, *S. tuberosum* *GLN* and maize *GS1.2*. Ireland & Lea, (1998), have reported a similar level of sequence identity. Together with *A. thaliana*, the GS from these species were distinctly divergent from the wheat GS. Overall, all the wheat *GS2D* alleles (except Kukri *GS2D*) were clustered genetically and separately from the genes of the other species (Fig. 3.6). All gene sequences shared a common ancestral root (Maize B73*GS2*) suggesting that they might have originally emerged from duplication of a common ancestor (Gautier et al. 2000).

Barley *GS2* was clearly divergent from *GS2* sequences of *G. max*, *B. napus* and *A. thaliana* that clustered together with the wheat *GS2B* sequences. Rice and sorghum *GS2B* was separated from the *GS2* homologues by a longer genetic distance across some plant species including maize B73, *A. thaliana*, *B. napus* and *G. max* (Fig. 3.6). In Fig. 6, the maize B73 *GS2* was linked directly to the base root of the phylogenetic tree and it was located on the node with the shortest genetic distance (0.17) from the ancestral root from which all the other *GS2* sequences (rice *GS2B* – 0.25; *Glycine max* – 0.31; Arabidopsis – 0.30; *Glycine max* – 0.31; *Solanum tuberosum* – 0.28) branched. The shorter genetic distance from the root and common phylogenetic link with maize B73*GS2* indicates that this maize lineage is independent. Some of the wheat *GS2* sequences were clustered next to the barley *GS2*, four of the wheat *GS2* sequences (Kukri *GS2D* – 0.34; RAC85 *GS2B* - 0.28; Gladius *GS2B* - 0.34; Excalibur *GS2B* - 0.36) were clustered together in the same node as the barley *GS2* sequence (0.32).



**Fig. 3.6** Dichotomy of *TaGS2* genes extracted in-vivo from spring wheat accessions (coloured) and from additional plant species (black). Genes of the same genome are similarly coloured.



### 3.3.2 Polymorphism between Wheat GS Alleles

Generally, there were four distinct phylogenetic clusters covering all the GS sequences analysed (Cluster 1: wheat *GS1.1*; Cluster 2: non-wheat *GS1.1*; Cluster 3: wheat *GS2*, and Cluster 4: non-wheat *GS2* (Fig. 3.4). The phylogenetic relationships and genetic location of the GS sequences from different the species are consistent with divergence of the cytosolic *GS1.1* and plastid *GS2* isoforms. Crucially, all the wheat GS alleles representing the *A*, *B* and *D* genomes branched from an ancestral root shared by the barley *GS1.1* gene (Fig. 3.5). The wheat *GS1.1B* sequences were closely clustered on a common branch shared by barley *GS1.1*. The wheat *GS1.1* sequences were closely clustered and separated from the *GS1.1D* sequences. The clustering information of the GS among the different species analysed can be utilised for hierarchical clustering of GS genes and elucidate genetic variation in different species (Gaur et al. 2012; Büchi et al. 2016). For example, the response to N treatment is specific in different accessions has been observed in *B. napus* (Orsel et al. 2014) in which the *GLN1* sequences were regulated according to the orthologous relationship among different accessions.

Both the Gladius *GS1.1A* and Drysdale *GS1.1A* sequences were located the same node as the wheat *GS1.1D* sequences. The Gladius *GS1.1A* and Drysdale *GS1.1A* sequences were clustered between Excalibur *GS1.1A* and Mace *GS1.1A* (Fig. 3.5). Phylogenetic differences between *GS1.1* and *GS2* have not previously been reported for spring wheat accessions.

This confirms the evidence of genetic divergence between cytosolic and plastid GS isoforms reported in maize (Gallais & Hirel, 2004; Martin et al. 2006). The analysis also shows that there is a close genetic relationship between different GS isoforms in different species. There were phylogenetic links between the *Brachipodium GS1.1*; sorghum *GS1.1* and barley *GS1.2*; *B. napus* GLN and Maize AF359511 *GS1.2*.

The next generation sequencing by *MiSeq* generated 45 complete full-length genomic sequences out of 48 GS sequences representing 9 wheat accessions across 6 GS genes. Generally, there were greater numbers of SNPs in intronic regions than in the exons. Within the complete genomic DNA sequences of *GS1.1A* (Size: 2.2kb, 11 exons) there were 87 SNPs in the introns and 15 at exons. Gladius, and Drysdale shared a high level of homology in both exon and intron domains. Excalibur *GS1.1A* and RAC875 *GS1.1A* shared identical homology; the two accessions differed by only 7 SNPS in the introns. Further, Scout shared close homology with the reference >lcl|6AL\_5820788 contig. Sequences of the A genome (*GS1.1A* and *GS2A*) showed more variation among accessions than genes of other genomes. However, this result may be genotype-specific as Mace *GS2A* sequence was diverged from the sequence Mace *GS1.1A* at a genetic distance of 0.162. This suggests high sequence homology and fewer nucleotide substitutions between cytosolic and plastid sequences of the same genome.

This according to the present knowledge is the first record of the existence of sequence similarity of GS genes in these accessions of hexaploid wheat. *GS2A* was the most closely clustered when sequences across the accessions were compared. Among the three *GS2* homologues, *GS2B* was the most divergent between the wheat accessions analysed, there were *GS2B* sequences in all three phylogenetic clusters of *GS2* (Fig. 3.6) when compared to the divergence recorded between the other wheat *GS2* alleles (*GS2A* and *GS2B*). This result suggests that the *GS2B* sequences were clearly genetically different from *GS2A* and *GS2B*. This difference may be due to genome differences between *GS2A* and the other *GS2* sequences (*GS2B* and *GS2D*) during speciation (Hewitt, 2001; Martin et al. 2006) or a consequence of polyploid ancestry (Buggs et al. 2008; Paun et al. 2009).

In the *GS1.1B* sequence (size: 7.5 kb, 11 exons), demonstrated a relatively fewer number of SNPs (47 in introns and only 8 at exons, which were identified in the corresponding mRNA - Fig. 3.7). With the exception of Drysdale, the accessions shared high level of homology in both exons and introns. Generally, there were observable SNPs unique to RAC875, Mace and Scout, which constituted a second *GS1.1B* haplotype. The *GS1.1D* allele (size: 4.4 kb, 11 exons) was relatively shorter in length compared to the other *GS1.1* sequences.

Table 3.1 The contig size, exon and number of single nucleotide polymorphism (SNP) identified in the exons and introns of cytosolic and plastic wheat GS sequences in hexaploid wheat accessions.

| <b>Gene</b>     | <b>Contig size (kb)</b> | <b>No. of Exons</b> | <b>No. of Exon SNP</b> | <b>No. of Intron SNP</b> |
|-----------------|-------------------------|---------------------|------------------------|--------------------------|
| <i>TaGS1.1A</i> | 11                      | 13                  | 65                     | 259                      |
| <i>TaGS1.1B</i> | 7.5                     | 11                  | 47                     | 8                        |
| <i>TaGS1.1D</i> | 4.4                     | 11                  | 120                    | 24                       |
| <i>TaGS2A</i>   | 6.2                     | 13                  | 65                     | 13                       |
| <i>TaGS2B</i>   | 11                      | 13                  | 1                      | 3                        |
| <i>TaGS2D</i>   | 9.5                     | 13                  | 5                      | 37                       |

As shown in Table 3.1, a total number of 120 SNPs occurred in introns and 24 in exons of *GS1.1D*. There was a high level of homology observed between Gladius and Drysdale particularly between exons 7-11. The two accessions were markedly identical to each other; however, they appeared to be markedly different from the other accessions in genomic regions spanning *GS1.1D* exons 1-6. There were SNPs observed between Gladius and Drysdale that clearly differentiated them from Excalibur, RAC875 and Mace.). Conversely, RAC875 and Scout sequences showed the same SNPs in both introns and exons of *GS1.1D*. The *GS2A* (size: 6.2kb, 13 exons) sequences were very diverse across accessions. There was a total of 259 SNPs in the introns and 65 in the exons. It was observed that Chinese spring and Mace shared homology at exons 1- 2. In addition, Drysdale, Mace and Scout shared homology in exons 3, 4 5, 6 and for Drysdale and Scout exon 6, 7 and 8. In addition, RAC875 and Scout consisted of identical SNPs at exons 11-13. *GS2B* (Size: 11kb, 13 exons).

There are very few SNPs in this gene (introns - 3; exons-1). Analysis confirms Exon 10 is missing from all the sequences and this was in agreement with the reference transcript that does not include exon 10. This *GS2D* allele (Size: 9.5kb, 13 exons) was the most conserved among six genes, 37 intron SNPs and 5 at exons.

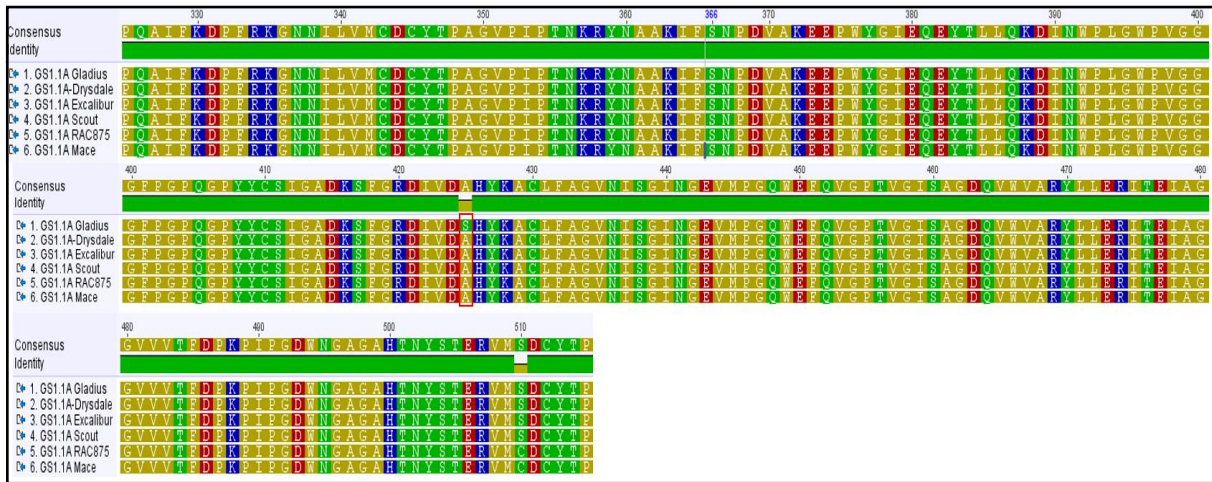
The alignment of the protein sequences corresponding to the conserved domains of the GS genes of the wheat accessions showed that, there was a high level of amino acid sequence similarity between the wheat accessions. The amino acids of the *GS1.1* and *GS2* sequences were isoform-specific and there were differences among the GS homologues unique to each GS isoform (Fig. 3.7, 3.8, 3.9 & 3.10). This highlighted the differences between *GS1.1* and *GS2* homologues within the conserved domains. Most of the SNPs observed were synonymous, but there were 4 non-synonymous amino acid variations in the DKSFGRDIVDSHYKA sequence (Fig. 3.7, 3.8, 3.9 & 3.10) within the conserved domain protein of the wheat *GS1.1A*, *GS1.1B* and *GS1.1D*. Fig. 3.7 shows that in *TaGS1.1A*, there is a change of the amino acid sequence between Gladius (DKSFGRDIVD**A**HYKA) and Drysdale, Excalibur, Scout, RAC875 and Mace (DKSFGRDIVD**S**HYKA). There was no change in *TaGS1.1B* in the amino acid sequence DKSFGRDIVD**S**HYKA between the wheat accessions (Fig. 3.8). However, in Fig. 3.9, the alignment of the protein sequences corresponding to the same conserved domains of *TaGS1.1D* homologues shows a variation between the Gladius and Drysdale *GS1.1D* DKSFGRDIVD**A**HYKA amino acid sequence

and the DKSFGRDIVD**S**HYKA sequence of Scout, Excalibur, RAC875 and Mace. Among the *TaGS2* sequences, there is an amino acid variation only in the GRSV**P**ARQPAG sequence. The GRSV**P**ARQPAG sequence is the same in *TaGS2B* and *TaGS2D* among the wheat sequences except Excalibur that has the sequence GRSV**P**AS**S**QPAG in *TaGS2A* (Fig. 3.10).

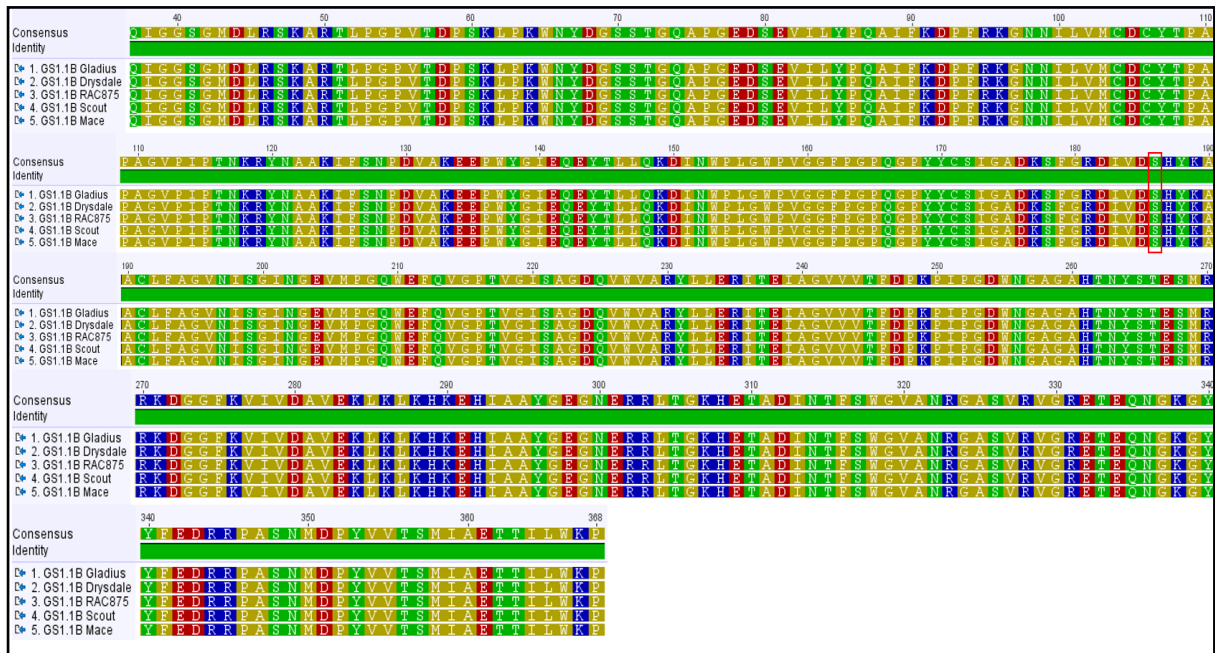
Besides highlighting the phylogenetic relationship, the sequence analysis also generated information on the amino acid substitution within the GS mRNA sequence sets queried. There were clearly distinct SNP based haplotypes of the wheat accessions within and among the different GS alleles suggesting clear patterns of genetic diversity of the wheat GS sequences. The active sites in all three *GS2* genes were anchored to exon 11. Differences exist between Excalibur, Scout and the other accessions on this exon, which carries a glutamic acid catalytic residue. The Excalibur *GS2A* was unique as it carried additional phosphorylation sites for one threonine residue on Exon 1 and three tyrosine residues on Exon 7 that were not present in *GS2B* and *GS2D*. The greater number of phosphorylation sites which were present in *GS2A* might explain the dominant functional role of *GS2A* in enzyme activity and metabolism reported in published studies (Gallais & Hirel, 2004; Martin et al 2006; Li et al. 2011).

As expected, amino acid variation in the mRNA sequences produced different protein structures between the different accessions sequenced. However, in the present study there was no experiment directly determining clear functional variation (with possible implication for NUE quantification) between *GS1.1* and

*GS2* alleles, though there were observable structural changes in their respective sequences. The results revealed clear differences in the modelled protein structure of the different alleles sequenced, a separate experiment needed to verify if these differences could result in changes to the primary function of the proteins (ATP-dependent glutamate-ammonia ligase activity). However, most allelic variation changes owing to synonymous SNPs may not result in considerable gene functional change (Zhang et al. 2014).

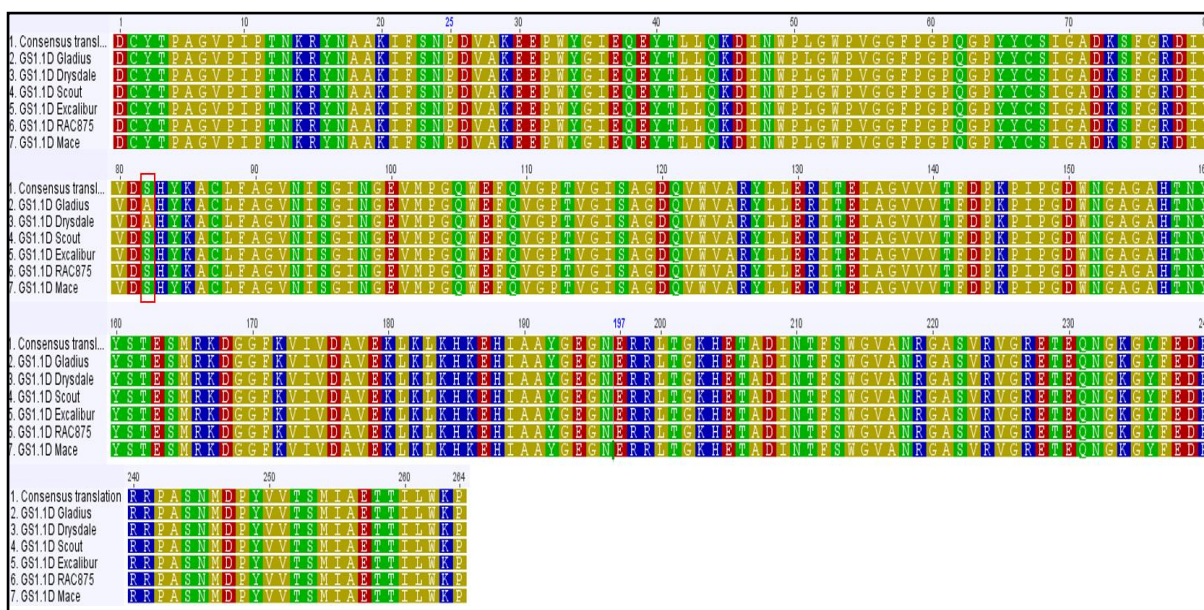


**Fig. 3.7** Alignment of the protein sequences corresponding to the conserved domains of *TaGS1.1A* homologues in six wheat accessions (Gladius, Drysdale, Excalibur, Scout, RAC875 and Mace). Similar colours show identical amino acids. Variation in the DKSFGRDIVDSHYKA sequence is highlighted by a red rectangle.

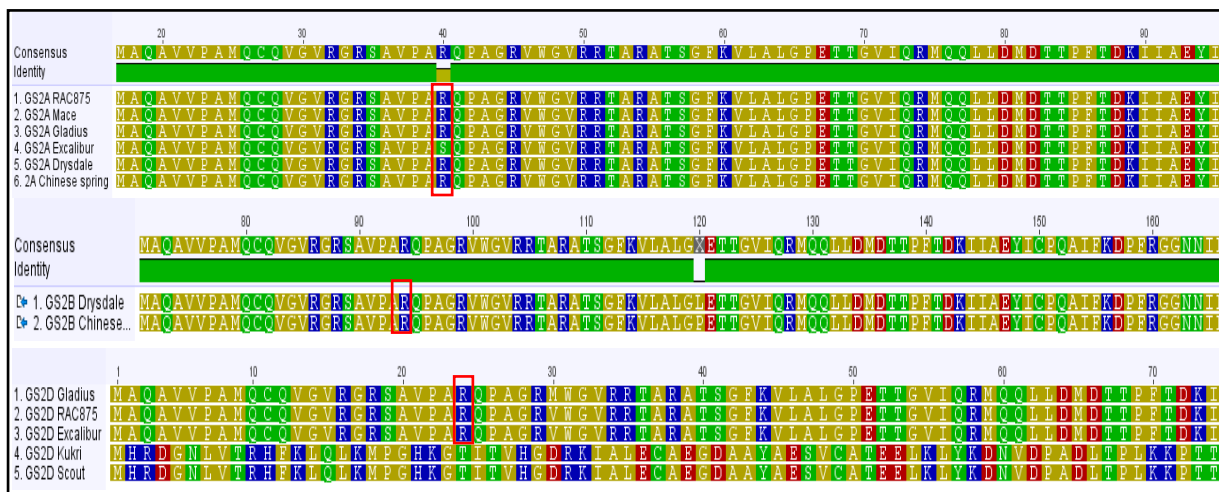


**Fig. 3.8** Alignment of the protein sequences corresponding to the conserved domains of *TaGS1.1B* homologues in five wheat accessions (Gladius, Drysdale, RAC875, Scout and Mace). Similar colours show identical amino acids. Non-variation in the DKSFGRDIVDSHYKA sequence is highlighted by a red rectangle.





**Fig. 3.9** Alignment of the protein sequences corresponding to the conserved domains of *TaGS1.ID* homologues in six wheat accessions (Gladius, Drysdale, Scout, Excalibur, RAC875 and Mace). Similar colours show identical amino acids. Variation in the DKSFG RDIVDSHYKA sequence is highlighted by a red rectangle.



**Fig. 3.10** Alignment of a sample of protein sequences corresponding to the conserved domains of three *GS2* homologues (*TaGS2A*, *TaGS2B* and *TaGS2D*) in six wheat accessions. Similar colours show identical amino acids. Variation in the GRSVAVPARQPAG sequence is highlighted by a red rectangle.

### 3.4 CONCLUSION

Detection of gene sequence homology has been revolutionised with the use of high throughput statistical tools (Hampson et al. 2003). However, here have been few studies that have analysed genomic level variation between GS alleles using full-length sequences of wheat GS and evaluated amino acids of corresponding GS proteins. GS sequences of the eight spring wheat accessions examined in this study demonstrated unique haplotypes with a high level of homology between exons 1-6 of *GDI.1D*. The phylogenetic analysis demonstrated that *GS1.1* and *GS2* are genetically different and highly divergent between different species and among wheat accessions. However, the short genetic distance observed between the wheat *GS.1.1* sequences and to a lesser extent between the wheat *GS2A* and *GS2D* alleles indicated high-level structural and functional conservation between the *GS1.1* sequences. This study of GS allelic variation provides an important resource for wheat genome wide association studies. It can be an important contribution in the characterisation of NUE in wheat. An approach to quantifying NUE in the wheat accessions will be to through expression analysis using cDNA templates of the wheat GS accessions. This will provide a means by which to quantify and potentially explain observed N response patterns.

## CHAPTER FOUR

### REGULATION OF GLUTAMINE SYNTHETASE GENES BY NITROGEN IN WHEAT

#### 4. ABSTRACT

The expression levels of six different glutamine synthetase (GS) homeologs was investigated under contrasting N regimes in hydroponic conditions. *In-silico* analysis of *TaGS1.1* and *TaGS2* expression with the novel tool POTAGE (PopSeq Ordered *Triticum aestivum* Gene Expression), showed differential expression of GS genes between wheat leaves and roots. The POTAGE analysis indicated that the *TaGS1.1* homeologs were highly expressed in roots and the *TaGS2* allelic variants were highly expressed in leaf tissues. The POTAGE results were confirmed in real-time quantitative polymerase chain reaction (qPCR) using gene specific primers specific to *GS1.1* and *GS2* genes in series of tissue samples obtained from Chinese Spring.

There was further analysis using qPCR techniques with different leaf and root tissue samples extracted from high and low N-treated plants of five additional wheat accessions. The analysis indicates that *TaGS1.1A* was down regulated in response to high N treatment and up regulated under low N in the young leaf of two wheat accessions (RAC875, Kukri). Also under low N, *TaGS1.1A* was up-regulated in the older leaf of three accessions (RAC875, Gladius, Drysdale) and older roots of two accessions (Gladius, Kukri) and in the

flag leaf stage roots of two accessions (Drysdale, Excalibur). Under high N, *TaGS2A* were up regulated in the flag leaf and down regulated in the roots harvested at flag leaf stage in RAC875, Excalibur and Drysdale. Similarly, the *TaGS2D* of RAC875 was up regulated in the flag leaf and down regulated in the roots. Generally, the transcript analysis and expression data demonstrated that the GS expression was genotype-specific and showed that there was no fixed pattern of gene expression in response to the N treatment.

#### **4.1 INTRODUCTION**

A greater proportion of N available (> 90 %) in the soil is in an organic state. A portion of this elemental N is mineralised into nitrate and ammonium that are two major sources of available mineral N for plant growth. Crop plants (Mohammed et al. 2013; Wei et al. 2014) do not take up approximately 60% of inorganic N added to soils. Because of the in-efficiency in crop N up-take in plants, there is on-going effort to increase grain yield in wheat and other cereal crops through enhanced breeding strategies that are targeted at specific agronomic traits. Some research strategies have focused on characterizing the physiological traits related to nitrogen use efficiency (NUE) such as grain yield (Hirel et al. 2007; Sylvester-Bradley & Kindred, 2009; Gaju et al. 2014; Cormier et al. 2013). For example, the ability of plants to assimilate N efficiently has been linked with grain yield variables (Purcino et al. 1998). Knowledge of N assimilation efficiency is crucial to breeding N-efficient accessions and making decisions on the amount of N fertiliser applicable to specific accessions.

Some researchers have developed molecular markers linked to genes controlling N assimilation. The aim was to identify metabolic functions of N-linked genes and characterise the agronomic traits linked to specific DNA markers. Based on the hypothesis that ammonium assimilation rate is important for NUE, Hirel et al. (2001) developed three GS markers (*gsy143b*; *gsy304*; *gsy52r*) on Chromosome 1 and two markers (*gsy343b*; *gsy258a*) on Chromosome 5 linked to leaf GS activity in maize. This study demonstrated that leaf GS activity was correlated with grain yield, under low N treatment. Also, using restriction fragment length polymorphisms (RFLP markers C560 and C1408), Obara et al. (2001) mapped one *GS1* transcript next to a locus for spikelet-weight in rice. This has important implications for NUE as the *GS1* gene plays a key role in primary N assimilation from inorganic sources (Kamachi et al. 1991; Guan & Schjørring, 2015; Guan et al. 2016). In addition, Yang et al. (2011; 2016) have demonstrated that polymorphic markers can be used as an effective tool in assessing gene expression. However, there is still relatively little information available relating to gene expression and NUE and more research is required to fully explain the relationship between expression and nitrogen regimes particularly at specific growth stages (Beatty et al. 2009).

Previous studies have sought to establish a link between N level and which genes N regulates in plants. The biological mechanisms and biochemical pathways involved in N related enzyme activity are considerably affected by the level of GS expression in different tissues (Fei et al. 2006; Avila-Ospina et al.

2014; 2015) and this has biological implications for grain yield (Valkama et al. 2013). However, there is strong evidence of GS expression (Habash et al. 2001, Grabowska et al. 2012; Yousfi et al. 2016) to suggest that under different N supplies, the level of GS expression in the leaf and root tissues becomes critical for enzyme activity (Masclaux et al. 2006; Guo et al. 2004; Martin et al. 2006; Orsel et al. 2014). Moreover, some researchers have attempted to understand the genetic basis of N uptake in crop plants. This has been by quantifying gene expression and linking expression with observable growth effects such as stress tolerance (Hoshida et al. 2000) seed germination (Glevarec et al. 2004), shoot development (Hirel et al. 2007; Can˜as et al. 2011), flowering (Grabowska et al. 2012) and grain yield (Tabuchi et al. 2005, Martin et al. 2006). Such expression analysis has produced information on the transcript abundance of GS genes in different organs of plants (Carvalho et al. 2000; Castaings et al. 2011). The levels of GS gene expression can change at different developmental stages of plants (Mifflin & Habash, 2002). Gene expression analysis is vital for confirming the cellular and tissue location within the plant tissues.

Genome-wide studies of *A. thaliana* have shown considerable variation in response to reduced N treatment and significant changes in the expression of key genes in plants (Wang et al. 2015; Kant et al. 2011; Araus et al. 2016). These genes have been shown to regulate the uptake, metabolism and assimilation of the mineral N (Tobin & Yamaya, 2001; Mifflin & Habash, 2002). Two sub-families of glutamine synthetase are constitutively expressed.

In terms of the complex interactive roles of the cytosolic and plastid GS genes, it appears that these two GS genes may be involved in biochemical processes that are much more complex and their combined metabolic reactions may have influence nitrogen uptake, synthesis and assimilation quite extensively (Oliveira et al. 2002; Bernard & Habash, 2009; Funayama et al. 2013).

The expression of GS genes is important for grain yield (Hirel et al. 2001, 2007; Obara et al. 2001; 2004; Hirel et al. 2007) as these genes play key roles in regulating the enzymes that catalyse important biochemical reactions in N metabolism. Generally cytosolic GS transcript abundance is high in roots than in the leaves (Ishiyama, 2004; Martin, 2006; Bernard et al. 1994; Goodall, 2013; Seabra, 2010). However, in rice, GS1 overexpression has been associated with increased enzyme activity in the leaves and total plant N (Cai et al. 2009). Primarily, the cytosolic GS enzyme catalyses the addition of ammonium to glutamate to form glutamine (Bernard & Habash, 2009). This is active in catalysing assimilation and remobilisation mechanisms to sustain and complete grain filling (Tabuchi et al. 2005; Martin et al. 2006) and play important roles in recycling N from senesced leaves (Martin et al. 2006; Bernard et al. 2008; Masclaux-Daubresse et al. 2008; 2010), redirecting this vital source of N towards grain filling and normal growth maintenance. Bernard and Habash (2009) reported that the N levels in the plant tissues and depending on the plant status influence cytosolic GS, GS regulation influences N assimilation (Ishiyama et al. 2004; Hirel et al. 2001; Asplund et al. 2016).

The plastid GS2 catalyses the conversion of glutamine to glutamate (key sources of organic N for protein and nucleic acids) in the GOGAT cycle which is important component of the nitrogen metabolic pathway (Lam et al. 1996; Fuentes et al. 2001). The role of the plastid GS2 is closely associated with ammonia metabolism in the oxidative photosynthetic carbon cycle (Wallsgrove et al. 1987; Lam et al. 1996). Within the chloroplasts and mitochondria GS2 acts in the conversion of glutamate to glutamine, using by-product ammonium generated from the activity of nitrate reductases and direct N uptake from soil (Xu et al. 2012).

Despite considerable efforts made to assess N-related agronomic traits in cereal crops such as maize (Hirel, 2001; Cañas - 2010, Gallais, 2004), there are questions remaining as to whether GS gene expression patterns explain the observed variation for NUE among wheat accessions. For example, a study of GS expression in wheat (Bernard et al. 2008), revealed that GS genes are differentially expressed in different plant organs, and that the expression of GS in the leaf is regulated according to developmental stage. However, there is limited knowledge about GS transcript expression in cereal species under different N treatments. Wheat GS studies involving the evaluation of transcript abundance have been limited. No study of wheat has yet directly linked genetic variation in GS genes to specific growth or developmental stages of plants that have been grown under differential N treatments of the wheat plants.



In the present study, the transcript abundance of six GS homeologs was investigated in qPCR reactions using cDNA samples extracted from five different wheat accessions as templates. The samples were taken from wheat plants that were grown under high and low N treatments in hydroponic units until the flag leaf stage. The tissue extracts from plants under both N treatments were taken from the young leaf, older leaf, flag leaf and the root tissues that corresponded to these developmental stages. The ultimate goal of this expression analysis is to identify significant genetic difference in gene expression that upon further experimentation could be linked to GS enzyme activities and improvement in grain yield per N applied. This analysis is of considerable agronomic importance and offers the potential to use such information in breeding N-efficient accessions (Edgerton, 2009).

## 4.2 MATERIALS AND METHODS

### 4.2.1 Experiment 1 - Gene Expression Analysis Using a Novel Tool

In the gene expression analysis (*Experiment 1*), the location and abundance *TaGS1* and *TaGS2* genes in tissue samples of different organs of hexaploid wheat plants was analysed in-silico with the POTAGE (PopSeq Ordered *Triticum aestivum* Gene Expression) – POTAGE programme (Schoppach et al. 2016). POTAGE was developed by the Bioinformatics Department of the Australian Centre for Plant Functional Genomics (ACPGF) in 2014, to integrate genome-specific map location of a gene with the expression and the inferred functional annotation.

POTAGE calculates transcripts in fragments per kilobase of exon per million fragments mapped (FPKM) per gene (Schoppach et al. 2016). POTAGE is a novel gene expression in-house inference tool that incorporates genomic reference sequences, partially ordered PopSeq data and RNA sequence mapped to the reference gene. In POTAGE, MIPS (Munich Information Centre for Protein Sequences) predicted genes on IWGSC (International Wheat Genome Sequencing Consortium) chromosome survey sequences using various types of data and tools. The expression values come from aligning RNA-Seq data to genomic regions corresponding to those predicted transcripts.

The reference is Chinese spring, the RNA-Seq reads come from various cultivars and the remaining data is ACPFG in-house. POTAGE is based on high confidence wheat gene prediction and is a tool designed for quick, preliminary selection of genes of interest and the values obtained are suitable to be used as evidence of differential expression. The POTAGE analysis largely relies on the correctness of MIPS whole genome analysis and predictions. POTAGE functionality depends on PopSeq and utilises whole genome sequencing to establish marker order from sequencing data. It provides also the genetic anchoring of the IWGSC survey sequences by population sequencing (Mascher, 2013). POTAGE also uses RNASeq data of Chinese spring library sequenced on Illumina HiSeq2000 2 x 100bp (PE) for 15 different conditions corresponding to extracts from the root, leaf, stem, spike, grain at three developmental stages.

#### **4.2.2. Experiment 2 - Confirmation of the POTAGE Analysis of GS Transcript Abundance**

In *Experiment 2* a series of cDNA samples (synthesised from extracted RNA) from the reference line Chinese spring was used. This comprising the root, embryo of germinating seed caryopsis (3-5 DAP - days after pollination), embryo (22 DAP) and endosperm (22 DAP), coleoptile, seedling root, crown, leaf, bracts, anthers, pistils, were sourced from in-house ACPFG stocks (Table 4.1) for a preliminary assessment of the GS expression levels. There were 3 replicates each of root, leaf, stem, spikelet and grain tissue samples.

**Table 4.1** Characteristics of the ACPFG in-house Chinese Spring/Cornerstone tissue series used experimentally to confirm accuracy of POTAGE inferences. Developmental stages marked (x) include three post-anthesis stages labelled DAA (days after anthesis). Source: ACPFG (<http://www.acpfg.com.au>). The Zadoks Growth Scale (Zadoks et al. 1974) determined growth stages.

| Stage         | Growth features                     | Zadoks scale | Leaves | Root | Stem | Spike | Grain |
|---------------|-------------------------------------|--------------|--------|------|------|-------|-------|
| Seedling      | First leaf through coleoptile       | 10           | x      | x    |      |       |       |
| Three leaves  | 3 leaves unfolded                   | 13           |        | x    |      |       |       |
| Three tillers | Main shoot and 3 tillers            | 23           | x      |      |      |       |       |
| Spike at 1 cm | Pseudo stem erection                | 30           |        |      | x    |       |       |
| Two nodes     | 2nd detectable node                 | 32           |        |      | x    | x     |       |
| Meiosis       | Flag leaf ligule and collar visible | 39           |        | x    |      | x     |       |
| Anthesis      | 1/2 of flag leaf flowering complete | 65           |        |      | x    | x     |       |
| 2 DAAs        |                                     |              |        |      |      |       |       |
| (50°C.days)   | Kernel (caryopsis) watery ripe      | 71           | x      |      |      |       | x     |
| 14 DAAs       |                                     |              |        |      |      |       |       |
| (350°C.days)  | Medium Milk                         | 75           |        |      |      |       | x     |
| 30 DAAs       |                                     |              |        |      |      |       |       |
| (700°C.days)  | Soft dough                          | 85           |        |      |      |       | x     |

#### **4.2.2.1 Primer Design, PCR Amplification and PCR Product Sequencing**

The transcript abundance of six GS genes (*GS1.1 6A*, *GS1.1 6B*, *GS1.1 6D* and *GS2A*, *GS2B*, *GS2D*) representing *TaGS1.1* and *TaGS2* homeologs was based on the mRNA sequences specific to the six GS genes. The mRNA sequences of the six GS genes were originally extracted from sequenced amplicons isolated with gene-specific primers in standard PCR reactions (Reddy et al. 2015). The transcript abundance data obtained from the qPCR was plotted in a graph. To amplify the GS, a set of three gene-specific primers (Table 4.2) were designed per each of the six GS genes. The three primer sets tested for successful amplification and one selected for the expression analysis per gene. This made it easy to capture each of the six genes in the expression analysis. Primers were designed to correspond to regions of mRNA sequences specific to each GS gene and polymerase chain reactions were conducted to confirm the amplification of the primers. The primers were designed using the *Geneious 9.1.3* (<http://www.geneious.com/>; Biomatters Limited) programme. The PCR reaction protocols (DNA polymerase, primer concentration and primer sequence) were entered into the New England BioLabs online T<sub>m</sub> Calculator (<https://www.neb.com/tools-and-resources/interactive-tools>) to select a suitable and optimal annealing temperature for the targeted amplicons. The primers were used in a standard PCR reaction on MJ Research Tetrad Peltier Thermal Cycler-

to confirm their suitability for amplification.

**Table 4.2** List and characteristics of primers used in quantifying the expression of six GS genes in wheat.

| Gene       | Accession Number | Sequence Length | Product Tm | Sense Primer            | Primer Length | Tm   | GC%  | Anti-sense Primer      | Sequence Length | Tm   | GC%  |
|------------|------------------|-----------------|------------|-------------------------|---------------|------|------|------------------------|-----------------|------|------|
| GS1.A 6A   | Ref. #000439_445 | 168             | 74.2       | ATGACGACAATGGAACTGAGAG  | 22            | 55.6 | 45.5 | ATGACGAGATGGCTGAGAGG   | 22              | 55.3 | 45.5 |
| GS1.A 6A   | Ref. #000439_445 | 196             | 73.4       | GCTCACTACGACGGACTAACG   | 22            | 58.6 | 54.5 | AGACAGCCAGCCCTATAAACC  | 21              | 58   | 52.4 |
| GS1.A 6A   | Ref. #000439_445 | 163             | 74.6       | CGATGACGACAATGGAACTGAG  | 22            | 56.9 | 50   | AGATGGCTGAGAAAGGAAGAGG | 22              | 56.9 | 50   |
| GS1.B 6B.L | Ref. #000440_445 | 174             | 75.8       | TGGTTCGTGGACTGTTCTG     | 22            | 56.2 | 45.5 | TGATAGGACAGCCGATAGAGG  | 21              | 56   | 52.4 |
| GS1.B 6B.L | Ref. #000440_445 | 106             | 75.6       | CTGGTCTCGTCTCGTGAATCATC | 24            | 58.7 | 50   | CACCCGTCCTCCGTAGC      | 18              | 59   | 72.2 |
| GS1.B 6B.L | Ref. #000440_445 | 177             | 77.9       | CTCTCTGTTGTGTGTAG       | 19            | 47.6 | 42.1 | GAATGTCCTGTATTGTAGC    | 19              | 47.4 | 42.1 |
| GS1.D 6D   | Ref. #000441_445 | 135             | 74.3       | TTTGCTCTGTGGGCTGTTTC    | 21            | 56.8 | 47.6 | GGGAGGGGTGTGGAATCTGC   | 19              | 56.7 | 63.2 |
| GS1.D 6D   | Ref. #000441_445 | 191             | 76.1       | AGCAAAGCCAGGTAAGATACAG  | 23            | 58   | 47.8 | GCACCAACCAGAGCAACAAC   | 20              | 57.3 | 55   |
| GS1.D 6D   | Ref. #000441_445 | 190             | 76         | GACAAAGCCAGGTAAGATAC    | 22            | 56.3 | 50   | ACCAACCAGAGCAACAACAG   | 20              | 55.6 | 50   |
| GS2.A 2A   | Ref. #000442_450 | 113             | 77.2       | GTCTCGGTTTGGTGGTGTG     | 21            | 58   | 52.4 | TTGTGCTTTGTGATCAGTAGC  | 24              | 58.1 | 41.7 |
| GS2.A 2A   | Ref. #000442_450 | 180             | 79.4       | TCCTGTCCGTCCTCTCTG      | 18            | 51.6 | 55.6 | CGCTGCTTACTTACTTACTC   | 20              | 50.7 | 45   |
| GS2.A 2A   | Ref. #000442_450 | 119             | 77         | CGGCTTCTCAICTCTG        | 18            | 49.3 | 50   | CGCTGCTTACTTACTTACTC   | 20              | 50.7 | 45   |
| GS2.B 2B   | Ref. #000443_450 | 167             | 78.7       | TTTGATAGACGGTGTGGAGAGAG | 24            | 58   | 45.8 | TGGATGGACGGAGCGGACTG   | 19              | 58   | 63.2 |
| GS2.B 2B   | Ref. #000443_450 | 112             | 76.9       | CAGTCGTCGGTCCATCCATC    | 21            | 59.8 | 61.9 | CCGCAAGCCAACCGCAAGC    | 18              | 60.5 | 66.7 |
| GS2.B 2B   | Ref. #000443_450 | 142             | 73.6       | CTGACCAACGACACAGG       | 19            | 53   | 52.6 | GATGGACAGGCCAATATGG    | 19              | 53.1 | 52.6 |
| GS2.D 2D   | Ref. #000444_450 | 133             | 79.8       | CACTGACTCCACAATCC       | 19            | 50.3 | 47.4 | CTGTAGGTTCTGTATCTGC    | 20              | 50.2 | 45   |
| GS2.D 2D   | Ref. #000444_450 | 184             | 77.8       | CTGTGAGGTGAGTTCTAGC     | 20            | 52.6 | 50   | CCGCTACTTACTTACTTACAGG | 22              | 53.3 | 45.5 |
| GS2.D 2D   | Ref. #000444_450 | 93              | 76.9       | CGAITTCAGTCAAGGTTCTGTAG | 24            | 55.8 | 41.7 | GTCCGAGCCGTCGCCATAC    | 18              | 56.2 | 66.7 |

The PCR reaction comprised DNA-1.5µl, pure water - 2.8µl, Phusion HF-2µl, DNTPs -1.25mM, forward and reverse primers - 2µl, Phusion Taq-0.1µl. The PCR protocol used was as follows: 98°C for 30s, 98°C for 10 s, annealing at 69°C for 30s, 72°C for 5 min, 34 cycles reset to step 2, 72°C for 10 min, 15°C for 15min. The DNA used as the template for the PCR reaction was extracted from the leaves of four-week old wheat seedlings by the freeze dried method and was purified with a Qiagen's Qiaquick PCR clean-up or Macherey-Nagel's NucleoSpin Gel and PCR Clean-up kit (<http://www.mn-net.com/>). The concentration of the purified DNA was verified by nanodrop on ND-1000 Spectrophotometer (<http://www.nanodrop.com/>).

The PCR products were purified using *Invitrogen Pure Link Quick PCR Purification* (<https://www.thermofisher.com/order/catalog/product/K310001>) and later sequenced. 3.0µl of purified PCR product was run on an ethidium bromide (EtBr) stained agarose DNA gel to confirm that there was a clear single band at the predicted molecular weight of the targeted amplicon. In addition, an aliquot of the purified PCR product and the forward or reverse primers were Sanger sequenced at the *Australian Genome Research Facility* (AGRF). The resultant gene-specific sequences were consolidated and verified for their homology with the reference gene sequences through sequence alignment using *Geneious 9.1.3*. The results confirmed that the amplified PCR products were correctly the amplicon matching the genes of interest.

### **4.2.3 Experiment 3 – Hydroponic Assessment of GS Expression in Plants at 60 Days after Seeding**

In *Experiment 3*, Samples of RNA was extracted from five wheat accessions (Gladius, Drysdale, Excalibur, RAC875, and Kukri). The above-mentioned wheat accessions were cultured in hydroponic solutions of high N (HN: 5.0mM NH<sub>4</sub><sup>+</sup>) and low N (LN: 0.5mM NH<sub>4</sub><sup>+</sup>) within a controlled environment room from seeding to 60 days after seeding and tissues were extracted from the plants in destructive sampling (Table 4.3). The composition of the nutrient solution is included in the Appendix. The controlled room was supplied with lighting from electric bulbs (250w 6400k) in a 12-hour photoperiod cycle alternated with total darkness, mean air temperature in the controlled room was 23.6°C, and the nutrient solution was kept at a pH of 6.5 and was replenished with fresh nutrient solution every three days.

Root and leaf samples from the plants in the hydroponic experiment were obtained for RNA extraction and subsequent complementary DNA (cDNA) synthesis. The first batch of tissue samples were harvested on September 22, 2014 and a second on October 13, 2014 (60 days after seeding- DAS) for QPCR analysis. The RNA was extracted using extraction method described in the Direct-zol RNA MiniPrep kit (Epigenetics Company, Zymo Research (<http://www.zymoresearch.com/epigenetics>) protocol. There were four distinct stages in the extraction process: (1). Homogenisation: 1.0g of fresh frozen extraction in 500.0µl Trizol, (2).



Phase separation and precipitation: samples were centrifuged in 1.5ml tubes 12,000 x 1g for 10 min in cold room (8°C). Washing the RNA pellet with 450.0µl of 100% ethanol, per 1.0ml of TRIZOL reagent used for the initial homogenization (3). DNase treatment (DNase – 5.0µl, 10 x DNase reaction buffer - 8µl, DNase/RNase Free water – 3.0µl, RNA Wash Buffer 1 – 64.0µl) (4). Prewash in 400.0µl Direct-zol RNA prewash and final washing in 700.0µl RNA Wash Buffer. The final RNA product was eluted in 50.0µl of DNase/RNase-Free Water in a sterile tube and stored in a pre-cooled rack (-75°C) and later stored at -80°C. The RNA quantity was checked by nanodrop (as above) and the RNA quality was verified by a run of RNA aliquot on a denaturing agarose gel stained with EtBr and confirmed by the appearance of two clear bands per each RNA sample on the agarose gel.

**Table 4.3** Characteristics of the tissue extracts obtained from nitrogen treated wheat plants from Gladius, Drysdale, Excalibur, RAC875, and Kukri that was cultivated under hydroponic conditions in a controlled environment growth chamber and harvested at Zadoks stage 39 (Flag leaf ligule and collar visible).

| Tissue type           | Tissue characteristics  | Nitrogen treatment                                 |   |
|-----------------------|---|--|---|
|                       |   | Low N<br>(0.5 mM<br>NH <sub>4</sub> <sup>+</sup> ) | High N<br>(5.0 mM<br>NH <sub>4</sub> <sup>+</sup> ) |
| Young leaf            | Youngest fully extended leaf next to the flag leaf                | x  | x   |
| Older leaf            | Oldest fully extended leaf about two times the size of young leaf | x  | x   |
| Older roots           | Uniform mixture of roots harvested same time as older leaf        | x  | x   |
| Flag leaf             | Fully extended flag leaf  | -  | x   |
| Flag leaf stage roots | Uniform mixture of roots harvested same time as flag leaf         | -  | x   |

#### **4.2.4 cDNA Synthesis from RNA Samples**

The RNA samples consisting of 4 biological replicates were used as a template for synthesis of the 48 cDNA samples. The cDNA reaction consisted of 1µg RNA + H<sub>2</sub>O (11.0µl), Oligo dT 20 (1.0µl), 10 mM each dNTP mix (1.0µl) making a total volume of 13.0µl. The reaction ran on Tetrad cyclers program using the programme, 65°C for 5 min, 50°C for 1 hr, 70°C for 15 min. After the programme started, the samples were loaded when the temperature reached 65 °C. After 5 min at 65°C, the samples were immediately placed on ice. Then the tetrad thermal cycler was paused (reducing the temperature to 50°C). The following mixture was constituted (5x First strand buffer- 4.0µl, 0.1M, DTT- 1.0µl, RNaseOUT-0.5µl, Reverse Transcriptase - 0.25µl, Water -1.25µl making a total volume of 7.0µl) and 7.0µl of the mixture was added to each tube and then reset on the tetrad thermal cycler (tube at 50 °C). The programme was re-started and terminated after 65°C for 5 min, 50°C for 1hr and 70°C for 15 min, the cDNA samples were finally stored at -30°C freezer.

#### **4.2.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

The qPCR run was performed on Rotor-Gene Qiagen 2 Plex platform (<https://www.qiagen.com>) complemented by generating a standard curve (Appendix Fig. A4.1) using Gene Works KAPA SYBR FAST Universal 2X qPCR Master Mix (1mL), KP-KK4600 ([www.geneworks.com.au](http://www.geneworks.com.au)). Three control genes (Actin, CyclophilinTa, GAPTa) were used in qPCR reactions to construct

the standard curve. These controls were high expression genes that could easily be detected in the qPCR. The PCR product was diluted in 1:100 in 1 X TE buffer (add 10.0 $\mu$ l of the purified PCR product to 990 $\mu$ l buffer), pH 7.5 with 10 $\mu$ g/ml of the cDNA. A 1:10 dilution series in the same buffer was used to prepare 500  $\mu$ l of the standards diluted in the ratios 1:50, 1:100, 1:500, 1:1000, and 1:10000 and stored at - 80°C. A qPCR was conducted on the 3 replicates of each standard (Vandesompele et al. 2002). In the main qPCR experiment test the wheat cDNA samples, the 10.0  $\mu$ l each of the template cDNA samples were loaded into qPCR plates and a master mix of SYBR Green Master Mix (2X) and forward and reverse primers (0.4 $\mu$ M, stock: 10.0 $\mu$ M) 10.0 $\mu$ l was added to each well of the qPCR plate. A volume of 10 $\mu$ l diluted cDNA sample was added to the SYBR Green Master Mix containing the primers. The standard samples were loaded into the qPCR plate and sealed with optical seal. The quantitative data analysis included melting curve data and transcript copy number and concentration (ng) of each sample.

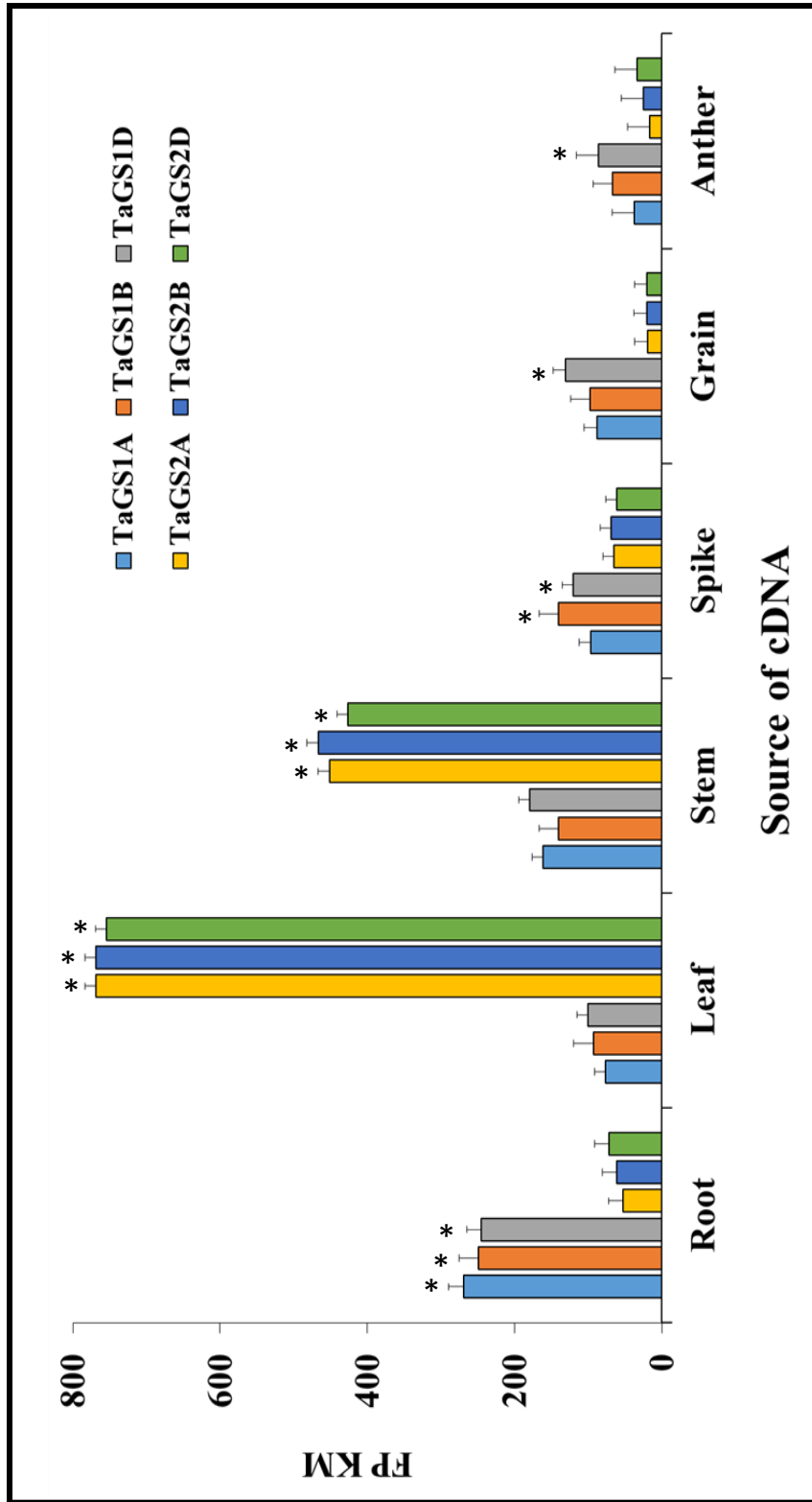
#### **4.2.6 Gene Expression Analysis**

Quantitative data of gene transcript number in wheat cDNA samples was obtained from the qPCR runs. The cDNA samples represented wheat tissues harvested at growth stages that were measured at different developmental stages. Independently for each gene, a linear mixed model analysis was conducted (West et al. 2015) to evaluate the effect of N treatment on gene expression across the different wheat cultivars and tissues. Preceding analysis, expression values were

appropriately log transformed adequately to satisfy model assumptions (<http://www.itl.nist.gov/div898/handbook/pmd/section6/pmd624.htm>). The linear mixed model contained a fixed component that estimated an individual mean for each combination of cultivar, tissue type and N treatment level. The model also contained a random effect to capture the variation attributed to technical replicates. From each of the models the means were extracted and a Tukey's Honest Significance Difference (Tukey, 1947) was calculated to aid in determining significant differences between means. Additionally, for each of the tissue types with both High and low N treatment levels, the differences in overall log mean expression levels between N treatments were determined using a Wald test (Engle, 1983). The Wald test accounts for a finite set of observed intensity or counts across a set of treatments, it is common to find a positive relationship between the intensity treatment means and the variance of the intensity treatment means. Without accounting for this relationship, the assumption of the statistical modelling process become invalid and further inference using model information, such as comparisons between means, may be dubious. A simple log transform of the intensity values mostly removes this dependency and ensures model assumptions are adequately satisfied. All further model inference, including comparisons between treatment means, should occur on the log scale.

### 4.3 RESULTS

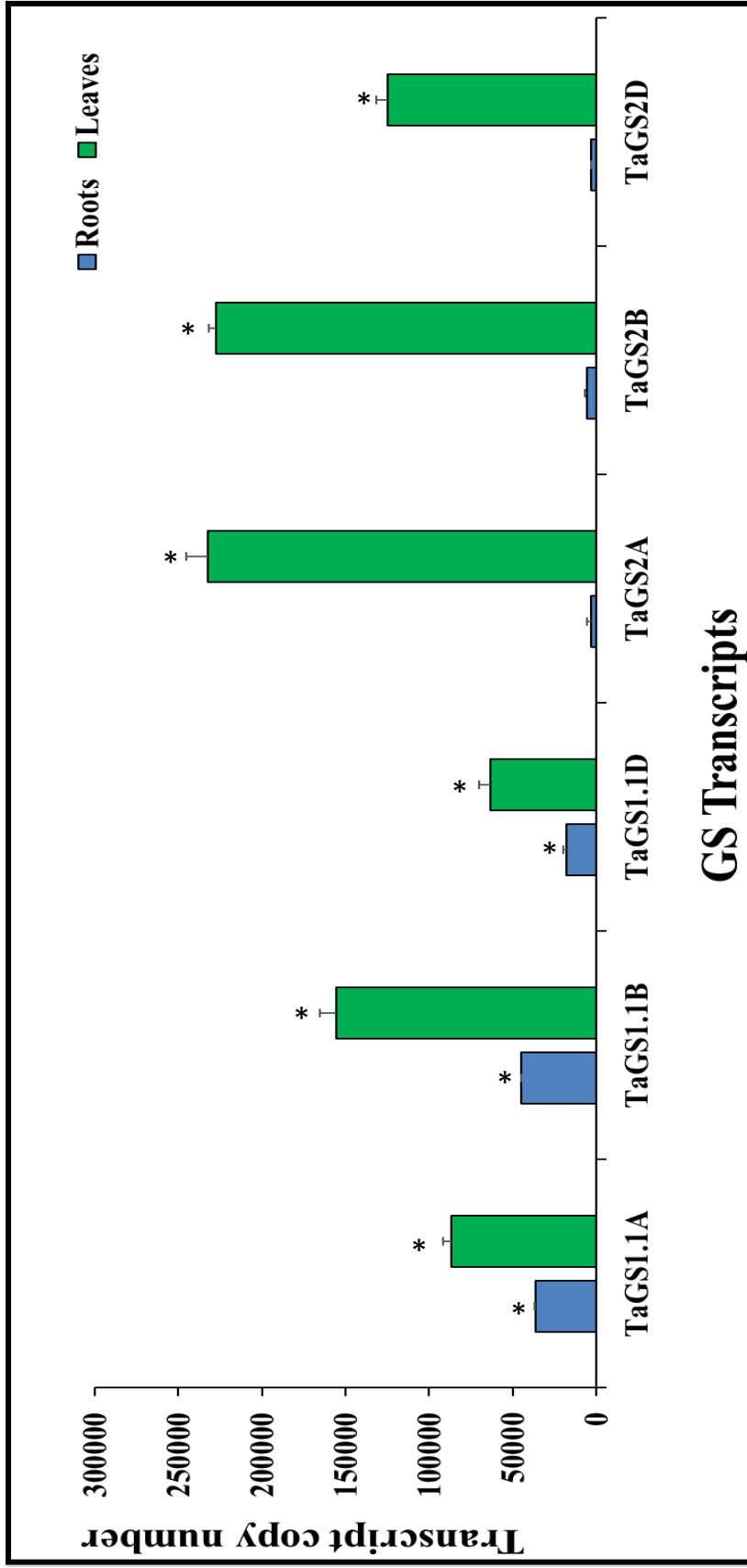
The POTAGE inference of gene expression produced contrasting levels of gene expression in FPKM for both sets of transcripts representative of the *TaGS1.1* and *TaGS2* homeologs. The three *GS1.1* transcripts *TaGS1.1A* (>lcl|6AL\_5820788), *TaGS1.1B* (>lcl|6BL\_227297) and *TaGS1.1D* (>lcl|6DL\_2494823 3419 46201) were found to produce significantly higher expression in root tissues than leaf tissues. In addition, the three *GS2* homeologs *TaGS2A* (>lcl|2AL\_6391136) and *TaGS2B* (>lcl|2BL\_7944286), *TaGS2D* (>2DL\_9842556) were expressed significantly higher expression in the leaves than in the roots. The pattern of expression revealed in the POTAGE results of *Experiment 1*, was consistent for all the GS transcripts examined across three sequences representative of both leaf and root tissues. The level of expression of six GS homeologs in 3 different replicates each of root, leaf, stem, spike and grain was quantified and values were plotted in graphs of Fig. 4.1. The *GS1.1* transcripts were highly expressed in the stem and spike but not in the grain (Fig. 4.1) and *GS2* transcripts were also highly expressed in the stem but comparatively low in the grains.



**Fig. 4.1** POTAGE in-silico sequence analysis and inference of six transcripts representing the expression of GS genes in Chinese spring root, leaf, stem, spike and grain tissues. Transcripts abundance is measured in fragments per kilobase of exon per million fragments (FPKM). Statistical significance ( $p$ -value,  $p < 0.05$ , Tukey test) is indicated by (\*), bars represent transcript abundance levels of errors of four biological replicates and error bars represent standard errors of the means.

The results (obtained through qPCR experiments using different Chinese spring cDNA samples) indicated that the expression levels of *GS1.1* and *GS2* genes were proportionally higher in leaves compared to roots. Among the series of wheat tissues, there were very low expression levels of both GS isoforms in the embryonic tissues (22 days after pollination). The transcript abundance of the *GS1.1* (*TaGS1.1A*, *TaGS1.1B* and *TaGS1.1D*) presented in Fig. 4.2, shows a significantly higher expression level in the root tissues than the *GS2* genes (*TaGS2A*, *TaGS2B* and *TaGS2D*). Conversely, the *GS2* transcript abundance levels in the leaf tissues were significantly higher than the *GS1.1* transcript abundance levels (Fig. 4.2). Overall, when comparing transcript abundance of root with that of leaf it was evident that both *GS1* and *GS2* homeologs were considerably higher in the leaf cDNA extracts (*TaGS1.1A* – 86587.9; *TaGS1.1B* – 155676.1; *TaGS1.1D* – 63170.3, *TaGS2A* – 232503.8; *TaGS2B* – 227152.4 and *TaGS2D* – 125044.5) when compared with the root samples (*TaGS1.1A* – 36351.5; *TaGS1.1B* – 44758.4; *TaGS1.1D* – 17937.5; *TaGS2A* – 3153.4; *TaGS2B* – 5852.3 and *TaGS2D* – 2998.0).



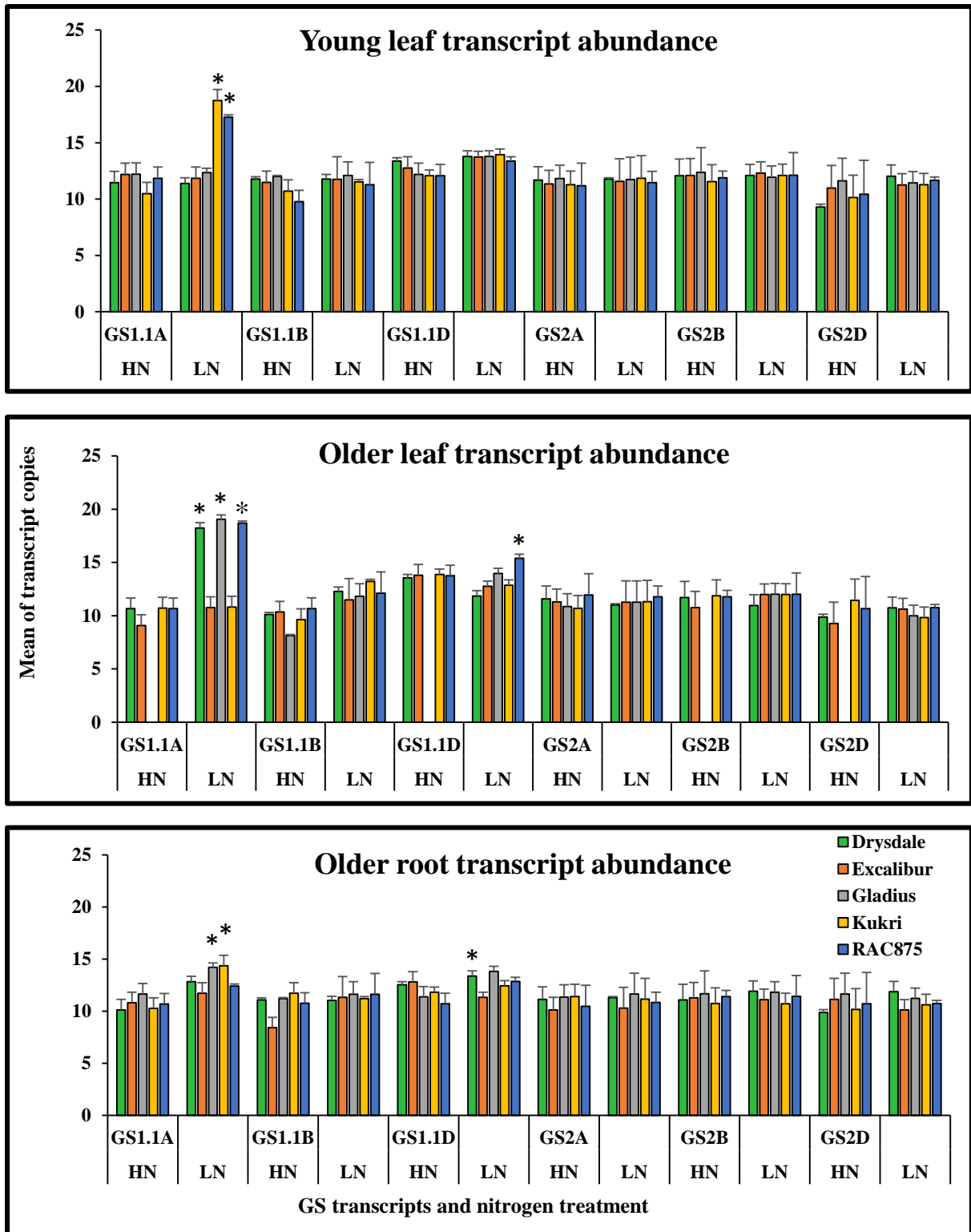


**Fig. 4.2** Real-time quantitative polymerase chain reaction analysis GS transcript abundance in Chinese spring cDNA synthesised from RNA extracted from leaf and root tissues. Statistical significance in comparison with other accessions in corresponding tissues under the same N treatment ( $p$ -value,  $p < 0.05$ , Tukey test) is indicated by (\*), bars represent transcript abundance levels of errors of four biological replicates and error bars represent standard errors of the means.

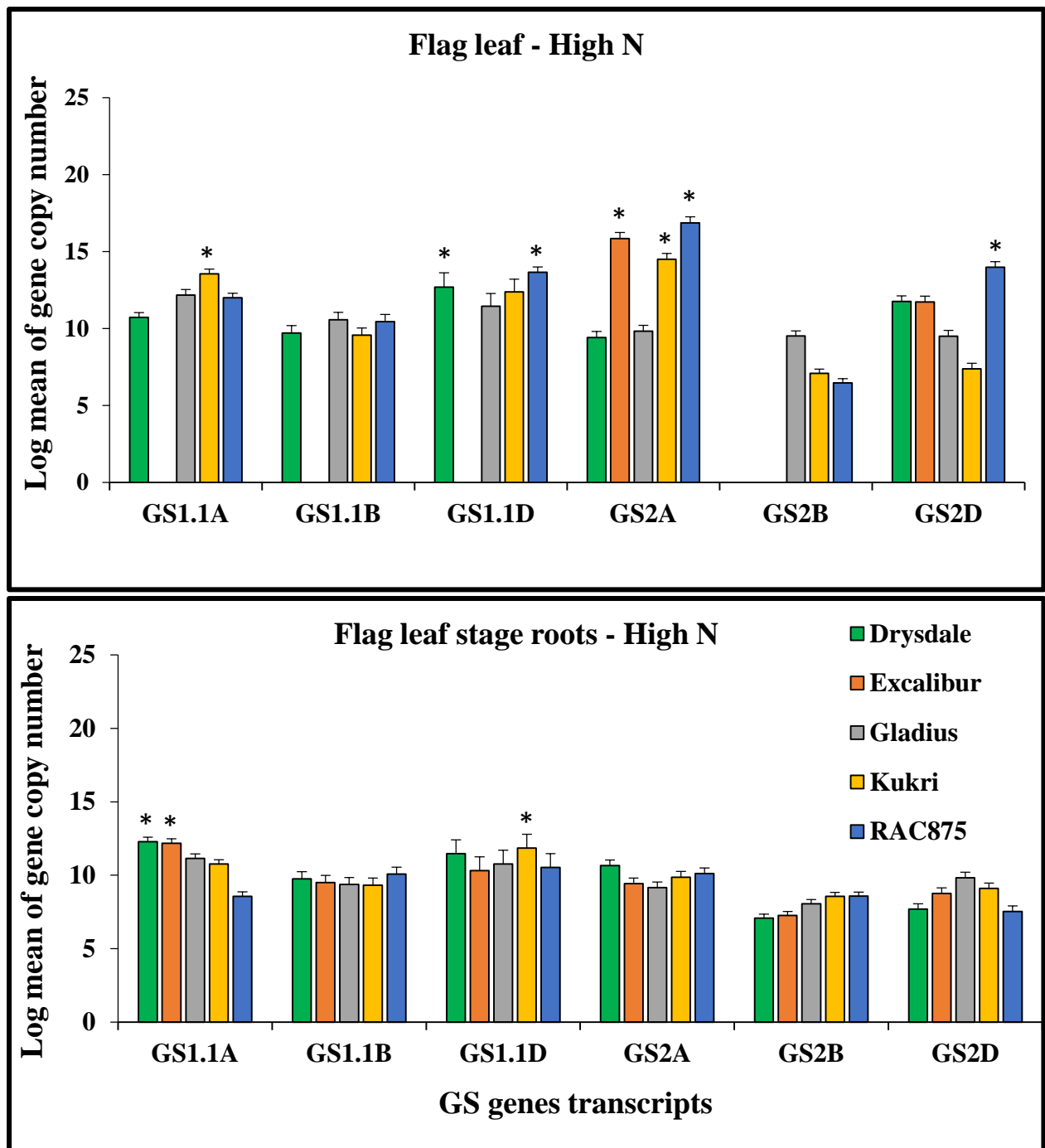
It was observed in *Experiment 3* that there were clearly elevated levels of transcript abundance in the root and leaf tissues of Gladius, Drysdale, Chinese Spring, Excalibur, RAC875, Kukri and Mace. When comparing the flag leaf in different accessions under high nitrogen treatment, gene expression was significantly higher for *TaGS1.1A* in Drysdale and Excalibur. Figure 4.3 shows that there was considerable expression of *TaGS1.1A* transcript abundance in young leaves, older leaves and older roots in four wheat accessions (RAC875, Kukri, Gladius and Drysdale) under low N treatment. In addition, a comparative analysis of the differences among the accessions showed that there was significantly higher *GS2A* expression in the flag leaf of Excalibur, RAC875 and Kukri. Moreover, between the accessions the *TaGS2B* and *TaGS2D* transcripts produced significantly higher expression values under high nitrogen in Gladius and RAC875 respectively (Fig. 4.4).

Under the low nitrogen treatment, there was no observable effect of N treatment on gene expression in the flag leaf. However, the low nitrogen treatment positively affected *TaGS1.1A* expression within the young leaf in Kukri and RAC875. There were parallel positive effects of low nitrogen treatment on *TaGS1.1A* expression when older leaf cDNA samples were quantified in Drysdale, Gladius and RAC875 and in the corresponding older roots for *TaGS1.1A* expression under similar low nitrogen treatment (Fig. 4.4). Generally, the *GS2* genes recorded low expression levels under both N treatments, except

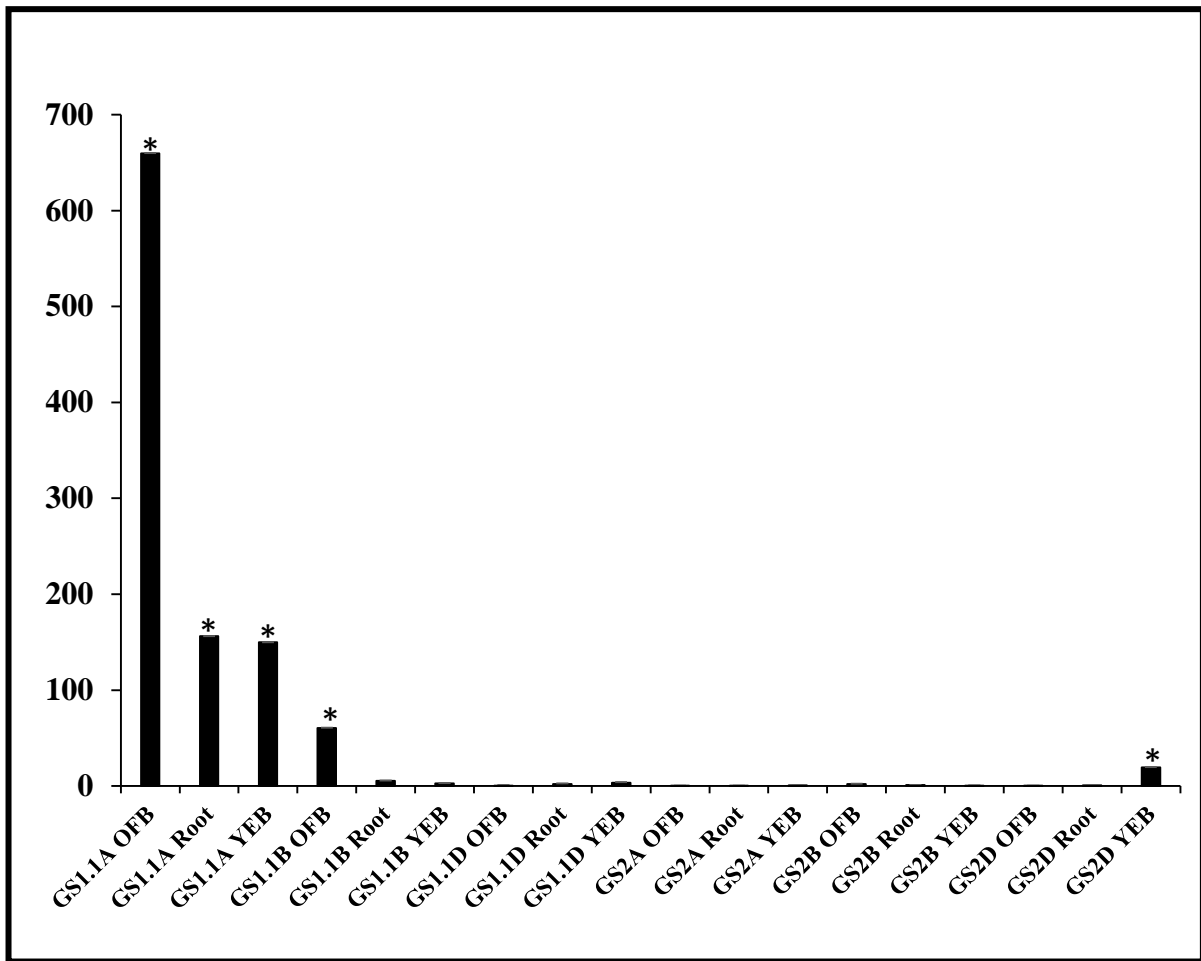
for some significant values in the flag leaf under high nitrogen. The Wald test revealed that there was a significant response of *TaGS1.1A* expression to high nitrogen treatment in young leaves, older leaves and roots (Fig. 4.4). The Wald test values of the GS transcripts presented in Fig. 4.5 show that *TaGS1.1A* was mostly expressed in the older leaves (659.8), root (156.2) and young leaf (149.9). *TaGS1.1B* (60.5) was similarly responsive to the N treatment in the older leaves and *TaGS2D* (19.5) in the young leaves. When reviewing the N treatment effect on transcript abundance in young and old leaf and root tissues, it was clear from the Wald statistical analysis of GS transcripts (statistical significance p-value,  $p < 0.05$ ) that a positive response of gene expression to N treatment was restricted to only three transcripts (*TaGS1.1A*, *TaGS1.1B* and *TaGS2D*).



**Fig. 4.3** Transcript abundance as copies of GS quantified in cDNA synthesised from young leaf, older roots and older leaf under low N (LN) and high N (HN) regimes in potted soil. Transcript abundance scores are log transformed. Statistical significance ( $p$ -value,  $p < 0.05$ , Tukey test) is indicated by (\*), bars represent transcript abundance levels of four biological replicates and error bars represent standard errors of the means.



**Fig. 4.4** Transcript abundance expressed as gene copy number of GS genes quantified in cDNA samples synthesised from flag leaf under high N treatment in potted soil. Transcript abundance scores are log transformed. Statistical significance in comparison with other accessions in corresponding tissues under the same N treatment ( $p$ -value,  $p < 0.05$ , Tukey test) is indicated by (\*), bars represent transcript abundance levels of errors of four biological replicates and error bars represent standard errors of the means.



**Fig. 4.5** Wald statistic profile of GS transcripts and the statistical significance (p-value,  $p < 0.05$ ) of the response to N treatment effect in young fully extended leaf (YEB) and old fully extended leaf (OFB) and root tissues. The asterisks indicate significance level between different accessions under the same N treatment at  $p < 0.001$ , Tukeys HSD.

## 4.4 DISCUSSION

Studies of gene expression in response to N treatment have been explored considerably in *A. thaliana* (Scheible et al. 2004; Schofield et al. 2009). However, reviews and experimental analyses of cereal crop N response and gene expression, provides compelling evidence that very little is known about the interaction of genes with N in terms of gene expression and tissue specific localisation of the genes (Beatty et al. 2009; Yang et al. 2011). An important finding from the present study is that the qPCR trial experiments confirmed that all the GS homeologs are expressed in both the root and shoot tissues of wheat. The POTAGE analysis in Experiment 1 showed higher *TaGS2* expression in leaves and similarly high *TaGS1.1* expression in roots. In Experiment 2, the POTAGE result was confirmed through *in-vivo* qPCR analysis of Chinese spring tissue cDNA samples matching the gene expression inference in the same tissue obtained through *in-silico* sequence analysis using POTAGE. These studies have shown that the expression of cytosolic GS homologs are differentially regulated in roots in response to N treatment.

### 4.4.1 Transcript Abundance of *TaGS1.1*

The mean of *TaGS1.1* expression also shows a similar transcript abundance values for older roots under low nitrogen conditions. This result projecting an increase in root *TaGS1.1* expression under reduced N is not surprising, as similar increases in root GS expression have been observed among barley plants

(Goodall, 2013). Through gene expression analysis of 2-week old barley seedlings Goodall et al. (2013) explained that except increase in *GS1.3*, most of the GS1 transcripts were least expressed in roots when N was increased but highly expressed when the N level was reduced to medium. However, within the leaves, there was increase in all GS transcripts abundance that was consistent with increased N treatment. *GS1.1* showed a reduction in transcript abundance under high ammonium nitrate treatment. In addition, a study of GS overexpression conducted on transgenic rice plants and compared with the wild type, (Cai et al. 2009) found that both *GS1.1* and *GS1.2* transcript abundance modified N metabolism by increasing leaf GS activity, the amino acid content and the N content of all tissues of the plant. This study also showed that overexpression of *GS 1.1* and *GS1.2* transcripts was congruent with a reduced grain yield and protein content when compared with the wild type rice. This pattern was consistent with higher tiller number and shoot biomass under moderate N treatment. These observations underscore the complexity of the effects of gene expression on N metabolism, enzyme activity and related physiological development.

#### **4.4.2 Transcript Abundance of *TaGS2***

The distinct expression of *GS2* genes in leaves is supported in related studies. These studies indicate that the expression of cytosolic and plastid GS isoforms is specific to specific tissues and is associated with distinct functions (Edwards et al. 1990; Pereira et al.1992; Kamachi et al.1991). Studies of maize



has shown that *GS2* was one of four genes that was expressed in roots in response to nitrate treatment (Redinbaugh & Campbell, 1993). This study of maize showed that *GS2* could actively be involved in the transmission of molecular signals from proteins for N-related biochemical reactions. In addition, higher levels of *GS2* mRNA was found in leaves of maize plants that were not treated with nitrate solution. It is clear that the tissue-specific expression of particular GS genes is linked directly to the function performed in that tissue. For example, it has been shown that the high expression of *GS2* in leaf mesophyll cells is consistent with assimilation of the by-product ammonium from photo-respiratory reactions (Migge et al. 2000). Also crucially, this analysis of gene expression in spring wheat accessions demonstrates that the expression levels of *TaGS2* was highest in leaf tissues, though expression levels of *TaGS2* in the leaf tissues were considerably higher than that of *TaGS1.1*.

#### **4.4.3 *GS1.1* and *GS2* Transcript Abundance in Wheat Tissues**

Interestingly, the present study shows that the GS genes were significantly altered when plants were exposed to contrasting N regimes under soil growth conditions. These results appear to establish a robust case for a positive N effect on GS expression. This is the case in spring wheat for the cytosolic GS transcript in both roots and leaves. There were significant positive effects of N treatment on the expression of three genes (*TaGS1.1A*, *TaGS1.1B* and *TaGS2D*). Uniquely, it was evident that there was significant ( $p < 0.05$ ) response of *TaGS1.1A* expression to N treatment in all the tissues quantified. This may suggest that

*TaGS1.1A* and *TaGS1.1B* could be important for N assimilation under high N conditions.

All five accessions showed statistically significant ( $p < 0.05$ ) gene expression patterns in the flag leaf under the high N treatment. However, there were inconsistent patterns showing that each accession responded to the N treatment differently. An evaluation of gene expression patterns in Fig. 10 and 11 shows there were significant increases in *TaGS1.1A* and in *TaGS1.1A* in the young leaf and older roots under low nitrogen, (expression levels in other Kukri tissues were not significant). In Gladius, there was a significant increase in only *TaGS1.1A* in older leaf and roots only under low nitrogen. Expression levels for Drysdale were only significant in *TaGS1.1A* (older leaf under low nitrogen and flag leaf-stage roots under high nitrogen). Excalibur showed significant increases only at the flag leaf stage in both the roots (*TaGS1.1A*).

Unlike the *TaGS1.1* transcripts, this study demonstrated that *TaGS2D* was responsive to N treatment in the young leaves. This is consistent with the result of gene expression of GS2 transcripts in both tobacco and tomato leaves reported by Becker et al. (1992). In a study of GS expression in rice, Zhao & Shi, (2006) found that low N treatment was consistent with higher expression of another cytosolic GS transcript (*GS1.2*). That study also demonstrated that *GS1.1* was significantly abundant than *GS1.2* in the rice leaves, suggesting that *GS1.1* and *GS1.2* may be up-regulated under low N. Tabuchi, (2005) showed that the rice

GS was down-regulated under low N treatment. Further analysis will clearly define the abundance of other cytosolic GS transcripts under low N.

These expression patterns were rather inconsistent between genes, accessions. For example, transcripts of three GS genes (*TaGS1.1A*-young leaf, *TaGS1.1D*-older leaf and *TaGS2A*-flag leaf) were highly expressed in RAC875 flag leaf, three in Kukri (*TaGS1.1A*-young leaf, older leaf, flag leaf, *TaGS1.1D*-flag leaf stage roots and *TaGS2A*-flag leaf) and one each in Gladius (*TaGS1.1A*-old leaf and older root, *TaGS2B*-flag leaf) and Excalibur (*TaGS2A*-flag leaf). When compared, the transcript abundance of the older leaf and older root tissues show that the *GS2* levels recorded expression levels under both N treatments were not significantly different between the tissues.

#### **4.4.4 Nitrogen Effect on GS Expression**

The study of barley leaves by Goodall et al. (2013) demonstrated the contrasting expression of *GS1.1*. In barley, the cytosolic GS expression was inhibited in the presence of increased N concentration, but the plastid genes were repressed. In rice *GS1.1* expression was found to be abundant in leaf tissue under similar N concentration (Tabuchi et al. 2005). This provides a clear indication that the effect of N treatment on gene expression is very complex and highly variable between species and tissue-dependent, and that the modulation of tissue specificity of GS genes is not constant when comparing different plant species. Sukanya et al. (1994) demonstrated that the cytosolic *GS 1.1* transcript expression was higher in roots than leaves and that *GS1.2* (another cytosolic GS) was higher

in leaves than in roots when maize seedlings were treated with 10 mM of potassium nitrate and 10 mM ammonium chloride solution. The same study also found unchanged patterns in the expression of cytosolic *GS1.3* though the plants were treated with the same nutrient solution. Prinsi & Espen (2015) demonstrated that the *GS1.1* increased in roots of maize plants that were treated with ammonium. These results indicate that N differentially regulated different GS homologs.

In the present study, N effect was clear in the patterns of *TaGS1.1A* expression and the GS activity observed in this accession. Therefore, the three highly expressed and N responsive GS genes (*TaGS1.1A*, *TaGS1.1B* and *TaGS2D*) are three potential genes that could be targeted for a detailed analysis of GS enzyme regulation in the RAC875 accession. Unlike the case of the flag leaf, evaluation of the older leaf and root data shows that significant gene expression was present for *TaGS1.1* gene only under LN treatment. This is considerable evidence suggesting that the plastid GS isoforms might not be expressive in response to reduced N in older leaf and root tissues, as opposed to their significant activities observable in young leaf and flag leaf tissues that were harvested at the same growth stage. The patterns of N treatment and gene expression explored in this study provides evidence of the intricate interaction of gene expression and gene specific response (Andrews et al. 2004; Tabuchi et al. 2005) to the N treatment assimilated in specific tissues.

Moreover, it is explainable in studies of barley mutants and other species that *GS1* genes could not compensate the loss of *GS2* enzymes in leaf cells (Lam et al. 1996). This suggests clearly distinct functional roles (Garnett et al. 2009; 2015) for the different enzymes encoded by the GS genes (Hirel & Gadai, 1980; Oliveira et al. 2002; Coruzzi et al. 2003; Thomsen et al. 2014) based on cell or tissue specific expression.

An outstanding observation about GS gene expression is that of the very labile and rather inconsistent patterns in expression recorded among different accessions. Grabowska et al. (2012) concluded that there is a likely affinitive interaction between GDH and *GS1* necessary for the biosynthesis of glutamine and glutamate. Hansen et al. (2009) have highlighted an earlier and corresponding observation respecting the cooperative roles of *GS1* and GDH during barley seed formation. It is clear from the present study of gene expression under contrasting N regimes that, there is the need for further quantitative analysis of gene expression within the framework of more stratified N regimes using different tissue templates. For future studies, the construction of larger cDNA libraries using accessions from a range of more diverse backgrounds may provide additional resources for examining and categorizing the scope of gene expression in hexaploid wheat. Such information may help pinpoint the genetic differences necessary for identifying genetic markers for NUE variables in wheat and other cereal crops.

## 4.5 CONCLUSION

Research targeting N related agronomic traits can be considered as very fundamental to cereal yield improvement. This is due to the significance of the biosynthetic mechanisms of N uptake and assimilation to plant growth and development. Among N-linked genes controlling these processes, GS genes feature prominently in the assimilation of ammonia and nitrate into roots and leaves. This study has used two standard genomic approaches (*in-silico* transcripts inference and qPCR analysis) to confirm the tissue specificity of GS genes by measuring the level of transcript abundance of *TaGS1* in roots and *TaGS2* in leaf tissues of the reference wheat accession Chinese spring under non-induced N treatment conditions. This demonstrated that syntenic groups of homeologs showed similar and consistent patterns of expression in similar plant tissues. Another confronting realisation was that there were no clear patterns in gene expression across different accessions. Neither was there uniform patterns in expression between the six different GS homeologs investigated. The evidence therefore suggests that, GS expression in different tissues was under targeted control in response to N among the different spring wheat accessions. There could be several genetic factors underpinning GS expression in relation to the N treatment.

The profile of gene expression in wheat and other plants might be much more complex and the biosynthetic mechanisms of N uptake and assimilation under different N treatments appears to be much more intricate than previously acknowledged or characterised. An effective approach to resolving the

confounding patterns evident in GS gene expression data may be to use high throughput quantitative and genomic techniques that will produce expression data on a larger set of GS genes and consequently identify genetic markers linking gene expression to specific N effects.

## CHAPTER FIVE

### GLUTAMINE SYNTHETASE ACTIVITY IN WHEAT AT HIGH AND LOW NITROGEN TREATMENTS

#### 5. ABSTRACT

Glutamine synthetase (GS, EC 6.3.1.2) activity in wheat accessions was used to investigate GS activity under high nitrogen and low nitrogen treatments. Seedlings of five spring wheat accessions lines and two mapping population were cultured in hydroponic units. Leaf and root tissues were harvested at 5 different growth points and assayed for GS activity. The tissue enzyme activity was analysed across genetically diverse accessions to explain patterns of metabolic activity within the wheat plants at different growth points. Analysis of total GS activity linked with ammonium and nitrate assimilation showed that GS activity was moderated by a number of genetic loci in two wheat mapping populations (Gladius x Drysdale, RAC875 x Kukri). The results however showed that enzyme activity was moderated under temporal control (significantly influenced by specific developmental changes on a temporal scale) as when harvesting date was standardised across different wheat accessions at Zadoks Scale 39 (Feeke's Scale 9) wheat growth stage. A significant QTL association for low N flag leaf GS activity was detected on *Chromosome 5A*, the locus at which the QTL mapped above the standardised threshold.



This suggests that this locus might be important for GS activity regulation under low N treatment. The results of the tissue enzyme assays and the significant changes in enzyme activity at tillering, stem elongation and anthesis reported in this study demonstrates that enzyme metabolic activity is developmentally regulated in hexaploid wheat.

## 5.1 INTRODUCTION

Glutamine synthetase catalyses important biochemical reactions associated with N assimilation and remobilisation in plants including cereal crops. Two notable GS isoforms (*GS1* and *GS2*) have been found to be active in the metabolic functions involving  $\text{NH}_4^+$  and  $\text{NO}_3^-$  as in the assimilation of inorganic N. *GS1* has been linked with the synthesis of glutamine essential for N remobilization (Lam et al. 1995; Li et al. 2011; Masclaux-Daubresse et al. 2001; 2006; Gaufichon et al. 2010). It has been demonstrated that, the cytosolic *GS1* catalyses  $\text{NH}_4^+$  to glutamine for further conversion to amino acids that eventually are assimilated into shoot tissues. The functional role of *GS1* has been investigated (Suarez, 2002) in the mesophyll and phloem cells in association with cytochemical roles in pine seedlings. The *GS2* gene is highly expressed in leaves and has been associated with photo-respiratory functions (Coschigano et al. 1998). Chloroplast and plastid *GS2* transcripts are actively involved in the glutamate 2-oxoglutarate transaminase (GOGAT EC 1.4.7.1) cycle converting glutamate to glutamine using  $\text{NH}_4^+$ . This is a critical biosynthetic and metabolic pathway for N-containing amino acids, alkaloids and polyamines.

It is evident that several biological and chemical factors mediate N uptake and assimilation in plants. Currently, reviews of published works (Mifflin & Habash, 2001; Swarbreck et al. 2011) reporting GS gene regulation and NUE variables in plant species, expose many knowledge gaps respecting GS regulation of ammonium assimilation, assimilation and remobilisation (Masclaux-Daubresse et al. 2008) in cereal crop species notably maize, rice and wheat. Due to the variety and complexity of the biological and chemical processes mediating NUE in plants, it is experimentally difficult quantitatively to verify the regulatory roles of individual genes. Unsurprisingly, a number of different approaches have been used in studying GS genes and profiling NUE in plants. Enzyme metabolic has been quantified for N-linked genes in *Arabidopsis thaliana* (Kant et al. 2008; 2011). This is the case with barley (Avila-Ospina et al. 2014; 2015), rice (Yamaya et al. 2002; Ishiyama et al. 2004), maize (Martin et al. 2006), wheat (Habash et al. 2001; Bernard et al. 2008, 2009; Yousfi et al. 2012; Wang et al. 2015) and other crop species (Brugiere et al. 2000). For example, tissue metabolic activity of the GS enzyme has been quantified in maize as a parameter to understand the variable response of accessions to fertilisation. Studies of maize have become the basis to explain the putative role of many enzymes (Rispaill et al, 2004) that mediate N metabolic reactions involving, uptake, utilization and remobilization. An improvement on classical physiological-agronomic studies of enzymes is the adoption of quantitative and genomic techniques including linkage analysis and quantitative trait loci mapping.

Quantification of GS activity is one of the effective approaches to investigate genotypic responses to N fertiliser treatment (Urbanczyk-Wochniak & Fernie, 2005) in cereal crops. In this study, GS activity was quantified as the total metabolic activity in 1.0 mg of homogenised tissue per incubation time of 30 minutes.

Generally, maize has represented a particularly important role in characterisation of the GS genes due to the dual merits of high rate of gene polymorphism (for detectability of genetic variation) and global cultivation status that can be a vital source of information on plant N efficiency in different environments (Masclaux et al. 2001). Cytosolic GS isoforms have been investigated in maize (Martin et al. 2006). These isoforms have been confirmed as important grain filling regulators (Martin et al. 2006). Different sampling time points have been used in evaluating GS activity in cereal species. For example, the effect of salinity on GS activity has been measured in 21 days old wheat shoot and root seedlings (Kwinta & Cal, 2004) after 14 days in source N solution. In addition, a study of *Hordeum vulgare* (L. cultivar Golden Promise) in a growth chamber, Avila-Ospina et al. (2015), investigated plants in pots supplied with either high or low nitrate nutrient solution. Leaves of seedlings 20 days after sowing (DAS) were harvested for GS activity analysis. All these studies reported significant differences in GS activity at the time points specified.

Therefore, there appears to be a wide range of time points for measuring GS activity. This will make it difficult to pre-select a particular measurement point that will show significant variation in enzyme activity in a wide panel of different species.

Generally, molecular characterisation of GS genes and GS activity in cereal crops have focused mainly on maize (Limami et al. 2002; Gallais & Hirel, 2004, Martin et al. 2006), barley (Avila-Ospina et al. 2015) and rice (Kusano et al. 2012). Limami et al. (2002) found one QTL for GS activity and thousand kernels weight. In addition, Gallais and Hirel (2004) detected QTLs for leaf GS activity accounting for 52.5% of the phenotypic variation between maize varieties. Three of the six QTLs coincided with cytosolic GS encoding maize *gln1*, *gln2* and *gln4*. *In-silico* sequence analysis, Swarbreck et al. (2010), reported significant variation (Pearson's correlation coefficient > 0.7) in the co-expression of *GS1* and *GS2*. This is important as it can serve as a reference for analysing *GS1* and *GS2* transcripts and the levels of enzyme activity linked to these transcripts.

In a study of maize GS activity, Martin et al. (2006) found that *Gln1.3* and *Gln1.4* mutants were associated with decreased GS activity compared with the wild type plants. In barley, Avila-Ospina et al. (2015) reported that under both low N and high N, GS activity was significantly higher in old leaves was significantly higher than the GS activity of young leaves. However, when comparing the first two corresponding lowest leaves on low N and high N plants, the total GS activity was higher in the low N leaf than the GS activity in HN leaf.

In rice, Funayama et al. (2013) detected that the *GS1* activity assayed in the roots of 18-day-old rice seedlings was lower than 50% of the GS activity recorded in roots of the wild type plants when all plants were grown under 1mM ammonium. These results indicate that cytosolic GS genes are important in the primary assimilation of ammonium and are strongly linked to GS activity as an integral component of the N metabolism. However, when comparing two rice varieties (IR-64 and Khitish), Singh and Ghosh (2013), concluded that a relatively lower root GS activity in IR-64 seedlings was due to decrease in GS2 activity under water deficit.

Despite the studies of maize, barley and rice GS activity, few studies have investigated NUE parameters like GS activity in spring wheat. Bernard et al. (2008) conducted the first characterisation of GS genes in wheat using mapping information, sequence analysis and phylogenetic analysis as a way of explaining gene function. However, that study did not include the analysis of GS activity under different N regimes. Habash et al. (2007), investigated GS isozymes and associated QTLs in two wheat cultivars (CS & SQ1). The study of GS isozymes (Habash. 2007) demonstrated that QTLs for flag leaf GS activity were linked with fresh weight and suggested that these QTLs may be linked with grain N. However, the approach used was limited by the sample size of tested accessions and in the scope of GS activity coverage respecting the early growth stages. There has not yet been a study of GS activity spanning a wider range of growth stages.

There is a need for such analysis to show the variation in metabolic activity in a larger number of wheat accessions. Such information will be important for understanding the N efficiency of plants at specific growth stages. The present study highlights the attempt to decipher total GS activity when comparing wheat accessions under high and low  $\text{NO}_3^-$  concentration levels in hydroponic solutions and over a specific time course. It is proposed that significant GS activity is related to specific and clearly defined developmental stages for each wheat genotype. For example, increased leaf metabolic activity (Oliveira et al. 2002) was observed to elevate sink-source transfer of N-compounds during grain filling (Racjan & Tollenaar, 1999).

The present study analysed tissue specific GS activity at distinct developmental stages from plants that have been hydroponically grown under high and low N. The GS activity assay and subsequent analysis of the metabolic activity showed consistent levels of enzyme activity in the flag leaf under high N treatment. However, contrasts in GS activity during anthesis and grain filling suggest specific genetic influence in accessions.

## 5.2 MATERIALS AND METHODS

A total of three experiments were conducted between 2013 and 2015 at the Plant Research Centre, University of Adelaide, Waite Institute, Australia.

### 5.2.1 Experiment 1: GS Activity at Four Growth Stages

*Experiment 1* consisted of two experiments: *Experiment 1(1)* was conducted using germplasm material that consisted of 8 hexaploid wheat accessions (Gladius, Drysdale, Excalibur, Kukri, RAC875, Scout, Espada, Chinese spring). The seedlings were cultured in hydroponic units until the fourth week after establishment. Hydroponic solutions of high N (HN: 5.0mM NH<sub>4</sub><sup>+</sup>) and low N (LN: 0.5mM NH<sub>4</sub><sup>+</sup>) were used as a medium to cultivate the seedlings until 46 DAS (days after seeding) within a controlled chamber that was lit from electric bulbs (250w 6400k) in a 12-hour photoperiod cycle that alternated with total darkness. The mean internal air temperature of the growth chamber was 23.6°C, the nutrient solution was replaced twice a week, and the pH was maintained at 6.5 by adding NaOH and HCl. The roots, young fully emerged leaf blade (YEB) and older fully formed leaf blade (OFB) were harvested at 18, 32 and 46 DAS. Harvesting dates were consistent for all accessions. At harvesting, phenotypic variables including SPAD, tiller number, shoot length, leaf number, fresh shoot and root weight and biomass were measured and analysed. *Experiment 1(2)* was established to test GS activity in five accessions (Gladius, Drysdale, Excalibur, Kukri and RAC875) that showed considerably high GS

activity in the *Experiment 1(1)*. The methods used in *Experiment 1(2)* were the same as used in *Experiment 1(1)* in design, but the harvesting dates included was extended to 60 DAS to assess GS activity at the flag leaf stage. Therefore, the harvesting dates were 18 DAS, 32 DAS, 46 DAS and 60 DAS. In the second experiment, the nutrient solution for the hydroponic medium was optimised by replacing pipe-borne water with reverse osmosis (RO) water. This was necessary to remove any chemical residues that may alter the nutrient composition of the growth medium. The shoot tissue material was assayed for GS activity levels in the young fully emerged leaf blade and older fully formed leaf blade, as well as in the roots.

## **5.2.2 Experiment 2: GS activity based on Zadoks Growth Stages**

### **5.2.2.1 Germplasm Material for Experiment 2**

The germplasm material consisted of hexaploid wheat accessions (Gladius, Drysdale, Excalibur, RAC875 and Kukri). The seeds were obtained from the Australian Centre for Plant Functional Genomics (ACPGF) stocks (<http://www.acpfg.com.au/index>). The material used also comprised one mapping population consisting of an F1 derived double haploid population (DH) from a cross between RAC875 x Kukri and second mapping population of recombinant in-bred lines (RILs) from a cross between Gladius x Drysdale.



### **5.2.2.2 Experiment 2 Activities**

The flag leaf of each plant was harvested synchronised with the emergence of the awns and clear visibility of the leaf ligule (Zadoks 39) in each genotype. It was important to harvest all accessions and mapping populations at precisely this same developmental stage to allow for uniform comparison of the GS activity at the same developmental stage across different accessions. There was a mean of 7 days' interval between the harvesting days of RAC875 (earlier) and the other accessions (Gladius, Drysdale, Excalibur, Espada, Mace, Chinese Spring). Kukri was the last to reach the developmental stages (14 days after the harvesting date of RAC875). The accessions were assessed for differences in GS activity. Significant differences among the accessions then served as a basis for choosing specific mapping populations that corresponded to the specific parents within the set of accessions.

Moreover, the flag leaf, leaf next to flag leaf (flag leaf-1) and root tissues were all sampled at six clearly distinct Zadoks wheat developmental stages (<https://www.agric.wa.gov.au/grains/zadoks-growth-scale>) for enzyme assays. The stages included Zadoks 23 (main stem and 3 tillers appear), Zadoks 31 (first node emerge / stem elongation), Zadoks 39 (flag leaf ligule just visible), Zadoks 49, (first awns emerge / heading (Zadoks 49), Zadoks 69 (anthesis (Zadoks 69) complete) and Zadoks 73 (early milk grain). There were 8 replicates of each variety in each treatment and the pots in the hydroponic units were arranged in 4 experimental blocks that represented the two N treatments.

Each block contained three sub-plots of each variety. Eight plants of each variety were sampled per treatment at the onset of distinct developmental stages that corresponded to clearly defined Zadoks growth stages.

### **5.2.2.3 Hydroponic System Set-up and Growth Conditions**

As shown in Appendix Fig. FA5.1 and Fig. A5.2, four large hydroponic units were set up in a randomised design in a controlled environment room or growth chamber (Appendix Fig. A5.1). The mean air temperature of the growth chamber was 22.6°C and relative humidity was 56.78% and light intensity 382.4lx. Each hydroponic unit treated as a block consisted of 120L plastic bin (set on a 1m high lab bench) connected to two upper 10L capacity buckets. The buckets and bins were supplied by pump-fitted plastic hoses (in-lets and out-lets) that served as drainage paths for the constant circulation of the nutrient solution between the bin and the buckets above bins (Fig. A5.1). Four replicates were established and 80 plants were set in each hydroponic unit. The nutrient solution supplied was constituted from inorganic N sources and trace elements (Appendix) formulated in RO water. Two different solutions (5.0mM NO<sub>3</sub><sup>-</sup>; HN; 0.5mM NO<sub>3</sub><sup>-</sup>; LN) representing two N treatments were prepared and applied separately. High and low N treatments were randomised in one block. Each unit was filled with polyvinyl plastic (PVP) tubes 3 x 14 cm. The pots were stabilised with plastic lid with circular holes carved to secure each pipe from swaying. Each PVP tube was fitted with a mesh set 6cm from the top of the PVP tube to serve as a platform for the seeds.

Seeds of each wheat accession were first set in filter paper embedded transparent petri-dishes and germinated in 60% RO water for three days at room temperature. The seeds were then transplanted into the pots (set in the hydroponic units) at the emergence of the radicle. The pH of the nutrient solution was maintained at 5.9 by balancing with 1M NaOH and 1M H<sub>2</sub>SO<sub>4</sub> and re-checked every 72 hours. The nutrient solution was renewed with fresh stock every 7 - 10 days.

#### **5.2.2.4 Buffer Preparation and Biochemical GS Assays**

The buffer for the reaction solution was made from 19.7g (125mM) Tris-HCl dissolved in 800ml H<sub>2</sub>O (milliQ), pH was adjusted to 7.6 (using 1M HCL/1M NaOH) and made up with H<sub>2</sub>O (milliQ) to 1000ml. The reaction solution (R-mix) was constituted from 36.97 (150mM) MgSO<sub>4</sub> .7 H<sub>2</sub>O (Mw: 246.48 g/mol), 88.28g (600mM) Glutamate (Mw: 147.1299g/mol), 3.13g (45mM) Hydroxylamine (Mw: 69.556g/mol), 11.16 g (30mM) EDTA - Na<sub>2</sub> (Mw: 372 g/mol). This solution was adjusted to pH 7.6 (1M HCl/1M NaOH) and H<sub>2</sub>O (MilliQ) added to 1000ml. Tissue samples (YEB, OLB and Flag leaf) were harvested and frozen in liquid N, crushed on a Geno grinder into fine powder and extracted in 100mg of 1ml extraction buffer. The extraction buffer was added in two 50µl samples separately into 2 adjacent wells on a 96-well microtitre plate: 50µl to the first well (ATP + mix) and 50µl to the second well (ATP - mix). The ATP + mix was a solution constituted from 6ml reaction buffer, 2ml R- mix and 2ml ATP. The ATP- mixture was constituted from 8ml reaction buffer and 2ml R- mix, minus ATP.

The ATP was a solution prepared from 3.3g (60mM) ATP (Mw: 551.1g/mol) that was dissolved in 100ml H<sub>2</sub>O (milliQ) and adjusted to a pH of 7.6. The next step in the GS assay process was to add 100µl of Mix 1 (ATP+ mix) with exactly 15-second intervals to the first well and thereafter 100µl of Mix 2 (ATP- mix) to the second well. The microtitre plate was then covered with fine parafilm and aluminum foil and incubated at 30°C for 30 min.

To stop GS activity 150.0µl stop solution was added to the plate in the same order (15-second intervals) as the addition of Mix 1 and Mix 2 solutions. The stop solution was made from 181.0g (0.67M) FeCl<sub>3</sub> · 6 H<sub>2</sub>O (Mw: 270.3g/mol), 32.7g (0.2 M) TCA (Mw: 163.39g/mol), 55 ml HCL (Mw: 36.45g/mol) dissolved and volume adjusted to 1000ml with H<sub>2</sub>O (milliQ). Seven standard (St) solutions (concentration in 200µl well, nmol/well - St1:0, St2:50, St3:100, St4:200, St5:400, St6:600, St7:800 - Table A5.3) was prepared from 100 nmol/µl stock of γ - glutamylhydroxamate (γ-Glutamic acid γ-monohydroxamate).

This was a mixture of 16.2 mg γ -glutamylhydroxamate mixed with 1ml H<sub>2</sub>O (milliQ). The standard solutions were added to the microtitre plate and centrifuged at 10000 rpm for 5 min. A total volume of 200µl was transferred from each well to a new plate using a multichannel pipette. The absorbance of all samples in the plate was measured at 540 nm on a microtitre plate reader using the MARS Data Analysis program (<http://www.bmglabtech.com/en/products/software/mars-data-analysis/>).

GS metabolic activity in the fresh wheat tissues was measured by spectrophotometric absorbance using Polarstar Optima BMG Labtech 2.20R2 ([www.labtechsoftware.com/](http://www.labtechsoftware.com/)) on a Greiner 96 U-Bottom plate. The absorbance settings used were Start concentration factor: 1, Start volume factor: 1; Excitation filter: Ex544, Emission filter 520; Shaking width: 150 rpm; Mode: double orbital).

### 5.2.2.5 GS Activity Determination

The absorbance values of 7 standard solutions (Table S5.3) were plotted in a linear regression and the function was used to calculate GS activity. GS activity absorbance values from the assay spectral measurements were treated as raw data for determining actual GS activity. The GS activity was calculated as the difference between the ATP+ and ATP- absorbance readings from a reaction/incubation time of 30min. In this study, we calculate this cumulative metabolic reaction in root and leaf tissues as a function of enzyme activity per incubation time of 30min and a dilution factor of the tissue extract.

GS activity was calculated as:

$$\frac{((X * (60 / \text{incubation time}) * (300 / 200)) / (\text{dil.factor} * 0.05)) / 1000}{(\text{incubation time}) * (60 / \text{incubation time}) * (300 \mu\text{l} / 200 \mu\text{l}) / (\text{dil.factor} \text{ g / ml} * (0.05 \text{ ml})) / 1000} \quad (X \text{ nmol / well / incubation time})$$

Each variable in the equation is defined as:

*(30/incubation time)*: incubation time, normally 30min

*(300/200)*: the reaction in 300µl but measured on 200µl per well.

*dil.factor*: sample ( $\text{g}^{-1}$  fresh weight) is diluted in extraction buffer in 1:10 ratio (0.1g/ml)

*0.05*: measurement done in 50 $\mu\text{l}$  (=0.05ml) extract in the final 300ul.

*1000*: conversion from *nmol* to  *$\mu\text{mol}$* .

The unit of GS activity calculation was stated as  *$\mu\text{molGHA/gFW/ h}$*  (glutamic acid reaction rate per gram fresh weight).

### **5.2.2.6 Genetic Analysis of GS Activity**

Prior to the overall trait analysis of the metabolic data, a phenotypic model was tested based on the non-replicated RIL and DH wheat accessions only. This increased the statistical strength of the data set. The trait data on GS activity was natural log transformed to increase the degree of normality within the data set (Yeo & Johnson, 2000; Kirisci et al. 2001). This improved the model diagnostics for a QTL analysis that utilised 1333 unique markers from a 90K SNP map dataset. A scanning method (i.e. one marker at a time) where each model included the relationship matrix for all the varieties was used (Verbyla et al. 2012; Bonneau et al. 2013). This was equivalent to keeping the whole genome marker set in each model. Before forming each relationship matrix, markers in a 20cM exclusion margin around the marker being tested was removed. This has been known to increase the power of the marker being tested (Wolfe et al. 2016; Gao et al. 2016). Plots of the Wald Statistic profile (similar to LOD profile) for each trait by treatment was analysed and spreadsheets for each trait by treatment with a test of each marker across the 1333 unique markers of the genome was constructed.

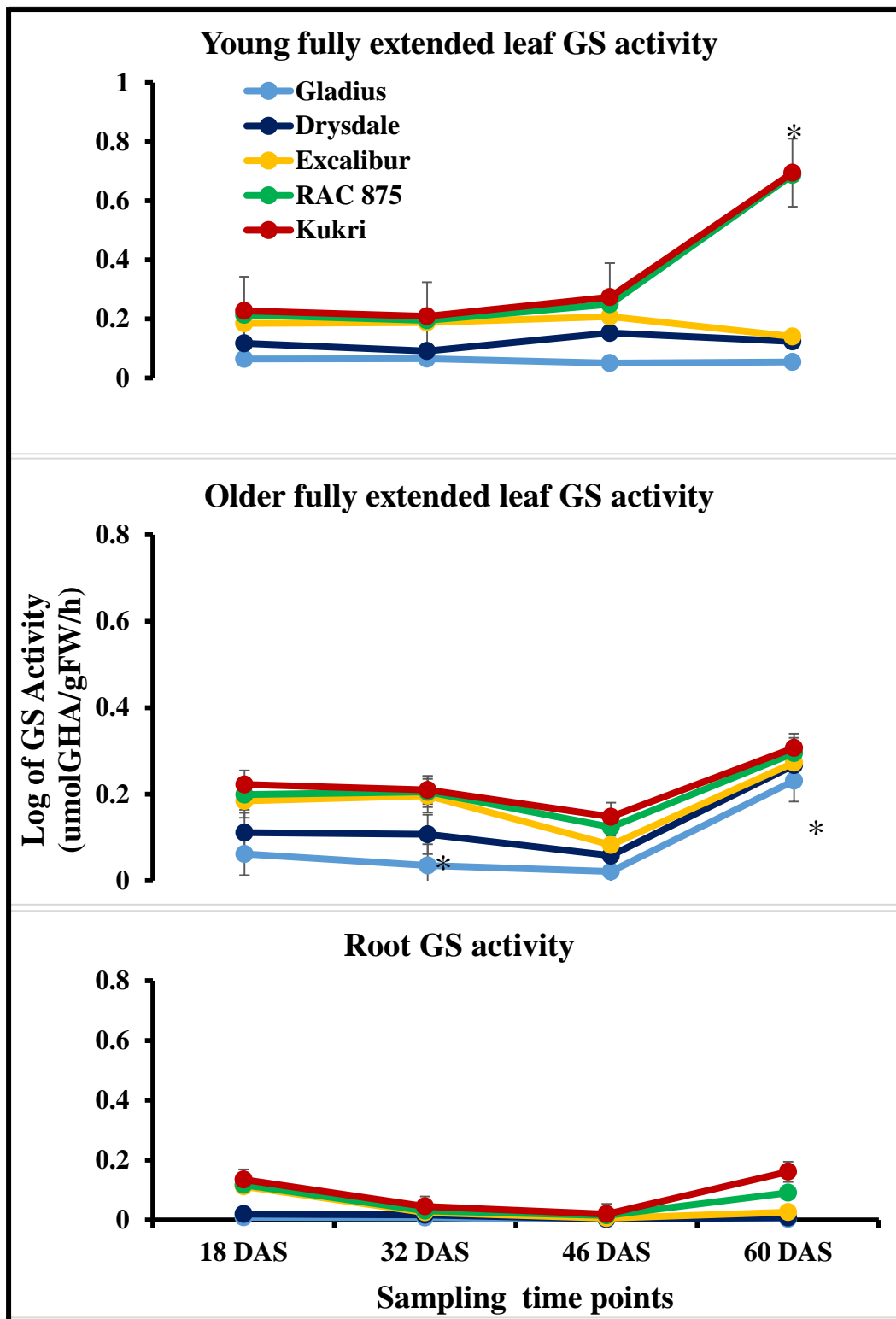
### **5.2.3 Experiment 3: GS Activity in Plants Grown in Soil**

In *Experiment 3* the plants were grown in pots filled with loamy soil. The nutrient composition of the growth medium was the same as the high and low treatments in the hydroponic experiment (5.0mM NO<sub>3</sub><sup>-</sup>; HN; 0.5mM NO<sub>3</sub><sup>-</sup>; LN) and micronutrients were of the same concentration in RO water as was used in the hydroponic experiments. The plants were supplied with the high and low N solutions twice a week. The plants were grown in the same growth chamber. The environmental conditions of the soil experiment were the same as that of the hydroponic experiment described above. Three accessions included two accessions that showed considerably high (RAC875) and low (Kukri) GS activity levels in the hydroponic experiments; and a third accession Mace which was found to have high N uptake in field studies (Mahrouidmajd et al. 2016). The flag leaf, fully extended leaf next to the flag leaf and roots were harvested per the developmental stages of tiller emergence, node (stem elongation), awn emergence, heading, anthesis and milk grain in each accession. The onset of these stages was varied from 7 days between RAC875 and Mace and 14 days between RAC875 and Kukri, as RAC875 matured faster. The GS assay and GS activity analysis was the same as described for the hydroponic experiments.

### 5.3 RESULTS

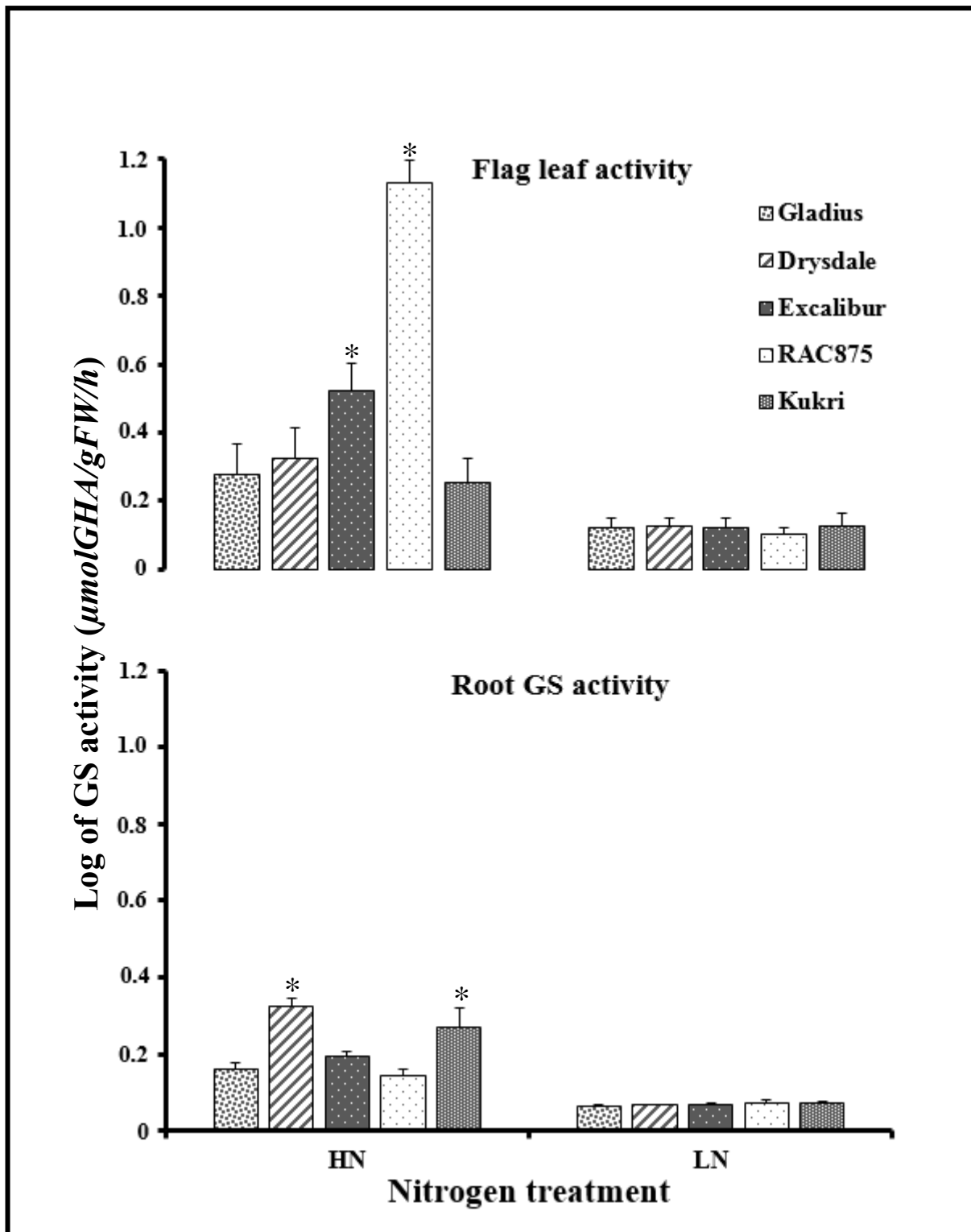
Overall, *Experiment 1(2)* with harvesting dates at 18 DAS, 32 DAS, 46 DAS and 60 DAS, GS activity results showed consistent across accessions, tissues and treatments. There were considerable differences in leaf GS activity between five accessions (Gladius, Drysdale, Excalibur, RAC875 and Kukri). Within these accessions, shoot GS activity was higher in the high N treatment. There was no significant variation in GS activity between accessions and N treatments at 18 DAS. Furthermore, at 32 DAS, GS activity was generally non-significant between treatments and accessions. However, the GS activity in OFB was significantly different between Excalibur, RAC875, Kukri ( $0.023 \mu\text{molGHA/gFW/h}$ ) and Gladius ( $0.003 \mu\text{molGHA/gFW/h}$ ) but not Drysdale ( $0.017 \mu\text{molGHA/gFW/h}$ ). There was consistent low to high distribution of GS activity between 46 DAS and 60 DAS in Kukri ( $0.024 - 0.075 \mu\text{molGHA/gFW/h}$ ) and RAC875 ( $0.022 - 0.075 \mu\text{molGHA/gFW/h}$ ) in all tissues. Significantly, higher levels of YEB GS activity were measured in RAC875 at 60 DAS and Kukri at 60 DAS, all other YEB GS activity measurements were lower than this level. With the exceptions of RAC875 and Kukri, GS activity in the HN and LN treatments were consistently low except at 60 DAS in the OFB (Fig. 5.1).





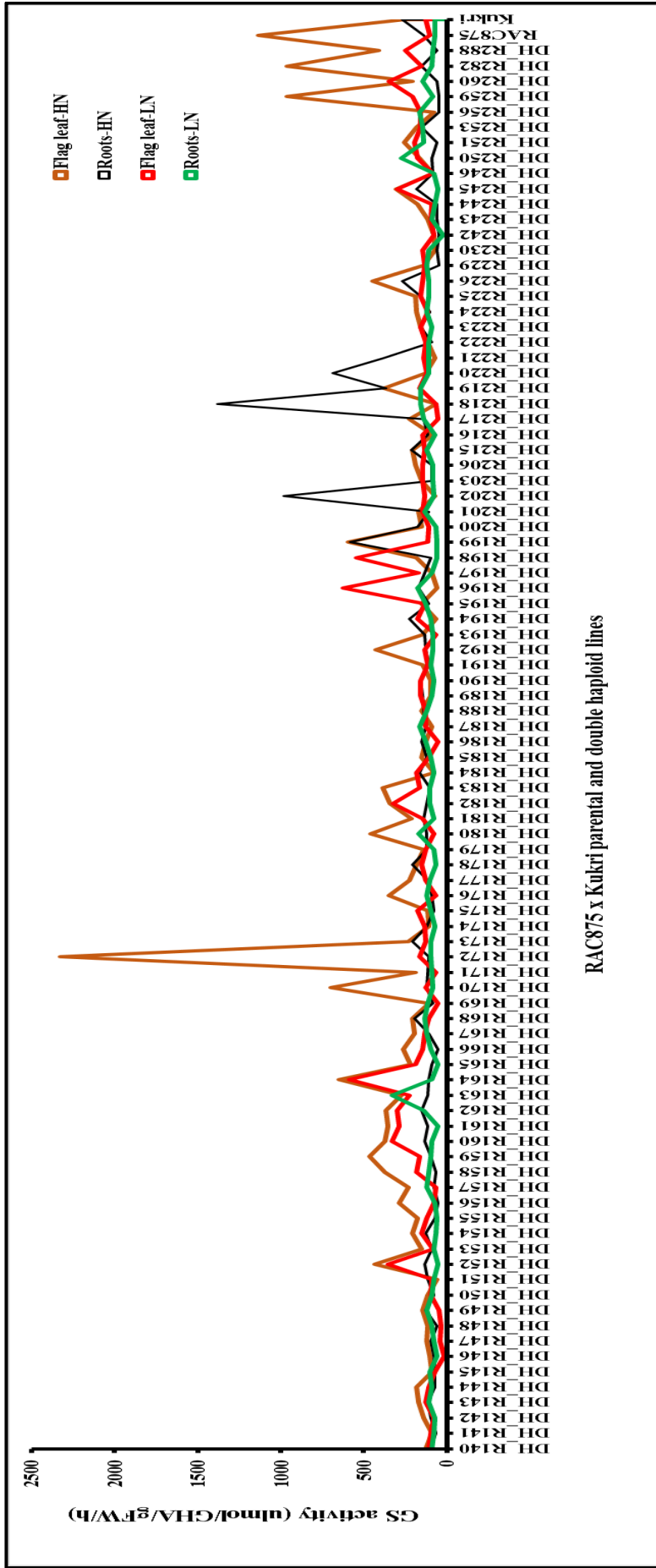
**Fig. 5.1** Experiment 1(2): Glutamine synthetase activity ( $\mu\text{molGHA/gFW/h}$ ) levels in 8 biological replicates of young fully extended leaves, older fully extended leaves and roots sampled at 18, 32, 46 and 60 days after seeding. Statistical significance ( $p$ -value,  $p < 0.05$ , Tukey test) between accessions in the same treatment and tissues is indicated by (\*).

In *Experiment 2* (in which GS activity was assayed at Zadoks 39), assay of the flag leaf produced clear high levels of GS activity in the high N than the low N treatments (Fig. 5.2). There was significantly high level of flag leaf GS activity in RAC875 and Excalibur. In the roots of Excalibur and Kukri, there were GS activity levels which corresponded to the GS activity in the flag leaf of these two accessions (Fig. 5.2) The GS activity levels under low n remained low in both flag leaf and root tissues.

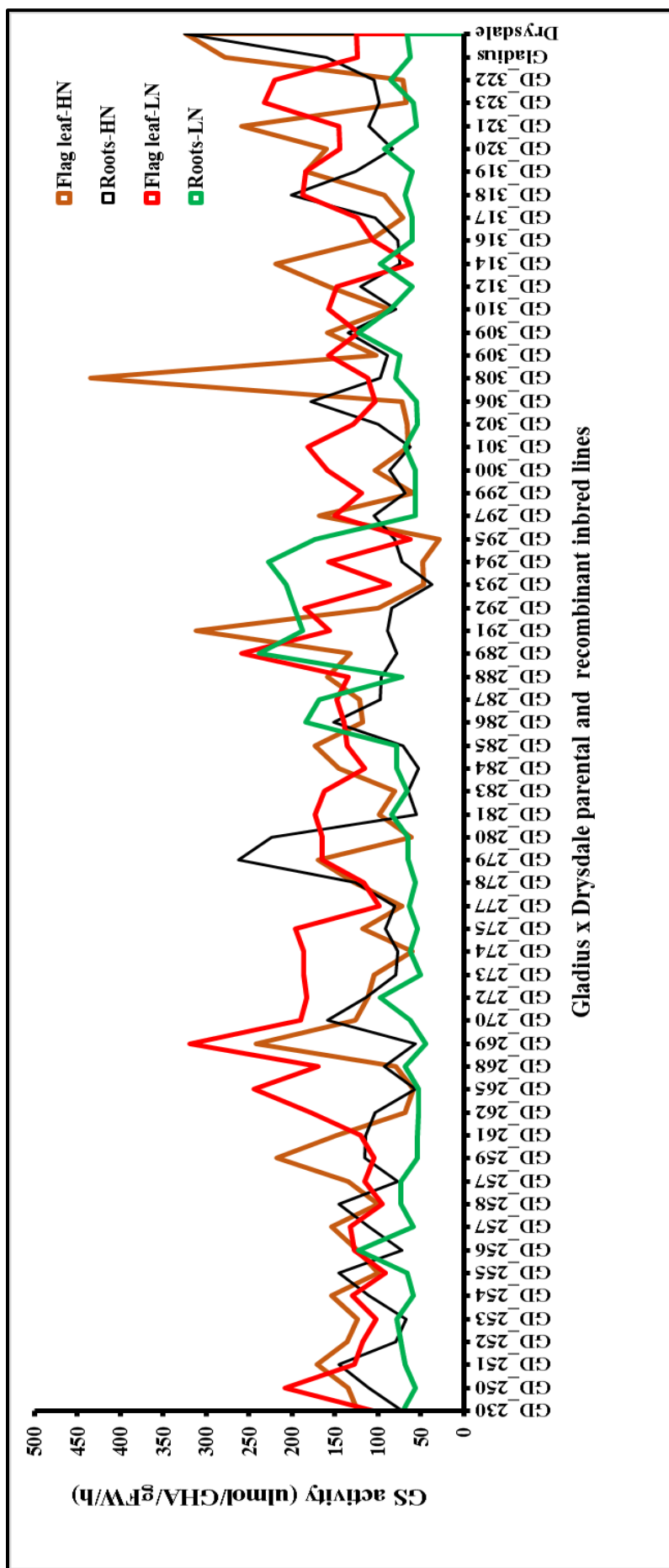


**Fig. 5.2** Experiment 2: Fully extended flag leaf Glutamine synthetase activity ( $\mu\text{molGHA/gFW/h}$ ) levels in 8 biological replicates of flag leaf and roots of five wheat accessions under high N (5mM  $\text{NO}_3^-$ ; HN) and low N (0.5mM  $\text{NO}_3^-$ ; LN) treatments sampled at Zadoks stage 39. Statistical significance ( $p$ -value,  $p < 0.05$ , Tukey test) between accessions in the same treatment and tissues is indicated by (\*).

Among the mapping population sets, the distribution curve of GS activity for the RAC875 x Kukri DH population shows about 50% of the double haploid lines produced higher root GS activity than the parental accessions RAC875 and Kukri (Fig. 5.3 & 5.4) under LN and HN. Conversely, RAC875 and Kukri recorded higher flag leaf GS activity than > 50% of the double haploid individuals within the RAC875 x Kukri DH population (Fig. 5.3 & 5.4) under both N treatments. Within the RAC875 x Kukri DH population there were lines that showed unique levels of GS activity in the flag leaf (DH-R172, DH-259, DH-R282, RAC875) and roots (DH-R202, DH-R202) under high N (Fig. 5.3). Within the Gladius x Drysdale recombinant inbred lines, there considerably high GS activity in some accessions in the flag leaf (Gladius, GD\_261, GD\_291, GD\_308, GD\_321) roots (Drysdale, GD\_271GD\_286, GD\_306, GD\_318) under high N treatment. There some notable GS activity levels in the flag (GD\_250, GD\_269, GD\_289 and GD\_323) and roots under low N treatment (Fig. 5.4).



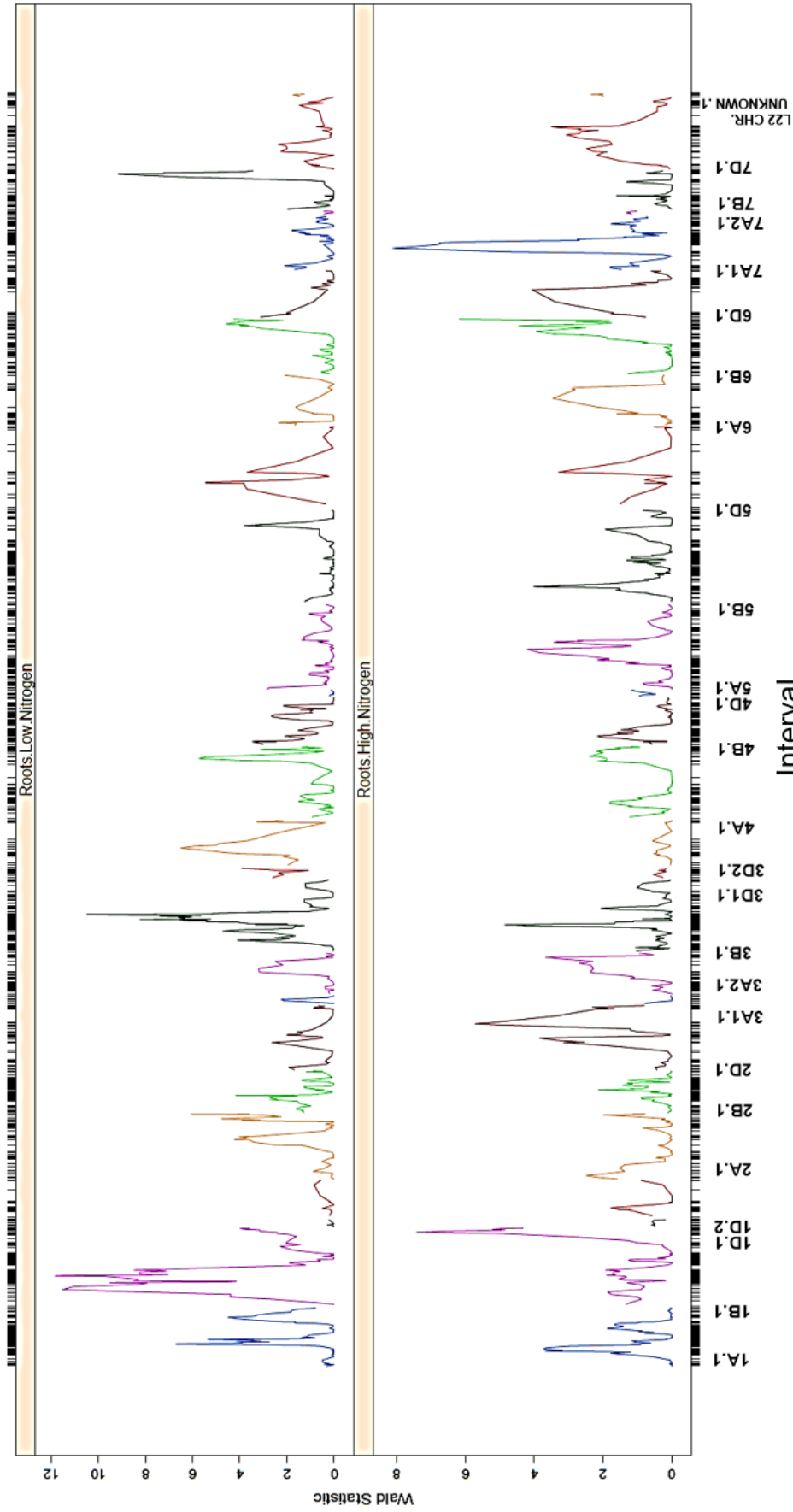
**Fig. 5.3** Glutamine synthetase activity ( $\mu\text{molGHA/gFW/h}$ ) distribution in accessions under high N ( $5\text{mM NO}_3^-$ ; HN) and low N ( $0.5\text{mM NO}_3^-$ ; LN) in RAC875 x Kukri mapping population set under high N ( $5\text{mM NO}_3^-$ ) treatments sampled at Zadoks stage 39.



**Fig. 5.4** Glutamine synthetase activity ( $\mu\text{molGHA/gFW/h}$ ) distribution in accessions under high N ( $5\text{mM NO}_3^-$ ; HN) and low N ( $0.5\text{mM NO}_3^-$ ; LN) in Gladius x Drysdale population set under high N ( $5\text{mM NO}_3^-$ ) treatments sampled at Zadoks stage 39.

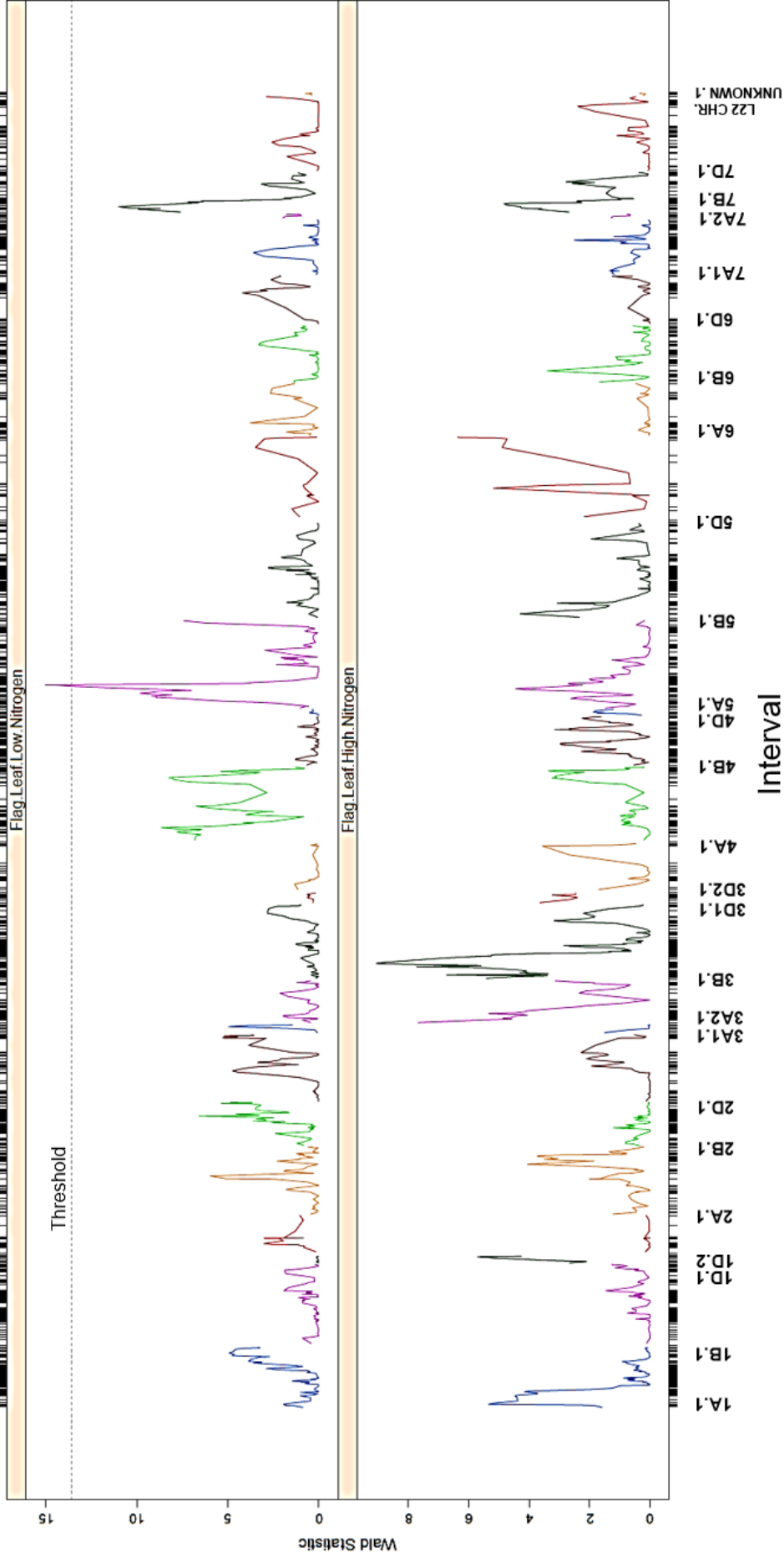
Regression analysis of the single treatment N effect on GS activity is presented in Figures 5.5, 5.6 and 5.7. Plots are produced to show possible QTL linkages in flag leaf and root tissues. There is a QTL association for low N root GS activity expression on the distal portion of *Chr.1B* - (Fig. 5.5) where this QTL maps close to the standardised threshold (13.59 - threshold at which the Wald statistic value of N responsiveness was accepted as significant). There was nothing significant over the threshold for the root trait. This was for either treatment, except flag leaf QTL on *Chr.5A* associated with the low N (Fig. 5.6). Most of the GS activity in the RAC875 x Kukri DH population did not rate above the 13.59 (Fig. 5.7).

In addition, a plot is presented to show a profile of the genetic responsiveness (how well the plants respond to the high nitrogen treatment given the low nitrogen treatment). This is essentially a more robust approach to determining whether there are QTL that express themselves differently depending on the treatment. A further analysis of genetic responsiveness highlighted on *Chr.5D.1*, shows that although the interval was not significant for either treatment, the change in the expression of GS activity was just below the threshold of significance (Fig. 5.7).

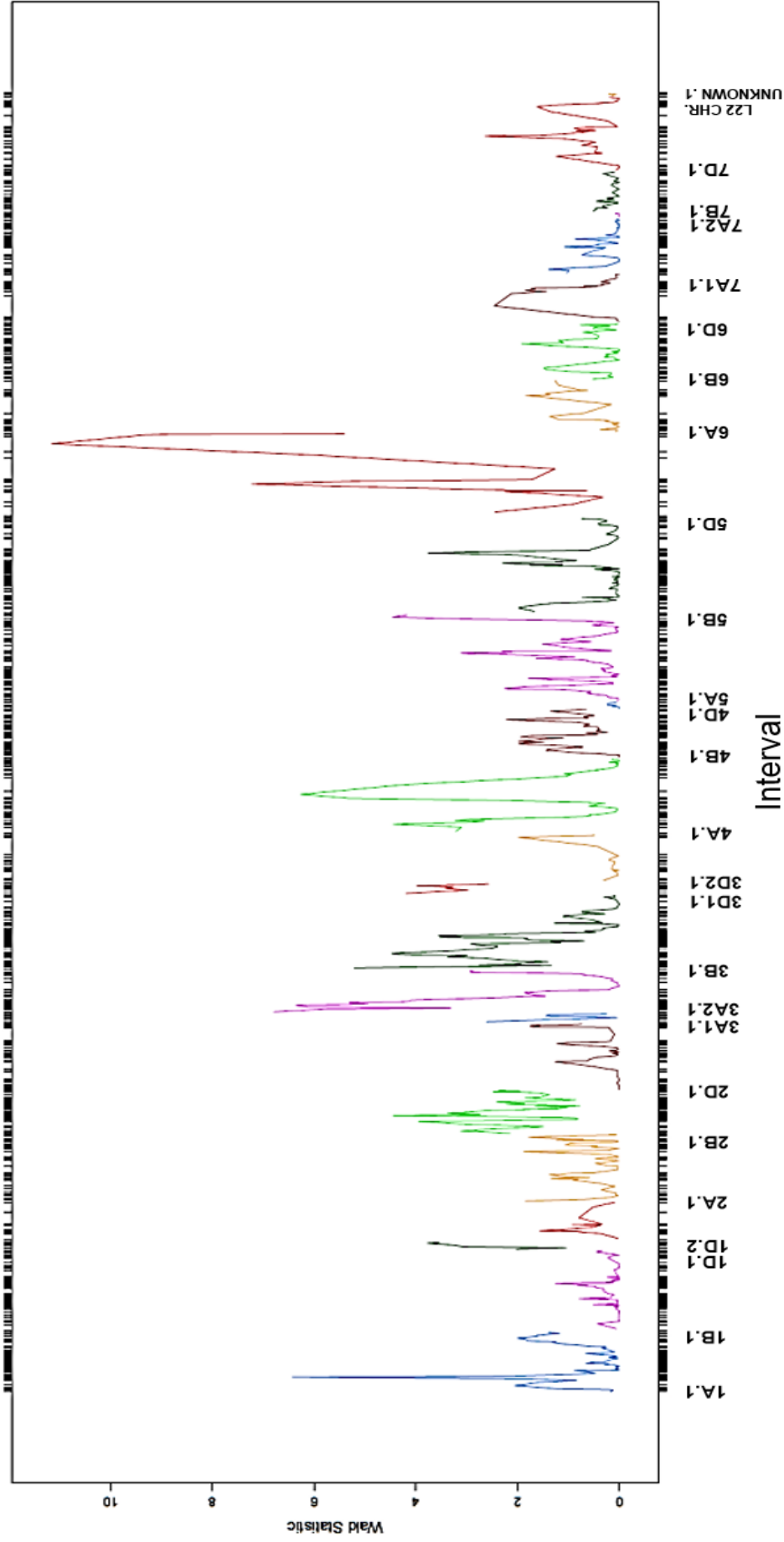


**Fig. 5.5** Plot of linkage analysis of RAC875 x Kukri recombinant inbred line population root Glutamine synthetase activity ( $\mu\text{molGHA/gFW/h}$ ) investigated under low N ( $0.5\text{mM NO}_3^-$ ) treatment.



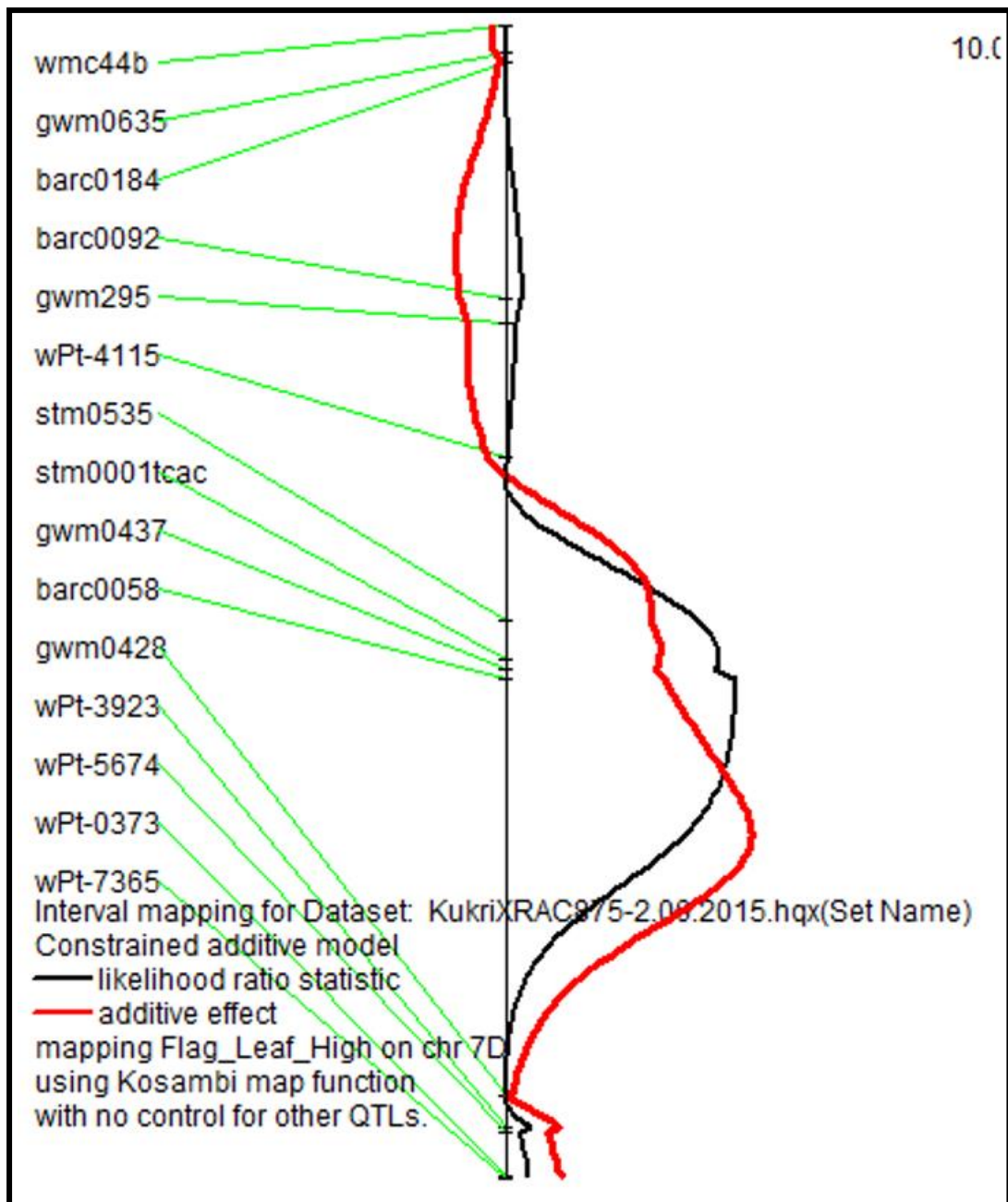


**Fig. 5.6** Plot of regression analysis of fully extended flag leaf Glutamine synthetase activity ( $\mu\text{molGHA/gFW/h}$ ) under low N ( $0.5\text{mM NO}_3^-$ ) treatment measured in RAC875 x Kukri mapping population set.

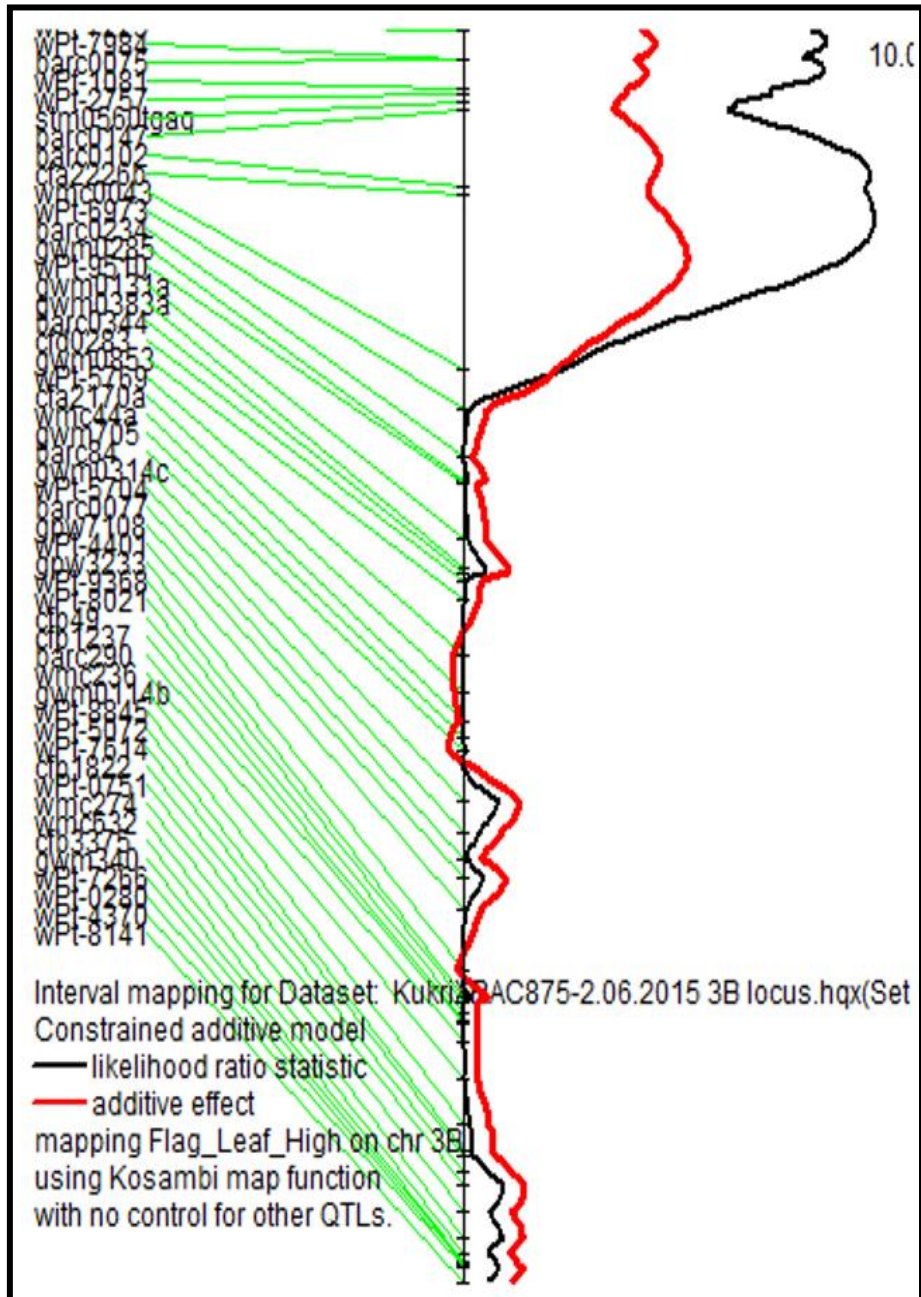


**Fig. 5.7** Genetic responsiveness of RAC875 x Kukri DH mapping population lines expressed as the degree of plant response to the high nitrogen treatment given the low nitrogen treatment.

Furthermore, quantitative mapping of flag leaf GS activity in the RAC875 x Kukri DH population under high N treatment showed many loci linked to GS activity including; *barc-0147*; *barc0102*, *wmc0043*, *barc0234*, *gwm0285*, and *wPt-9510* on *Chr.3D* (Fig. 5.8) and *barc0058*, *wPt-3923*, *wPt-5674*, *wPt-5674*, *wPt-0373* and *wPt-7365* on *Chr.7D* (Fig. 5.9).



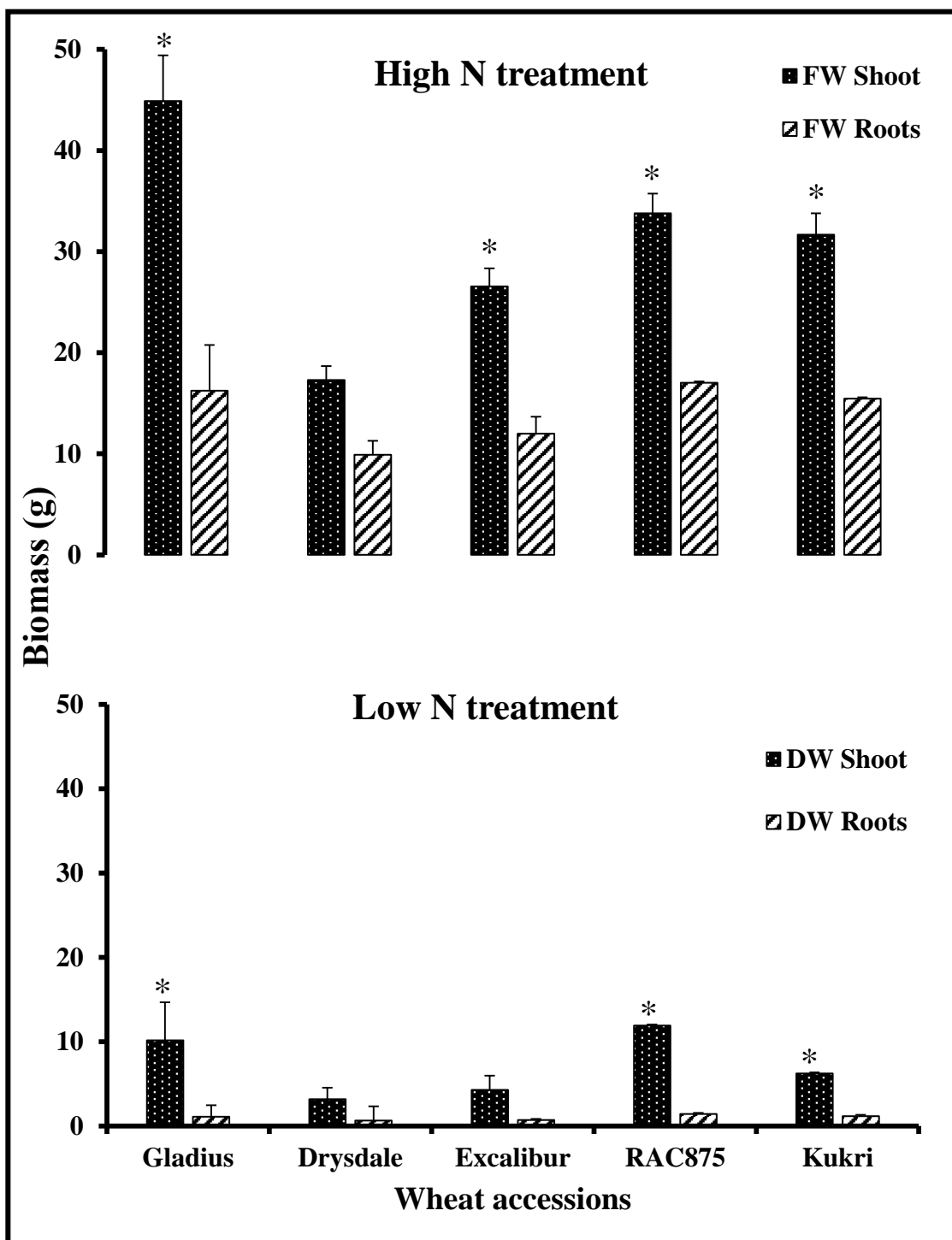
**Fig. 5.8** Quantitative mapping of flag leaf GS activity under high N treatment within the RAC875 x Kukri double haploid population shows eight significant loci on Chromosome. 7D.



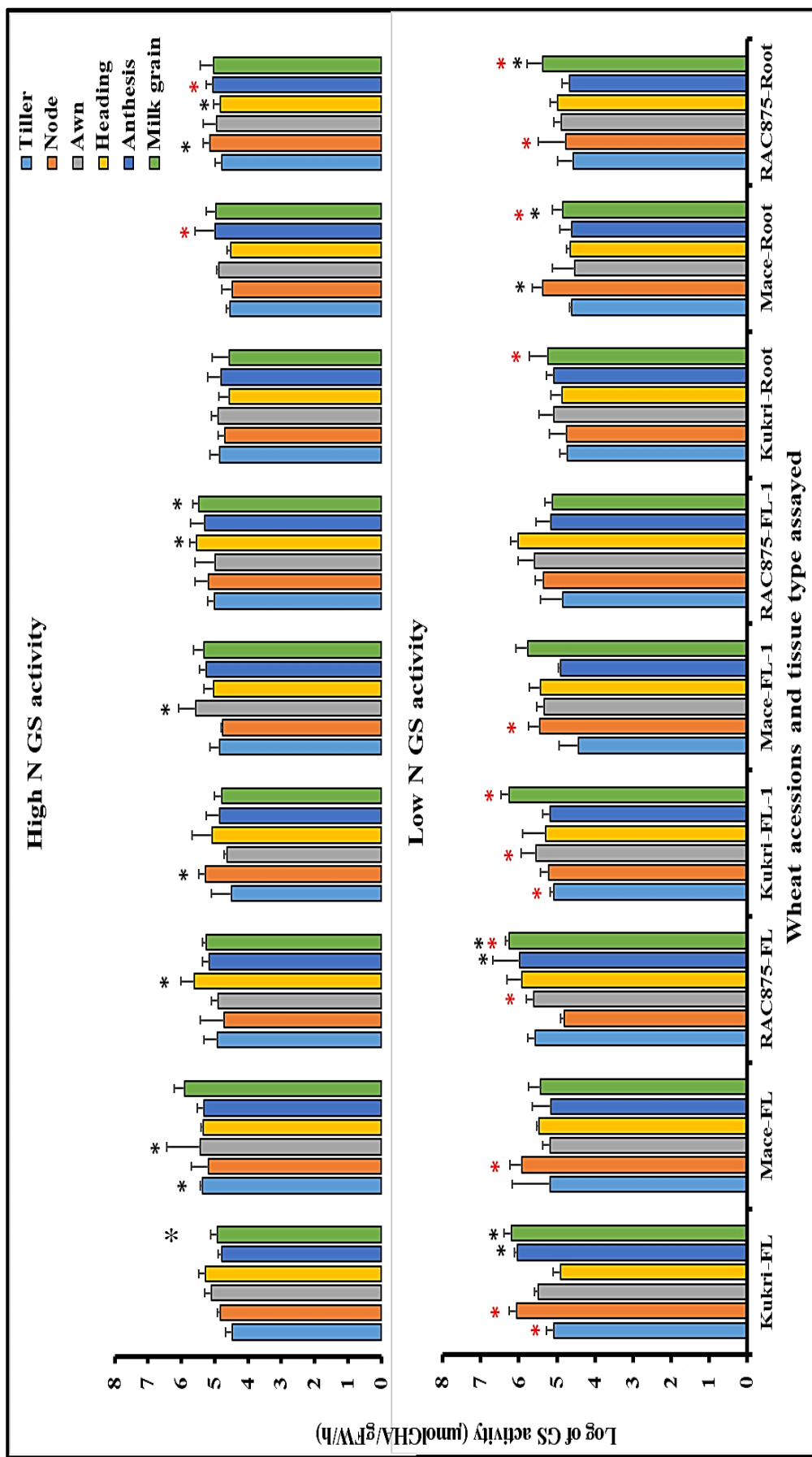
**Fig. 5.9** Quantitative mapping of flag leaf GS activity under high N treatment within the RAC875 x Kukri double haploid population shows significant loci on Chromosome 3B.

When the dry weight (g) of the shoot and roots were compared, there was significantly higher dry weight in shoots than roots under the high N treatment in comparison with the low N treatment in Gladius, RAC875 and Kukri (Fig. 5.10). Similar pattern of differences between shoots and roots was recorded in fresh weight measurements of the three accessions (Fig. 5.10).

In *Experiment 3*, the results of enzyme assay in tissues extracted from soil cultivated wheat plants (Fig. 5.11) showed that GS activity was under temporal control. There were significant differences in GS activity which when tissue sampling time was standardised across different wheat accessions at specific developmental stages on Zadoks Scale (23, 31, 39, 49, 69 and 73). In Figure 5.11, the coloured red asterisks refer to significant differences ( $p$ -value,  $p < 0.05$ , Tukey test) between high nitrogen and LN treatment within the same genotype and the black; refer to significant differences between wheat accessions under similar nitrogen treatment and in corresponding tissues.



**Fig. 5.10** Variation in biomass change at full flag leaf extension (Zadoks 39) expressed as fresh weight (FW) and dry weight (DW) in 8 biological replicates of accessions under high N (5mM NO<sub>3</sub><sup>-</sup>) and under low N (0.5mM NO<sub>3</sub><sup>-</sup>) treatments. Statistical significance (*p*-value, *p* < 0.05, Tukey test) between accessions in the same treatment and tissues is indicated by (\*).



**Fig. 5.11.** Experiment 3: GS activity ( $\mu\text{molGHA/gFW/h}$ ) profile of three wheat accessions assessed under low N ( $0.5\text{mM NO}_3^-$ ) and high ( $5.0\text{mM NO}_3^-$ ) treatment measured at six growth stages in 8 biological replicates of flag leaf (FL), leaf next to flag leaf (FL-1) and roots of plants grown in pot soil within a controlled environment room. Red-coloured asterisks show significant difference between N treatments involving corresponding tissues and black asterisks represent significant differences ( $p < 0.0001$ , Tukey test) between wheat accessions in the same tissue under the same N treatment.



There were no significant differences in enzyme activity in roots at Zadoks 23 and in flag leaf-1 at Zadoks 69 (Fig. 5.11) between RAC875, Mace and Kukri. Apart from Mace, the effect of nitrogen treatment on enzyme activity was not significantly different between two N treatments. However, there were significant differences in flag leaf GS activity between RAC875, Kukri and Mace at Zadoks 69 (anthesis) and Zadoks 73 (milk grain) stages under the low N and between RAC875 and the other two accessions under the high N at Zadoks 49 (heading). The assay results in *Experiment 3* shows that RAC875 and Kukri showed significant differences in enzyme activity between high and low N treatments during the reproductive stages (Zadoks 49, Zadoks 69 and Zadoks 73).

## 5.4 DISCUSSION

The aim of the GS assay was to determine if GS activity was linked with some GS isoforms under contrasting N treatments (measurement of GS isoforms transcripts was in *Chapter 3*). In *Experiment 1(2)*, there was an increase in GS activity between 46DAS and 60DAS. The plants were developing through the reproductive stage between these time points and the upsurge in GS activity suggests there was an increase in N-related metabolic activity in consistent with the *GSI* (Rolny et al. 2016) demands of developing tissues such as in the young leaves at critical developmental stages.

In *Experiment 2*, tissues sampled for the GS assay were harvested from young leaves, older leaves and roots at 18, 32, 46 and 60 DAS. The biological growth stage of the wheat accessions at harvest was neither synchronised nor uniform at these growth points. Three of the wheat accessions produced larger flag leaf under high nitrogen treatment, significantly higher in some accessions under low nitrogen treatment. There was increased GS activity in the roots of Excalibur and Drysdale and high GS activity in the YEB of RAC875 and Gladius. The GS activity levels increased between 18 and 46 DAS in most accessions and there was no variation in leaf and root GS activity. Again, this is consistent with the findings of (de Oliveira et al. 2016) elevated tissue GS enzyme activity in young *Byrsonima crassifolia* plants progressed to maturity. While there was no clear pattern to the increase and decline in GS activity between accessions and in different tissues across treatments, it was observed that consistent variation in GS

activity levels occurred within individual accessions at the four growth points. For example, two accessions (Gladius and RAC875) showed significant increase in GS activity in young fully emerged leaves at 60 DAS. This may point to differing control mechanisms underlying both GS activity and quantitative differences underlying contrasting N treatments. The differential control mechanisms support the finding that, increased GS activity in the shoot was highly correlated with increased N concentration in the hydroponic growth medium and agrees with the work of Hirel et al. (2001) and Mattsson (1998).

The biological growth stage of the accessions at harvest was neither synchronised nor uniform at these growth points. The growth stage varied considerably between accessions and among mapping population lines. It was observed that RAC875 reached anthesis (Zadoks 69) and initiated grain filling from 2-3 weeks earlier than Mace and Kukri. While a positive correlation has been established between GS activity and grain yield and leaf  $\text{NO}_3^-$  content under low N supply conditions (Habash et al. 2001, Hirel et al. 2001, Masclaux et al. 2001, Martin et al. 2006), in this study, the high nitrogen treatments produced elevated levels of GS activity in both flag leaf and roots.

Distribution curves of GS activity among mapping population lines show similar pattern of GS activity between high nitrogen and LN treatments. The high nitrogen treatments produced elevated levels of GS activity in both flag leaf and root GS activity.

The increased GS activity was consistent with biomass data from fresh weight and dry weight measurements in shoots and roots of all accessions under high nitrogen and LN treatments (Fig. 11). The biomass change in wheat was different to the GS activity contribution to biomass observed in non-cereal plant species. For example, when assessing the overexpression of GS in *Nicotiana tabacum*, transgenic plants produced higher shoot and greater root biomass and leaf surface area under depleted levels of N in comparison with the wild type under the same N condition (Fuentes et al. 2001). It can be deduced that the effect of GS activity on biomass yield may be significantly influenced by gene expression levels of different GS alleles (present in some species and organs) which mediate N – linked traits (Cañas, 2016, Zhou et al. 2016).

In addition, GS activity can be linked to a stay green phenotype (sustained greenness of the shoot especially leaves and stem) in wheat. This may be explained by the effect of increased N remobilisation leading to a stay-green phenotype (Sinclair & De Wit, 1975; Masclaux et al. 2001; Spano et al. 2003). Loci for a stay-green phenotype has also been associated with drought tolerance and anthesis (Thomas & Howarth, 1999). GS activity may be linked to stay-green trait through induced N uptake and remobilisation due to pre-anthesis (Zadoks 69) N contribution to improved photosynthesis. Consequently, it is critical to examine the effect of N supply on stay-green in the paradigm of enhanced GS activity or otherwise.

The results so far have indicated a considerable YEB GS activity increase at 60 DAS (which is an average of 3 days after the start of grain filling among the accessions). The analysis presented in this paper shows that GS activity was under temporal control as per the standardisation of harvesting date and flag leaf GS activity. This can serve as useful benchmark in breeding for N-linked traits in hexaploid in wheat. A more robust approach to deciphering N-linked traits will be to combine enzyme assay analysis with assisted marker selection and targeted genotyping of GS activity in a wider pool of genetic variants under more stratified and contrasted N treatment regimes, possibly in the context of natural field conditions.

The analysis of flag leaf GS activity under low N in the RAC875 x Kukri DH population identified a QTL on *Chr. 5* on the A - genome. In the soil based GS activity assays involving Mace, RAC875 and Kukri, there were significant differences at six Zadoks developmental stages investigated. It was noted that in all three varieties, low nitrogen plants matured faster and reached each developmental stage faster and earlier (app. 2-3 weeks) than high nitrogen plants. The faster growth and maturity might be an adaptation by plants to complete each of the growth stages and ultimately the life cycle as a response to limited N supply. Nitrogen treatment clearly has an influence on GS activity and this was clearly observed in the leaf tissues. Generally, in the soil experiment Mace, RAC875 and Kukri showed increased enzyme activity under low nitrogen.

Though this trend contrasts the hydroponic GS activity results (which showed increased GS activity under high N), the soil-based analysis is consistent with previous studies that confirm up-regulation of GS activity under low nitrogen. In the early developmental stages (tiller and node initiation), the varieties showed no difference in GS activity between N treatments in the flag leaf. Overall, the identification of a QTL for flag leaf GS activity in the RAC875 x Kukri DH population as was observed under low N. This has implications for identifying genetic variation in GS activity in different wheat accessions and mapping populations.

Consistent levels of GS activity in the YEB and roots of RAC875, Kukri was used as a parameter in selecting these accessions for further analysis in *Experiment 3* (soil-based analysis) using the same accessions. This experiment included analysis of the fully extended flag leaf GS activity at Zadoks 39 identical to a study of GS isoforms in which flag leaf GS activity was measured (Habash et al. 2006) and due to the considerable abundance of plastid GS in the flag leaf. The flag leaf synthesises about 70% of carbohydrates stored in the grain, and is linked to elevated nitrogen remobilisation towards grain filling (Lopes et al. 2006).

In this study, it was anticipated that any significant variation in flag leaf GS activity among the different accessions could indicate the capacity to assimilate N efficiently and convert N resources into grain filling (Lopes et al. 2006; Rolny et al. 2016). These differences are consistent with the previous results from the

hydroponic *Experiment 1(2)* and *Experiment 2*. In both experiments, RAC875 produced significantly higher metabolic activity than Kukri. This indicates that there may be a genetic basis for enzyme activity differences. This is due to differences reflected in the levels of gene expression differences between the two accessions. Evidently, the GS activity levels of RAC875 depicted at Zadoks 49 was more consistent than that of Kukri and Mace as there were significant enzyme activity in the flag leaf, flag leaf-1 and root tissues at this stage, but not in the other two accessions.

The distribution of GS activity between varieties was clearly differentiated in the flag leaf and likewise the effect of N treatment in the second leaf (flag leaf-1) however, activity was largely unchanged in the roots of the different varieties. In the reproductive stages (anthesis - Zadoks 69, and milk grain stage), analysis of the first leaf and flag leaf-enzyme activity between the varieties revealed that though GS activity was significantly higher under low nitrogen, the varieties that showed higher GS activity under low nitrogen produced lower GS activity under high nitrogen. Conversely, under high nitrogen there was an increase in GS activity. This was in varieties that had shown lower GS activity under low nitrogen. The consistent flag leaf-enzyme activity during reproductive stage point to N metabolism during reproductive development (Rolny et al. 2016; Zhou et al. 2016).

The sharply contrasting varietal responses suggest genetic differences underlie the consistently contrasted GS activity during reproduction, especially during grain filling. RAC875 and Kukri showed higher GS activity under low nitrogen, this suggests that there is a GS activity response to reduced N supply. GS activity appears to be down regulated in the presence of increased N supply, as result of reduced demand for resources. Mace showed a significantly higher GS activity under high nitrogen and reduced GS activity under low nitrogen. This might indicate reduced N uptake and synthesis in comparison with RAC875 and Kukri, contrasts RAC875 and Kukri. However, the onset of grain filling may be varied between cultivars with the same seeding date, a measure of leaf GS activity (Habash et al. 2006) between different accessions on the same harvesting date will yield contrasting levels of enzyme activity (Masclaux-Daubresse et al. 2006), thereby compromising any comparative analysis of the accessions. The variation in enzyme activity may be due to the interactive roles of different GS alleles which may be differentially localised (sub-cellular and tissue specific) and differentially regulated at different developmental stages.



## 5.5 CONCLUSION

In the hydroponic analysis, there was elevated GS activity under high N and an observation of faster growth and maturity which was not observed under low nitrogen which is a direct response to a nitrogen deficit. Though there were inconsistent GS activity patterns between specific developmental stages of vegetative growth, there was a consistent level of enzyme activity in the flag leaf during the reproductive stage. This may reflect the importance of flag leaf N metabolism during the reproductive stage of the plant. The sharply contrasting varietal responses suggest genetic differences underlie the consistently contrasted GS activity during reproduction, particularly during grain filling. In the soil experiment, there was a general increase in GS activity in the glag leaf, flag leaf-1 and roots under low N. This shows that the wheat accessions responded differently to the N treatment in the hydroponic and soil growth media.

## CHAPTER SIX

### GENERAL OVERVIEW AND FUTURE PERSPECTIVES FOR GLUTAMINE SYNTHETASE CHARACTERISATION

#### 6. INTRODUCTION

Prior to this study, Glutamine synthetase (GS) has been investigated in non-cereal crops mostly using *Arabidopsis thaliana*, *Brassica napus*, *Medicago truncatula* (Lima et al. 2006) and *Nicotiana tabacum* (Lothier et al. 2011; Guan et al. 2014; Orsel et al. 2014). Among cereal crop species, efforts have been made to explain the role of GS transcripts in physiological processes such as seed germination, growth rate and grain yield and protein content (Tabuchi et al. 2005; Martin et al. 2006; Gadaleta et al. 2014; Guan et al. 2014).

Other studies have examined the performance of the GS transcripts in nitrogen, assimilation, remobilisation (Hirel et al. 2001; Coque et al. 2008; Masclaux-Daubresse et al. 2008) and nitrogen use efficiency (Avila-Ospina et al. 2014; Melino et al. 2015). Other important contributions include the characterisation and sequencing of GS genes in the model legume *M. truncatula* (Lima et al. 2006; Torreira, 2014) and *Zea mays* (Hirel et al. 2001; Gallais et al. 2006) which are documented in the *Protein Data Bank* (<http://www.wwpdb.org/>).

In addition, there have been attempts to elucidate GS pathways in maize, barley, rice and recently wheat (Habash et al. 2001; Tabuchi et al. 2005; Bernard et al. 2008; Goodall et al. 2013; Thomsen et al. 2014; Melino et al. 2015).

However, the results of most GS studies have largely not been consistent in their findings. This is especially the case regarding the trends in gene expression and enzyme activity. A possible explanation of this may be related to the complex nature of the underlying metabolic processes (Thomsen et al. 2014). Possibly, the effect of environmental variables such as the level of nitrogen nutrition and the rate of senescence (Kichey et al. 2005; Lothier et al. 2011), photo-respiratory factors (Oliveira et al. 2002) and the variable nature of the agronomic practices adopted in growing the experimental plants. The current study has however, expanded the scope of the study of GS genes on both terminal and temporal scales using a combination of marker development and mapping, gene sequencing, expression analysis, enzyme activity profiling and phylogenetic analysis as a way of understanding the genetic basis for nitrogen use efficiency in spring wheat varieties.

While the present study does not claim to resolve all the questions pertaining to the genetic characteristics of GS in hexaploid wheat, and many issues involving the intricacies of nitrogen uptake and metabolism, the study does provide some useful insights into deciphering these important themes.

## 6.1 ADVANCES IN KNOWLEDGE FROM THE PRESENT STUDY

The major findings of the present study are summarised in the following points:

- Analysis of Chinese spring aneuploid DNA samples confirm that *TaGS1.1A*, *TaGS1.1B* *TaGS1.1D* are located on Chr. 6 and *TaGS2A*, *TaGS2B* *TaGS2D* are on Chr. 2 of their respective genomes.
- Transcript abundance analysis of GS shows that the cytosolic GS (*TaGS1.1*) is highly expressed in roots and the plastid GS (*TaGS2*) is highly expressed in the leaves.
- Phylogenetic analysis shows a clear dichotomy between *TaGS1.1* and *TaGS2*; and between the wheat GS and GS sequences of other cereal and non-cereal species.
- SNP markers in the conserved domains of GS sequences were found to be polymorphic among wheat accessions in two double haploid populations and a recombinant inbred-line set.
- Principal component analysis show the bi-allelic polymorphism of the SNP markers conforms to clearly distinct groups of wheat accessions with different nitrogen response.
- GS activity analysis does not reveal any clear pattern of enzyme activity among the wheat accessions, except when the accessions were evaluated at specific temporal stages of growth.

- The nitrogen treatment influenced GS activity differentially in corresponding tissues within the same accession.
- A quantitative trait locus for flag leaf GS activity under low nitrogen treatment was identified on Chr. 5A.

The PCR amplification of DNA from Chinese spring aneuploid lines confirmed that all six GS gene sequences were located on the predicted chromosomes and respective genomes. Furthermore, the sequence alignment of the *TaGS1.1* and *TaGS2* homologues of the wheat lines confirmed a high level of sequence similarity to the reference GS sequences. Additionally, the sequence alignment of the conserved regions of *TaGS1.1* and *TaGS2* genes demonstrated that the exons in these regions shared a 98% sequence similarity. The localisation of the six GS genes to their respective chromosomes indicates that both cytosolic and plastid genes in wheat are syntenic to GS genes in barley maize and other cereals. This may explain why there is a strongly observed sequence homology between the GS genes of the different species. This the first known reported characterisation of *GS1.1* and *GS2* loci in hexaploid wheat.

In this study, polymorphic markers were detected as single nucleotide polymorphisms (SNPs) which amplified from one parental genotype but did not amplify from the other parent. Several polymorphic markers of *TaGS1.1A*, *TaGS1.1B*, *TaGS2A* and *TaGS2B* were mapped across three wheat mapping populations.

The SNP genotyping demonstrated that these markers showed a clear bi-allelic variation with a total of 20 polymorphic marker loci able to be identified within the diversity panel. These indicates that both accessions within the mapping population lines were genetically linked to the SNPs for which these markers mapped. These markers could be used to determine parental segregation for GS genes in other cereal species, within specific mapping populations.

A principal component analysis of the SNP markers genotyped for the diversity panel identified distinct groups of accessions that corresponded to responsiveness to nitrogen treatment. In addition to analysis of SNP variation, the assessment of sequence variation provides a further insight into the genetic diversity and possibly gene flow within wheat accessions.

It was demonstrated through phylogenetic analysis of the wheat sequences that, the *GS2* alleles were genetically diverse from *GS1.1* allele. This diversity is observed amongst different species and between the different wheat accessions examined in this study. There was low level of observed genetic divergence between each of the wheat *GS1.1* alleles that largely explains the high level of sequence conservation between the *GS1.1* allele. Phylogenetic analysis demonstrates that the *Brachypodium distachyon* *GS1.1* and the *Oryza sativa* *GS1.1* sequences were clustered together but genetically distant from barley and maize. Previous study of genetic relationship among GS genes in other species suggest that the barley and maize GS genes were genetically related to each other (Avila-Ospina et al. 2015).

A study of *Populus trichocarpa* by Castro-Rodríguez et al. (2011) suggests that extensive GS gene duplication has occurred during species divergence while the phylogenetic analysis of GS sequences of maize, rice, barley and *B. distachyon* indicate that they are orthologous or even paralogous to the wheat *GS1.1* allele. Assessment of the wheat GS2 sequences indicates a common link with Maize GS locus B73GS2 and possibly suggests that the wheat sequences may have originated from the duplication a maize ancestral GS gene.

The phylogenetic analysis of plastid GS sequences of both wheat and barley clearly demonstrates that they are genetically divergent from *Glycine max*, *B. napus* and *A. thaliana*. Furthermore, the comparison of Arabidopsis GS sequences confirms that the *GS1* sequences are distinct from *GS1* sequences of cereal species (Thomsen *et al.* 2014). This may imply that there may be different functions of the *GS1* genes in Arabidopsis compared to the GS1 genes in cereals. When comparing genomes, there was clear evidence to suggest that among the plastid GS homologues, the D-genome homologues contained the highest level of genetic similarity across the wheat accessions indicating that the *GS2D* genes shared a common gene background. There was wider genetic divergence among the wheat *GS2A* and *GS2B* sequences, which was not present among the *GS2D* sequences. This genetic variation suggests that the *GS2D* sequences are highly conserved in comparison with the *GS2A* and *GS2B* sequences.

There is clear genetic divergence between the GS1 homologues of wheat and both other cereals and non-cereal species. In addition, there is also genetic significance between *GS1.1* and *TaGS2* homologues in wheat. This difference in genetic divergence is one of the key genetic factors contributing to the different biological functions of the plastid and cytosolic GS enzymes. An example of this is their differing roles in N assimilation during leaf senescence. Besides the observed sequence variation of these GS loci, there were clearly different patterns of gene expression with a highly differentiated expression of cytosolic *GS1.1* in roots and plastid *GS2* in the leaves.

Based on POTAGE sequence analysis of Chinese spring cDNA samples it was predicted that *TaGS1* would be higher in roots and that the *TaGS2* transcripts would be highly expressed in leaves. When the expression levels in Gladius, Drysdale, Excalibur, RAC875 and Kukri were analysed, three cytosolic GS transcripts were found to be highly expressed in root tissues than leaf tissues. Similarly, three plastid *GS2* transcripts were measured to be expressed significantly higher expressed in leaves than in the roots. This confirmed the differential expression of the GS loci in root and leaf tissues as predicted by POTAGE and clearly confirms previous tissue-specific localisation in GS expression studies (Fuentes et al. 2001; Bernard et al. 2008; Castro-Rodríguez et al. 2011; Wang et al. 2015).



When GS expression levels were measured in plants that have been treated with high and low nitrogen, there was no significant observed effect of low N treatment on gene expression in the flag leaf. There was however, some evidence to suggest that GS expression in different tissues was modified in response to nitrogen treatment. However, this gene expression did not follow a consistent response pattern that suggests that the expression patterns recorded may be influenced significantly by the expression of other genes. The expression of genes involved in N assimilation and metabolism may be expressed differentially in response to the changing N status within the tissues. The present study also suggests that GS expression could be integral to the complex series of many biosynthetic mechanisms mediating N uptake and assimilation and that these mechanisms could be influenced under different N treatments.

The analysis of flag leaf activity demonstrated a strong effect of low N treatment on GS activity in the soil-based experiment. This effect was significantly higher than that of a high nitrogen treatment. Generally, plants receiving the low nitrogen treatment grew faster and attained maturity and developmental organs earlier than those in the high nitrogen treatment did. In the soil-based assessment, GS was down regulated in response to increased N supply in RAC875, Kukri and Mace. Within both the flag leaf and flag-1, GS activity was consistently higher under low N and lower under high N. Generally, leaf GS activity was comparable to root GS activity during the early development (tiller and node initiation, awning emergence); however, leaf GS activity significantly

increased and remained consistently higher than root GS activity during reproductive development. This effect may be correlated with photosynthesis and increased demand for N resources during reproductive development. The pattern of GS activity in the flag leaf was comparable to the root GS activity during the reproductive development that may indicate that GS activity in roots and leaves may be co-regulated at the reproductive stage. The variation of GS activity between RAC875, Kukri and Mace suggests that GS activity is modulated temporally and that there is genetic variation for the controlling mechanism between these varieties. This evidence therefore, strongly supports the prognosis that complex interaction between nitrogen and genetic factors may regulate GS activity and N assimilation during the reproductive stage. It also implies that GS activity as a biological activity is highly regulated by the nitrogen resources accessible to specific accessions. It is therefore clear that not all accessions respond to nitrogen treatments in the same pattern, even when expressing similar levels of GS transcripts. GS activity is clearly a highly complex biosynthetic process that is mediated by several genetic factors including the activity of GOGAT, aminotransferases and possibly yet uncharacterized factors. It was clear that Kukri and RAC875 respond differently depict differently to nitrogen treatment when compared to Mace. This provides clear evidence of the role of genetic effects on GS activity at specific developmental stages.

## 6.2 FUTURE DIRECTIONS FOR GS CHARACTERISATION IN WHEAT

The development of next generation sequencing has contributed to the precision and rapid sequencing of the genomes of species such as maize, rice and Arabidopsis. In addition to DNA sequencing molecular marker tools for hexaploid wheat (Bertin, 2000; 2001; Ahn et al. 2006; Garnett et al. 2015) has further facilitated genomic studies (Avila-Ospina et al. 2015). However, a complete genome is yet to be completed for wheat. This has limited the research activities and the characterisation of specific genes and enzymes in wheat such as GS genes.

The first cloning and study of GS genes in wheat was conducted by Bernard et al. (2008) to confirm the role of cytosolic GS enzymes in assimilating and remobilising ammonia during leaf senescence. Recently, new wheat GS isoforms (*GS<sub>I</sub>*, *GS<sub>II</sub>* and *GS<sub>III</sub>*) and three new cytosolic GS sub-units (*GS1*, *GSr1*, and *GSr2*) were characterised and localized to the leaf tissue (Wang et al. 2015). However, until recently, very few studies have been undertaken to characterise variation between wheat GS alleles and none have employed the full-length sequences of the six GS homologues analysed in the present study. Consequently, very little is known about the specific characteristics and functions associated with individual GS genes and the enzymes they encode. Despite the absence of a complete wheat genome, SNP-based molecular markers will continue to be utilised in identifying new regulatory loci and in characterizing currently identified genes (Cavanagh et al. 2013).

In addition to direct sequence identification from laboratory experiments, the International Wheat Sequencing Consortium (IWGSC) database on hexaploid wheat contains vital sequence data that constitute an important resource for studying gene sequence conservation and conduct homology analysis.

The aspects of GS characterisation presented in the current study may serve as the basis for further exploration of the GS enzyme and its associated functions in hexaploid wheat. QTL mapping of traits such as nitrogen uptake and nitrogen remobilisation is necessary to dissect the genetic basis of nitrogen use efficiency in wheat. This will also present the opportunity to identify specific GS haplotypes in existing collections of wheat such as CIMMYT's Wheat Genetic Resources programme of FIGS (Focussed Identification of Germplasm Strategy) sets developed for nitrogen use efficiency.

Another area of potential investigation is the relationship between nitrogen treatment and plant morphological changes that appear in response. In the present study, there was considerable variation in flag leaf morphology between N treatments and between different accessions. This suggests a possible effect of nitrogen treatment on flag leaf morphology among the wheat accessions examined. However, from the current study, it is inconclusive as to whether the differences in flag leaf morphology were due solely to the effect of nitrogen regime or in part that to genotypic variances among the wheat accessions. There is a further need to investigate the extent to which nitrogen treatment affects flag leaf morphology and to determine what, if any role the GS enzymes play in flag

leaf morphological variation. If it could be determined confirmed that flag leaf morphology can be used as a reliable indicator of nitrogen response in wheat, this could significantly aid in the determination of nitrogen efficient accessions.

The present study has also demonstrated that both GS expression and GS activity is significantly increased by the low nitrogen treatment. A future challenge is to verify the consistency of these results by conducting GS expression and GS activity experiments under standard agronomic practices in field experiments. Such an approach could be utilised to capture the full scope of the response to nitrogen treatments, and experimental protocols could be designed to include fertiliser formulations that includes a broader scope of nitrate and ammonium concentrations supplied.

Additionally, different strategies could be applied during the application of fertilisers taking into consideration developmental stage effects. Such fertiliser application protocols could be implemented under different growth conditions including different soils, hydroponic systems and glasshouses and possibly in the context of natural field conditions. Some of the accessions examined in this study have complex genetic and breeding backgrounds. For example, Gladius that was used in this study was derived from a cross, involving Excalibur, Kukri, RAC875, Krichauff and a derivative of Trident. In order to understand the genetic control of nitrogen response in agronomically appropriate situations, it is important to explore the nitrogen response of these and other varieties such as Wyalkatchem, Frame and Yipti which are widely cultivated in South Australia.

Since these varieties are adapted to the agronomic practices of South Australian wheat cultivation systems, it is also critical that their nitrogen responses are investigated considering the effect of drought stress conditions, as it is not clear how the patterns of GS expression respond to the drought prone agronomic environment prevalent in the wheat cultivation area within South Australia. Cytosolic GS genes are induced during senescence (Masclaux et al. 2000; Guo et al. 2004; Martin et al. 2006). Therefore, it is crucial to characterise the regulatory mechanisms that mediate the induction of these genes in response to drought-induced leaf senescence. Moreover, in some cereal crops such as rice, there is a direct relationship between the overexpression of non - GS genes such as the *PEPc* gene (Thomsen et al. 2014), involved in nitrogen metabolism and the upregulation of GS activity. Therefore, future study of GS and non - GS gene interactions may be required to explore the regulatory pathways involving non - GS genes that indirectly mediate GS activity in wheat. Such analysis may help to clarify the intricate mechanisms underlying these gene interactions.

Furthermore, to understand the roles of GS enzymes in nitrogen metabolism, there is a need to investigate the post-translational modification of GS proteins including phosphorylation and nitration by nitric oxide. Such efforts will explain differences in gene function associated with specific GS proteins that may be encoded by specific GS homologues.

### 6.3 CONCLUDING REMARKS

There is increased emphasis on developing accessions with the capacity for N uptake and assimilation to increase yield output or maintain high grain yield under limited N supply regimes. Glutamine synthetase biosynthesis is certainly an integral component of nitrogen metabolism as the enzyme is involved in the ATP-dependent conversion of glutamate to glutamine. The regulation of the GS enzyme occurs at the level of the genes, transcript and protein. The GS enzyme also catalyses the release of ammonium for nitrogen remobilisation during senescence and especially, to boost grain filling. In the present study, the six GS alleles investigated among nine spring wheat accessions give some useful insights into the genetic diversity at both the sequence and amino acid level, and highlight aspects of GS expression and GS activity that are influenced by nitrogen treatment or deprivation. The analysis presented in this study also shows that GS activity was under temporal control when standardisation of harvesting stage was controlled. This can serve as useful reference in breeding for nitrogen use efficiency in hexaploid wheat.

There are however, several questions relating to the characterisation of GS genes. These include uncertainties about the exact role of specific GS homologues have in mediating nitrogen uptake within genetically diverse wheat accessions, the effect of different nitrogen concentrations on gene expression and GS activity in different accessions and the level of GS contribution to grain filling and grain protein content.

The exploration of the underlying mechanisms of these characteristics and the genetic variables of the GS enzymes in diverse accessions is one of the key strategies for discovering the genetic potential existing in these wheat accessions for nitrogen use efficiency. This study contributed to the identification of genetic diversity in hexaploid wheat by creating 20 new SNP markers for GS alleles. This GS characterisation may provide a useful set of tools for wheat breeding targeting the optimal use of nitrogen fertilisers and ensuring sustainable wheat crop production.



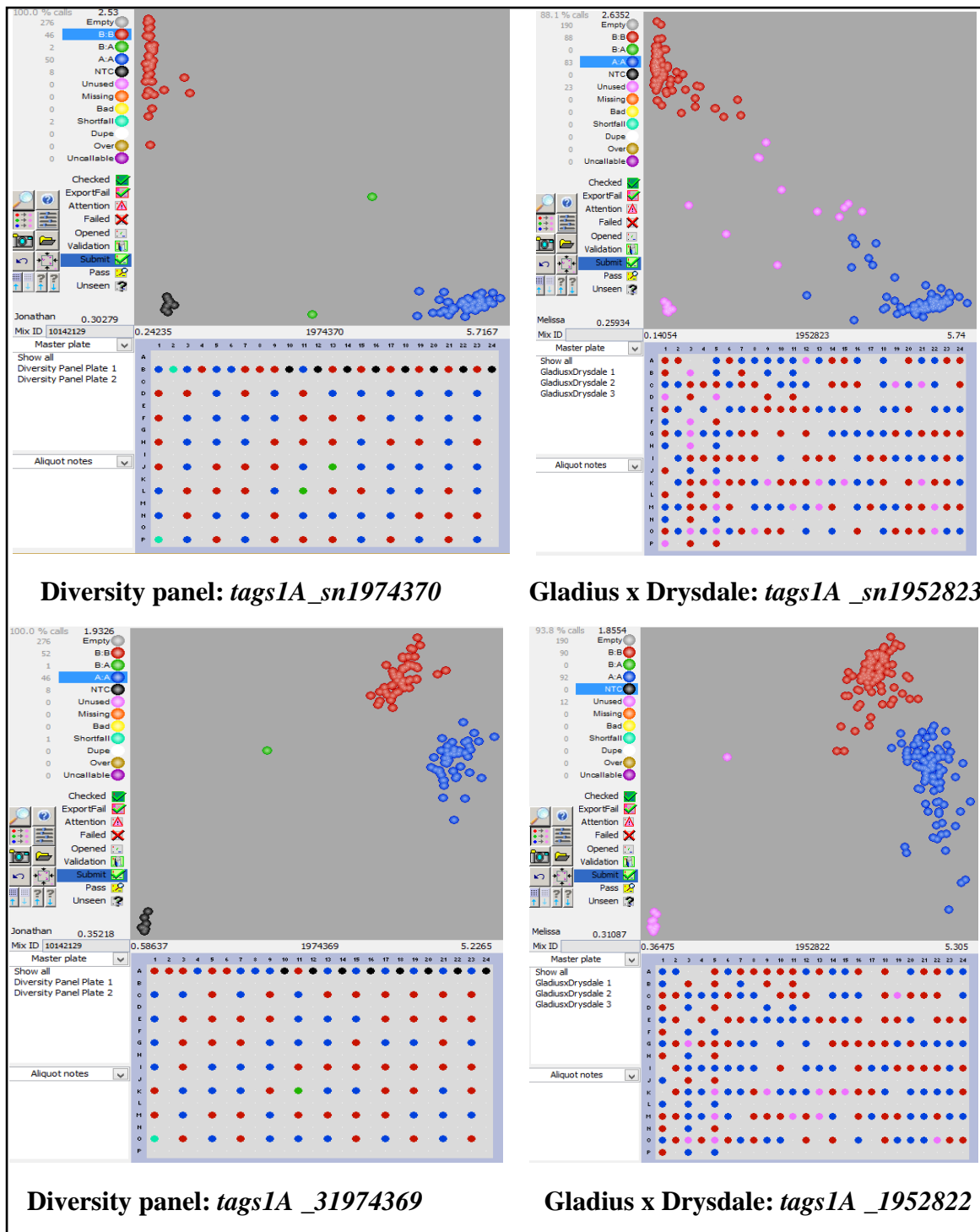
## APPENDIX

**Table A2.1** Characteristics of oligonucleotide pairs used to amplify the *TaGS1.1* conserved domain sequences among accessions.

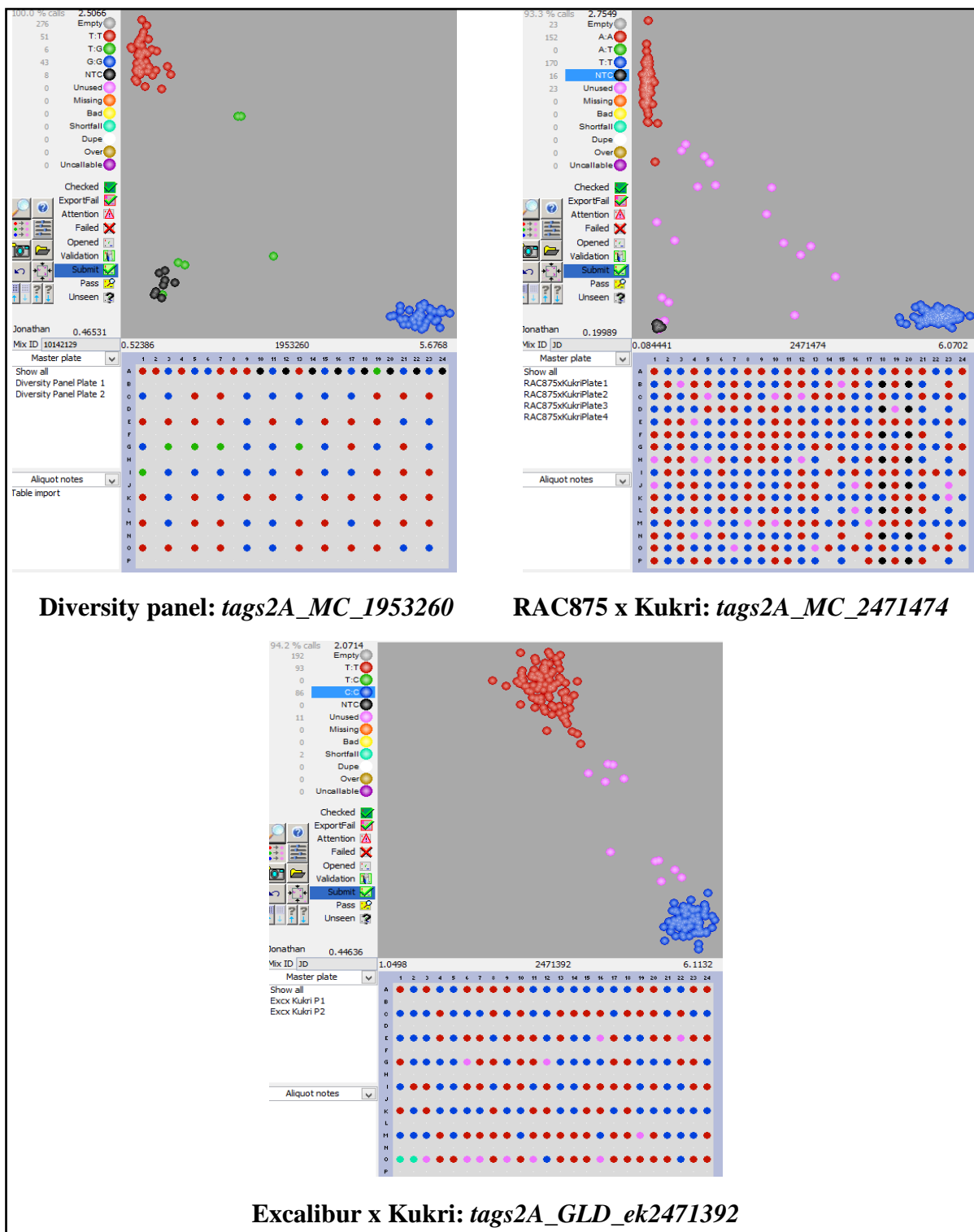
| Sequence Name        | Primer Name | Primer Sequence           | Length / bp | Direction | %GC  | Hairpin T <sub>m</sub> | Self-Dimer T <sub>m</sub> | T <sub>m</sub> | Seq. size/bp |
|----------------------|-------------|---------------------------|-------------|-----------|------|------------------------|---------------------------|----------------|--------------|
| 6AL_5748909-5820788_ | 4,694 F     | CTGGGTCGTCGCCATTATATA     | 21          | forward   | 47.6 | None                   | None                      | 57.1           |              |
| 6AL_5748909-5820788  | 5,093 R     | CTAACAGCGCAAACACTACGAC    | 20          | reverse   | 50   | None                   | None                      | 57.2           | 428          |
| 6AL_5748909-5820788_ | 5,235 F     | GTTTCAGTTCAGTTCGGTCCCGT   | 22          | forward   | 50   | None                   | 11                        | 61.1           |              |
| 6AL_5748909-5820788_ | 5,757 R     | CTTGCTCCTGAGATCCATGC      | 20          | reverse   | 55   | None                   | 0.6                       | 58.4           | 562          |
| 6AL_5748909-5820788  | 5,845 F     | CTGCCGTGCTACCTAGTTATCCA   | 23          | forward   | 52.2 | None                   | None                      | 61.8           |              |
| 6AL_5748909-5820788_ | 6,406 R     | GCTTAAGTTGATCTCCCCTCTT    | 22          | reverse   | 45.5 | None                   | 4                         | 57.5           | 683          |
| 6AL_5748909-5820788  | 6,375 F     | GAGCATCATTAAAGAGGGGAGATCA | 24          | forward   | 45.8 | None                   | None                      | 59.7           |              |
| 6AL_5748909-5820788_ | 6,838 R     | GAACGGAGGGAGTAGATCAT      | 20          | reverse   | 50   | None                   | None                      | 55.8           | 489          |
| 6AL_5748909-5820788  | 7,231 F     | CTACTGTAGTATTGGTGTGAC     | 22          | forward   | 45.5 | None                   | 6.7                       | 56             |              |
| 6AL_5748909-5820788  | 8,019 R     | CAGATGATCCAGAACTAGAAGG    | 22          | reverse   | 45.5 | None                   | None                      | 55.4           | 859          |
| 6AL_5748909-5820788_ | 7,009 F     | GATTGACTGATCGCGTTGTT      | 19          | forward   | 45   | None                   | None                      | 56.8           |              |
| 6AL_5748909-5820788_ | 8,019 R     | CAGATGATCCAGAACTAGAAGG    | 21          | reverse   | 45.5 | None                   | None                      | 55.4           | 1104         |
| 6BL_227297r-866323_  | 749 F       | CTGTTGCTGCTCTGGTTGGT      | 20          | forward   | 55   | None                   | None                      | 60.8           |              |
| 6BL_227297r-866323_  | 1,626 R     | GCAGCGTTGTATCTCTTGTAGTG   | 24          | reverse   | 45.8 | None                   | None                      | 60.2           | 910          |
| 6BL_227297r-866323_  | 1,785 F     | GTACGGTATTGAGCAGGAGTAC    | 22          | forward   | 50   | None                   | None                      | 58             |              |
| 6BL_227297r-866323_  | 2,266 R     | CTCAAGAAGGTAGCGAGCAA      | 19          | reverse   | 50   | None                   | None                      | 57.6           | 534          |
| 6DL_2494823          | 401 F       | CTCTTGTTTCAGTTCAGTCCCGT   | 22          | forward   | 45.5 | None                   | None                      | 58.5           |              |
| 6DL_2494823          | 930 R       | CTGGCTTTGCTCCTGAGATC      | 20          | reverse   | 55   | None                   | None                      | 58.3           | 564          |
| 6DL_2494823          | 2,984 F     | CGCCTTCTAGTCTGAATCATC     | 22          | forward   | 45.5 | None                   | None                      | 56.7           |              |
| 6DL_2494823          | 3,269 R     | CAACCCATCACTGATCATCG      | 20          | reverse   | 50   | None                   | None                      | 56.3           | 395          |
| 6DL_2494823          | 2,001 F     | GATTGATTGACTGGTCGTGT      | 20          | forward   | 45   | None                   | None                      | 55.8           |              |
| 6DL_2494823          | 3,005 R     | GATGATTCAGAACTAGAAGGCG    | 22          | reverse   | 45.5 | None                   | None                      | 56.7           | 1109         |

**Table A2.2** Characteristics of oligonucleotide pairs used to amplify the *TaGS2* conserved domain sequences among accessions.

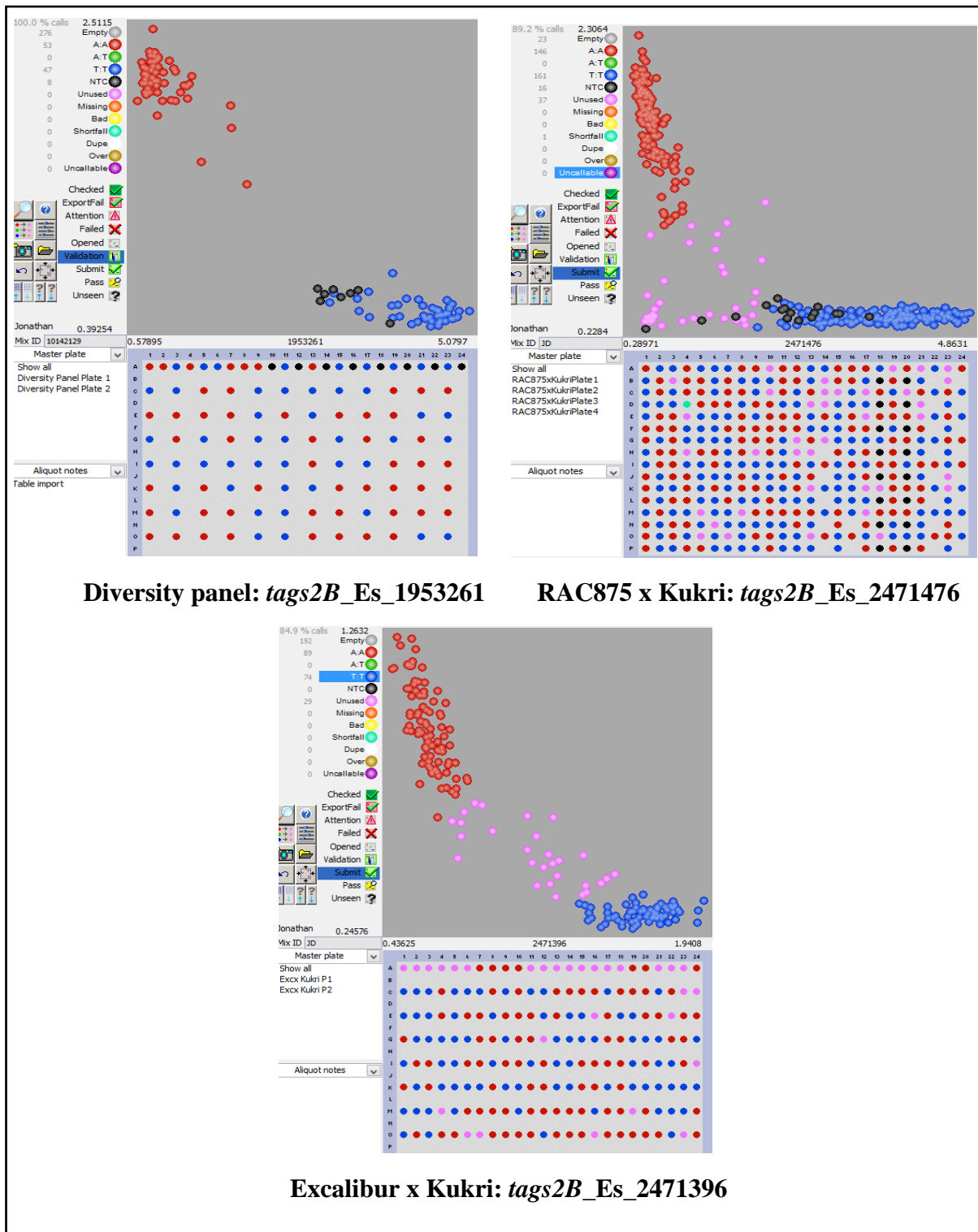
| Sequence Name          | Primer Name | Primer Sequence          | Length | Direction | %GC  | Hairpin Tm | Self-Dimer Tm | Tm   | Ampli-con size/bp |
|------------------------|-------------|--------------------------|--------|-----------|------|------------|---------------|------|-------------------|
| 2AL_6391136 (reversed) | 1,115 F     | GCGGAGTAAGTAAGTAAGCAGC   | 22     | forward   | 50   | None       | None          | 59.1 |                   |
| 2AL_6391136 (reversed) | 1,491 R     | CTGTGCTCTCATGGAAGTCAATC  | 23     | reverse   | 47.8 | None       | None          | 59.4 | 402               |
| 2AL_6391136 (reversed) | 1,561 F     | GATTAGATTAGCACGGAGGGA    | 21     | forward   | 47.6 | None       | None          | 56.7 |                   |
| 2AL_6391136 (reversed) | 1,905 R     | CCTTGATTGCTTCTGAGGTCA    | 22     | reverse   | 45.5 | None       | 0.4           | 58.6 | 408               |
| 2AL_6391136 (reversed) | 1,933 F     | CACAGTTATCGATGTTTCCTTGCA | 24     | forward   | 41.7 | None       | None          | 59.8 |                   |
| 2AL_6391136 (reversed) | 2,671 R     | CAAATGTTCAACTTCGTGTGC    | 22     | reverse   | 40.9 | None       | None          | 57.6 | 797               |
| 2AL_6391136 (reversed) | 2,438 F     | CTGGTTACATGTGTATTTGGC    | 22     | forward   | 40.9 | None       | None          | 56   |                   |
| 2AL_6391136 (reversed) | 3,037 R     | CATGCACCCATATAAATAAGGAGG | 24     | reverse   | 41.7 | None       | None          | 57.2 | 727               |
| 2BL_7944286            | 3,837 F     | GAATGCCCTTCGTAAATACCAT   | 22     | forward   | 40.9 | None       | None          | 56.6 |                   |
| 2BL_7944286            | 4,181 R     | CACCAGATGAACAAATATTAACAG | 24     | reverse   | 33.3 | None       | None          | 54.3 | 360               |
| 2BL_7944286            | 4,477 F     | CGTGTGTCTTGTGTGGCA       | 20     | forward   | 45   | None       | None          | 57.7 |                   |
| 2BL_7944286            | 4,868 R     | CTGCATTCCCAACCATTGCA     | 20     | reverse   | 50   | None       | None          | 59.4 | 394               |
| 2DL_9714273            | 2,175 F     | CAGGCCTGTAGTGAGCATC      | 20     | forward   | 55   | None       | None          | 58.3 |                   |
| 2DL_9714273            | 2,606 R     | CCTTGATTGCTCCTGAGGTCA    | 22     | reverse   | 50   | None       | None          | 60.3 | 445               |
| 2DL_9714273            | 3,314 F     | GTTGGAGGATCTGGGATTGAC    | 21     | forward   | 52.4 | None       | None          | 58.1 |                   |
| 2DL_9714273            | 3,799 R     | CAGTATGTTGTTGCCTCCTC     | 20     | reverse   | 50   | None       | None          | 56.1 | 499               |
| 2DL_9714273            | 4,425 F     | GAAGCTTGATTACCAGATGACAA  | 24     | forward   | 37.5 | None       | None          | 57.5 |                   |
| 2DL_9714273            | 4,855 R     | CATGGACTTATGGGATCTGATG   | 22     | reverse   | 45.5 | None       | None          | 56.1 | 522               |
| 2DL_9714273            | 6,269 F     | CAGTGATTGTACAGACCCAACAG  | 23     | forward   | 47.8 | None       | None          | 59.3 |                   |
| 2DL_9714273            | 6,690 R     | CCTTGC GCGATT CATTATCCT  | 22     | reverse   | 45.5 | None       | None          | 59.5 | 432               |



**Fig. A2.1** Comparison of marker based segregation of *TaGS1.1A* alleles between the Gladius x Drysdale recombinant inbred lines and different spring wheat varieties in the diversity panel. Blue and red groups represent genotype clustering per allelic variation at the SNP locus.



**Fig. A2.2** Marker based segregation of *TaGS2A* alleles in different spring wheat varieties double haploid mapping populations and diversity panel lines. Blue and red groups represent genotype clustering per allelic variation at the SNP locus.



**Fig. A2.3** Marker based segregation of *TaGS2B* alleles in different spring wheat varieties double haploid mapping populations and diversity panel lines. Blue and red groups represent genotype clustering per allelic variation at the SNP locus.

## S.2.1

>gi|321531576|gb|HQ840647.1|*Triticum aestivum* cultivar Yumai 49  
glutamine synthetase (Bd3)

TCGCACCACCCCAACCCGCTTCCTTCCTGCCTTCCTGCCCTCAGTCAGCCGGAGCCGGATT  
GCTCCTCCCGCCATGGCGCTCCTCACCGATCTCCTCAACCTCGACCTCACCGACTCCACGGA  
GAAGATCATCGCCGAGTACATATGGATCGGCGGATCTGGCATGGATCTCAGGAGCAAGGCCA  
GGACCCCTCCCGGCCCCGGTACCGGATCCCAGCAAGCTGCCCAAGTGGAACACTACGACGGCTCC  
AGCACCGGCCAGGCCCCCGGGCAGGACAGCGAGGTCATCCTGTACCCACAGGCCATCTTCAA  
GGACCCGTTTACAGGAAGGGCAACAACATCCTTGTATGTGCGATTGCTACACCCAGCTGGAG  
TGCCAATCCCCACCAACAAGAGATAACAACGCTGCCAAGATCTTTAGCAACCCTGATGTTGCC  
AAGGAGGAGCCATGGTATGGTATTGAGCAGGAGTACACCCCTCCTACAGAAGGACATCAACTG  
GCCTCTCGGCTGGCCTGTTGGTGGATTCCCTGGTCCTCAGGGTCCTTACTACTGTAGTATTG  
GTGCTGACAAGTCGTTTGGGCGTGACATAGTTGACGCTCACTACAAGGCCCTGCCTCTTTGCC  
GGTGTCAACATCAGTGGCATCAATGGCGAGGTCATGCCCGGACAGTGGGAGTTCCAAGTCGG  
CCCAGCTGTTGGCATTCTGCTGGCGACCAAGTGTGGGTGCTCGCTACCTTCTTGAGAGGA  
TCACTGAGATCGCCGGAGTTGTCGTCACATTTGACCCCAAGCCATCCCAGGCGACTGGAAC  
GGTGTGGTGCTCACACAACTACAGTACCGAGTCGATGAGGAAGGACGGCGGGTTCAAGGT  
CATCGTGGATGCCGTGGAGAAGCTCAAGCTCAAGCACAAGGAGCACATCGCCGCCACGGCG  
AGGGCAACGAGCGCCGCTCACCGGCAAGCACGAGACCGCCGACATCAACACCTTCAGCTGG  
GGCGTCGCAAACCGTGGCGCGTCCGTGCGCGTGGGCCGGGAGACGGAGCAGAACGGCAAGGG  
CTACTTCGAGGACCGCCGGCCGGCGTCCAACATGGACCCATACGTGGTTCACCTCGATGATCG  
CCGAGACCACCATCCTGTGGAAGCCCAGCCGATCGGCGCCGATCGCCGTGCGATGATCA  
GTGATGGGTTGGGTTGGATTTGGTGGGTGGCCACTGGAGGATTTGGACGAAATTCCTCCAAC  
ATATTTGCTTTTCCGTTTGGTTGCATACTACGCGTAGTCCGCTTAGGTAGGTGCGATCATCG  
TCGTCGTCGTCATGCATCAGGGTGTCTGGTCGCTCTTCTCTCGTCTCTGGGTGGTGGGTGGC  
AAGAGGCGTGTCAAACACACAAGGCTACTATTGATTGTTTTCCCTTGCCGTTTGGGTGCTCT  
GGTCCCTCGTGTAATCATCAATTGATATGGTAATAAAATGGTCCGTACAGCTGCCGTGGTACC  
ACACCA

## S.2.1

>gi|321531576 protein 6AL\_5820788 GLN1.2

MALLTDLLNLDLTDSTEKIIAEYIWIWGGSGMDLRSKARTLPGPVTDPSKLPKWNVDGSSTGQ  
APGEDSEVILYPQAIKDFRKGNNILVMDCYTPAGVPIPTNKRYNAAKIFSNPDVAKEEP  
WYGIEQEYTLQKNDINWPLGWPVGGFPGPQGPYYCSIGADKSFGRDIVDAHYKACLFAGVNI  
SGINGEVMPGQWEFQVGPVGI SAGDQVWVARYLLERITEIAGVVVTFDPKPIPGDWNGAGA  
HTNYSTESMRKDGGFKVIVDAVEKLLKHKHEHIAAYGEGNERRLTGKHETADINTFSWGVAN  
RGASVRVGRETEQNGKGYFEDRRPASNMDPYVVTSMIAETTILWKP\*

## S.2.2

### >lcl|6BL\_227297 rev comp.

This is Homoeologue on 6BL– this contig is incomplete with respect to the gene (incomplete sequence at 5')>lcl|tplb0036k03 GLN1.1 (Bd3), color coding shows start and end of genomic sequence overlap.

```
GGACTCGCACCACCCCCACCCCCACCCGCTTCCTTCCTCCGCGACTTGCCTGCCCGCCCTC
AGTCAGCCGGCCATGGCGCTCCTCACCGATCTCCTCAACCTCGACCTCACCGACTCCACGGA
GAGGATCATCGCCGAGTACATATGGATCGGCGGATCTGGCATGGATCTCAGGAGCAAGGCCA
GGACCCCTCCCCAGCCCGGTACCCGATCCCAGCAAGCTGCCCAAGTGGAACACGACGGCTCC
AGCACCGGCCAGGCCCCCGGCGAGGACAGCGAGGTCATCCTGTACCCACAGGCCATCTTCAA
GGACCCGTTTCAGGAAGGGCAACAACATCCTTGTATGTGCGATTGCTACACCCAGCTGGAG
TGCCAATCCCCACTAACAAGAGATAACAACGCTGCTAAGATCTTTAGCAACCCTGATGTTGCC
AAGGAGGAGCCATGGTACGGTATTGAGCAGGAGTACACCCCTCCTACAGAAGGACATCAACTG
GCCTCTCGGCTGGCCTGTTGGTGGCTTCCCTGGTCCTCAGGGTCCTTACTACTGTAGTATCG
GTGCTGACAAGTCGTTTCGGGCGTGACATAGTTGACTCCCCTACAAGGCCCTGCCTCTTTGCC
GGCGTCAACATCAGTGGCATCAACGGCGAGGTCATGCCCGGACAGTGGGAGTTCCAAGTTGG
CCCAGCTGTTGGCATTCTGCTGGTGACCAAGTGTGGGTTGCTCGCTACCTTCTTGAGAGGA
TCACCGAGATCGCCGGAGTTGTCGTCACATTTGACCCCAACCCCATCCCAGGCGACTGGAAC
GGTGCTGGTGCTCACACAAACTACAGTACCGAGTCGATGAGGAAGGACGGCGGGTTCAAGGT
CATCGTGGACGCCGTCGAGAAGCTCAAGCTGAAGCACAAGGAGCACATCGCCGCCACGGCG
AGGGCAACGAGCGCCGTCTGACCGGCAAGCACGAGACCGCCGACATCAACACCTTCAGCTGG
GGTGTGCGCAACCGTGGCGCGTCCGTGCGCGTGGGCCGGGAGACGGAGCAGAACGGCAAGGG
CTACTTCGAGGACCGCCGGCCGGCGTCCAACATGGACCCCTACGTGGTCACCTCGATGATCG
CCGAGACCACCATCCTGTGGAAGCCCTGAGCCGATCAGCGCCGATCGCCGTGCGATGATCA
GTGGTGGGTTGGGTTTGGTGGTGGCCATTGGAGGATTTGGACGAAATTCCTTCCAAGATATTT
CCTTTTCCGTTTGGTTGCATACTACGCGTAGTCCGTTTAGGTAGGTCACATCATCGTCGTCG
TCATCAGGGTGTCTGGTCTCTCTCTCTCGTCTTTGGGTGGTGGGTGGCAAGAGGCGTGTCA
AACACACAGGACTACTATTGATTGTTTTCCCTTGCCGTCTGGGTGCTCTGGTCTCGTCTCGT
GTAATCATCAATTGATATGGTAATAAAAATGGTCCGTACCGCAGCCGTGGTACCACGCAAAAA
AAAAAAAAA
```

## S.2.3

### >tplb0036k03 deduced protein 6BL

```
MALLTDLLNLDLTDSTERI IAEYIWIWIGSGMDLRSKARTLPSVTDPSKLPKWNVDGSSTGQ
APGEDSEVILYPQAI FKPDPFRKGNNI LVMDCYTPAGVPIPTNKRYNAAKIFSNPDVAKEEP
WYGIEQEYTL LQKDINWPLGWPVGGFPGPQGPYYCSIGADKSFGRDIVDSHYKACLFAGVNI
SGINGEVMPGQWEFQVGPVVGISAGDQVWVARYLLERITEIAGVVVTFDPNPIPGDWNNGAGA
HTNYSTESMRKDGGFKVIVDAVEKLLKHKHEHIAAYGEGNERRLTGKHETADINTFSWGVAN
RGASVRVGRETEQNGKGYFEDRRPASNMDPYVVTSMIAETTILWKP*
```

## S.2.4

### >6DL\_2494823 derived CDS: (Bd3)

ATCGCGCTCCTCACCGATCTCCTCAACCTCGACCTCACCGACTCCACGGAGAAGATCATCGC  
CGAGTACATATGGATCGGCGGATCTGGCATGGATCTCAGGAGCAAAGCCAGGACCCTCCCCG  
GCCCCGGTCACCGACCCCAGCAAGCTGCCAAGTGGAACCTACGACGGCTCCAGCACCGGCCAG  
GCCCCCGGCGAGGACAGCGAGGTCATCCTGTACCCACAGGCCATCTTCAAGGACCCGTTTCAG  
GAAGGGCAACAACATCCTTGTTCATGTGCGATTGCTACACCCCAGCTGGAGTGCCAATCCCCA  
CCAACAAGAGATAACAACGCTGCCAAGATCTTTAGCAACCCTGATGTTGCCAAGGAGGAGCCA  
TGGTACGGTATCGAGCAGGAGTACACCCTCCTACAGAAGGACATCAACTGGCCTCTCGGCTG  
GCCTGTTGGTGGATTCCCTGGTCCTCAGGGTCCTTACTACTGTAGTATTGGTGCTGACAAGT  
CGTTTGGGCGTGACATAGTTGACTCCCCTACAAGGCCTGCCTCTTTGCCGGCGTCAACATC  
AGTGGCATCAACGGCGAGGTCATGCCCGGACAGTGGGAGTTCCAAGTTGGCCCGACTGTCGG  
CATCTCTGCTGGTGACCAAGTGTGGGTTGCTCGCTACCTTCTTGAGAGGATCACTGAGATCG  
CCGGAGTTGTCGTCACATTTGACCCCAAGCCATCCCAGGCGACTGGAACGGTGTGGTGCT  
CACACAACTACAGTACCGAGTCGATGAGGAAGGACGGCGGGTTCAAGGTCATCGTGGACGC  
TGTCGAGAAGCTCAAGCTGAAGCACAAGGAGCACATCGCCGCCTACGGCGAGGGCAACGAGC  
GCCGTCTCACCGGCAAGCACGAAACCGCCGACATCAACACCTTCAGCTGGGGTGTGCGGAAC  
CGTGGCGCGTGGTGC GCGTGGGACGGGAGACGGAGCAGAACGGCAAGGGCTACTTCGAGGA  
CCGCCGGCCGGCGTCCAACATGGACCCCTACGTGGTCACCTCCATGATCGCCGAGACCACCA  
TCCTGTGGAAGCCC TGA

## S.2.5

### >6DL\_2494823 derived protein

MALLTDLLNLDLTDSTEKI IAEYIWIWGGSGMDLRSKARTLPGPVTDPSKLPKWNVDGSSTGQ  
APGEDSEVILYPQAI FKPFRKGNNILVMDCYTPAGVPIPTNKRYNAAKIFSNPDVAKEEP  
WYGIEQEYTLQKDINWPLGWPVGGFPGPQGPYYCSIGADKSFGRDIVDSHYKACLFAGVNI  
SGINGEVMPGQWEFQVGPTVGISAGDQVWVARYLLERITEIAGVVVTFDPKPIPGDWNAGGA  
HTNYSTESMRKDGGFKVIVDAVEKLLKHKHEHIAAYGEGNERRLTGKHETADINTFSWGVAN  
RGASVRVGRETEQNGKGYFEDRRPASNMDPYVVTSMIAETTILWKP\*



## S.2.6

### >2AL\_6391136 Deduced CDS:

ATCGCGCAGGCGGTGGTGCCGGCGATGCAGTGCCAGGTGGGCGTGCGGGGCAGGTCGGCCGT  
CCCGGCGAGGCAGCCCGCGGGCAGGGTGTGGGGCGTCAGGAGGACCGCCCGTGCCACCTCCG  
GCTTCAAGGTGCTGGCCCTCGGCCCGGAGACCACCGGCGTCATCCAGAGGATGCAGCAGCTG  
CTCGACATGGACACCACGCCCTTACCCGACAAGATCATCGCCGAGTACATCTGGGTTGGAGG  
ATCTGGAATTGACCTCAGAAGCAAATCAAGGACGATTTTGAAGCCAGTGGAGGACCCGTCAG  
AGCTACCCAAATGGAATTATGACGGATCGAGCACCGGGCAGGCTCCTGGAGAAGACAGTGAA  
GTCATTCTATACCCACAGGCCATATTCAAGGACCCATTCCGAGGAGGCAACAACATCCTGGT  
TATCTGTGACACCTACACGCCACAAGGGGAACCCATCCCTACTAACAAGCGACACATGGCTG  
CACAAATCTTCAGTGACCCCAAGGTCACTGCACAAGTGCCATGGTTTGGAAATCGAACAGGAG  
TACACTCTGATGCAGAGGGATGTGAACTGGCCTCTTGGCTGGCCTGTTGGAGGGTACCCTGG  
CCCCCAGGGTCCATACTACTGCGCCGTAGGATCAGACAAGTCATTTGGCCGTGACATATCCG  
ATGCTCACTACAAGGCCTGCCTTTACGCGGGAATTGAAATCAGTGGAACAAACGGGGAGGTC  
ATGCCTGGTCAAGTACATTCTCGAGAGAATCACGGAGCAAGCTGGTGTGGTGCTCACCCTTG  
ACCCAAAACCAATCCAGGGTGACTGGAATGGAGCTGGCTGCCACACAAATTACAGTACACTG  
AGCATGCGCGAAGATGGAGGTTTCGACGTGATCAAGAAGGCAATCCTGAACCTTTCACCTTCG  
CCATGACTTGCACATAGCCGCATATGGTGAAGGGAACGAGCGGAGGTTGACAGGGCTACACG  
AGACAGCTAGCATTTCAGACTTCTCATGGGTATGGGGTGTGCGAACCCTGGCTGCTCTATT  
CGTGTGGGCCGAGAGACCGAGGCAAAGGGCAAAGGATACCTGGAGGACCGTCGCCCGGCGTC  
GAACATGGACCCGTACACCGTGACGGCGCTGCTGGCCGAGACCACGATCCTGTGGGAGCCGA  
CCCTCGAGGCGGAGGCCCTCGCTGCCAAGAAGCTGGCGCTGAAGGTA TGA

## S.2.7

### >2AL\_6391136 protein

MAQAVVPAMQCQVGVRRGRSAVPARQPAGRVWGVRRRTARATSGFKVLALGPETTGVIRQMQQL  
LDMDTTPFTDKIIAEYIWVGGSGIDLRKSRITISKVEDPSELPKWNYDGSSTGQAPGEDSE  
VILYPQAI FKDFRGGNNILVICDTYTPQGEPIPTNKRHMAAQIFSDPKVTAQVPWFGIEQE  
YTLMQRDVNWPLGWPVGGYPGPQGPYCAVGS DKSFRDISDAHYKACLYAGIEISGTNGEV  
MPGQWEYQVGPSVVIDAGDHIWASRYILERITEQAGVVLTLDPKPIQGDWNGAGCHTNYSTL  
SMREDGGFDVIKKAILNLSLRHDLHIAAYGEGNERRLTGLHETASISDFSWVWGVANRGC SI  
RVGRETEAKGKGYLEDRRPASNMDPYTVTALLAETTILWEPTLEAEALAAKKLALKV\*

## S.2.8

### >2BL\_7944286 deduced CDS

ATCGCGCAGGCGGTGGTGCCGGCGATGCAGTGCCAGGTGGGCGTGCGGGGCAGGTCGGCCGT  
CCCGGCGAGGCAGCCCGCGGGCAGGGTGTGGGGCGTCAGGAGGACCGCCCGCGCCGCTCCG  
GCTTCAAGGTGCTCGCCCTCGGCCCGGAGACCACCGGCGTCATCCAGAGGATGCAGCAGCTG  
CTCGACATGGACACCACGCCCTTACCCGACAAGATCATCGCCGAGTACATCTGGGTTGGAGG  
ATCTGGAATTGACCTCAGAAGCAAATCAAGGACGATTTTGAAGCCAGTGGAGGACCCGTCAG  
AGCTACCGAAATGGAACACGATGGATCGAGCACAGGCCAGGCTCCTGGAGAAGACAGTGAA  
GTCATCCTATACCACAGGCCATATTC AAGGACCCATTCCGAGGAGGCAACAACATACTGGT  
TATCTGTGACACCTACACACCACAAGGGGAACCCATCCCTACTAACAACGACACATGGCTG  
CACAAATCTTCAGTGACCCCAAGGTCACTTCACAAGTGCCATGGTTTGG AATCGAACAGGAG  
TAACTCTGATGCAGAGGGATGTGAACTGGCCTCTTGGCTGGCCTGTTGGAGGGTACCCTGG  
CCCCAGGGTCCATACTACTGCGCCGTAGGATCAGACAAGTCATTTGGCCGTGACATTTAG  
ATGCTCACTACAAGGCATGCCTTTACGCCGGAATTGAAATCAGTGGAACAAACGGGGAGGTC  
ATGCCTGGTCAGTGGGAGTACCAGGTGGACCCAGCGTTGGTATTGATGCAGGAGATCACAT  
ATGGGCTTCAAGATACATTTCTCGAGAGAATCACGGAGCAAGCTGGTGTCTGCTCACCCTTG  
ACCCAAAACCAATCCAGGGTGAAGTGGAGCTGGCTGCCACACAAATTACAGCACATTG  
AGCATGCGTGAGGATGGAGGTTTCGACGTGATCAAGAAGGCAATCCTGAACCTTTCACTCCG  
CCATGACTTGCACATAGCCGCATATGGTGAAGGAAACGAACGGAGGTTGACAGGGCTACATG  
AGACAGCTAGCATATCAGACTTCTCATGGGGTGTGCGGAATCGTGGCTGCTCTATTCGTGTG  
GGCGAGAGACTGAGGCAAAGGGCAAAGGATACCTGGAGGACCGTCGCCCGGCCTCGAACAT  
GGACCCATACACCGTGACGGCGCTGCTGGCCGAGACCACGATCCTGTGGGAGCCGACCCTCG  
AGGCAGAGGCCCTCGCTGCCAAGAAGCTGGCGCTGAAGGTA TGA

## S.2.9

### >2BL\_7944286 deduced protein

MAQAVVPAMQCQVGVGRSAV PARQPAGRVWGVRRRTARAASGFKVLALGPETTGV IQRMQQL  
LDMDTTPFTDKIIAEYIWVGGSGIDLRSKSRITISKVEDPSELPKWNYDGSSTGQAPGEDSE  
VILYPQAI FKDFFRGGNNILVICDTYTPQGEPIPTNKRHMAAQIFSDPKVTSQVPWFGIEQE  
YTLMQRDVNWPLGWPVGGYPGPQGPYCAVGS DKSFRDISDAHYKACLYAGIEISGTNGEV  
MPGQWEYQVGPSVGIDAGDHIWASRYILERITEQAGVVLTLDPKPIQGDWNGAGCHTNYSTL  
SMREDGGFDVIKKAILNLSLRHDLHIAAYGEGNERRLTGL

## S.2.10

### >2DL\_9842556 deduced CDS

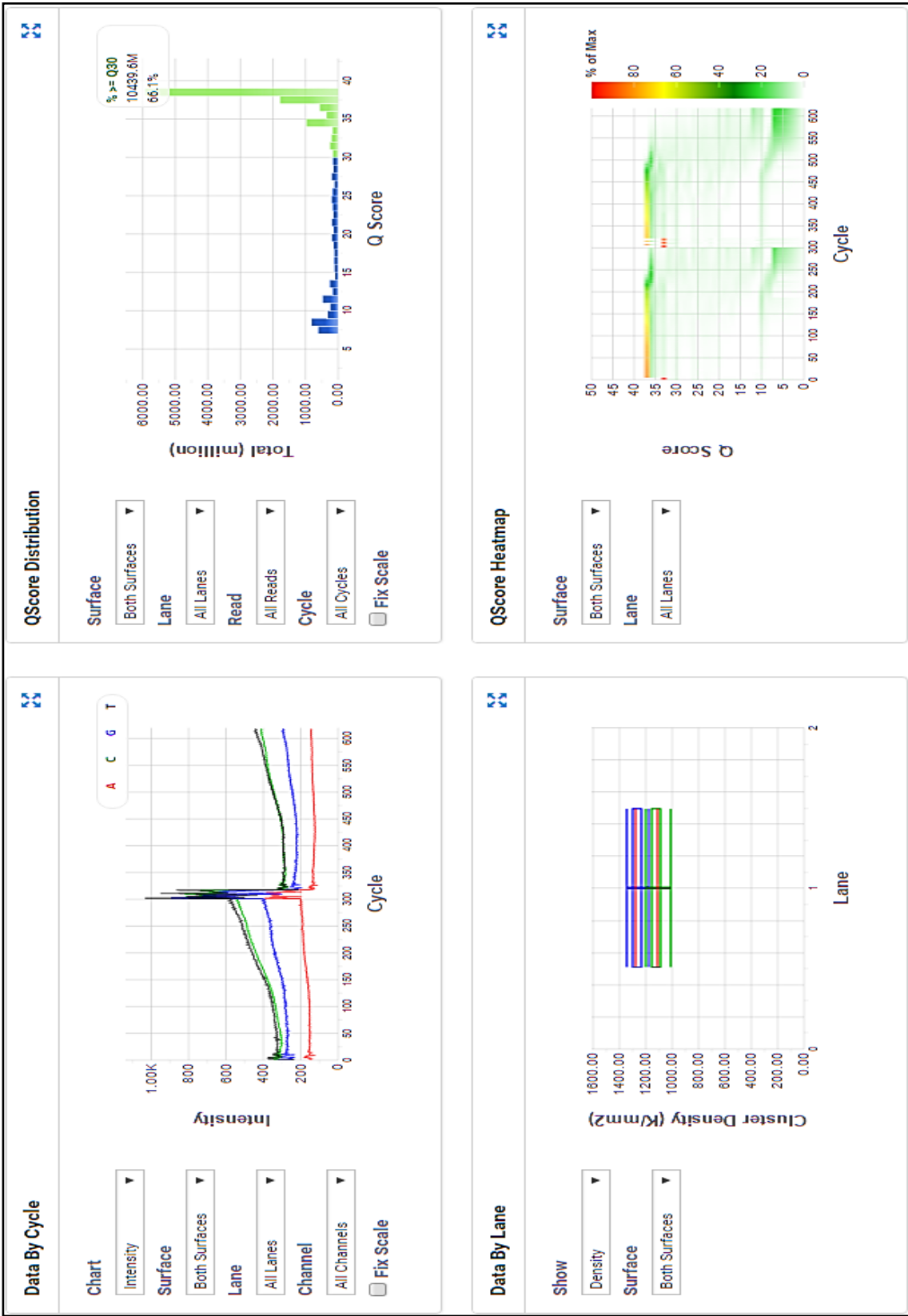
>lcl|tplb0004c07 No definition line found

GCTTGGCCCCCTTCCCTCCGTCCCCTCCTCCCTCGTCTCGTCCGCGTCCTTGTTCAGGTTTGGAG  
GGCGGCAGAGTACCTGTAAGTAAGTAAGTAGCGGCGATGCGCAGGCGGTGGTGCCGGCGAT  
GCAGTGCCAGGTGGGCGTGCGGGGCAGGTCTGCCGTCCCGGCGAGGCAGCCCGGGGCAGGG  
TGTGGGGCGTGAGGAGGACCGCCCGGCCACCTCCGGGTTCAAGGTGCTGGCCCTCGGCCCG  
GAGACCACCGGCGTCATCCAGAGGATGCAGCAGCTGCTCGACATGGACACCACGCCCTTCAC  
CGACAAGATCATCGCCGAGTACATCTGGGTTGGAGGATCTGGGATTGACCTCAGGAGCAAAT  
CAAGGACGATTTCAAAGCCAGTGGAGGACCCATCAGAGCTACCGAAATGGAACCTACGACGGA  
TCGAGCACAGGGCAGGCTCCTGGAGAAGACAGTGAAGTCATCCTATAACCCACAGGCCATATT  
CAAGGACCCATTCCGAGGAGGCAACAACATACTGGTTATCTGTGACACCTACACGCCACAAG  
GGGAACCCATCCCTACTAACAAGCGACACATGGCTGCACAAATCTTCAGTGACCCCAAGGTC  
ACTGCACAAGTGCCATGGTTTGAATCGAACAGGAGTACACTCTGATGCAGAGGGATGTGAA  
CTGGCCTCTTGGCTGGCCTGTTGGAGGGTACCCTGGCCCCAGGGTCCATACTACTGCGCCG  
TAGGATCAGACAAGTCATTTGGCCGTGACATATCCGATGCTCACTACAAGGCCTGCCTTTAC  
GCGGGAATTGAAATCAGTGAACAAACGGGGAGGTCATGCCTGGTCAGTGGGAGTACCAGGT  
TGGACCTAGCGTTGGTATTGATGCGGGAGACCACATATGGGCTTCAAGATACATTCTCGAGA  
GAATCACGGAGCAAGCTGGTGTGGTGTCCACCCTTGACCCAAAACCAATCCAGGGTGACTGG  
AATGGAGCTGGCTGCCACACAAATTACAGCACATTGAGCATGCGCGAGGATGGAGGTTTCGA  
CGTGATCAAGAAGGCAATCCTGAACCTTTCACCTCGCCATGACTTGCACATAGCCGCATATG  
GTGAAGGAAACGAACGGAGGCTGACAGGGCTACATGAGACAGCTAGCATATCAGACTTCTCA  
TGGGGCGTCGCGAACCGTGGCTGCTCTATTCTGTGTGGGGCGAGAAACCGAGGCAAAGGGCAA  
AGGATACCTGGAGGACCGTCCGCCGCGTCCGAACATGGACCCGTACACCGTGACGGCGCTGC  
TGGCCGAGACCACGATCCTGTGGGAGCCGACCCTCGAGGCGGAGGCCCTCGCTGCCAAGAAG  
CTGGCGCTGAAGGTAAGGACCTGAAAAGGCCGAATTTCTCCGGGGAAAAGAAAATAAAT  
CGGCGGCGGCGAGACCGTTCGTCCGTGTCATTTCTTGTGATCCTGTGGTTCCGTCGGGGC  
ACTGTCTGTACAAAATCCTCACGGTTTGTAGAACCGTCCGCATGTTTTTTCGCTTGAAC TG  
AGTCCATTTGATCTGTTGGGTCTGTACAATCACTGTACCTGAGTCCATTCGGAGA ACTACGT  
TATTAAAAGGATAATGAATCGCGCAAAAAAAAAAAAAAAAAACGA

## S.2.11

### >2DL\_protein

MAQAVVPAMQCQVGVGRSAVPARQPAGRVWGVRRRTARATSGFKVLALGPETTGIQRMQQL  
LDMDTTPFTDKIIAEYIWVGGSGIDLRSKSRITISKVEDPSELPKWNYDGSSTGQAPGEDSE  
VILYPQAI FKDPFRGGNNILVICDTYTPQGEPIPTNKRHMAAQIFSDPKVTAQVPWFGIEQE  
YTLMQRDVNWPLGWPVGGYPGPQGPYYCAVGS DKSFRDISDAHYKACLYAGIEISGTNGEV  
MPGQWEYQVGPSVGIDAGDHIWASRYILERITEQAGVVLTLDPKPIQGDWNGAGCHTNYSTL  
SMREDGGFDVIKKAILNLSLRHDLHIAAYGEGNERRLTGLHETASISDFSWGVANRGC SIRV  
GRETEAKGKGYLED RRPASNMDPYTVTALLAETTILWEPTLEAEALAAKKLALKV



**Fig. A3.1** Score distribution data of *TaGS1.1* and *TaGS2* allele sequences from wheat samples.

**Table. A3.1** The complete profile of next generation sequencing reads and cycles involving *TaGSI.1* and *TaGS2* alleles

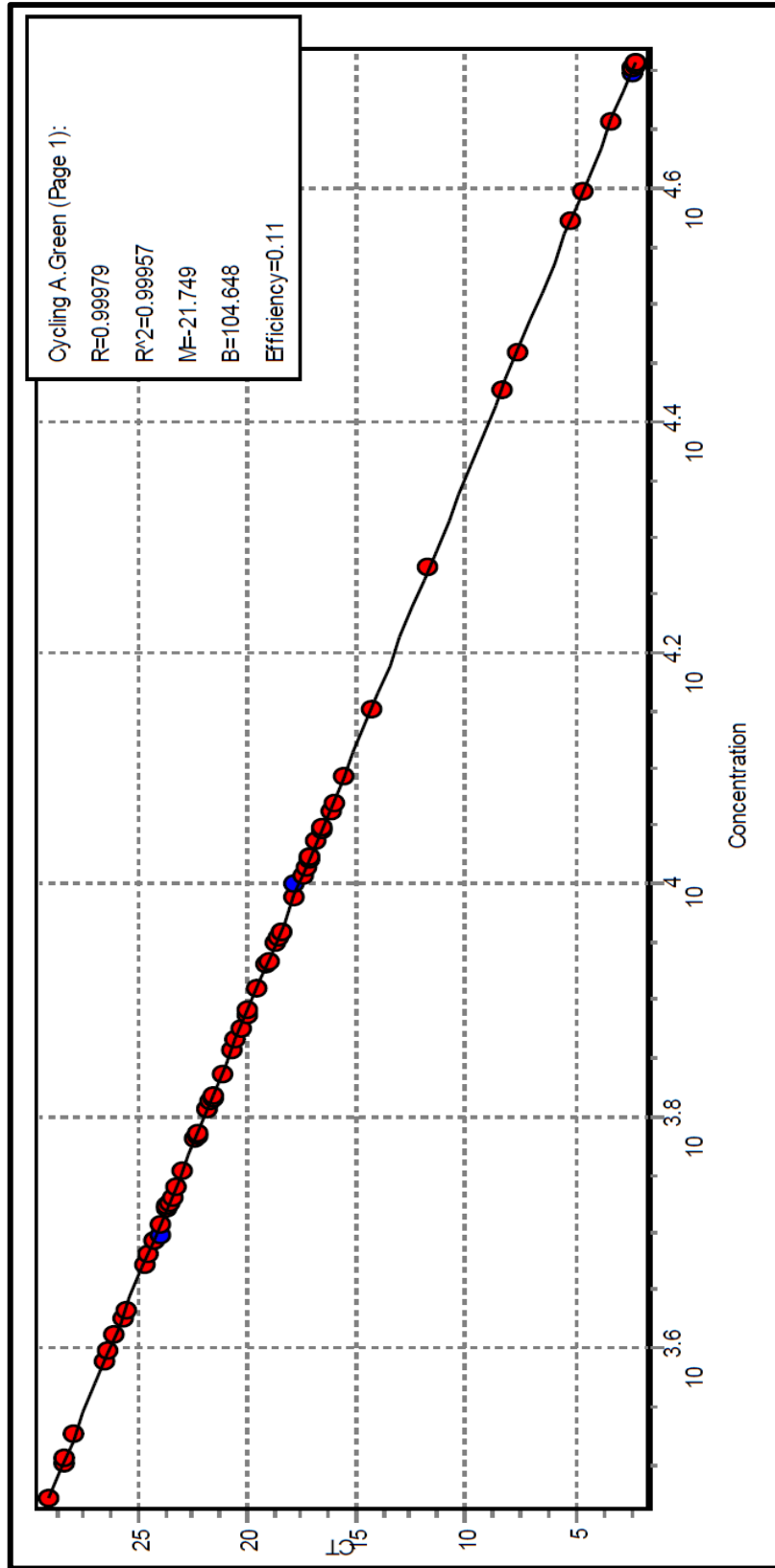
|                       |       | CYCLES     | YIELD                          | PROJECTED YIELD  | ALIGNED (%)      | ERROR RATE (%) | INTENSITY CYCLE 1 | %≥300        |            |                  |             |                |                          |                          |                           |                   |        |
|-----------------------|-------|------------|--------------------------------|------------------|------------------|----------------|-------------------|--------------|------------|------------------|-------------|----------------|--------------------------|--------------------------|---------------------------|-------------------|--------|
| READ 1                |       | 301        | 7.67 Gbp                       | 7.67 Gbp         | 3.28             | 3.17           | 153               | 74.70        |            |                  |             |                |                          |                          |                           |                   |        |
| READ 2 (I)            |       | 8          | 178.92 Mbp                     | 178.92 Mbp       | 0.00             | 0.00           | 394               | 93.94        |            |                  |             |                |                          |                          |                           |                   |        |
| READ 3 (I)            |       | 8          | 178.92 Mbp                     | 178.92 Mbp       | 0.00             | 0.00           | 315               | 92.95        |            |                  |             |                |                          |                          |                           |                   |        |
| READ 4                |       | 301        | 7.67 Gbp                       | 7.67 Gbp         | 3.21             | 4.25           | 140               | 56.45        |            |                  |             |                |                          |                          |                           |                   |        |
| NON-INDEX READS TOTAL |       | 602        | 15.33 Gbp                      | 15.34 Gbp        | 3.25             | 3.71           | 146               | 65.58        |            |                  |             |                |                          |                          |                           |                   |        |
| <b>TOTALS</b>         |       | <b>618</b> | <b>15.69 Gbp</b>               | <b>15.69 Gbp</b> | <b>3.25</b>      | <b>3.71</b>    | <b>250</b>        | <b>66.22</b> |            |                  |             |                |                          |                          |                           |                   |        |
| LANE                  | READ  | TILES      | DENSITY (K / MM <sup>2</sup> ) | CLUSTER PF (%)   | PHAS/PREPHAS (%) | READS          | READS PF          | %≥300        | YIELD      | CYCLES ERR RATED | ALIGNED (%) | ERROR RATE (%) | ERROR RATE 35 CYCLES (%) | ERROR RATE 75 CYCLES (%) | ERROR RATE 100 CYCLES (%) | INTENSITY CYCLE 1 | STATUS |
| 1                     | 1     | 38         | 1,272 ±47                      | 87.95 ±1.48      | 0.159 / 0.038    | 29,058,052     | 25,560,108        | 74.70        | 7.67 Gbp   | 300              | 3.28 ±0.14  | 3.17 ±0.07     | 0.11 ±0.01               | 0.18 ±0.02               | 0.28 ±0.02                | 153 ±21           | --     |
|                       | 2 (I) | 38         | 1,272 ±47                      | 87.95 ±1.48      | 0.000 / 0.000    | 29,058,052     | 25,560,108        | 93.94        | 178.92 Mbp | 0                | 0.00 ±0.00  | 0.00 ±0.00     | 0.00 ±0.00               | 0.00 ±0.00               | 0.00 ±0.00                | 394 ±45           |        |
|                       | 3 (I) | 38         | 1,272 ±47                      | 87.95 ±1.48      | 0.000 / 0.000    | 29,058,052     | 25,560,108        | 92.95        | 178.92 Mbp | 0                | 0.00 ±0.00  | 0.00 ±0.00     | 0.00 ±0.00               | 0.00 ±0.00               | 0.00 ±0.00                | 315 ±34           |        |
|                       | 4     | 38         | 1,272 ±47                      | 87.95 ±1.48      | 0.253 / 0.063    | 29,058,052     | 25,560,108        | 56.45        | 7.67 Gbp   | 300              | 3.21 ±0.12  | 4.25 ±0.24     | 0.19 ±0.02               | 0.31 ±0.04               | 0.39 ±0.04                | 140 ±17           |        |

**Table A3.2** Index profile of GS genes tagged and sequenced in 8 wheat accessions coded as; G: Gladius, D: Drysdale, E: Excalibur, R: RAC875, K: Kukri, C: Chinese spring, M: Mace and S: Scout.

| INDEX NUMBER | ALLELE CODE | GENE          | INDEX 1  | INDEX 2  | % READS IDENTIFIED (PF) |
|--------------|-------------|---------------|----------|----------|-------------------------|
| 1            | 1_GS1.1A_G  | <i>GS1.1A</i> | TAAGGCGA | TAGATCGC | 0.1529                  |
| 2            | 2_GS1.1A_D  | <i>GS1.1A</i> | TAAGGCGA | CTCTCTAT | 0.2495                  |
| 3            | 3_GS1.1A_E  | <i>GS1.1A</i> | TAAGGCGA | TATCCTCT | 0.0777                  |
| 4            | 4_GS1.1A_R  | <i>GS1.1A</i> | TAAGGCGA | AGAGTAGA | 0.6613                  |
| 5            | 5_GS1.1A_K  | <i>GS1.1A</i> | TAAGGCGA | GTAAGGAG | 0.3379                  |
| 6            | 6_GS1.1A_C  | <i>GS1.1A</i> | TAAGGCGA | ACTGCATA | 0.3827                  |
| 7            | 7_GS1.1A_M  | <i>GS1.1A</i> | TAAGGCGA | AAGGAGTA | 3.5186                  |
| 8            | 8_GS1.1A_S  | <i>GS1.1A</i> | TAAGGCGA | CTAAGCCT | 4.6953                  |
| 9            | 9_GS1.1B_G  | <i>GS1.1B</i> | CGTACTAG | TAGATCGC | 0.0025                  |
| 10           | 10_GS1.1B_D | <i>GS1.1B</i> | CGTACTAG | CTCTCTAT | 1.9041                  |
| 11           | 11_GS1.1B_E | <i>GS1.1B</i> | CGTACTAG | TATCCTCT | 0.7432                  |
| 12           | 12_GS1.1B_R | <i>GS1.1B</i> | CGTACTAG | AGAGTAGA | 0.1411                  |
| 13           | 13_GS1.1B_K | <i>GS1.1B</i> | CGTACTAG | GTAAGGAG | 0.0007                  |
| 14           | 14_GS1.1B_C | <i>GS1.1B</i> | CGTACTAG | ACTGCATA | 1.8222                  |
| 15           | 15_GS1.1B_M | <i>GS1.1B</i> | CGTACTAG | AAGGAGTA | 0.0214                  |
| 16           | 16_GS1.1B_S | <i>GS1.1B</i> | CGTACTAG | CTAAGCCT | 5.5252                  |
| 17           | 17_GS1.1D_G | <i>GS1.1D</i> | AGGCAGAA | TAGATCGC | 6.2027                  |
| 18           | 18_GS1.1D_D | <i>GS1.1D</i> | AGGCAGAA | CTCTCTAT | 2.9388                  |
| 19           | 19_GS1.1D_E | <i>GS1.1D</i> | AGGCAGAA | TATCCTCT | 0.6010                  |
| 20           | 20_GS1.1D_R | <i>GS1.1D</i> | AGGCAGAA | AGAGTAGA | 5.5697                  |
| 21           | 21_GS1.1D_K | <i>GS1.1D</i> | AGGCAGAA | GTAAGGAG | 0.8231                  |
| 22           | 22_GS1.1D_C | <i>GS1.1D</i> | AGGCAGAA | ACTGCATA | 0.7071                  |
| 23           | 23_GS1.1D_M | <i>GS1.1D</i> | AGGCAGAA | AAGGAGTA | 5.8994                  |
| 24           | 24_GS1.1D_S | <i>GS1.1D</i> | AGGCAGAA | CTAAGCCT | 6.5856                  |
| 25           | 25_GS2A_G   | <i>GS2A</i>   | TCCTGAGC | TAGATCGC | 0.2473                  |
| 26           | 26_GS2A_D   | <i>GS2A</i>   | TCCTGAGC | CTCTCTAT | 5.0630                  |
| 27           | 27_GS2A_E   | <i>GS2A</i>   | TCCTGAGC | TATCCTCT | 1.9108                  |
| 28           | 28_GS2A_R   | <i>GS2A</i>   | TCCTGAGC | AGAGTAGA | 2.8281                  |
| 29           | 29_GS2A_K   | <i>GS2A</i>   | TCCTGAGC | GTAAGGAG | 3.1456                  |
| 30           | 30_GS2A_C   | <i>GS2A</i>   | TCCTGAGC | ACTGCATA | 2.4464                  |
| 31           | 31_GS2A_M   | <i>GS2A</i>   | TCCTGAGC | AAGGAGTA | 1.6683                  |
| 32           | 32_GS2A_S   | <i>GS2A</i>   | TCCTGAGC | CTAAGCCT | 0.6201                  |
| 33           | 33_GS2B_G   | <i>GS2B</i>   | GGACTCCT | TAGATCGC | 1.1561                  |
| 34           | 34_GS2B_D   | <i>GS2B</i>   | GGACTCCT | CTCTCTAT | 6.5931                  |
| 35           | 35_GS2B_E   | <i>GS2B</i>   | GGACTCCT | TATCCTCT | 0.0905                  |
| 36           | 36_GS2B_R   | <i>GS2B</i>   | GGACTCCT | AGAGTAGA | 0.7018                  |
| 37           | 37_GS2B_K   | <i>GS2B</i>   | GGACTCCT | GTAAGGAG | 3.3915                  |
| 38           | 38_GS2B_C   | <i>GS2B</i>   | GGACTCCT | ACTGCATA | 3.0078                  |
| 39           | 39_GS2B_M   | <i>GS2B</i>   | GGACTCCT | AAGGAGTA | 0.2723                  |
| 40           | 40_GS2D_G   | <i>GS2D</i>   | GGACTCCT | CTAAGCCT | 9.6201                  |
| 41           | 41_GS2D_E   | <i>GS2D</i>   | TAGGCATG | TAGATCGC | 0.1936                  |
| 42           | 42_GS2D_R   | <i>GS2D</i>   | TAGGCATG | CTCTCTAT | 0.2493                  |
| 43           | 43_GS2D_K   | <i>GS2D</i>   | TAGGCATG | TATCCTCT | 0.2024                  |
| 44           | 44_GS2D_C   | <i>GS2D</i>   | TAGGCATG | AGAGTAGA | 0.0919                  |
| 45           | 45_GS2D_S   | <i>GS2D</i>   | TAGGCATG | GTAAGGAG | 1.4184                  |

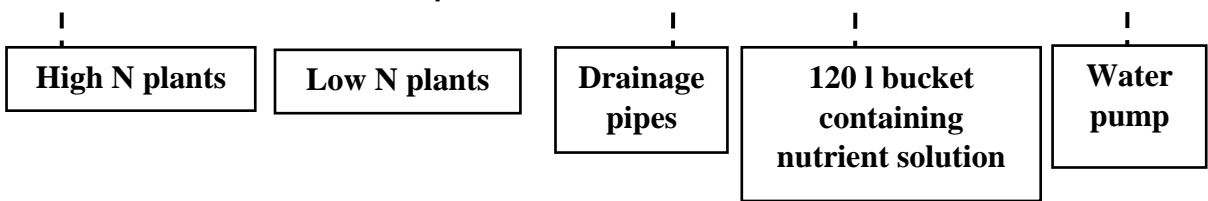
**Table A3.3.** The gene sequences of plant species included in phylogenetic analysis including six reference sequences (6AL\_5748909; 6BL\_227297; 6DL\_249482; 2AL\_6391136; 2BL\_7944286 and 2DL\_9714273).

| <b>Plant species</b>           | <b>Gene</b>   | <b>Contig</b>                   |
|--------------------------------|---------------|---------------------------------|
| <i>Arabidopsis thaliana</i>    | <i>GLN</i>    | gi 240256493:13830429-13833542  |
| <i>Brassica napus</i>          | <i>GLN</i>    | ZS11GLN 919448883:311056-314033 |
| <i>Brachipodium distachyon</i> | <i>GS1.1</i>  | gi 357138209 ref XM_003570642.1 |
| <i>Glycine max</i>             | <i>GS2</i>    | <i>Glycine max GS2</i>          |
| <i>Hordeum vulgare</i>         | <i>GS1.1</i>  | JX878489                        |
| <i>Hordeum vulgare</i>         | <i>GS1.2</i>  | JX878490                        |
| <i>Hordeum vulgare</i>         | <i>GS1.3</i>  | JX878491                        |
| <i>Hordeum vulgare</i>         | <i>GS2</i>    | Modified Barley GS2             |
| <i>Oryza sativa</i>            | <i>GS1.1</i>  | Modified Rice GS 1.1            |
| <i>Oryza sativa</i>            | <i>GS1.1</i>  | Rice GS 1.1 AB037664            |
| <i>Oryza sativa</i>            | <i>GS2</i>    | Rice GS2B                       |
| <i>S. bicolor bicolor</i>      | <i>GS1.1</i>  | gi 194326197 emb FM196538.1     |
| <i>Solanum tuberosum</i>       | <i>GLN</i>    | gi 564112171:561508-566660      |
| <i>S. bicolor bicolor</i>      | <i>GS1.1</i>  | Modified S. bicolor GS 1.1      |
| <i>S. bicolor bicolor</i>      | <i>GS2</i>    | Modified S. bicolor GS 1.1      |
| <i>Zea mays</i>                | <i>GLN1.4</i> | Mo17 GLN 1.4 EU369651           |
| <i>Zea mays</i>                | <i>GS1.2</i>  | AF359511                        |
| <i>Zea mays</i>                | <i>GLN</i>    | 1-3-4 HM371365                  |
| <i>Zea mays</i>                | <i>GLN</i>    | 1-3-3 HM371364                  |
| <i>Zea mays</i>                | <i>GLN</i>    | 1-3-2 HM371363                  |
| <i>Zea mays</i>                | <i>GLN</i>    | 1-3-1 HM371362                  |
| <i>Zea mays</i>                | <i>GS2</i>    | B73 GS2                         |
| <b>Reference Contigs</b>       |               |                                 |
| <i>Hordeum vulgare</i>         | <i>GS1.1A</i> | 6AL_5748909                     |
| <i>Hordeum vulgare</i>         | <i>GS1.1B</i> | 6BL_227297                      |
| <i>Hordeum vulgare</i>         | <i>GS1.1D</i> | 6DL_249482                      |
| <i>Hordeum vulgare</i>         | <i>GS2A</i>   | 2AL_6391136                     |
| <i>Hordeum vulgare</i>         | <i>GS2B</i>   | 2BL_7944286                     |
| <i>Hordeum vulgare</i>         | <i>GS2D</i>   | 2DL_9714273                     |

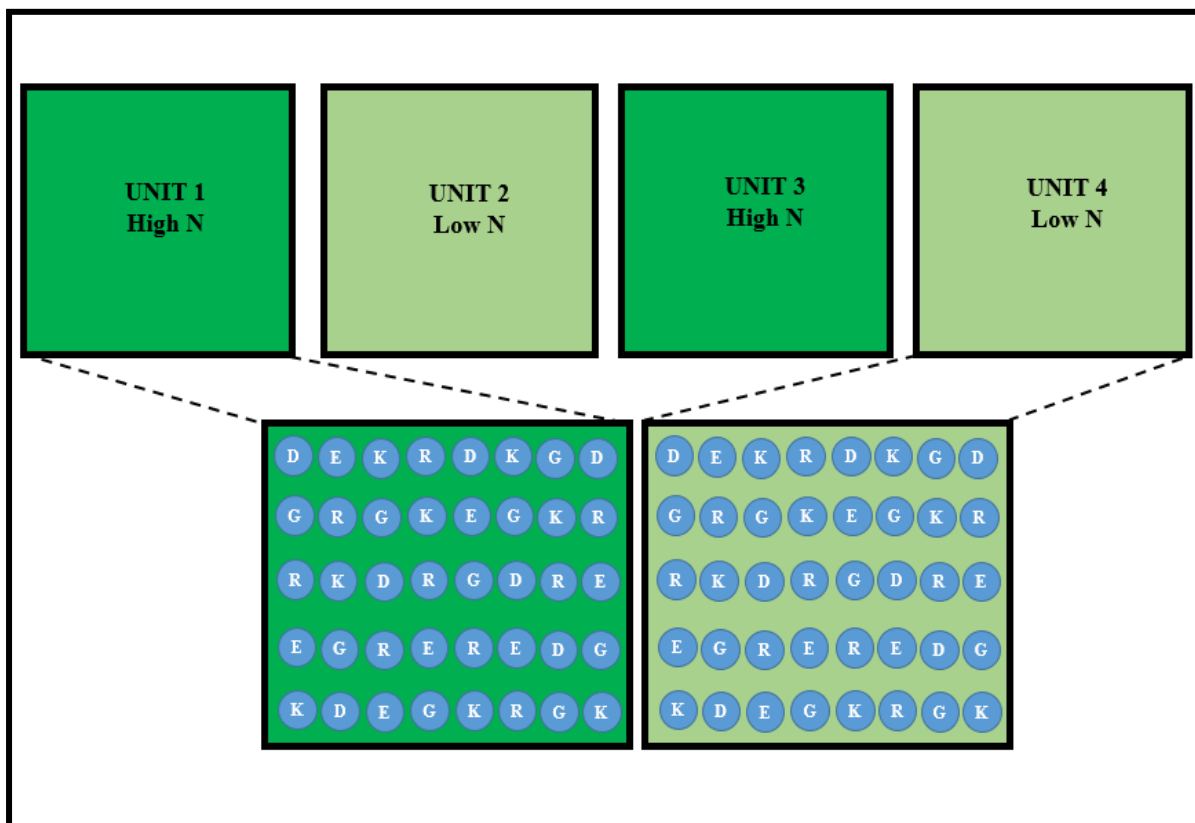


**Fig. A4.1** Standard curve to normalize quantitative PCR template cDNA concentrations of selected wheat samples.





**Fig. A5.1** Hydroponic set-up in controlled environment. Growth chamber used in phenotyping wheat accessions under low and high nitrogen treatment in a controlled environment room. Clear phenotypic differences are observable between low and high treatments. Tissue extracts were used in GS activity assays and transcript abundance analysis.



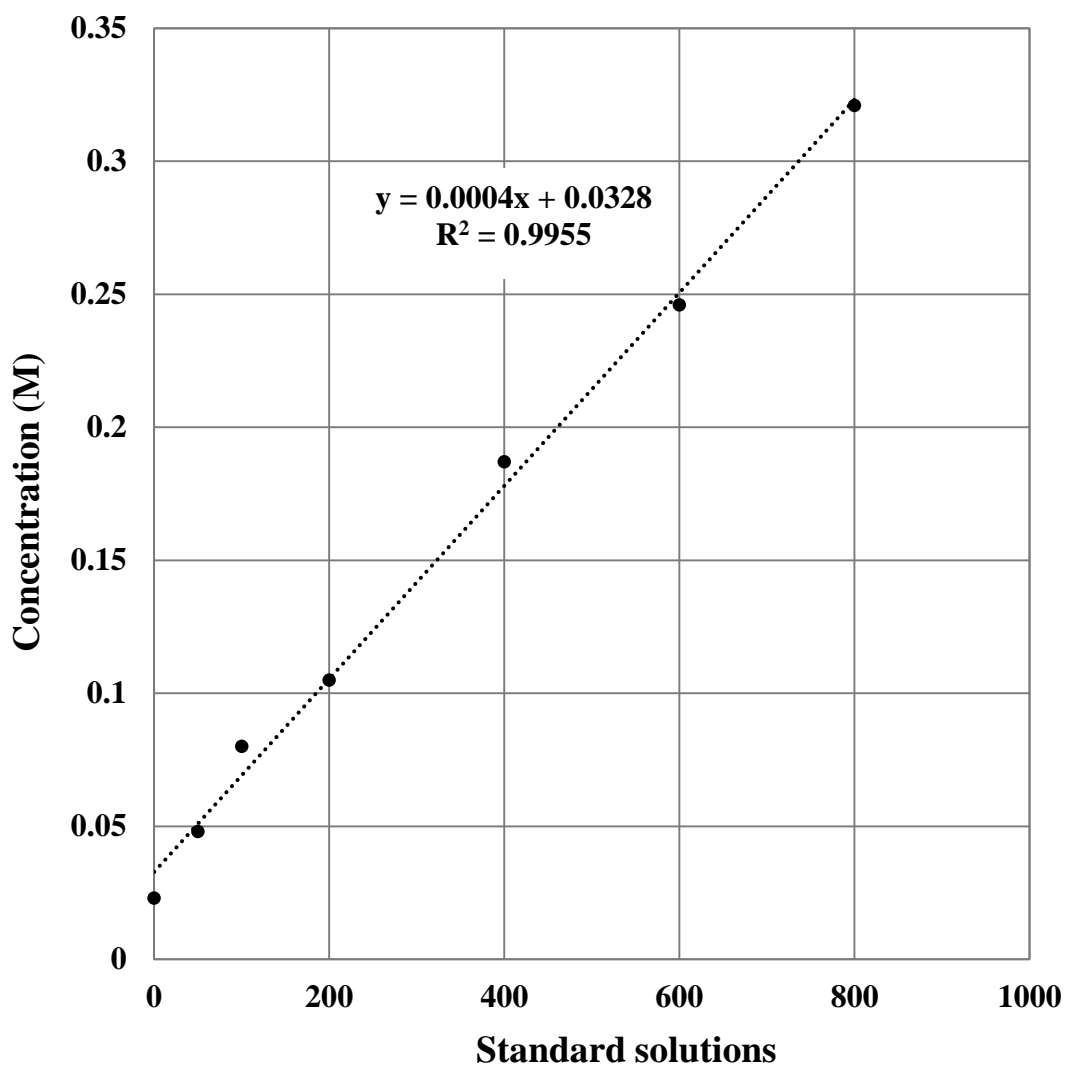
**Fig. A5.2** Randomised design of *Experiment 2 and 3*, utilised in analysing GS activity in five spring wheat accessions under low and high N treatment in four hydroponic units. Key terms: Gladius - G; Drysdale - D; Excalibur - E; RAC875 - R; Kukri - K. Blue circles represent the arrangement of pots within each hydroponic unit.

**Table A5.1** Composition of nutrient solution and mineral constituents of hydroponic solution. The final concentration was  $\text{NO}_3^-$  (4.55 mM) Final  $\text{NH}_4^+$  0.45 mM) in the high N and  $\text{NO}_3^-$  (0.455 mM) and  $\text{NH}_4^+$  (0.045 mM) in the low N treatment. For the high N treatment the final volume of the constituents was adjusted as follows:  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (198.0 ml)  $\text{KNO}_3$  (150.0 ml),  $(\text{NH}_4)_2\text{SO}_4$  (27.0 ml). No amount of  $\text{K}_2\text{SO}_4$  and  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  was added.

| Chemical  | MW       | Stock [M] | g / L stock | mls stock/ 1.0 L growth soln. | Soln. Vol.(L) | mls stock to add | Final Soln. [mM] |  |                    |                           |
|---|----------|-----------|-------------|-------------------------------|---------------|------------------|------------------|--|--------------------|---------------------------|
| <b>Stock Solutions</b>  |          |           |             |                               |               |                  |                  |  |                    |                           |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$                               | 246.4700 | 1.0000    | 246.4700    | 0.50                          | 120           | 60.000           | 0.5000           |  |                    |                           |
| $\text{KH}_2\text{PO}_4$  | 136.0900 | 1.0000    | 136.0900    | 0.50                          | 120           | 60.000           | 0.5000           |  |                    |                           |
| <b>Trace Elements</b>   |          |           |             |                               |               |                  |                  |  |                    |                           |
| $\text{H}_3\text{BO}_3$   | 61.8300  | 0.0250    | 1.5458      |                               |               |                  | 0.0250           |  | N as $\text{NO}_3$ | Final total mM            |
| $\text{MnSO}_4 \cdot \text{H}_2\text{O}$                                | 151.0000 | 0.0020    | 0.3020      |                               |               |                  | 0.0020           |  | P                  | Final total $\mu\text{M}$ |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$                               | 287.5400 | 0.0020    | 0.5751      | 1.00                          | 120           | 120.000          | 0.0020           |  | K                  |                           |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$                               | 249.6800 | 0.0005    | 0.1248      |                               |               |                  | 0.0005           |  | Mg                 |                           |
| $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$                     | 241.9500 | 0.0005    | 0.1210      |                               |               |                  | 0.0005           |  | Cl                 |                           |
| KCl   | 74.5500  | 0.0500    | 3.7275      |                               |               |                  | 0.0500           |  | S                  |                           |
| <b>Fe-Stock</b>   |          |           |             |                               |               |                  |                  |  |                    |                           |
| Fe-EDTA   | 367.1000 | 0.1000    | 36.7100     | 1.00                          | 120           | 120.000          | 0.1000           |  | Mn                 |                           |
| FeEDDHA   |          | 0.1000    | 43.4800     | 0.00                          | 120           | 0.000            | 0.0000           |  | Zn                 |                           |
| <b>N Stocks</b>   |          |           |             |                               |               |                  |                  |  |                    |                           |
| $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$                    | 236.1500 | 1.0000    | 236.1500    | 0.17                          | 120           | 19.800           | 0.1650           |  | B                  |                           |
| $\text{KNO}_3$  | 101.1000 | 1.0000    | 101.1000    | 0.13                          | 120           | 15.000           | 0.1250           |  | Cu                 |                           |
| $\text{NH}_4\text{NO}_3$  | 80.04    | 1.0000    | 80.0400     | 0.00                          | 120           | 0.000            | 0.0000           |  | Mo                 |                           |
| $(\text{NH}_4)_2\text{SO}_4$  | 132.1400 | 1.0000    | 132.1400    | 0.02                          | 120           | 2.700            | 0.0225           |  | Fe                 |                           |
|   |          |           |             |                               |               |                  |                  |  | Ca                 |                           |
|   |          |           |             |                               |               |                  |                  |  | N as $\text{NH}_4$ |                           |
| <b>No Nitrogen or <math>\text{NO}_3</math> (K &amp; Ca supplements)</b> |          |           |             |                               |               |                  |                  |  |                    |                           |
| $\text{K}_2\text{SO}_4$   | 174.2500 | 0.5000    | 87.1250     | 1.00                          | 120           | 120.000          | 0.5000           |  |                    |                           |
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$                               | 147.0100 | 1.0000    | 147.0100    | 0.50                          | 120           | 60.000           | 0.5000           |  |                    |                           |

**Table A5.2** Composition of nutrient solution and mineral constituents of hydroponic solution. The final concentration was  $\text{NO}_3^-$  (4.55 mM Final  $\text{NH}_4^+$  0.45 mM) in the high N and  $\text{NO}_3^-$  (4.55 mM) and  $\text{NH}_4^+$  (0.45 mM) in the high N treatment. For the high N treatment the final volume of the constituents was adjusted as follows:  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (198.0 ml)  $\text{KNO}_3$  (150.0 ml),  $(\text{NH}_4)_2\text{SO}_4$  (27.0 ml). No amount of  $\text{K}_2\text{SO}_4$  and  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  was added.

| Chemical  | MW       | Stock [M] | g / L stock | mls stock/ 1.0 L growth soln. | Soln. Vol. (L) | mls stock to add | Final Soln. [mM] | Final                |                           |
|---|----------|-----------|-------------|-------------------------------|----------------|------------------|------------------|----------------------|---------------------------|
|   |          |           |             |                               |                |                  |                  | total mM             | Final total $\mu\text{M}$ |
| <b>Stock Solutions</b>  |          |           |             |                               |                |                  |                  |                      |                           |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$                                 | 246.4700 | 1.0000    | 246.4700    | 0.50                          | 25             | 12.500           | 0.5000           |                      |                           |
| $\text{KH}_2\text{PO}_4$  | 136.0900 | 1.0000    | 136.0900    | 0.50                          | 25             | 12.500           | 0.5000           |                      |                           |
| <b>Trace Elements</b>   |          |           |             |                               |                |                  |                  |                      |                           |
| $\text{H}_3\text{BO}_3$   | 61.8300  | 0.0250    | 1.5458      |                               |                |                  | 0.0250           | N as $\text{NO}_3^-$ | 4550.0                    |
| $\text{MnSO}_4 \cdot \text{H}_2\text{O}$                                  | 151.0000 | 0.0020    | 0.3020      |                               |                |                  | 0.0020           | P                    | 500.0                     |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$                                 | 287.5400 | 0.0020    | 0.5751      | 1.00                          | 25             | 25.000           | 0.0020           | K                    | 1800.0                    |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$                                 | 249.6800 | 0.0005    | 0.1248      |                               |                |                  | 0.0005           | Mg                   | 500.0                     |
| $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$                       | 241.9500 | 0.0005    | 0.1210      |                               |                |                  | 0.0005           | Cl                   | 50.0                      |
| KCl   | 74.5500  | 0.0500    | 3.7275      |                               |                |                  | 0.0500           | S                    | 729.5                     |
| <b>Fe-Stock</b>   |          |           |             |                               |                |                  |                  |                      |                           |
| Fe-EDTA   | 367.1000 | 0.1000    | 36.7100     |                               |                |                  |                  | Mn                   | 0.002                     |
| FeEDDHA   |          | 0.1000    | 43.4800     |                               |                |                  |                  | Zn                   | 0.002                     |
| <b>N Stocks</b>   |          |           |             |                               |                |                  |                  |                      |                           |
| $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$                      | 236.1500 | 1.0000    | 236.1500    | 1.65                          | 25             | 41.250           | 1.6500           | B                    | 0.025                     |
| $\text{KNO}_3$  | 101.1000 | 1.0000    | 101.1000    | 1.25                          | 25             | 31.250           | 1.2500           | Cu                   | 0.001                     |
| $\text{NH}_4\text{NO}_3$  | 80.04    | 1.0000    | 80.0400     | 0.00                          | 25             | 0.000            | 0.0000           | Mo                   | 0.001                     |
| $(\text{NH}_4)_2\text{SO}_4$  | 132.1400 | 1.0000    | 132.1400    | 0.23                          | 25             | 5.625            | 0.2250           | Fe                   | 0.100                     |
| <b>No Nitrogen or <math>\text{NO}_3^-</math> (K &amp; Ca supplements)</b> |          |           |             |                               |                |                  |                  |                      |                           |
| $\text{K}_2\text{SO}_4$   | 174.2500 | 0.5000    | 87.1250     | 0.00                          | 25             | 0.000            | 0.0000           | Ca                   | 1.650                     |
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$                                 | 147.0100 | 1.0000    | 147.0100    | 0.00                          | 25             | 0.000            | 0.0000           | N as $\text{NH}_4$   | 0.450                     |



**Fig. A5.3** Linear regression plots of different concentrations of seven standard solutions used in standardizing absorbance data from 4-glutamylhydroxamate (L-Glutamic acid  $\gamma$ -monohydroxamate).

**Table A4.3**  $\gamma$ -glutamylhydroxamate standards used in GS assay.

|  | <b>St 1</b> | <b>St 2</b>  | <b>St 3</b>    | <b>St 4</b> | <b>St 5</b> | <b>St 6</b> | <b>St 7</b> |
|--|-------------|--------------|----------------|-------------|-------------|-------------|-------------|
| Standard concentration in 200 $\mu$ L well (nmol/well) | 0           | 50<br>(53.3) | 100<br>(106.7) | 200         | 400         | 600         | 800         |
| Standard concentration in 300 $\mu$ L well (nmol/well) | 0           | 80           | 160            | 300         | 600         | 900         | 1200        |
| 100nmol/ $\mu$ l stock ( $\mu$ L)                      | 0           | 0.80         | 1.6            | 3           | 6           | 9           | 12          |
| Extraction buffer ( $\mu$ L)                           | 50          | 49.2         | 48.4           | 47          | 44          | 41          | 38          |
| Mix 1 (ATP positive ( $\mu$ L)                         | 100         | 100          | 100            | 100         | 100         | 100         | 100         |
| Stop-solution ( $\mu$ L)                               | 150         | 150          | 150            | 150         | 150         | 150         | 150         |

## LIST OF REFERENCES

- Agrama, H, Zakaria, A, Said, F & Tuinstra, M 1999**, 'Identification of quantitative trait loci for nitrogen use efficiency in maize', *Molecular Breeding*, vol. 5, no. 2, pp. 187-195.
- Ahn, S, Anderson, J, Sorrells, M & Tanksley, S 1993**, 'Homoeologous relationships of rice, wheat and maize chromosomes', *Molecular and General Genetics MGG*, vol. 241, no. 5-6, pp. 483-490.
- An, D, Su, J, Liu, Q, Zhu, Y, Tong, Y, Li, J, Jing, R, Li, B & Li, Z 2006**, 'Mapping QTLs for nitrogen uptake in relation to the early growth of wheat (*Triticum aestivum* L.)', *Plant and Soil*, vol. 284, no. 1-2, pp. 73-84.
- Anbessa, Y, Juskiw, P, Good, A, Nyachiro, J & Helm, J 2009**, 'Genetic variability in nitrogen use efficiency of spring barley', *Crop Science*, vol. 49, no. 4, pp. 1259-1269.
- Andrews, M, Lea, PJ, Raven, J & 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*, vol. 145, no. 1, pp. 25-40.
- Araus, V, Vidal, EA, Puelma, T, Alamos, S, Mieulet, D, Guiderdoni, E & Gutiérrez, RA 2016**, 'Members of BTB gene family regulate negatively nitrate uptake and nitrogen use efficiency in *Arabidopsis thaliana* and *Oryza sativa*', *Plant Physiology*, p. pp. 01731.02015.

**Asplund, L, Bergkvist, G & Weih, M 2016**, 'Functional traits associated with nitrogen use efficiency in wheat', *Acta Agriculturae Scandinavica, Section B-Soil & Plant Science*, vol. 66, no. 2, pp. 153-169.

**Avice, J-C & Etienne, P 2014**, 'Leaf senescence and nitrogen remobilization efficiency in oilseed rape (*Brassica napus* L.)', *Journal of Experimental Botany*, vol. 65, no. 14, pp. 3813-3824.

**Avila-Ospina, L, Marmagne, A, Talbotec, J, Krupinska, K & Masclaux-Daubresse, C 2015**, 'The identification of new cytosolic glutamine synthetase and asparagine synthetase genes in barley (*Hordeum vulgare* L.), and their expression during leaf senescence', *Journal of Experimental Botany*, p. 3.

**Avila-Ospina, L, Moison, M, Yoshimoto, K & Masclaux-Daubresse, C 2014**, 'Autophagy, plant senescence, and nutrient recycling', *Journal of Experimental Botany*, p. 39.

**Bailey, KJ & Leegood, RC 2016**, 'Nitrogen recycling from the xylem in rice leaves: dependence upon metabolism and associated changes in xylem hydraulics', *Journal of Experimental Botany*, p. 132.

**Baker, A, Pearson, T, Price, EP, Dale, J, Keim, P, Hornstra, H, Greenhill, A, Padilla, G & Warner, J 2011**, 'Molecular phylogeny of *Burkholderia pseudomallei* from a remote region of Papua New Guinea', *PLOS One*, vol. 6, no. 3, p. 18343.



**Balotf, S, Kavooosi, G & Kholdebarin, B 2015**, 'Nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase expression and activity in response to different nitrogen sources in nitrogen-starved wheat seedlings', *Biotechnology and Applied Biochemistry*, p. 3.

**Bao, A, Zhao, Z, Ding, G, Shi, L, Xu, F & Cai, H 2014**, 'Accumulated expression level of cytosolic glutamine synthetase 1 gene (OsGS1; 1 or OsGS1; 2) alter plant development and the carbon-nitrogen metabolic status in rice', *PloS One*, vol. 9, no. 4, p. 95581.

**Barutçular, C, Yıldırım, M, Koç, M, Akıncı, C, Toptaş, I, Albayrak, O, Tanrikulu, A & El Sabagh, A 2016**, 'Evaluation of SPAD chlorophyll in spring wheat accessions under different environments', *Fresenius Environmental Bulletin*, vol. 25, no. 4, pp. 1258-1266.

**Basuchaudhuri, P 2016**, *Nitrogen Metabolism in Rice*, CRC Press.

**Beatty, PH, Shrawat, AK, Carroll, RT, Zhu, T & Good, AG 2009**, 'Transcriptome analysis of nitrogen-efficient rice over-expressing alanine aminotransferase', *Plant Biotechnology Journal*, vol. 7, no. 6, pp. 562-576.

**Bennett, JR & Mathews, S 2006**, 'Phylogeny of the parasitic plant family Orobanchaceae inferred from phytochrome A', *American Journal of Botany*, vol. 93, no. 7, pp. 1039-1051.

**Berkman, PJ, Lai, K, Lorenc, MT & Edwards, D 2012**, 'Next-generation sequencing applications for wheat crop improvement', *American Journal of Botany*, vol. 99, no. 2, pp. 365-371.

**Bernard, SM & Habash, DZ 2009**, 'The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling', *New Phytologist*, vol. 182, no. 3, pp. 608-620.

**Bernard, SM, Møller, ALB, Dionisio, G, Kichey, T, Jahn, TP, Dubois, F, Baudo, M, Lopes, MS, Tercé-Laforgue, T & Foyer, CH 2008**, 'Gene expression, cellular localisation and function of glutamine synthetase isozymes in wheat (*Triticum aestivum* L.)', *Plant Molecular Biology*, vol. 67, no. 1-2, pp. 89-105.

**Bernard, SM, Møller, ALB, Dionisio, G, Kichey, T, Jahn, TP, Dubois, F, Bernhard, WR & Matile, P 1994**, 'Differential expression of glutamine synthetase genes during the senescence of *Arabidopsis thaliana* rosette leaves', *Plant Science*, vol. 98, no. 1, pp. 7-14.

**Bertin, P & Gallais, A 2000**, 'Genetic variation for nitrogen use efficiency in a set of recombinant maize inbred lines. I. Agrophysiological Results', *Maydica*, vol. 45, no. 1, pp. 53-66.

**Bertin, P & Gallais, A 2001**, 'Physiological and genetic basis of nitrogen use efficiency in maize. II. QTL detection and coincidences', *Maydica*, vol. 46, pp. 53-68.

**Betti, M, García-Calderón, M, Pérez-Delgado, CM, Credali, A, Pal'ove-Balang, P, Estivill, G, Repčák, M, Vega, JM, Galván, F & Márquez, AJ 2014**, 'Reassimilation of ammonium in *Lotus japonicus*', *Journal of Experimental Botany*, vol. 65, no. 19, pp. 5557-5566.

**Bishop, PE, Guevara, JG, Engelke, JA & Evans, HJ 1976**, 'Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*', *Plant Physiology*, vol. 57, no. 4, pp. 542-546.

**Bonneau, J, Taylor, JD, Parent, B, Reynolds, M, Feuillet, C, Langridge, P, Mather, D, 2013**, 'Multi-environment analysis and fine mapping of a yield related QTL on chromosome 3B of wheat', *Theoretical and Applied Genetics*, vol. 126, pp.747-761.

**Bortiri, E, Oh, S-H, Gao, F-Y & Potter, D 2002**, 'The phylogenetic utility of nucleotide sequences of sorbitol 6-phosphate dehydrogenase in *Prunus* (Rosaceae)', *American Journal of Botany*, vol. 89, no. 10, pp. 1697-1708.

**Bouwman, L, Goldewijk, KK, Van Der Hoek, KW, Beusen, AH, Van Vuuren, DP, Willems, J, Rufino, MC & Stehfest, E 2013**, 'Exploring global changes in nitrogen and phosphorus cycles in agriculture induced by livestock production over the 1900-2050 period', *Proceedings of the National Academy of Sciences*, vol. 110, no. 52, pp. 20882-20887.

**Brauer, EK & Shelp, BJ 2010**, 'Nitrogen use efficiency: re-consideration of the bioengineering approach', *Botany*, vol. 88, no. 2, pp. 103-109.

**Brugière, N, Dubois, F, Limami, AM, Lelandais, M, Roux, Y, Sangwan, RS & Hirel, B 1999**, 'Glutamine synthetase in the phloem plays a major role in controlling proline production', *The Plant Cell*, vol. 11, no. 10, pp. 1995-2011.

**Büchi, L, Charles, R, Schneider, D, Sinaj, S, Maltas, A, Fossati, D & Mascher, F 2016**, 'Performance of eleven winter wheat varieties in a long term experiment on mineral nitrogen and organic fertilisation', *Field Crops Research*, vol. 191, pp. 111-122.

**Buggs, RJ, Soltis, PS, Mavrodiev, EV, Symonds, VV & Soltis, DE 2008**, 'Does phylogenetic distance between parental genomes govern the success of polyploids', *Castanea*, vol. 73, no. 2, pp. 74-93.

**Burge, C, Karlin S 1997**, 'Prediction of complete gene structures in Human genomic DNA', *Journal of Molecular Biology*. p. 268: 78-94

**Butterly, CR, Armstrong, R, Chen, D & Tang, C 2015**, 'Carbon and nitrogen partitioning of wheat and field pea grown with two nitrogen levels under elevated CO<sub>2</sub>', *Plant and Soil*, vol. 391, no. 1-2, pp. 367-382.

**Cai, H, Zhou, Y, Xiao, J, Li, X, Zhang, Q & Lian, X 2009**, 'Overexpressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice', *Plant Cell Reports*, vol. 28, no. 3, pp. 527-537.

**Campbell, P & Braam, J 1999**, 'Xyloglucan endotransglycosylases: diversity of genes, enzymes and potential wall-modifying functions', *Trends in Plant Science*, vol. 4, no. 9, pp. 361-366.

**Cañas, RA, Quilleré, I, Lea, PJ & Hirel, B 2010**, 'Analysis of amino acid metabolism in the ear of maize mutants deficient in two cytosolic glutamine synthetase isoenzymes highlights the importance of asparagine for nitrogen translocation within sink organs', *Plant Biotechnology Journal*, vol. 8, no. 9, pp. 966-978.

**Cao, W, Scoles, G, Hucl, P & Chibbar, R 2000**, 'Phylogenetic relationships of five morphological groups of hexaploid wheat (*Triticum aestivum* L.) based on RAPD analysis', *Genome*, vol. 43, no. 4, pp. 724-727.

**Carvalho, H, Lescure, N, de Billy, F, Chabaud, M, Lima, L, Salema, R & Cullimore, J 2000**, 'Cellular expression and regulation of the *Medicago truncatula* cytosolic glutamine synthetase genes in root nodules', *Plant Molecular Biology*, vol. 42, no. 5, pp. 741-756.

**Castaigns, L, Marchive, C, Meyer, C & Krapp, A 2011**, 'Nitrogen signaling in *Arabidopsis*: how to obtain insights into a complex signaling network', *Journal of Experimental Botany*, vol. 62, no. 4, pp. 1391-1397.

**Castro-Rodríguez, V, García-Gutiérrez, A, Canales, J, Cañas, RA, Kirby, EG, Avila, C & Canovas, FM 2016**, 'Poplar trees for phytoremediation of high levels of nitrate and applications in bioenergy', *Plant Biotechnology Journal*, vol. 14, no. 1, pp. 299-312.

**Causse, M, Rocher, J, Henry, A, Charcosset, A, Prioul, J & De Vienne, D 1995**, 'Genetic dissection of the relationship between carbon metabolism and early growth in maize, with emphasis on key-enzyme loci', *Molecular Breeding*, vol. 1, no. 3, pp. 259-272.

**Cavanagh, CR, Chao, S, Wang, S, Huang, BE, Stephen, S, Kiani, S, Forrest, K, Saintenac, C, Brown-Guedira, GL & Akhunova, A 2013**, 'Genome-wide comparative diversity uncovers multiple targets of selection for improvement in hexaploid wheat landraces and cultivars', *Proceedings of the National Academy of Sciences*, vol. 110, no. 20, pp. 8057-8062.

**Chao, J, Zhang, S, Chen, Y & Tian, W-M 2016**, 'Isolation and characterization of a plastidic glutamine synthetase (GS) gene in laticifers of *Hevea brasiliensis* Muell. Arg', *Acta Physiologiae Plantarum*, vol. 38, no. 4, pp. 1-11.

**Chapman, JA, Mascher, M, Buluç, A, Barry, K, Georganas, E, Session, A, Strnadova, V, Jenkins, J, Sehgal, S & Olliker, L 2015**, 'A whole-genome shotgun approach for assembling and anchoring the hexaploid bread wheat genome', *Genome Biology*, vol. 16, no. 1, p. 1.

**Chatterjee, A, Cattanach, N, Awale, R & Thapa, R 2016**, 'Can We Reduce Rainfed Maize (*Zea mays* L.) Nitrogenous Fertilizer Application Rate with Addition of Nitrpyrin?', *Communications in Soil Science and Plant Analysis*, no. just-accepted.

**Chen, G, Chen, Y, Zhao, G, Cheng, W, Guo, S, Zhang, H & Shi, W 2015**, 'Do high nitrogen use efficiency rice cultivars reduce nitrogen losses from paddy fields?', *Agriculture, Ecosystems & Environment*, vol. 209, pp. 26-33.

**Chen, G, Guo, S, Kronzucker, HJ & Shi, W 2013**, 'Nitrogen use efficiency (NUE) in rice links to NH<sub>4</sub><sup>+</sup> toxicity and futile NH<sub>4</sub><sup>+</sup> cycling in roots', *Plant and Soil*, vol. 369, no. 1-2, pp. 351-363.

**Chen, X, Cho, Y & McCouch, S 2002**, 'Sequence divergence of rice microsatellites in *Oryza* and other plant species', *Molecular Genetics and Genomics*, vol. 268, no. 3, pp. 331-343.

**Chen, X, Cui, Z, Fan, M, Vitousek, P, Zhao, M, Ma, W, Wang, Z, Zhang, W, Yan, X & Yang, J 2014**, 'Producing more grain with lower environmental costs', *Nature*, vol. 514, no. 7523, pp. 486-489.

**Chester, N & Marshak, DR 1993**, 'Dimethyl sulfoxide-mediated primer T m reduction: a method for analyzing the role of renaturation temperature in the polymerase chain reaction', *Analytical Biochemistry*, vol. 209, no. 2, pp. 284-290.

**Ching, A, Caldwell, KS, Jung, M, Dolan, M, Smith, O, Tingey, S, Morgante, M & Rafalski, AJ 2002**, 'SNP frequency, haplotype structure and linkage disequilibrium in elite maize inbred lines', *BMC Genetics*, vol. 3, no. 1, p. 1.

**Coque, M & Gallais, A 2006**, 'Genomic regions involved in response to grain yield selection at high and low nitrogen fertilization in maize', *Theoretical and Applied Genetics*, vol. 112, no. 7, pp. 1205-1220.

**Cormier, F, Foulkes, J, Hirel, B, Gouache, D, Moënne-Loccoz, Y & Le Gouis, J 2016**, 'Breeding for increased nitrogen-use efficiency: a review for wheat (*Triticum aestivum* L.)', *Plant Breeding*, vol. 135, no. 3, pp. 255-278.

**Coruzzi, GM 2003**, 'Primary N-assimilation into amino acids in Arabidopsis', *The Arabidopsis Book*, p. 0010.

**Coschigano, KT, Melo-Oliveira, R, Lim, J & Coruzzi, GM 1998**, 'Arabidopsis gls mutants and distinct Fd-GOGAT genes: implications for photorespiration and primary nitrogen assimilation', *The Plant Cell*, vol. 10, no. 5, pp. 741-752.

**Crossa, J & Franco, J 2004**, 'Statistical methods for classifying genotypes', *Euphytica*, vol. 137, no. 1, pp. 19-37.

**Cullimore, J, Gebhardt, C, Saarelainen, R, Miflin, B, Idler, K & Barker, R 1983**, 'Glutamine synthetase of *Phaseolus vulgaris* L.: organ-specific expression of a multigene family', *Journal of Molecular and Applied Genetics*, vol. 2, no. 6, pp. 589-599.

**Czechowski, T, Bari, RP, Stitt, M, Scheible, WR & Udvardi, MK 2004**, 'Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root-and shoot-specific genes', *The Plant Journal*, vol. 38, no. 2, pp. 366-379.

**Dawson, JC, Huggins, DR & Jones, SS 2008**, 'Characterizing nitrogen use efficiency in natural and agricultural ecosystems to improve the performance of cereal crops in low-input and organic agricultural systems', *Field Crops Research*, vol. 107, no. 2, pp. 89-101.



**de la Vega, AJ, Hall, AJ & Kroonenberg, PM 2002**, 'Investigating the physiological bases of predictable and unpredictable genotype by environment interactions using three-mode pattern analysis', *Field Crops Research*, vol. 78, no. 2, pp. 165-183.

**de Oliveira Neto, CF, de Oliveira, TB, Silva, KRS, dos Santos Nogueira, GA, Maltarolo, BM, Costa, TC, Cardoso, KPS, de Souza, LC, da Silva, RTL & Siqueira, JAM 2016**, 'Nitrogen compounds and enzyme activity in young muruçi (*Byrsonima crassifolia* L.) plant subjected to water stress', *Australian Journal of Crop Science*, vol. 10, no. 1, p. 111.

**Dell'Acqua, M, Gatti, DM, Pea, G, Cattonaro, F, Coppens, F, Magris, G, Hlaing, AL, Aung, HH, Nelissen, H & Baute, J 2015**, 'Genetic properties of the MAGIC maize population: a new platform for high definition QTL mapping in *Zea mays*', *Genome Biology*, vol. 16, no. 1, p. 1.

**Deulvot, C, Charrel, H, Marty, A, Jacquin, F, Donnadieu, C, Lejeune-Hénaut, I, Burstin, J & Aubert, G 2010**, 'Highly-multiplexed SNP genotyping for genetic mapping and germplasm diversity studies in pea', *BMC Genomics*, vol. 11, no. 1, p. 1.

**Döring, F, Streubel, M, Bräutigam, A & Gowik, U 2016**, 'Most photorespiratory genes are preferentially expressed in the bundle sheath cells of the C4 grass *Sorghum bicolor*', *Journal of Experimental Botany*, p. erw041.

**Dragičević, M, Todorović, S, Bogdanović, M, Filipović, B, Mišić, D & Simonović, A 2014**, 'Knockout mutants as a tool to identify the subunit composition of Arabidopsis glutamine synthetase isoforms', *Plant Physiology and Biochemistry*, vol. 79, pp. 1-9.

**Dubois, F, Brugière, N, Sangwan, RS & Hirel, B 1996**, 'Localization of tobacco cytosolic glutamine synthetase enzymes and the corresponding transcripts shows organ-and cell-specific patterns of protein synthesis and gene expression', *Plant Molecular Biology*, vol. 31, no. 4, pp. 803-817.

**Edgerton, MD 2009**, 'Increasing crop productivity to meet global needs for feed, food, and fuel', *Plant Physiology*, vol. 149, no. 1, pp. 7-13.

**Edwards, JW, Walker, EL & Coruzzi, GM 1990**, 'Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase', *Proceedings of the National Academy of Sciences*, vol. 87, no. 9, pp. 3459-3463.

**Eisenberg, D, Gill, HS, Pfluegl, GM & Rotstein, SH 2000**, 'Structure-function relationships of glutamine synthetases', *Biochimica et Biophysica Acta (BBA)- Protein Structure and Molecular Enzymology*, vol. 1477, no. 1, pp. 122-145.

**Elazab, A, Ordóñez, RA, Savin, R, Slafer, GA & Araus, JL 2016**, 'Detecting interactive effects of N fertilization and heat stress on maize productivity by remote sensing techniques', *European Journal of Agronomy*, vol. 73, pp. 11-24.

**Endo, TR 2015**, 'New Aneuploids of Common Wheat', in *Advances in Wheat Genetics: From Genome to Field*, Springer, pp. 73-81.

**Engel, B & Keen, A 1994**, 'A simple approach for the analysis of generalized linear mixed models', *Statistica Neerlandica*, vol. 48, no. 1, pp. 1-22.

**Feder, JL, Egan, SP & Nosil, P 2012**, 'The genomics of speciation-with-gene-flow', *Trends in Genetics*, vol. 28, no. 7, pp. 342-350.

**Fei, H, Chaillou, S, Hirel, B, Mahon, JD & Vessey, KJ 2003**, 'Overexpression of a soybean cytosolic glutamine synthetase gene linked to organ-specific promoters in pea plants grown in different concentrations of nitrate', *Planta*, vol. 216, no. 3, pp. 467-474.

**Fei, H, Chaillou, S, Hirel, B, Polowick, P, Mahon, JD & Vessey, JK 2006**, 'Effects of the overexpression of a soybean cytosolic glutamine synthetase gene (GS15) linked to organ-specific promoters on growth and nitrogen accumulation of pea plants supplied with ammonium', *Plant Physiology and Biochemistry*, vol. 44, no. 10, pp. 543-550.

**Feller, U & Fischer, A 1994**, 'Nitrogen metabolism in senescing leaves', *Critical Reviews in Plant Sciences*, vol. 13, no. 3, pp. 241-273.

**Finnemann, J & Schjoerring, JK 2000**, 'Post-translational regulation of cytosolic glutamine synthetase by reversible phosphorylation and 14-3-3 protein interaction', *The Plant Journal*, vol. 24, no. 2, pp. 171-181.

**Fossati, D, Fossati, A & Feil, B 1993**, 'Relationship between grain yield and grain nitrogen concentration in winter triticale', *Euphytica*, vol. 71, no. 1-2, pp. 115-123.

**Fowler, D, Pyle, JA, Raven, JA & Sutton, MA 2013**, 'The global nitrogen cycle in the twenty-first century: introduction', *Philosophical Transactions Royal Society B*, vol. 368, no. 1621, p. 20130165.

**Fraisier, V, Gojon, A, Tillard, P & Daniel-Vedele, F 2000**, 'Constitutive expression of a putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source', *The Plant Journal*, vol. 23, no. 4, pp. 489-496.

**Fraser, AR & Ridley, SM 1984**, 'Direct measurement of enzyme activities and inhibition kinetics within isolated asparagus cells using a freeze-thaw technique', in *Advances in photosynthesis research*, Springer, pp. 589-592.

**Frey, B & Suppmann, B 1995**, 'Demonstration of the Expand™ PCR system's greater fidelity and higher yields with a lacI-based PCR fidelity assay', *Biochemica*, vol. 2, pp. 8-9.

**Fuentes, SI, Allen, DJ, Ortiz-Lopez, A & Hernández, G 2001**, 'Over-expression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations', *Journal of Experimental Botany*, vol. 52, no. 358, pp. 1071-1081.

**Funayama, K, Kojima, S, Tabuchi-Kobayashi, M, Sawa, Y, Nakayama, Y, Hayakawa, T & Yamaya, T 2013**, 'Cytosolic glutamine synthetase1;2 is responsible for the primary assimilation of ammonium in rice roots', *Plant and Cell Physiology*, p. 46.

**Gadaleta, A, Nigro, D, Giancaspro, A & Blanco, A 2011**, 'The glutamine synthetase (GS2) genes in relation to grain protein content of durum wheat', *Functional & Integrative Genomics*, vol. 11, no. 4, pp. 665-670.

**Gadaleta, A, Nigro, D, Marcotuli, I, Giancaspro, A, Giove, SL & Blanco, A 2014**, 'Isolation and characterisation of cytosolic glutamine synthetase (GSe) genes and association with grain protein content in durum wheat', *Crop and Pasture Science*, vol. 65, no. 1, pp. 38-45.

**Gaju, O, Allard, V, Martre, P, Le Gouis, J, Moreau, D, Bogard, M, Hubbart, S & Foulkes, MJ 2014**, 'Nitrogen partitioning and remobilization in relation to leaf senescence, grain yield and grain nitrogen concentration in wheat cultivars', *Field Crops Research*, vol. 155, pp. 213-223.

**Gallais, A & Hirel, B 2004**, 'An approach to the genetics of nitrogen use efficiency in maize', *Journal of Experimental Botany*, vol. 55, no. 396, pp. 295-306.

**Gallais, A, Coque, M, Quilléré, I, Prioul, JL & Hirel, B 2006**, 'Modelling postsilking nitrogen fluxes in maize (*Zea mays*) using <sup>15</sup>N-labelling field experiments', *New Phytologist*, vol. 172, no. 4, pp. 696-707.

**Gao, H, Tang, H & Bustamante, C 2016**, 'Increasing the Efficiency of Genome-wide Association Mapping via Hidden Markov Models', *BioRxiv*, p. 039099.

**Garcia-Lor, A, Curk, F, Snoussi-Trifa, H, Morillon, R, Ancillo, G, Luro, F, Navarro, L & Ollitrault, P 2013**, 'A nuclear phylogenetic analysis: SNPs, indels and SSRs deliver new insights into the relationships in the 'true citrus fruit trees' group (Citrinae, Rutaceae) and the origin of cultivated species', *Annals of Botany*, vol. 111, no. 1, pp. 1-19.

**Garnett, T, Conn, V & Kaiser, BN 2009**, 'Root based approaches to improving nitrogen use efficiency in plants', *Plant, Cell & Environment*, vol. 32, no. 9, pp. 1272-1283.

**Garnett, T, Plett, D, Heuer, S & Okamoto, M 2015**, 'Genetic approaches to enhancing nitrogen-use efficiency (NUE) in cereals: challenges and future directions', *Functional Plant Biology*, vol. 42, no. 10, pp. 921-941.

**Gaufichon, L, Marmagne, A, Yoneyama, T, Hase, T, Clément, G, Trassaert, M, Xu, X, Shakibaei, M, Najihi, A & Suzuki, A 2016**, 'Impact of the Disruption of ASN3-Encoding Asparagine Synthetase on Arabidopsis Development', *Agronomy*, vol. 6, no. 1, p. 12.

**Gaufichon, L, Masclaux-Daubresse, C, Tcherkez, G, Reisdorf-Cren, M, Sakakibara, Y, Hase, T, Clement, G, Avice, JC, Grandjean, O & Marmagne, A 2013**, 'Arabidopsis thaliana ASN2 encoding asparagine synthetase is involved in the control of nitrogen assimilation and export during vegetative growth', *Plant, Cell & Environment*, vol. 36, no. 2, pp. 328-342.

**Gautier, M-F, Cosson, P, Guirao, A, Alary, R & Joudrier, P 2000**, 'Puroindoline genes are highly conserved in diploid ancestor wheats and related species but absent in tetraploid *Triticum* species', *Plant Science*, vol. 153, no. 1, pp. 81-91.

**Gelli, M, Mitchell, SE, Liu, K, Clemente, TE, Weeks, DP, Zhang, C, Holding, DR & Dweikat, IM 2016**, 'Mapping QTLs and association of differentially expressed gene transcripts for multiple agronomic traits under different nitrogen levels in sorghum', *BMC Plant Biology*, vol. 16, no. 1, p. 1.

**Ghosh, G & Adams, JA 2011**, 'Phosphorylation mechanism and structure of serine-arginine protein kinases', *FEBS Journal*, vol. 278, no. 4, pp. 587-597.

**Giambalvo, D, Ruisi, P, Di Miceli, G, Frenda, AS & Amato, G 2010**, 'Nitrogen use efficiency and nitrogen fertilizer recovery of durum wheat accessions as affected by interspecific competition', *Agronomy Journal*, vol. 102, no. 2, pp. 707-715.

**Gigova, LG & Ivanova, NJ 2015**, 'Microalgae respond differently to nitrogen availability during culturing', *Journal of Giosciences*, vol. 40, no. 2, pp. 365-374.

**Gill, K, Lubbers, E, Gill, B, Raupp, W & Cox, T 1991**, 'A genetic linkage map of *Triticum tauschii* (DD) and its relationship to the D genome of bread wheat (AABBDD)', *Genome*, vol. 34, no. 3, pp. 362-374.

**Gioia, T, Nagel, KA, Beleggia, R, Fragasso, M, Ficco, DBM, Pieruschka, R, De Vita, P, Fiorani, F & Papa, R 2015**, 'Impact of domestication on the phenotypic architecture of durum wheat under contrasting nitrogen fertilization', *Journal of Experimental Botany*, vol. 66, no. 18, pp. 5519-5530.

**Glass, NL & Donaldson, GC 1995**, 'Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes', *Applied and Environmental Microbiology*, vol. 61, no. 4, pp. 1323-1330.

**Glevarec, G, Bouton, S, Jaspard, E, Riou, M-T, Cliquet, J-B, Suzuki, A & Limami, AM 2004**, 'Respective roles of the glutamine synthetase/glutamate synthase cycle and glutamate dehydrogenase in ammonium and amino acid metabolism during germination and post-germinative growth in the model legume *Medicago truncatula*', *Planta*, vol. 219, no. 2, pp. 286-297.

**Good, AG, Shrawat, AK & Muench, DG 2004**, 'Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production?', *Trends in Plant Science*, vol. 9, no. 12, pp. 597-605.

**Goodall, AJ, Kumar, P & Tobin, AK 2013**, 'Identification and expression analyses of cytosolic glutamine synthetase genes in barley (*Hordeum vulgare* L.)', *Plant and Cell Physiology*, vol. 54, no. 4, pp. 492-505.

**Gouia, H, Suzuki, A, Brulfert, J & Ghorbal, MH 2003**, 'Effects of cadmium on the co-ordination of nitrogen and carbon metabolism in bean seedlings', *Journal of Plant Physiology*, vol. 160, no. 4, pp. 367-376.



**Grabowska, A, Kwinta, J & Bielawski, W 2012**, 'Glutamine synthetase and glutamate dehydrogenase in triticale seeds: molecular cloning and genes expression', *Acta Physiologiae Plantarum*, vol. 34, no. 6, pp. 2393-2406.

**Grant, CA, O'Donovan, JT, Blackshaw, RE, Harker, KN, Johnson, EN, Gan, Y, Lafond, GP, May, WE, Turkington, TK & Lupwayi, NZ 2016**, 'Residual effects of preceding crops and nitrogen fertilizer on yield and crop and soil N dynamics of spring wheat and canola in varying environments on the Canadian prairies', *Field Crops Research*, vol. 192, pp. 86-102.

**Greenough, L, Schermerhorn, KM, Mazzola, L, Bybee, J, Rivizzigno, D, Cantin, E, Slatko, BE & Gardner, AF 2016**, 'Adapting capillary gel electrophoresis as a sensitive, high-throughput method to accelerate characterization of nucleic acid metabolic enzymes', *Nucleic Acids Research*, vol. 44, no. 2, pp. e15-e15.

**Griffiths, A, Robinson, LA & Willett, P 1984**, 'Hierarchic agglomerative clustering methods for automatic document classification', *Journal of Documentation*, vol. 40, no. 3, pp. 175-205.

**Griffiths, AJ, Miller, JH, Suzuki, DT, Lewontin, RC & Gelbart, WM 2000**, 'Aneuploidy', *NCBI Bookshelf*, p. 3.

**Gu, L, Liu, T, Jun, Z, Dong, S, Liu P 2015**, 'Nitrate leaching of winter wheat grown in lysimeters as affected by fertilizers and irrigation on the North China Plain', *Journal of Integrative Agriculture* vol. 14 no. 2, pp. 374-388.

**Guan, M, de Bang, T, Pedersen, C & Schjoerring, JK 2016**, 'Cytosolic glutamine synthetase Gln1; 2 is the main isozyme contributing to GS1 activity in Arabidopsis shoots and can be up-regulated to relieve ammonium toxicity', *Plant Physiology*, p. pp. 01195.02016.

**Guan, M, Møller, IS & Schjørring, JK 2014**, 'Two cytosolic glutamine synthetase isoforms play specific roles for seed germination and seed yield structure in Arabidopsis', *Journal of Experimental Botany*, p. 411.

**Guan, M, Møller, IS & Schjørring, JK 2015**, 'Two cytosolic glutamine synthetase isoforms play specific roles for seed germination and seed yield structure in Arabidopsis', *Journal of Experimental Botany*, vol. 66, no. 1, pp. 203-212.

**Guiboileau, A, Avila-Ospina, L, Yoshimoto, K, Soulay, F, Azzopardi, M, Marmagne, A, Lothier, J & Masclaux-Daubresse, C 2013**, 'Physiological and metabolic consequences of autophagy deficiency for the management of nitrogen and protein resources in Arabidopsis leaves depending on nitrate availability', *New Phytologist*, vol. 199, no. 3, pp. 683-694.

**Guo, B, Gao, H, Tang, C, Liu, T & Chu, G 2015**, 'Response of water coupling with N supply on maize nitrogen uptake, water and N use efficiency, and yield in drip irrigation condition', *Ying yong sheng tai xue bao= The Journal of Applied Ecology/Zhongguo sheng tai xue xue hui, Zhongguo ke xue yuan Shenyang ying yong sheng tai yan jiu suo zhu ban*, vol. 26, no. 12, pp. 3679-3686.

**Guo, Y, Cai, Z & Gan, S 2004**, 'Transcriptome of Arabidopsis leaf senescence', *Plant, Cell & Environment*, vol. 27, no. 5, pp. 521-549.

**Gupta, P, Rustgi, S, Sharma, S, Singh, R, Kumar, N & Balyan, H 2003**, 'Transferable EST-SSR markers for the study of polymorphism and genetic diversity in bread wheat', *Molecular Genetics and Genomics*, vol. 270, no. 4, pp. 315-323.

**Gupta, R & Dhugga, KS 2013**, 'Manipulation of glutamine synthetases (GS) to improve nitrogen use efficiency and grain yield in higher plants', *Google Patents* p. 3.

**Habash, D, Massiah, A, Rong, H, Wallsgrove, R & Leigh, R 2001**, 'The role of cytosolic glutamine synthetase in wheat', *Annals of Applied Biology*, vol. 138, no. 1, pp. 83-89.

**Habash, DZ, Bernard, S, Schondelmaier, J, Weyen, J & Quarrie, SA 2007**, 'The genetics of nitrogen use in hexaploid wheat: N utilisation, development and yield', *Theoretical and Applied Genetics*, vol. 114, no. 3, pp. 403-419.

**Hampson, S, McLysaght, A, Gaut, B & Baldi, P 2003**, 'Line-Up: statistical detection of chromosomal homology with application to plant comparative genomics', *Genome Research*, vol. 13, no. 5, pp. 999-1010.

**Hanley, S, Edwards, D, Stevenson, D, Haines, S, Hegarty, M, Schuch, W & Edwards, KJ 2000**, 'Identification of transposon-tagged genes by the random sequencing of Mutator-tagged DNA fragments from *Zea mays*', *The Plant Journal*, vol. 23, no. 4, pp. 557-566.

**Hayashi, H & Chino, M 1990**, 'Chemical composition of phloem sap from the uppermost internode of the rice plant', *Plant and Cell Physiology*, vol. 31, no. 2, pp. 247-251.

**Hewitt, GM 2001**, 'Speciation, hybrid zones and phylogeography-or seeing genes in space and time', *Molecular Ecology*, vol. 10, no. 3, pp. 537-549.

**Hirel, B & Gadal, P 1980**, 'Glutamine synthetase in rice a comparative study of the enzymes from roots and leaves', *Plant Physiology*, vol. 66, no. 4, pp. 619-623.

**Hirel, B & Gadal, P 1982**, 'Glutamine synthetase isoforms in leaves of a C<sub>4</sub> plant: *Sorghum vulgare*', *Physiologia Plantarum*, vol. 54, no. 1, pp. 69-74.

**Hirel, B & Gallais, A 2006**, 'Rubisco synthesis, turnover and degradation: some new thoughts on an old problem', *New Phytologist*, vol. 169, no. 3, pp. 445-448.

**Hirel, B, Bertin, P, Quilleré, I, Bourdoncle, W, Attagnant, C, Dellay, C, Gouy, A, Cadiou, S, Retailiau, C & Falque, M 2001**, 'Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize', *Plant Physiology*, vol. 125, no. 3, pp. 1258-1270.

**Hirel, B, Le Gouis, J, Ney, B & Gallais, A 2007**, 'The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches', *Journal of Experimental Botany*, vol. 58, no. 9, pp. 2369-2387.

**Hirel, B, Tétu, T, Lea, PJ & Dubois, F 2011**, 'Improving nitrogen use efficiency in crops for sustainable agriculture', *Sustainability*, vol. 3, no. 9, pp. 1452-1485.

**Horton, RM, Hunt, HD, Ho, SN, Pullen, JK & Pease, LR 1989**, 'Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension', *Gene*, vol. 77, no. 1, pp. 61-68.

**Hoshida, H, Tanaka, Y, Hibino, T, Hayashi, Y, Tanaka, A, Takabe, T & Takabe, T 2000**, 'Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase', *Plant Molecular Biology*, vol. 43, no. 1, pp. 103-111.

**Hruz, T, Wyss, M, Docquier, M, Pfaffl, MW, Masanetz, S, Borghi, L, Verbrugghe, P, Kalaydjieva, L, Bleuler, S & Laule, O 2011**, 'Reference Genes: identification of reliable and condition specific reference genes for RT-qPCR data normalization', *BMC Genomics*, vol. 12, no. 1, p. 156.

**Hsu, P-K & Tsay, Y-F 2013**, 'Two phloem nitrate transporters, NRT1. 11 and NRT1. 12, are important for redistributing xylem-borne nitrate to enhance plant growth', *Plant Physiology*, vol. 163, no. 2, pp. 844-856.

**Ishimaru, K, Hirotsu, N, Madoka, Y, Murakami, N, Hara, N, Onodera, H, Kashiwagi, T, Ujiie, K, Shimizu, B-i & Onishi, A 2013**, 'Loss of function of the IAA-glucose hydrolase gene TGW6 enhances rice grain weight and increases yield', *Nature Genetics*, vol. 45, no. 6, pp. 707-711.

**Ishiyama, K, Inoue, E, Tabuchi, M, Yamaya, T & Takahashi, H 2004**, 'Biochemical background and compartmentalized functions of cytosolic glutamine synthetase for active ammonium assimilation in rice roots', *Plant and Cell Physiology*, vol. 45, no. 11, pp. 1640-1647.

**Ishiyama, K, Inoue, E, Watanabe-Takahashi, A, Obara, M, Yamaya, T & Takahashi, H 2004**, 'Kinetic properties and ammonium-dependent regulation of cytosolic isoenzymes of glutamine synthetase in Arabidopsis', Journal of Biological Chemistry, vol. 279, no. 16, pp. 16598-16605.

**Ishiyama, K, Kojima, S, Takahashi, H, Hayakawa, T & Yamaya, T 2003**, 'Cell type distinct accumulations of mRNA and protein for NADH-dependent glutamate synthase in rice roots in response to the supply of NH<sub>4</sub><sup>+</sup>', Plant Physiology and Biochemistry, vol. 41, no. 6, pp. 643-647.

**Jia, J, Zhao, S, Kong, X, Li, Y, Zhao, G, He, W, Appels, R, Pfeifer, M, Tao, Y & Zhang, X 2013**, '*Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation', Nature, vol. 496, no. 7443, pp. 91-95.

**Kaiser, J & Lewis, O 1984**, 'Nitrate reductase and glutamine synthetase activity in leaves and roots of nitrate-fed *Helianthus annuus* L', Plant and Soil, vol. 77, no. 1, pp. 127-130.

**Kamachi, K, Yamaya, T, Mae, T & Ojima, K 1991**, 'A role for glutamine synthetase in the remobilization of leaf nitrogen during natural senescence in rice leaves', Plant Physiology, vol. 96, no. 2, pp. 411-417.

**Kant, P, Gordon, M, Kant, S, Zolla, G, Davydov, O, Heimer, YM, Chalifa-Caspi, V, Shaked, R & Barak, S 2008**, 'Functional-genomics-based identification of genes that regulate Arabidopsis responses to multiple abiotic stresses', Plant, Cell & Environment, vol. 31, no. 6, pp. 697-714.

**Kant, S, Bi, Y-M & Rothstein, SJ 2010**, 'Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency', *Journal of Experimental Botany*, p. 297.

**Kant, S, Bi, Y-M & Rothstein, SJ 2011**, 'Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency', *Journal of Experimental Botany*, vol. 62, no. 4, pp. 1499-1509.

**Kant, S, Bi, Y-M, Weretilnyk, E, Barak, S & Rothstein, SJ 2008**, 'The Arabidopsis halophytic relative *Thellungiella halophila* tolerates nitrogen-limiting conditions by maintaining growth, nitrogen uptake, and assimilation', *Plant Physiology*, vol. 147, no. 3, pp. 1168-1180.

**Kar, M & Feierabend, J 1984**, 'Changes in the activities of enzymes involved in amino acid metabolism during the senescence of detached wheat leaves', *Physiologia Plantarum*, vol. 62, no. 1, pp. 39-44.

**Karin, M & Hunter, T 1995**, 'Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus', *Current Biology*, vol. 5, no. 7, pp. 747-757.

**Kaur, B & Asthir, B 2016**, 'Hydroponic Culturing Upregulates Sucrose and Glutamine Metabolism by Enhancing Their Utilization via Intermediates of Aerobic Pathway in Wheat', *Cereal Research Communications*, pp. 1-12.

**Kaur, H, Ram, H, Sikka, R & Kaur, H 2016**, 'Productivity, agronomic efficiency and quality of bread wheat [*Triticum aestivum* (L.)] cultivars in relation to nitrogen', International Journal of Agriculture, Environment and Biotechnology, vol. 9, no. 1, p. 101.

**Kearse, M, Moir, R, Wilson, A, Stones-Havas, S, Cheung, M, Sturrock, S, Buxton, S, Cooper, A, Markowitz, S & Duran, C 2012**, 'Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data', Bioinformatics, vol. 28, no. 12, pp. 1647-1649.

**Kebrom, TH & Mullet, JE 2016**, 'Transcriptome profiling of tiller buds provides new insights into PhyB regulation of tillering and indeterminate growth in sorghum', Plant Physiology, vol. 170, no. 4, pp. 2232-2250.

**Kelley, LA, Mezulis, S, Yates, CM, Wass, MN & Sternberg, MJE 2015**, 'The Phyre2 web portal for protein modeling, prediction and analysis', Nature Protocols, vol. 10, no. 6, 06//print, pp. 845-858.

**Kichey, T, Heumez, E, Pocholle, D, Pageau, K, Vanacker, H, Dubois, F, Le Gouis, J & Hirel, B 2006**, 'Combined agronomic and physiological aspects of nitrogen management in wheat highlight a central role for glutamine synthetase', New Phytologist, vol. 169, no. 2, pp. 265-278.

**Kichey, T, Le Gouis, J, Sangwan, B, Hirel, B & Dubois, F 2005**, 'Changes in the cellular and subcellular localization of glutamine synthetase and glutamate dehydrogenase during flag leaf senescence in wheat (*Triticum aestivum* L.)', Plant and Cell Physiology, vol. 46, no. 6, pp. 964-974.



**Kienzler, KM, Rudenko, I, Ruzimov, J, Ibragimov, N & Lamers, JP 2011**, 'Winter wheat quantity or quality? Assessing food security in Uzbekistan', *Food Security*, vol. 3, no. 1, pp. 53-64.

**Kindu, GA, Tang, J, Yin, X & Struik, PC 2014**, 'Quantitative trait locus analysis of nitrogen use efficiency in barley (*Hordeum vulgare* L.)', *Euphytica*, vol. 199, no. 1-2, pp. 207-221.

**Kirisci, L, Hsu, T-c & Yu, L 2001**, 'Robustness of item parameter estimation programs to assumptions of unidimensionality and normality', *Applied Psychological Measurement*, vol. 25, no. 2, pp. 146-162.

**Kjaer, B & Jensen, J 1995**, 'The inheritance of nitrogen and phosphorus content in barley analysed by genetic markers', *Hereditas*, vol. 123, no. 2, pp. 109-119.

**Kong, L, Xie, Y, Hu, L, Feng, B & Li, S 2016**, 'Remobilization of vegetative nitrogen to developing grain in wheat (*Triticum aestivum* L.)', *Field Crops Research*. pp. 1-4.

**Kozaki, A & Takeba, G 1996**, 'Photorespiration protects C3 plants from photooxidation'. pp. 1-5.

**Krapp, A, Berthomé, R, Orsel, M, Mercey-Boutet, S, Yu, A, Castaings, L, Elftieh, S, Major, H, Renou, J-P & Daniel-Vedele, F 2011**, 'Arabidopsis roots and shoots show distinct temporal adaptation patterns toward nitrogen starvation', *Plant Physiology*, vol. 157, no. 3, pp. 1255-1282.

**Kricka, LJ & Fortina, P 2016**, '16 Future Perspectives on Nucleic Acid Testing', *Nucleic Acid Testing for Human Disease*, p. 413.

**Kusano, M, Tabuchi, M, Fukushima, A, Funayama, K, Diaz, C, Kobayashi, M, Hayashi, N, Tsuchiya, YN, Takahashi, H & Kamata, A 2011,** 'Metabolomics data reveal a crucial role of cytosolic glutamine synthetase 1; 1 in coordinating metabolic balance in rice', *The Plant Journal*, vol. 66, no. 3, pp. 456-466.

**Kwinta, J & Cal, K 2005,** 'Effects of salinity stress on the activity of glutamine synthetase and glutamate dehydrogenase in triticale seedlings', *Polish Journal of Environmental Studies*, vol. 14, no. 1, pp. 125-130.

**Ladha, J, Tirol-Padre, A, Reddy, C, Cassman, K, Verma, S, Powlson, D, van Kessel, C, Richter, DdB, Chakraborty, D & Pathak, H 2016,** 'Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems', *Scientific Reports*, vol. 6.

**Lam, H-M, Coschigano, K, Schultz, C, Melo-Oliveira, R, Tjaden, G, Oliveira, I, Ngai, N, Hsieh, M-H & Coruzzi, G 1995,** 'Use of Arabidopsis mutants and genes to study amide amino acid biosynthesis', *The Plant Cell*, vol. 7, no. 7, p. 887.

**Lam, H-M, Wong, P, Chan, H-K, Yam, K-M, Chen, L, Chow, C-M & Coruzzi, GM 2003,** 'Overexpression of the ASN1 gene enhances nitrogen status in seeds of Arabidopsis', *Plant Physiology*, vol. 132, no. 2, pp. 926-935.

**Lan, Q, Feng, B, Xu, Z, Zhao, G & Wang, T 2013**, 'Molecular cloning and characterization of five novel low molecular weight glutenin subunit genes from Tibetan wheat landraces (*Triticum aestivum* L.)', *Genetic Resources and Crop Evolution*, vol. 60, no. 2, pp. 799-806.

**Laperche, A, Brancourt-Hulmel, M, Heumez, E, Gardet, O, Hanocq, E, Devienne-Barret, F & Le Gouis, J 2007**, 'Using genotype× nitrogen interaction variables to evaluate the QTL involved in wheat tolerance to nitrogen constraints', *Theoretical and Applied Genetics*, vol. 115, no. 3, pp. 399-415.

**Law, C, Snape, J & Worland, A 1987**, 'Aneuploidy in wheat and its uses in genetic analysis', in *Wheat Breeding*, Springer, pp. 71-108.

**Le Gouis, J, Béghin, D, Heumez, E & Pluchard, P 2000**, 'Genetic differences for nitrogen uptake and nitrogen utilisation efficiencies in winter wheat', *European Journal of Agronomy*, vol. 12, no. 3, pp. 163-173.

**Le Gouis, J, Beghin, D, Heumez, E & Pluchard, P 2002**, 'Diallel analysis of winter wheat at two nitrogen levels', *Crop Science*, vol. 42, no. 4, pp. 1129-1134.

**Lea, P & Azevedo, R 2007**, 'Nitrogen use efficiency. 2. Amino acid metabolism', *Annals of Applied Biology*, vol. 151, no. 3, pp. 269-275.

**Lea, P & Forde, B 1994**, 'The use of mutants and transgenic plants to study amino acid metabolism', *Plant, Cell & Environment*, vol. 17, no. 5, pp. 541-556.

**Lea, P 2014**, 'Ammonia assimilation and amino acid biosynthesis', *Techniques in Bioproductivity and Photosynthesis: Pergamon International Library of Science, Technology, Engineering and Social Studies*, p. 173.

**Lea, PJ & Azevedo, RA 2006**, 'Nitrogen use efficiency. 1. Uptake of nitrogen from the soil', *Annals of Applied Biology*, vol. 149, no. 3, pp. 243-247.

**Lea, PJ & Miflin, BJ 2003**, 'Glutamate synthase and the synthesis of glutamate in plants', *Plant Physiology and Biochemistry*, vol. 41, no. 6, pp. 555-564.

**Leonforte, A, Sudheesh, S, Cogan, NO, Salisbury, PA, Nicolas, ME, Materne, M, Forster, JW & Kaur, S 2013**, 'SNP marker discovery, linkage map construction and identification of QTLs for enhanced salinity tolerance in field pea (*Pisum sativum* L.)', *BMC Plant Biology*, vol. 13, no. 1, p. 1.

**Li, JH, Zhang, J, Li, WJ, Xu, DH, Knops, JM & Du, GZ 2016**, 'Plant functional groups, grasses versus forbs, differ in their impact on soil carbon dynamics with nitrogen fertilization', *European Journal of Soil Biology*, vol. 75, pp. 79-87.

**Li, R-J, Hua, W & Lu, Y-T 2006**, 'Arabidopsis cytosolic glutamine synthetase AtGLN1; 1 is a potential substrate of AtCRK3 involved in leaf senescence', *Biochemical and Biophysical Research Communications*, vol. 342, no. 1, pp. 119-126.

**Li, XP, Zhao, XQ, He, X, Zhao, GY, Li, B, Liu, DC, Zhang, AM, Zhang, XY, Tong, YP & Li, ZS 2011**, 'Haplotype analysis of the genes encoding glutamine synthetase plastic isoforms and their association with nitrogen-use-and yield-related traits in bread wheat', *New Phytologist*, vol. 189, no. 2, pp. 449-458.

**Li, Y, Wang, M, Zhang, F, Xu, Y, Chen, X, Qin, X & Wen, X 2016**, 'Effect of post-silking drought on nitrogen partitioning and gene expression patterns of glutamine synthetase and asparagine synthetase in two maize (*Zea mays* L.) varieties', *Plant Physiology and Biochemistry*, vol. 102, pp. 62-69.

**Liaw, SH, Villafranca, JJ & Eisenberg, D 1993**, 'A model for oxidative modification of glutamine synthetase, based on crystal structures of mutant H269N and the oxidized enzyme', *Biochemistry*, vol. 32, no. 31, pp. 7999-8003.

**Lima, L, Seabra, A, Melo, P, Cullimore, J & Carvalho, H 2006**, 'Phosphorylation and subsequent interaction with 14-3-3 proteins regulate plastid glutamine synthetase in *Medicago truncatula*', *Planta*, vol. 223, no. 3, pp. 558-567.

**Lima, L, Seabra, A, Melo, P, Cullimore, J & Carvalho, H 2006**, 'Post-translational regulation of cytosolic glutamine synthetase of *Medicago truncatula*', *Journal of Experimental Botany*, vol. 57, no. 11, pp. 2751-2761.

**Limami, AM & de Vienne, D 2001**, 'Natural genetic variability in nitrogen metabolism', in *Plant Nitrogen*, Springer, pp. 369-378.

**Limami, AM, Rouillon, C, Glevarec, G, Gallais, A & Hirel, B 2002**, 'Genetic and physiological analysis of germination efficiency in maize in relation to nitrogen metabolism reveals the importance of cytosolic glutamine synthetase', *Plant Physiology*, vol. 130, no. 4, pp. 1860-1870.

**Liu, H, Wang, Z, Yu, R, Li, F, Li, K, Cao, H, Yang, N, Li, M, Dai, J & Zan, Y 2016**, 'Optimal nitrogen input for higher efficiency and lower environmental impacts of winter wheat production in China', *Agriculture, Ecosystems & Environment*, vol. 224, pp. 1-11.

**Lopes, MS, Cortadellas, N, Kichey, T, Dubois, F, Habash, DZ & Araus, JL 2006**, 'Wheat nitrogen metabolism during grain filling: comparative role of glumes and the flag leaf', *Planta*, vol. 225, no. 1, pp. 165-181.

**Loridon, K, McPhee, K, Morin, J, Dubreuil, P, Pilet-Nayel, M, Aubert, G, Rameau, C, Baranger, A, Coyne, C & Lejeune-Henaut, I 2005**, 'Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.)', *Theoretical and Applied Genetics*, vol. 111, no. 6, pp. 1022-1031.

**Lothier, J, Gauffichon, L, Sormani, R, Lemaître, T, Azzopardi, M, Morin, H, Chardon, F, Reisdorf-Cren, M, Avice, J-C & Masclaux-Daubresse, C 2011**, 'The cytosolic glutamine synthetase GLN1;2 plays a role in the control of plant growth and ammonium homeostasis in *Arabidopsis* rosettes when nitrate supply is not limiting', *Journal of Experimental Botany*, vol. 62, no. 4, pp. 1375-1390.

**Luo, J, Qin, J, He, F, Li, H, Liu, T, Polle, A, Peng, C & Luo, Z-B 2013**, 'Net fluxes of ammonium and nitrate in association with H<sup>+</sup> fluxes in fine roots of *Populus popularis*', *Planta*, vol. 237, no. 4, pp. 919-931.

**Lutts, S, Majerus, V & Kinet, JM 1999**, 'NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings', *Physiologia Plantarum*, vol. 105, no. 3, pp. 450-458.

**Lutz, MW, Crenshaw, D, Welsh-Bohmer, KA, Burns, DK & Roses, AD 2016,** 'New Genetic Approaches to AD: Lessons from APOE-TOMM40 Phylogenetics', *Current Neurology and Neuroscience Reports*, vol. 16, no. 5, pp. 1-9.

**Lv, Y, Liang, Z, Ge, M, Qi, W, Zhang, T, Lin, F, Peng, Z & Zhao, H 2016,** 'Genome-wide identification and functional prediction of nitrogen-responsive intergenic and intronic long non-coding RNAs in maize (*Zea mays* L.)', *BMC genomics*, vol. 17, no. 1, p. 1.

**Mahjourimajd, S, Kuchel, H, Langridge, P & Okamoto, M 2016,** 'Evaluation of Australian wheat genotypes for response to variable nitrogen application', *Plant and Soil*, vol. 399, no. 1-2, pp. 247-255.

**Malek, L, Bogorad, L, Ayers, AR & Goldberg, AL 1984,** 'Newly synthesized proteins are degraded by an ATP-stimulated proteolytic process in isolated pea chloroplasts', *FEBS Letters*, vol. 166, no. 2, pp. 253-257.

**Manly, KF, Cudmore Jr, RH & Meer, JM 2001,** 'Map Manager QTX, cross-platform software for genetic mapping', *Mammalian Genome*, vol. 12, no. 12, pp. 930-932.

**Marras, SA, Kramer, FR & Tyagi, S 2002,** 'Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes', *Nucleic Acids Research*, vol. 30, no. 21, pp. 122.

**Martin, A, Lee, J, Kichey, T, Gerentes, D, Zivy, M, Tatout, C, Dubois, F, Balliau, T, Valot, B & Davanture, M 2006**, 'Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production', *The Plant Cell*, vol. 18, no. 11, pp. 3252-3274.

**Martre, P, He, J, Le Gouis, J & Semenov, MA 2015**, 'In silico system analysis of physiological traits determining grain yield and protein concentration for wheat as influenced by climate and crop management', *Journal of Experimental Botany*, p. 49.

**Martre, P, Porter, JR, Jamieson, PD & Triboï, E 2003**, 'Modeling grain nitrogen accumulation and protein composition to understand the sink/source regulations of nitrogen remobilization for wheat', *Plant Physiology*, vol. 133, no. 4, pp. 1959-1967.

**Masalkar, PD & Roberts, DM 2015**, 'Glutamine synthetase isoforms in nitrogen-fixing soybean nodules: distinct oligomeric structures and thiol-based regulation', *FEBS Letters*, vol. 589, no. 2, pp. 215-221.

**Mascher, M, Muehlbauer, GJ, Rokhsar, DS, Chapman, J, Schmutz, J, Barry, K, Muñoz-Amatriaín, M, Close, TJ, Wise, RP & Schulman, AH 2013**, 'Anchoring and ordering NGS contig assemblies by population sequencing (POPSEQ)', *The Plant Journal*, vol. 76, no. 4, pp. 718-727.



**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*, vol. 138, no. 1, pp. 69-81.

**Masclaux-Daubresse, C, Daniel-Vedele, F, Dechorgnat, J, Chardon, F, Gaufichon, L & Suzuki, A 2010**, 'Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture', *Annals of Botany*, vol. 105, no. 7, pp. 1141-1157.

**Masclaux-Daubresse, C, Reisdorf-Cren, M & Orsel, M 2008**, 'Leaf nitrogen remobilisation for plant development and grain filling', *Plant Biology*, vol. 10, no. s1, pp. 23-36.

**Masclaux-Daubresse, C, Reisdorf-Cren, M, Pageau, K, Lelandais, M, Grandjean, O, Kronenberger, J, Valadier, M-H, Feraud, M, Jouglet, T & Suzuki, A 2006**, 'Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco', *Plant Physiology*, vol. 140, no. 2, pp. 444-456.

**Mattsson, M & Schjoerring, JK 2002**, 'Dynamic and steady-state responses of inorganic nitrogen pools and NH<sub>3</sub> exchange in leaves of *Lolium perenne* and *Bromus erectus* to changes in root nitrogen supply', *Plant Physiology*, vol. 128, no. 2, pp. 742-750.

**Mcallister, C, Beatty, P, D'entremont, J & Good, A 2016**, 'Plants having enhanced nitrogen use efficiency and methods of producing the same', US Patent 20,160,010,102.

**McAllister, CH & Good, AG 2015**, 'Alanine Aminotransferase Variants Conferring Diverse NUE Phenotypes in *Arabidopsis thaliana*', *PloS One*, vol. 10, no. 4, p. 121830.

**McAllister, CH, Wolansky, M & Good, AG 2016**, 'The impact on nitrogen-efficient phenotypes when aspartate aminotransferase is expressed tissue-specifically in *Brassica napus*', *New Negatives in Plant Science*, vol. 3, pp. 1-9.

**McNally, SF, Hirel, B, Gadal, P, Mann, AF & Stewart, GR 1983**, 'Glutamine synthetases of higher plants evidence for a specific isoform content related to their possible physiological role and their compartmentation within the leaf', *Plant Physiology*, vol. 72, no. 1, pp. 22-25.

**Melino, VJ, Fiene, G, Enju, A, Cai, J, Buchner, P & Heuer, S 2015**, 'Genetic diversity for root plasticity and nitrogen uptake in wheat seedlings', *Functional Plant Biology*, vol. 42, no. 10, pp. 942-956.

**Miflin, BJ & Habash, DZ 2002**, 'The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops', *Journal of Experimental Botany*, vol. 53, no. 370, pp. 979-987.

**Migge, A, Carrayol, E, Hirel, B & Becker, TW 2000**, 'Leaf-specific overexpression of plastidic glutamine synthetase stimulates the growth of transgenic tobacco seedlings', *Planta*, vol. 210, no. 2, pp. 252-260.

**Mitchelson, KR 2001**, 'Overview: The Application of Capillary Electrophoresis for DNA Polymorphism Analysis', *Capillary Electrophoresis of Nucleic Acids: vol. I: Introduction to the Capillary Electrophoresis of Nucleic Acids*, pp. 3-26.

**Mitchelson, KR, Hawkes, DB, Turakulov, R & Men, AE 2007**, 'Overview: developments in DNA sequencing', *Perspectives in Bioanalysis*, vol. 2, pp. 3-44.

**Miyashita, Y & Good, AG 2008**, 'Glutamate deamination by glutamate dehydrogenase plays a central role in amino acid catabolism in plants', *Plant Signaling & Behavior*, vol. 3, no. 10, pp. 842-843.

**Mochida, K, Yamazaki, Y & Ogihara, Y 2004**, 'Discrimination of homoeologous gene expression in hexaploid wheat by SNP analysis of contigs grouped from a large number of expressed sequence tags', *Molecular Genetics and Genomics*, vol. 270, no. 5, pp. 371-377.

**Mohammed, YA, Kelly, J, Chim, BK, Rutto, E, Waldschmidt, K, Mullock, J, Torres, G, Desta, KG & Raun, W 2013**, 'Nitrogen fertilizer management for improved grain quality and yield in winter wheat in Oklahoma', *Journal of Plant Nutrition*, vol. 36, no. 5, pp. 749-761.

**Molnár, I, Vrána, J, Farkas, A, Kubaláková, M, Cseh, A, Molnár-Láng, M & Doležel, J 2015**, 'Flow sorting of C-genome chromosomes from wild relatives of wheat *Aegilops markgrafii*, *Ae. triuncialis* and *Ae. cylindrica*, and their molecular organization', *Annals of Botany*, p. 073.

**Mon, J, Bronson, K, Hunsaker, D, Thorp, K, White, J & French, A 2016,** 'Interactive effects of nitrogen fertilization and irrigation on grain yield, canopy temperature, and nitrogen use efficiency in overhead sprinkler-irrigated durum wheat', *Field Crops Research*, vol. 191, pp. 54-65.

**Moore, KL, Tosi, P, Palmer, R, Hawkesford, MJ, Grovenor, CR & Shewry, PR 2016,** 'The Dynamics of Protein Body Formation in Developing Wheat Grain', *Plant Biotechnology Journal*, p. 4.

**Moorhead, G, Douglas, P, Cotellet, V, Harthill, J, Morrice, N, Meek, S, Deiting, U, Stitt, M, Scarabel, M & Aitken, A 1999,** 'Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins', *The Plant Journal*, vol. 18, no. 1, pp. 1-12.

**Mueller, ND, West, PC, Gerber, JS, MacDonald, GK, Polasky, S & Foley, JA 2014,** 'A tradeoff frontier for global nitrogen use and cereal production', *Environmental Research Letters*, vol. 9, no. 5, p. 054002.

**Namai, S, Toriyama, K & Fukuta, Y 2009,** 'Genetic variations in dry matter production and physiological nitrogen use efficiency in rice (*Oryza sativa* L.) varieties', *Breeding Science*, vol. 59, no. 3, pp. 269-276.

**Nazemi, G & Salvi, S 2016,** 'Genetic Bases of Root System Architecture in Durum Wheat Seedlings', *International Journal of Life Sciences*, vol. 10, no. 1, pp. 25-28.

**Neilson, EH, Edwards, A, Blomstedt, C, Berger, B, Møller, BL & Gleadow, R 2015**, 'Utilization of a high-throughput shoot imaging system to examine the dynamic phenotypic responses of a C4 cereal crop plant to nitrogen and water deficiency over time', *Journal of Experimental Botany*, vol. 66, no. 7, pp. 1817-1832.

**Nigro, D, Fortunato, S, Giove, SL, Paradiso, A, Gu, YQ, Blanco, A, de Pinto, MC & Gadaleta, A 2016**, 'Glutamine synthetase in durum wheat: genotypic variation and relationship with Grain Protein Content', *Frontiers in Plant Science*, vol. 7.p. 4.

**Nigro, D, Gu, Y, Huo, N, Marcotuli, I, Blanco, A & Kashkush, K 2013**, 'Structural Analysis of the Wheat Genes Encoding NADH-Dependent Glutamine-2-oxoglutarate'. *PLOS One*, p. 2.

**Novitskaya, L, Trevanion, S, Driscoll, S, Foyer, C & Noctor, G 2002**, 'How does photorespiration modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis', *Plant, Cell & Environment*, vol. 25, no. 7, pp. 821-835.

**Novotny, AM, Schade, JD, Hobbie, SE, Kay, AD, Kyle, M, Reich, PB & Elser, JJ 2007**, 'Stoichiometric response of nitrogen-fixing and non-fixing dicots to manipulations of CO<sub>2</sub>, nitrogen, and diversity', *Oecologia*, vol. 151, no. 4, pp. 687-696.

**Nowak, M, Leśniowska-Nowak, J, Zapalska, M, Banaszak, Z, Kondracka, K, Dudziak, K & Kowalczyk, K 2014**, 'Analysis of VRN1 gene in triticale and common wheat genetic background', *Scientia Agricola*, vol. 71, no. 5, pp. 380-386.

**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*, vol. 52, no. 359, pp. 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*, vol. 110, no. 1, pp. 1-11.

**Ogihara, Y, Mochida, K, Nemoto, Y, Murai, K, Yamazaki, Y, Shin-I, T & Kohara, Y 2003**, 'Correlated clustering and virtual display of gene expression patterns in the wheat life cycle by large-scale statistical analyses of expressed sequence tags', *The Plant Journal*, vol. 33, no. 6, pp. 1001-1011.

**Oliveira, IC, Brears, T, Knight, TJ, Clark, A & Coruzzi, GM 2002**, 'Overexpression of cytosolic glutamine synthetase. Relation to nitrogen, light, and photorespiration', *Plant Physiology*, vol. 129, no. 3, pp. 1170-1180.

**Orsel, M, Moison, M, Clouet, V, Thomas, J, Leprince, F, Canoy, A-S, Just, J, Chalhoub, B & Masclaux-Daubresse, C 2014**, 'Sixteen cytosolic glutamine synthetase genes identified in the *Brassica napus* L. genome are differentially regulated depending on nitrogen regimes and leaf senescence', *Journal of Experimental Botany*, vol. 65, no. 14, pp. 3927-3947.

**Ortega, JL, Moguel-Esponda, S, Potenza, C, Conklin, CF, Quintana, A & Sengupta-Gopalan, C 2006**, 'The 3' untranslated region of a soybean cytosolic glutamine synthetase (GS1) affects transcript stability and protein accumulation in transgenic alfalfa', *The Plant Journal*, vol. 45, no. 5, pp. 832-846.

**Ortega, JL, Roche, D & Sengupta-Gopalan, C 1999**, 'Oxidative turnover of soybean root glutamine synthetase. In vitro and in vivo studies', *Plant Physiology*, vol. 119, no. 4, pp. 1483-1496.

**Ortega, JL, Wilson, OL & Sengupta-Gopalan, C 2012**, 'The 5' untranslated region of the soybean cytosolic glutamine synthetase  $\beta$ 1 gene contains prokaryotic translation initiation signals and acts as a translational enhancer in plants', *Molecular Genetics and Genomics*, vol. 287, no. 11-12, pp. 881-893.

**Otie, V, Ping, A, John, N & Eneji, AE 2016**, 'Interactive Effects of Plant Growth Regulators and Nitrogen on Corn Growth and Nitrogen Use Efficiency', *Journal of Plant Nutrition*, no. just-accepted, pp. 00-00.

**Pang, J, Milroy, SP, Rebetzke, GJ & Palta, JA 2015**, 'The influence of shoot and root size on nitrogen uptake in wheat is affected by nitrate affinity in the roots during early growth', *Functional Plant Biology*, vol. 42, no. 12, pp. 1179-1189.

**Pantoja-Uceda, D, Neira, JL, Saelices, L, Robles-Rengel, R, Florencio, FJ, Muro-Pastor, MI & Santoro, J 2016**, 'Dissecting the binding between glutamine synthetase and its two natively unfolded protein inhibitors', *Biochemistry*, p. 4.

**Pathak, H, Mohanty, S, Jain, N & Bhatia, A 2010**, 'Nitrogen, phosphorus, and potassium budgets in Indian agriculture', *Nutrient Cycling in Agroecosystems*, vol. 86, no. 3, pp. 287-299.

**Paun, O, Forest, F, Fay, MF & Chase, MW 2009**, 'Hybrid speciation in angiosperms: parental divergence drives ploidy', *New Phytologist*, vol. 182, no. 2, pp. 507-518.

**Peltonen-Sainio, P, Jauhiainen, L & Nissilä, E 2012**, 'Improving cereal protein yields for high latitude conditions', *European Journal of Agronomy*, vol. 39, pp. 1-8.

**Pereira, S, Carvalho, H, Sunkel, C & Salema, R 1992**, 'Immunocytolocalization of glutamine synthetase in mesophyll and phloem of leaves of *Solanum tuberosum* L', *Protoplasma*, vol. 167, no. 1-2, pp. 66-73.

**Pérez-Delgado, CM, García-Calderón, M, Márquez, AJ & Betti, M 2015**, 'Reassimilation of photorespiratory ammonium in *Lotus japonicus* plants deficient in plastidic glutamine synthetase', *PloS One*, vol. 10, no. 6, p. e0130438.



**Peterman, TK & Goodman, HM 1991**, 'The glutamine synthetase gene family of *Arabidopsis thaliana* light-regulation and differential expression in leaves, roots and seeds', *Molecular and General Genetics MGG*, vol. 230, no. 1-2, pp. 145-154.

**Pineda-Hidalgo, KV, Méndez-Marroquín, KP, Alvarez, EV, Chávez-Ontiveros, J, Sánchez-Peña, P, Garzón-Tiznado, JA, Vega-García, MO & López-Valenzuela, JA 2013**, 'Microsatellite-based genetic diversity among accessions of maize landraces from Sinaloa in México', *Hereditas*, vol. 150, no. 4-6, pp. 53-59.

**Plaschke J, Ganai, MW, Röder, MS 1995**, Detection of genetic diversity in closely related bread wheat using microsatellite markers. *Theoretical and Applied Genetics*, vol. 91(6), pp.1001-1007.

**Plett, D, Holtham, L, Baumann, U, Kalashyan, E, Francis, K, Enju, A, Toubia, J, Roessner, U, Bacic, A & Rafalski, A 2016**, 'Nitrogen assimilation system in maize is regulated by developmental and tissue-specific mechanisms', *Plant Molecular Biology*, pp. 1-20.

**Pommel, B, Gallais, A, Coque, M, Quilleré, I, Hirel, B, Prioul, J, Andrieu, B & Floriot, M 2006**, 'Carbon and nitrogen allocation and grain filling in three maize hybrids differing in leaf senescence', *European Journal of Agronomy*, vol. 24, no. 3, pp. 203-211.

**Presterl, T, Seitz, G, Landbeck, M, Thiemt, E, Schmidt, W & Geiger, H 2003,** 'Improving nitrogen-use efficiency in European maize', *Crop Science*, vol. 43, no. 4, pp. 1259-1265.

**Prinsi, B & Espen, L 2015,** 'Mineral nitrogen sources differently affect root glutamine synthetase isoforms and amino acid balance among organs in maize', *BMC Plant Biology*, vol. 15, no. 1, p. 1.

**Pumphrey, M, Bai, J, Laudencia-Chingcuanco, D, Anderson, O & Gill, BS 2009,** 'Nonadditive expression of homoeologous genes is established upon polyploidization in hexaploid wheat', *Genetics*, vol. 181, no. 3, pp. 1147-1157.

**Purcino, A, Arellano, C, Athwal, G & Huber, S 1998,** 'Nitrate effect on carbon and nitrogen assimilating enzymes of maize hybrids representing seven eras of breeding', *Maydica*, vol. 43.

**Quail MA, Smith M, Coupland P, Otto DT, Harris RS, Connor RT, Bertoni A, Swerdlow PH, Yong Gu 2012,** 'A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers', *BMC Genomics*, p.13:341.

**Quan, X, Zeng, J, Ye, L, Chen, G, Han, Z, Shah, JM & Zhang, G 2016,** 'Transcriptome profiling analysis for two Tibetan wild barley accessions in responses to low nitrogen', *BMC Plant Biology*, vol. 16, no. 1, p. 1.

**Raina, S, Rani, V, Kojima, T, Ogihara, Y, Singh, K & Devarumath, R 2001,** 'RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species', *Genome*, vol. 44, no. 5, pp. 763-772.

**Rajcan, I & Tollenaar, M 1999,** 'Source: sink ratio and leaf senescence in maize: II. Nitrogen metabolism during grain filling', *Field Crops Research*, vol. 60, no. 3, pp. 255-265.

**Rawat, SR, Silim, SN, Kronzucker, HJ, Siddiqi, MY & Glass, AD 1999,** 'AtAMT1 gene expression and NH<sub>4</sub><sup>+</sup> uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels', *The Plant Journal*, vol. 19, no. 2, pp. 143-152.

**Reddy, MM & Ulaganathan, K 2015,** 'Nitrogen Nutrition, Its Regulation and Biotechnological Approaches to Improve Crop Productivity', *American Journal of Plant Sciences*, vol. 6, no. 18, p. 2745.

**Redinbaugh, MG & Campbell, WH 1993,** 'Glutamine synthetase and ferredoxin-dependent glutamate synthase expression in the maize (*Zea mays*) root primary response to nitrate (evidence for an organ-specific response)', *Plant Physiology*, vol. 101, no. 4, pp. 1249-1255.

**Reguera, M, Peleg, Z, Abdel-Tawab, YM, Tiimimbang, EB, Delatorre, CA & Blumwald, E 2013,** 'Stress-Induced Cytokinin Synthesis Increases Drought Tolerance through the Coordinated Regulation of', *Plant Physiology*, pp. 2.4.

**Rentsch, D, Schmidt, S & Tegeder, M 2007**, 'Transporters for uptake and allocation of organic nitrogen compounds in plants', *FEBS Letters*, vol. 581, no. 12, pp. 2281-2289.

**Rhodes, D, Rendon, G & Stewart, G 1975**, 'The control of glutamine synthetase level in *Lemna minor* L', *Planta*, vol. 125, no. 3, pp. 201-211.

**Rial-Lovera, K, Davies, W, Cannon, N & Conway, J 2016**, 'Influence of tillage systems and nitrogen management on grain yield, grain protein and nitrogen-use efficiency in UK spring wheat', *The Journal of Agricultural Science*, pp. 1-16.

**Riedel, J, Tischner, R, Mack, G 2001**, 'The chloroplastic glutamine synthetase (GS-2) of tobacco is phosphorylated and associated with 14-3-3 proteins inside the chloroplast', *Planta*, p. 213, 396-40.

**Rieseberg, LH & Blackman, BK 2010**, 'Speciation genes in plants', *Annals of Botany*, p. mcq126.

**Rieseberg, LH & Blackman, BK 2010**, 'Speciation genes in plants', *Annals of Botany*, p. mcq126.

**Rigby, H, Clarke, BO, Pritchard, DL, Meehan, B, Beshah, F, Smith, SR & Porter, NA 2016**, 'A critical review of nitrogen mineralization in biosolids-amended soil, the associated fertilizer value for crop production and potential for emissions to the environment', *Science of The Total Environment*, vol. 541, pp. 1310-1338.

**Rispail, N, Cliquet, J-B, Ourry, A & Le Deunff, E 2004**, 'Putative role of GABA as a long distance signal in up-regulation of nitrate uptake in *Brassica napus* L. Plant Cell Environ', Plant, Cell and Environment, vol. 27, pp. 1035-1046.

**Rochat, C & Boutin, JP 1992**, 'Temporary storage compounds and sucrose-starch metabolism in seed coats during pea seed development (*Pisum sativum*)', Physiologia Plantarum, vol. 85, no. 4, pp. 567-572.

**Rodríguez-Lázaro, D, Lombard, B, Smith, H, Rzezutka, A, D'Agostino, M, Helmuth, R, Schroeter, A, Malorny, B, Miko, A & Guerra, B 2007**, 'Trends in analytical methodology in food safety and quality: monitoring microorganisms and genetically modified organisms', Trends in Food Science & Technology, vol. 18, no. 6, pp. 306-319.

**Rolny, N, Bayardo, M, Guiamet, JJ & Costa, L 2016**, 'Nitrogen fertilization increases ammonium accumulation during senescence of barley leaves', Acta Physiologiae Plantarum, vol. 38, no. 4, pp. 1-11.

**Rost, B, Yachdav, G & Liu, J 2004**, 'The predictprotein server', Nucleic Acids Research, vol. 32, no. supplementary 2, pp. W321-W326.

**Sakakibara, H, Shimizu, H, Hase, T, Yamazaki, Y, Takao, T, Shimonishi, Y & Sugiyama, T 1996**, 'Molecular identification and characterization of cytosolic isoforms of glutamine synthetase in maize roots', Journal of Biological Chemistry, vol. 271, no. 47, pp. 29561-29568.

**Salichos, L & Rokas, A 2013**, 'Inferring ancient divergences requires genes with strong phylogenetic signals', *Nature*, vol. 497, no. 7449, pp. 327-331.

**Salo, T, Palosuo, T, Kersebaum, K, Nendel, C, Angulo, C, Ewert, F, Bindi, M, Calanca, P, Klein, T & Moriondo, M 2016**, 'Comparing the performance of 11 crop simulation models in predicting yield response to nitrogen fertilization', *The Journal of Agricultural Science*, pp. 1-23.

**Scheible, W-R, Morcuende, R, Czechowski, T, Fritz, C, Osuna, D, Palacios-Rojas, N, Schindelasch, D, Thimm, O, Udvardi, MK & Stitt, M 2004**, 'Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen', *Plant Physiology*, vol. 136, no. 1, pp. 2483-2499.

**Schena, M, Shalon, D, Davis, RW & Brown, PO 1995**, 'Quantitative monitoring of gene expression patterns with a complementary DNA microarray', *Science*, vol. 270, no. 5235, p. 467.

**Schildhauer, J, Wiedemuth, K & Humbeck, K 2008**, 'Supply of nitrogen can reverse senescence processes and affect expression of genes coding for plastidic glutamine synthetase and lysine-ketoglutarate reductase/saccharopine dehydrogenase', *Plant Biology*, vol. 10, no. s1, pp. 76-84.

**Schoppach, R, Taylor, JD, Majerus, E, Claverie, E, Baumann, U, Suchecki, R, Fleury, D & Sadok, W 2016**, 'High resolution mapping of traits related to whole-plant transpiration under increasing evaporative demand in wheat', *Journal of Experimental Botany*, vol. 67, no. 9, pp. 2847-2860.

**Scofield, SR, Huang, L, Brandt, AS & Gill, BS 2005**, 'Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway', *Plant Physiology*, vol. 138, no. 4, pp. 2165-2173.

**Seabra, AR & Carvalho, HG 2015**, 'Glutamine synthetase in *Medicago truncatula*, unveiling new secrets of a very old enzyme', *Frontiers in Plant Science*, vol. 6.p. 5.

**Seabra, AR, Vieira, CP, Cullimore, JV & Carvalho, HG 2010**, '*Medicago truncatula* contains a second gene encoding a plastid located glutamine synthetase exclusively expressed in developing seeds', *BMC Plant Biology*, vol. 10, no. 1, p. 183.

**Serrago, RA, Alzueta, I, Savin, R & Slafer, GA 2013**, 'Understanding grain yield responses to source–sink ratios during grain filling in wheat and barley under contrasting environments', *Field Crops Research*, vol. 150, pp. 42-51.

**Shavrukov, Y 2016**, 'Comparison of SNP and CAPS markers application in genetic research in wheat and barley', *BMC Plant Biology*, vol. 16, no. 1, p. 47.

**Sheldrick, WF 1987**, *World Nitrogen Survey*, The World Bank.

**Shrawat, AK, Carroll, RT, DePauw, M, Taylor, GJ & Good, AG 2008**, 'Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase', *Plant Biotechnology Journal*, vol. 6, no. 7, pp. 722-732.

**Silveira, JAG, de Almeida Viégas, R, da Rocha, IMA, Moreira, ACdOM, de Azevedo Moreira, R & Oliveira, JTA 2003**, 'Proline accumulation and glutamine synthetase activity are increased by salt-induced proteolysis in cashew leaves', *Journal of Plant Physiology*, vol. 160, no. 2, pp. 115-123.

**Sinclair, T & de Wit, Ct 1975**, 'Photosynthate and nitrogen requirements for seed production by various crops', *Science*, vol. 189, no. 4202, pp. 565-567.

**Singh, D & Singh, S 2016**, 'Leaf area index retrieval using Hyperion EO-1 data-based vegetation indices in Himalayan forest system', in *SPIE Asia-Pacific Remote Sensing*, International Society for Optics and Photonics, pp. 98800U-98800U-98811.

**Singh, KK & Ghosh, S 2013**, 'Regulation of glutamine synthetase isoforms in two differentially drought-tolerant rice (*Oryza sativa* L.) cultivars under water deficit conditions', *Plant Cell Reports*, vol. 32, no. 2, pp. 183-193.

**Smith, D & Cooper, M 2004**, 'Changes in performance, parentage, and genetic diversity of successful corn hybrids, 1930-2000', *Corn: Origin, History, Technology, and Production*, vol. 4, p. 65.



**Smith, S, John, B, Smith, F & Nicholas, D 1985**, 'Activity of glutamine synthetase and glutamate dehydrogenase in *Trifolium subterraneum* L. and *Allium cepa* L: effects of mycorrhizal infection and phosphate nutrition', *New Phytologist*, vol. 99, no. 2, pp. 211-227.

**Somerville, CR & Ogren, WL 1980**, 'Inhibition of photosynthesis in *Arabidopsis* mutants lacking leaf glutamate synthase activity', *AGRIS*, p. 6.

**Sonoda, Y, Ikeda, A, Saiki, S, Yamaya, T & Yamaguchi, J 2003**, 'Feedback regulation of the ammonium transporter gene family AMT1 by glutamine in rice', *Plant and Cell Physiology*, vol. 44, no. 12, pp. 1396-1402.

**Spano, G, Di Fonzo, N, Perrotta, C, Platani, C, Ronga, G, Lawlor, D, Napier, J & Shewry, P 2003**, 'Physiological characterization of 'stay green' mutants in durum wheat', *Journal of Experimental Botany*, vol. 54, no. 386, pp. 1415-1420.

**Stadtman, ER 2001**, 'The story of glutamine synthetase regulation', *Journal of Biological Chemistry*, vol. 276, no. 48, pp. 44357-44364.

**Stewart, G & Rhodes, D 1978**, 'Nitrogen metabolism of halophytes III. Enzymes of ammonia assimilation', *New Phytologist*, vol. 80, no. 2, pp. 307-316.

**Suárez, MF, Avila, C, Gallardo, F, Cantón, FR, García-Gutiérrez, A, Claros, MG & Cánovas, FM 2002**, 'Molecular and enzymatic analysis of ammonium assimilation in woody plants', *Journal of Experimental Botany*, vol. 53, no. 370, pp. 891-904.

**Sukanya, R, Li, M-g & Snustad, DP 1994**, 'Root-and shoot-specific responses of individual glutamine synthetase genes of maize to nitrate and ammonium', *Plant Molecular Biology*, vol. 26, no. 6, pp. 1935-1946.

**Sullivan, JP, Lavoué, S & Hopkins, CD 2002**, 'Discovery and phylogenetic analysis of a riverine species flock of African electric fishes (Mormyridae: Teleostei)', *Evolution*, vol. 56, no. 3, pp. 597-616.

**Swarbreck, SM, Defoin-Platel, M, Hindle, M, Saqi, M & Habash, DZ 2011**, 'New perspectives on glutamine synthetase in grasses', *Journal of Experimental Botany*, vol. 62, no. 4, pp. 1511-1522.

**Swigoňová, Z, Lai, J, Ma, J, Ramakrishna, W, Llaca, V, Bennetzen, JL & Messing, J 2004**, 'Close split of sorghum and maize genome progenitors', *Genome Research*, vol. 14, no. 10a, pp. 1916-1923.

**Sylvester-Bradley, R & Kindred, DR 2009**, 'Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency', *Journal of Experimental Botany*, vol. 60, no. 7, pp. 1939-1951.

**Tabuchi, M, Abiko, T & Yamaya, T 2007**, 'Assimilation of ammonium ions and reutilization of nitrogen in rice (*Oryza sativa* L.)', *Journal of Experimental Botany*, vol. 58, no. 9, pp. 2319-2327.

**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', *The Plant Journal*, vol. 42, no. 5, pp. 641-651.

**Taira, M, Valtersson, U, Burkhardt, B & Ludwig, RA 2004**, '*Arabidopsis thaliana* GLN2-encoded glutamine synthetase is dual targeted to leaf mitochondria and chloroplasts', *The Plant Cell*, vol. 16, no. 8, pp. 2048-2058.

**Tanksley, SD & McCouch, SR 1997**, 'Seed banks and molecular maps: unlocking genetic potential from the wild', *Science*, vol. 277, no. 5329, pp. 1063-1066.

**Taulemesse, F, Le Gouis, J, Gouache, D, Gibon, Y & Allard, V 2016**, 'Bread Wheat (*Triticum aestivum* L.) Grain Protein Concentration Is Related to Early Post-Flowering Nitrate Uptake under Putative Control of Plant Satiety Level', *PloS One*, vol. 11, no. 2, p. 0149668.

**Taylor, JR, Johnson, SK, Taylor, J, Njila, S & Jackaman, C 2016**, 'Oxidation of commercial ( $\alpha$ -type) zein with hydrogen peroxide improves its hydration and dramatically increases dough extensibility even below its glass transition temperature', *Journal of Cereal Science*, vol. 70, pp. 108-115.

**Team, RC 2013**, 'R: A language and environment for statistical computing', R-Project, p. 1-13.

**Temple, SJ, Knight, TJ, Unkefer, PJ & Sengupta-Gopalan, C 1993**, 'Modulation of glutamine synthetase gene expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: molecular and biochemical analysis', *Molecular and General Genetics MGG*, vol. 236, no. 2-3, pp. 315-325.

**Tercé-Laforgue, T, Mäck, 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*, vol. 120, no. 2, pp. 220-228.

**Thompson, JD, Gibson, TJ, Plewniak, F, Jeanmougin, F & Higgins, DG 1997**, 'The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools', *Nucleic Acids Research*, vol. 25, no. 24, pp. 4876-4882.

**Thomsen, HC, Eriksson, D, Møller, IS & Schjoerring, JK 2014**, 'Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency?', *Trends in Plant Science*, vol. 19, no. 10, pp. 656-663.

**Tian, F, Bradbury, PJ, Brown, PJ, Hung, H, Sun, Q, Flint-Garcia, S, Rocheford, TR, McMullen, MD, Holland, JB & Buckler, ES 2011**, 'Genome-wide association study of leaf architecture in the maize nested association mapping population', *Nature Genetics*, vol. 43, no. 2, pp. 159-162.

**Tian, G-W, Mohanty, A, Chary, SN, Li, S, Paap, B, Drakakaki, G, Kopec, CD, Li, J, Ehrhardt, D & Jackson, D 2004**, 'High-throughput fluorescent tagging of full-length Arabidopsis gene products in planta', *Plant Physiology*, vol. 135, no. 1, pp. 25-38.

**Tian, H, Fu, J, Drijber, RA & Gao, Y 2015**, 'Expression patterns of five genes involved in nitrogen metabolism in two winter wheat (*Triticum aestivum* L.) accessions with high and low nitrogen utilization efficiencies', *Journal of Cereal Science*, vol. 61, pp. 48-54.

**Tian, Y-S, Xu, J, Zhao, W, Xing, X-J, Fu, X-Y, Peng, R-H & Yao, Q-H 2015**, 'Identification of a phosphinothricin-resistant mutant of rice glutamine synthetase using DNA shuffling', *Scientific Reports*, vol. 5.

**Tobin, AK & Yamaya, T 2001**, 'Cellular compartmentation of ammonium assimilation in rice and barley', *Journal of Experimental Botany*, vol. 52, no. 356, pp. 591-604.

**Tobin, EM & Silverthorne, J 1985**, 'Light regulation of gene expression in higher plants', *Annual Review of Plant Physiology*, vol. 36, no. 1, pp. 569-593.

**Torreira, E, Seabra, AR, Marriott, H, Zhou, M, Llorca, Ó, Robinson, CV, Carvalho, HG, Fernández-Tornero, C & Pereira, PJB 2014**, 'The structures of cytosolic and plastid-located glutamine synthetases from *Medicago truncatula* reveal a common and dynamic architecture', *Acta Crystallographica Section D: Biological Crystallography*, vol. 70, no. 4, pp. 981-993.

**Tsukaguchi, T, Nitta, S & Matsuno, Y 2016**, 'Cultivar differences in the grain protein accumulation ability in rice (*Oryza sativa* L.)', *Field Crops Research*, vol. 192, pp. 110-117.

**Tukey, JW 1947**, 'Non-Parametric Estimation II. Statistically Equivalent Blocks and Tolerance Regions-The Continuous Case', *The Annals of Mathematical Statistics*, pp. 529-539.

**Udvardi, MK, Czechowski, T & Scheible, W-R 2008**, 'Eleven golden rules of quantitative RT-PCR', *The Plant Cell*, vol. 20, no. 7, pp. 1736-1737.

**Unno, H, Uchida, T, Sugawara, H, Kurisu, G, Sugiyama, T, Yamaya, T, Sakakibara, H, Hase, T & Kusunoki, M 2006**, 'Atomic Structure of Plant Glutamine synthetase a key enzyme for plant productivity', *Journal of Biological Chemistry*, vol. 281, no. 39, pp. 29287-29296.

**Urbanczyk-Wochniak, E & Fernie, AR 2005**, 'Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (*Solanum lycopersicum*) plants', *Journal of Experimental Botany*, vol. 56, no. 410, pp. 309-321.

**Urriola, J & Rathore, KS 2015**, 'Overexpression of a glutamine synthetase gene affects growth and development in sorghum', *Transgenic Research*, vol. 24, no. 3, pp. 397-407.

**Valkama, E, Salo, T, Esala, M & Turtola, E 2013**, 'Nitrogen balances and yields of spring cereals as affected by nitrogen fertilization in northern conditions: A meta-analysis', *Agriculture, Ecosystems & Environment*, vol. 164, pp. 1-13.

**Van Drecht, G, Bouwman, A, Knoop, J, Beusen, A & Meinardi, C 2003**, 'Global modeling of the fate of nitrogen from point and nonpoint sources in soils, groundwater, and surface water', *Global Biogeochemical Cycles*, vol. 17, no. 4.

**Van Nguyen, T, Lee, JE, Sweredoski, MJ, Yang, S-J, Jeon, S-J, Harrison, JS, Yim, J-H, Lee, SG, Handa, H & Kuhlman, B 2016**, 'Glutamine Triggers Acetylation-Dependent Degradation of Glutamine Synthetase via the Thalidomide Receptor Cereblon', *Molecular Cell*, vol. 61, no. 6, pp. 809-820.

**Van Sanford, D & MacKown, C 1986**, 'Variation in nitrogen use efficiency among soft red winter wheat accessions', *Theoretical and Applied Genetics*, vol. 72, no. 2, pp. 158-163.

**Vandesompele, J, De Preter, K, Pattyn, F, Poppe, B, Van Roy, N, De Paepe, A & Speleman, F 2002**, 'Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes', *Genome Biology*, vol. 3, no. 7, p. 1.

**Verbyla, AP, Taylor, JD, Verbyla, KL, 2012**, 'RWGAIM: An efficient high dimensional random whole genome average (QTL) interval mapping approach', *Genetics Research*, vol.94, pp. 291-306.

**Vicente, R, Pérez, P, Martínez-Carrasco, R, Usadel, B, Kostadinova, S & Morcuende, R 2015**, 'Quantitative RT-PCR Platform to Measure Transcript Levels of C and N Metabolism-Related Genes in Durum Wheat: Transcript Profiles in Elevated [CO<sub>2</sub>] and High Temperature at Different Levels of N Supply', *Plant and Cell Physiology*, vol. 56, no. 8, pp. 1556-1573.

**Vincent, R, Fraiser, V, Chaillou, S, Limami, MA, Deleens, E, Phillipson, B, Douat, C, Boutin, J-P & Hirel, B 1997**, 'Overexpression of a soybean gene encoding cytosolic glutamine synthetase in shoots of transgenic *Lotus corniculatus* L. plants triggers changes in ammonium assimilation and plant development', *Planta*, vol. 201, no. 4, pp. 424-433.

**Vitousek, PM, Aber, JD, Howarth, RW, Likens, GE, Matson, PA, Schindler, DW, Schlesinger, WH & Tilman, DG 1997**, 'Human alteration of the global nitrogen cycle: sources and consequences', *Ecological Applications*, vol. 7, no. 3, pp. 737-750.

**Wallsgrave, RM, Turner, JC, Hall, NP, Kendall, AC & Bright, SW 1987**, 'Barley mutants lacking chloroplast glutamine synthetase - biochemical and genetic analysis', *Plant Physiology*, vol. 83, no. 1, pp. 155-158.

**Wang, H-Z, Feng, S-G, Lu, J-J, Shi, N-N & Liu, J-J 2009**, 'Phylogenetic study and molecular identification of 31 *Dendrobium* species using inter-simple sequence repeat (ISSR) markers', *Scientia Horticulturae*, vol. 122, no. 3, pp. 440-447.

**Wang, Q, Zhao, Y, Luo, W, Li, R, He, Q, Fang, X, De Michele, R, Ast, C, von Wirén, N & Lin, J 2013**, 'Single-particle analysis reveals shutoff control of the *Arabidopsis* ammonium transporter AMT1; 3 by clustering and internalization', *Proceedings of the National Academy of Sciences*, vol. 110, no. 32, pp. 13204-13209.



**Wang, S, Wong, D, Forrest, K, Allen, A, Chao, S, Huang, BE, Maccaferri, M, Salvi, S, Milner, SG & Cattivelli, L 2014**, 'Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array', *Plant Biotechnology Journal*, vol. 12, no. 6, pp. 787-796.

**Wang, X, Wei, Y, Shi, L, Ma, X & Theg, SM 2015**, 'New isoforms and assembly of glutamine synthetase in the leaf of wheat (*Triticum aestivum* L.)', *Journal of Experimental Botany*, p. 388.

**Wang, Y, Fu, B, Pan, L, Chen, L, Fu, X & Li, K 2013**, 'Overexpression of Arabidopsis Dof1, GS1 and GS2 enhanced nitrogen assimilation in transgenic tobacco grown under low-nitrogen conditions', *Plant Molecular Biology Reporter*, vol. 31, no. 4, pp. 886-900.

**Wei, G, Zhou, Z, Guo, Y, Dong, Y, Dang, H, Wang, Y & Ma, J 2014**, 'Long-term effects of tillage on soil aggregates and the distribution of soil organic carbon, total nitrogen, and other nutrients in aggregates on the semi-arid loess plateau, China', *Arid Land Research and Management*, vol. 28, no. 3, pp. 291-310.

**Wei, S, Wang, X, Shi, D, Li, Y, Zhang, J, Liu, P, Zhao, B & Dong, S 2016**, 'The mechanisms of low nitrogen induced weakened photosynthesis in summer maize (*Zea mays* L.) under field conditions', *Plant Physiology and Biochemistry*, vol. 105, pp. 118-128.

**Weissmann, S, Ma, F, Furuyama, K, Gierse, J, Berg, H, Shao, Y, Taniguchi, M, Allen, DK & Brutnell, TP 2016**, 'Interactions of C4 subtype metabolic activities and transport in maize are revealed through the characterization of *dct2* mutants', *The Plant Cell*, vol. 28, no. 2, pp. 466-484.

**West, BT, Welch, KB & Galecki, AT 2014**, *Linear mixed models: a practical guide using statistical software*, CRC Press.

**Wheeler, QD 2000**, 'A Critique from the Wheeler and Platnick Phylogenetic Species Concept Perspective: Problems with Alternative Concepts of Species', *Species concepts and phylogenetic theory: A Debate*, p. 133.

**Winfield, MO, Allen, AM, BurrIDGE, AJ, Barker, GL, Benbow, HR, Wilkinson, PA, Coghill, J, Waterfall, C, Davassi, A & Scopes, G 2015**, 'High-density SNP genotyping array for hexaploid wheat and its secondary and tertiary gene pool', *Plant Biotechnology Journal*, pp. 2-6.

**Wolfe, MD, Rabbi, IY, Egesi, C, Hamblin, M, Kawuki, R, Kulakow, P, Lozano, R, Carpio, DPD, Ramu, P & Jannink, J-L 2016**, 'Genome-Wide Association and Prediction Reveals Genetic Architecture of Cassava Mosaic Disease Resistance and Prospects for Rapid Genetic Improvement', *The Plant Genome*, p. 4-6.

**Woolfolk, C & Stadtman, E 1967**, 'Regulation of glutamine synthetase: III. Cumulative feedback inhibition of glutamine synthetase from *Escherichia coli*', *Archives of Biochemistry and Biophysics*, vol. 118, no. 3, pp. 736-755.

**Xie, W & Nevo, E 2008**, 'Wild emmer: genetic resources, gene mapping and potential for wheat improvement', *Euphytica*, vol. 164, no. 3, pp. 603-614.

**Xu, G, Fan, X & Miller, AJ 2012**, 'Plant nitrogen assimilation and use efficiency', *Annual Review of Plant Biology*, vol. 63, pp. 153-182.

**Xu, H, Liu, C, Lu, R, Guo, G, Chen, Z, He, T, Gao, R, Li, Y & Huang, J 2016**, 'The difference in responses to nitrogen deprivation and re-supply at seedling stage between two barley genotypes differing nitrogen use efficiency', *Plant Growth Regulation*, vol. 79, no. 1, pp. 119-126.

**Xu, J, H & Messing, J 2006**, 'Maize haplotype with a helitron-amplified cytidine deaminase gene copy', *BMC Genetics*, vol. 7, no. 1, p. 52.

**Xu, Y, Zhang, R, Joachimiak, A, Carr, PD, Huber, T, Vasudevan, SG & Ollis, DL 2004**, 'Structure of the N-terminal domain of *Escherichia coli* glutamine synthetase adenylyltransferase', *Structure*, vol. 12, no. 5, pp. 861-869.

**Yamaya, T & Kusano, M 2014**, 'Evidence supporting distinct functions of three cytosolic glutamine synthetases and two NADH-glutamate synthases in rice', *Journal of Experimental Botany*, p. 103.

**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', *Journal of Experimental Botany*, vol. 53, no. 370, pp. 917-925.

**Yang, M, Dong, J, Zhao, W & Gao, X 2016**, 'Characterization of proteins involved in early stage of wheat grain development by iTRAQ', *Journal of Proteomics*, vol. 136, pp. 157-166.

**Yang, Y-C, Zhang, M, Zheng, L, Cheng, D-D, Liu, M & Geng, Y-Q 2011,** 'Controlled release urea improved nitrogen use efficiency, yield, and quality of wheat', *Agronomy Journal*, vol. 103, no. 2, pp. 479-485.

**Yeo, IK & Johnson, RA 2000,** 'A new family of power transformations to improve normality or symmetry', *Biometrika*, vol. 87, no. 4, pp. 954-959.

**Yousfi, S, Márquez, AJ, Betti, M, Araus, JL & Serret, MD 2016,** 'Gene expression and physiological responses to salinity and water stress of contrasting durum wheat genotypes', *Journal of Integrative Plant Biology*, vol. 58, no. 1, pp. 48-66.

**Yuan, H, Cheung, C, Poolman, MG, Hilbers, PA & Riel, NA 2016,** 'A genome-scale metabolic network reconstruction of tomato (*Solanum lycopersicum* L.) and its application to photorespiratory metabolism', *The Plant Journal*, vol. 85, no. 2, pp. 289-304.

**Zhang S, Chen W, Xin, L, Gao Z, Hou Y, Yu, X, Zhang, Z, Shenchun Qu, S 2014,** 'Genomic variants of genes associated with three horticultural traits in apple revealed by genome re-sequencing', *Horticulture Research*, p. 3.

**Zhang, L, Liu, D, Guo, X, Yang, W, Sun, J, Wang, D, Zhang, A & Zhang, A 2010,** 'Distribution in genome of Quantitative trait loci (QTL) for yield and yield-related traits in common wheat (*Triticum aestivum* L.)', *J. Integrated Plant Biology*, vol. 52, pp. 996-1007.

**Zhang, Y, Dai, X, Jia, D, Li, H, Wang, Y, Li, C, Xu, H & He, M 2016,** 'Effects of plant density on grain yield, protein size distribution, and bread making quality

of winter wheat grown under two nitrogen fertilisation rates', *European Journal of Agronomy*, vol. 73, pp. 1-10.

**Zhao, X-Q & Shi, W-M 2006**, 'Expression analysis of the glutamine synthetase and glutamate synthase gene families in young rice (*Oryza sativa*) seedlings', *Plant Science*, vol. 170, no. 4, pp. 748-754.

**Zheng, J, Zhang, Y & Wang, C 2015**, 'Molecular functions of genes related to grain shape in rice', *Breeding Science*, vol. 65, no. 2, p. 120.

**Zhou, B, Serret, MD, Elazab, A, Bort Pie, J, Araus, JL, Aranjuelo, I & Sanz-Sáez, Á 2016**, 'Wheat ear carbon assimilation and nitrogen remobilization contribute significantly to grain yield', *Journal of Integrative Plant Biology*.

**Zhu, CQ, Zhu, XF, Hu, AY, Wang, C, Wang, B, Dong, XY & Shen, RF 2016**, 'Differential effects of nitrogen forms on cell wall phosphorus remobilization in rice (*Oryza sativa*) are mediated by nitric oxide, pectin content and the expression of the phosphate transporter OsPT2', *Plant Physiology*, p. pp. 00176.02016.

## Internet Web Links Cited

[http://modraseeds.com.au/pdf/AGT\\_Gladius.pdf](http://modraseeds.com.au/pdf/AGT_Gladius.pdf)

[http://tmcalculator.neb.com/#/!](http://tmcalculator.neb.com/#/)

<http://www.acpfg.com.au/index>

<http://www.bioline.com/au/immolase-dna-polymerase.html>

<http://www.bio-rad.com/en-au/category/gel-imaging-systems>

<http://www.gcbiotech.com/liquidhandlingautomation?pid=56&sid=116:HydroCycler-16>

<http://www.geneious.com>

<http://www.geneious.com>, Kearse et al. 2012

<http://www.geneious.com/>; Biomatters Limited

<http://www.gmi-inc.com/mj-research-tetrad-ptc-225-thermal-cycler.html>

[http://www.illumina.com/products/nextera\\_dna\\_library\\_prep\\_kit.html](http://www.illumina.com/products/nextera_dna_library_prep_kit.html)

<http://www.itl.nist.gov/div898/handbook/pmd/section6/pmd624.htm>

<http://www.lgcgroup.com/products>

<http://www.lgcgroup.com/products/genotyping-instruments/>

<http://www.mn-net.com>

<http://www.nanodrop.com/>

<https://www.thermofisher.com/order/catalog/product/K310001>

<http://www.ncbi.nlm.nih.gov/gene>

<https://phytozome.jgi.doe.gov/pz/portal.html>

<https://www.agrf.org.au/services/sanger-sequencing>

<https://www.arabidopsis.org>

<https://www.ebi.ac.uk/Tools/msa/clustalw2/>

<https://www.ebi.ac.uk/Tools/msa/mafft/>), ClustalW

<https://www.geneworks.com.au>

<https://www.neb.com/tools-and-resources/interactive-tools>

<https://www.qiagen.com>

<https://www.thermofisher.com/>

<https://www.thermofisher.com/order/catalog/product/K310001>

<https://www.thermofisher.com/order/catalog/product/ND-2000>

<https://www.thermofisher.com/order/catalog/product/S33102>

<https://www.wageningenur.nl/en/show/Mapchart-2.30.htm>