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Investigating root traits for drought adaptation in barley: an insight into the genetics influencing root system architecture

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Abstract

Environmental events, such as drought, are predicted to increase in frequency and duration, and coupled with an expanding global population, improving cereal crop productivity and yield stability is crucial. A body of research suggests that roots may be fundamental to increasing crop yields, whereby optimised root systems could capture more water and nutrients with minimal metabolic costs. For efficient resource capture in most water-limited environments, a narrow and deep root architecture is likely advantageous. The central importance of the root system in plant productivity cannot be underestimated, yet the functional and genetic basis of root system architecture in cereal crops is relatively unknown.

In this thesis, the genetics underpinning root system architecture in barley (*Hordeum vulgare* L.) were investigated and the value of roots as a drought adaptive trait in barley was examined. In addition, the genetic variation in delayed-foliar senescence and flowering time were also explored to investigate any shared genetic control between above- and below-ground drought adaptation traits. Through the characterisation of three diverse barley populations, key genes influencing root system architecture were identified. Preliminary evidence for shared genetic control between delayed foliar senescence and root architecture was observed through co-located quantitative trait loci (QTL), specifically the gibberellic acid biosynthesis gene, *Hv20ox1*. Preliminary associations between time to flowering and seminal root traits were confirmed through the identification of *VERNALIZATION1* (*VRN1*) as a major gene influencing root architecture in barley. Whereby, *VRN1* is a key regulator of flowering behaviour in cereal crops. The research described in this thesis provides novel insight into the genetic control of root system architecture and reveals a new role for the previously described pathway for regulation of flowering in modulating the largely unexplored genetic architecture of root development in barley. The knowledge generated from this research may be harnessed in barley breeding programs to assist in the development of robust cultivars better adapted to the increasingly variable future climate.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Research Involving Human or Animal Subjects

No animal or human subjects were involved in this research

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Keywords

Barley, drought adaptation, root system architecture, stay-green, flowering time, *VERNALIZATION1*, quantitative trait loci, linkage mapping, genome-wide association mapping, breeding

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FoR code: 0604, Genetics, 50%
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List of abbreviations

2D	2-dimensional
3D	3-dimensional
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
AIC	Akaike information criteria
AM	Association mapping
BLUE	Best linear unbiased estimator
BLUP	Best linear unbiased predictor
Cas9	CRISPR-associated Protein9
CIM	Composite interval mapping
CMLM	Compressed mixed linear model
CO	CONSTANS
CRISPR	Clustered regularly interspaced short palindromic repeats
DAF	Department of Agriculture and Fisheries
DArT	Diversity Arrays Technology
DH	Doubled haploid
DRO1	*DEEPER ROOTING1*
DTA	Days to anthesis
ET	Environment type
FA	Factor analytic
FDR	False discovery rate
FT	*FLOWERING LOCUS T*
GA	Gibberellic acid
GAPIT	Genome association and prediction integrated tool
GBS	Genotype-by-sequencing
GPC	Grain protein content
GS	Genomic selection
GWAS	Genome-wide association study
HSD	Honest significant difference
ICIM	Inclusive composite interval mapping
LD	Linkage disequilibrium
MAGIC	multi-parent advance generation inter-cross

MAS	Marker-assisted selection
MEF	Management environment facilities
MET	Multi-environment trial
NAM	Nested-association mapping
NDVI	Normalised difference vegetative index
NGS	Next generation sequencing
NIL	Near-isogenic line
NRB	Northern region barley
PCA	Principal component analysis
QTL	Quantitative trait loci
RA	Root angle
RAPD	Random amplification of polymorphic DNA
RDW	Root dry weight
REML	Residual maximum likelihood
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred line
RL	Root length
RN	Root number
RS	Rate of senescence
RSR	Root-to-shoot ratio
RSS	Root system size
RV	Root volume
RWC	Relative water content
SGI	Stay-green integral
SNP	single nucleotide polymorphism
SUPER	Settlement of mixed linear model under progressively exclusive relationship
TFN10	Time from anthesis until senescence conclusion
UQ	University of Queensland
VRN1	*VERNALIZATION1*
VRN2	*VERNALIZATION2*
VRN3	*VERNALIZATION3*
WSC	Water soluble carbohydrate

Chapter 1

GENERAL INTRODUCTION

1.1 Imminent challenges for cereal crop production

The challenges ahead for cereal crop production cannot be underestimated, with a rising population and increasing call for animal products exacerbating the current demands on global production. During the mid-90s a similar situation was occurring in developing parts of the world, where there was wide-spread concern of famine and inability to feed the growing population. Fortunately, the Green Revolution negated these concerns with cereal crop production tripling in developing countries while the land area for cultivation only increased by approximately one-third (Pingali 2012). The green revolution was largely a product of adopting high-input systems and the introduction of short stature genes to improve lodging resistance in combination with genes conferring yield stability (Khush 1999). Plateauing yield gains in conjunction with water stress becoming one of the greatest yield constraints globally, the viability of these intensively managed systems is becoming increasingly uncertain (Bishopp and Lynch 2015). Thus, despite the gains of the Green Revolution, many challenges prevail and once again there is concern that crop production cannot meet the food availability needs of the expanding global population (Tilman et al. 2011). Couple this with a changing climate, where the duration and intensity of drought and warming are predicted to increase (Lobell et al. 2011; Dai 2013; Asseng et al. 2015; Lobell et al. 2015), improvement of cereal crop production and yield stability is vital.

Drought not only reduces cereal crop production, but also negatively impacts the import/export trade, inflates food prices, and can trigger or worsen famine in developing countries. Actual yields are dependent on seasonal and local environmental factors, but one of the more critical factors in Australia is rainfall. Annual crop yields decline under water-limited conditions (Lobell et al. 2015), a result of stress and the lack of drought-adapted

cultivars. Water stress not only impacts yield, but also grain quality, where a water deficit during the grain-filling period can alter grain morphology and protein content (Gilliham et al. 2017). Globally, our ability to meet the demands of the growing population and protect the grain quality of high value crops will depend on the development of cereal cultivars better adapted to less productive environments.

1.2 Barley, the widely adaptable cereal crop

Barley (*Hordeum vulgare*. L) is one of the oldest cultivated crops and the fourth largest cereal crop produced per tonne worldwide, with on average 135 million tonnes of barley produced per annum estimated in 2013 (Stanca et al. 2016). Approximately 50 million hectares of barley are grown annually in 106 countries (Stanca et al. 2016). Australia is one of the top 10 producers, with 3-4 million hectares of land harvested and an average barley production of 8 million tonnes annually. The gross production value of barley worldwide in 2014 was 30 billion US dollars, with Australia having the third highest production value. Australia is also the largest exporter of barley globally, responsible for more than 30% of the worldwide malting barley trade and 20% of the feed trade (Gordon 2016).

Animal feed is the most common use for barley, with 80-90% of worldwide barley production being utilised for this purpose. Following feed, 10% of barley is malted for use in alcoholic beverage production, and only a very small portion is consumed as human food (Stanca et al. 2016). Barley is an important food source in a number of African countries, with the population consuming almost half the barley produced for human consumption (Stanca et al. 2016). The threat from a changing climate and a predicted rise in the global population will likely lead to increased pressure on food security issues in these developing countries (Newton et al. 2011).

Barley is the most popular raw material for malting and beer production, with malt barley generally providing the greatest returns. Australia's malting selection rate is one of the highest worldwide, with 30-35% of the national crop meeting malt quality standards, resulting in an average of 2.3 million tonnes of malt barley produced annually. Grain quality is critical for malting and production of high quality malt. Grain protein content (GPC) is a key determinant of grain quality for malting and high GPC is undesirable. For example, to meet the malt 1 grade, GPC must be > 9% and ≤ 12% (Emebiri 2015). High GPC adversely effects the grain malting process by 1) increasing steeping time and the uneven uptake of water during steeping, 2) reduced malt due to uneven germination during malting, 3)

excessive enzymatic activity, 4) low extract yields, and 5) undesirable haze formation in finished beer (Burger et al. 1979). Too little GPC is also undesirable, leading to difficulties during the fermentation process (Emebiri 2015). It is important to note that beer can be produced from other grains, such as wheat, sorghum and millet, as well as from alternative starch sources.

Malt barley cultivars predominately grown in Australia are historically based on European germplasm, which was traditionally bred and developed for higher rainfall environments. Hence, Australian barley cultivars are relatively susceptible to drought stress. It is important to note that breeding efforts in Australia have led to the development of improved barley cultivars with significant yield advantages.

1.3 The complexity of drought adaptation

Drought stress pre- and post-anthesis can significantly influence the grain protein content and thus grain quality in barley. Pre-anthesis drought stress can cause reduced nitrogen uptake during the vegetative stage, decreasing yield potential and increasing the availability of nitrogen throughout grain-filling. Similarly, post-anthesis stress may lead to premature senescence, which can limit carbohydrate uptake and the resultant dilution of grain protein content. Previous research in Australian barley has shown that even mild post-anthesis drought stress leads to a reduced quantity and quality of starch (Savin and Nicolas 1999). Drought is defined as any environment where soil moisture is inadequate and cannot meet the transpiration needs of a crop throughout development (Tuberosa 2012). The coping mechanisms for plants under drought stress is so complex that it has been likened to cancer in mammalian biology, whereby crops have evolved intricate mechanisms encompassing various traits to cope (Pennis 2008). Traits that contribute to drought adaptation aim to increase water availability through conservation and improved access to water. Many drought adaptation traits have been described in cereal crops, including transpiration efficiency (Condon et al. 2002; Rebetzke et al. 2002; Richards et al. 2002; Krzeminska and Gorny 2003; Barbour et al. 2010), osmotic adjustment (Ludlow and Muchow 1990; Morgan 1995; Teulat et al. 1997; Gonzalez et al. 1999), reduced tillering (Dabbert et al. 2010; Kebrom and Richards 2013; Mitchell et al. 2013), early vigour (Rebetzke and Richards 1999; Rebetzke et al. 2004), lower canopy temperature (Elsayed et al. 2015), flowering time (Cattivelli et al. 2008; Shavrukov et al. 2017), delayed foliar senescence (Borrell and Hammer 2000; Borrell

et al. 2001; Christopher et al. 2008; Borrell et. al. 2014 a, b; Christopher et al. 2014; Christopher et al. 2018) and root system architecture (Manschadi et al. 2006; Manschadi et al. 2008; Manschadi et al. 2010; Tuberosa 2012; Christopher et al. 2013).

Despite the current research into drought adaptation, the genetic gain and heritability for yield in water-limited environments is relatively low, due to the annual variability of the crop water supply and the complex interaction between the genetics driving the inbuilt plant adaptation mechanisms and the environment (genotype × environment; Fischer et al. 2014). Previous research in barley, examining a large number of breeding lines, demonstrated that lines which were high yielding in high-input environments were low yielding under minimal inputs, thus highlighting the importance of G x E (Ceccarelli et al. 1992). A holistic approach combining drought adaptation traits and investigating the genotype × environment interactions may be more appropriate to improve genetic gain. Many studies propose that root traits are essential for drought adaptation and will pave the way to a second green revolution in low-input systems. Whereby, root systems are developed to capture more water and nutrients for minimal metabolic costs, thus freeing up energy for the crop to invest in other developmental process, such as above-ground biomass (Lynch 2007, 2013, 2015; Herder et al. 2010; Bishopp and Lynch 2015; Topp et al. 2016).

1.4 Project objectives

The overall objective of this PhD project is to better understand the genetic control of drought adaptation in barley, more specifically root system architecture, where current knowledge is limited. Previous studies in cereal crops indicate that root traits expressed at early plant developmental stages, such as a narrow seminal root angle, are associated with improved access to water stored deep in the soil profile. Furthermore, the relationship between root system architecture and yield is yet to be explored in barley.

Using a recently developed high-throughput phenotyping technique for root traits (i.e. the ‘clear pot’ method; Richard et al. 2015) and low-cost high-throughput genotyping, this study will investigate the genetics of seminal root traits in a large doubled haploid (DH) population, a panel of elite breeding lines, and a subset of lines from a nested-association mapping (NAM) population. The project will then explore the genetic relationship between seminal root traits and yield across 20 environments throughout the northern grain-growing region of Australia. Expanding on this, the possibility for shared genetic control between

seminal root traits and above-ground drought adaptation traits (i.e. delayed foliar senescence) will be explored in an attempt to provide greater insight into the genetic drivers of root system architecture.

Overall, this study aims to investigate root traits for drought adaptation in a holistic manner, examining roots in combination with above-ground canopy traits measured in the field. This study will provide a deeper understanding of the underlying genetics of both above- and below-ground traits related to water-use and water-access in barley. Information generated in this study can be harnessed in barley breeding programs to develop more robust cultivars for water-limited environments in Australia and around the world.

1.5 Thesis outline

This thesis encompasses a literature review (Chapter 2), four research chapters (Chapters 3 – 6) and a general discussion (Chapter 7). Chapter 2 briefly reviews the literature pertaining to roots as an important drought adaptive trait, root phenotyping methods, and genomic regions influencing roots in barley. In accordance with the project's objective to examine roots in a holistic manner, the review also details literature surrounding delayed canopy senescence and flowering time. Previous research in cereal crops has found associations between these two drought-adaptive traits and roots. To summarise, the most up-to-date molecular technology and its use for trait introgression in breeding programs is described.

The core of this thesis is constructed around four research chapters, where results from two out of the four chapters (Chapter 3 and Chapter 6) have been published in international referred journals (i.e. The Plant Genome and Molecular Plant, respectively). Chapter 3 investigates the genomic regions influencing seminal root traits in the ND24260 × Flagship DH population. Further, root trait QTL are aligned with previously reported QTL for abiotic stress tolerance to highlight important regions potentially underpinning drought adaptation in barley. Chapter 4 also maps QTL for seminal root traits, but this time in a panel of elite breeding lines developed for the northern grain-growing region of Australia with greater genetic diversity. This chapter also explores the genetic relationship between seminal root traits and yield in the breeding population across 20 environments spanning three growing seasons. To investigate any shared genetic control between delayed foliar senescence (stay-green) and root architecture, Chapter 5 details the characterisation of stay-green in a subset of the ND24260 × Flagship DH population and in a selection of lines from the multi-parent reference NAM

population across five field environments. QTL are then mapped for stay-green traits and aligned with previously detected root trait QTL in these two populations. Similarly, time to anthesis is also characterised in Chapter 5. Chapter 6 delves deeper into the relationship between flowering time and root system architecture by investigating the influences of *VERNALIZATION1* (*VRN1*), a key regulator of flowering time, on root system architecture in barley.

In the final chapter, Chapter 7, the major outcomes from all four research chapters are summarised. The implications of these findings are discussed from the perspective of pre-breeding and delivery of outcomes to the Australian barley breeding industry. The key limitations of the research are also discussed, along with strategies for future research expanding on the thesis outcomes.

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Chapter 2

LITERATURE REVIEW

2.1 Roots and drought adaptation

2.1.1 Root system architecture — an important drought adaptation trait

Roots play a vital role in resource acquisition and plant growth regulation by being the primary interface for water and nutrient capture. In addition, roots provide anchorage and interact with symbiotic organisms in the soil. Defined as the spatial distribution of roots throughout the soil space, root system architecture is a multifaceted plastic trait with many underlying processes, such as root elongation, curving and branch (Lynch 1995; Rich and Watt 2013). Furthermore, the root system architecture of a plant has been shown to influence the efficiency and timing of water capture and extraction in cereal crops (Kondo et al. 2000; Pennisi 2008). The fibrous root system of cereals is broadly divided into seminal roots, emerging from the primordia in the embryo of the seed, and nodal or secondary roots, developing from the lower nodal regions of the culm throughout tillering (Forster et al. 2007). The growth angle between the first pair of emerging seminal roots, described as the seminal root angle, was found to be representative of the mature root system architecture in wheat (Oyanagi et al. 1993; Manschadi et al. 2008; Manschadi et al. 2010). As a result, seminal root angle is considered a proxy trait for mature root system architecture in wheat (Sanguineti et al. 2007; Hamada et al. 2012; Christopher et al. 2013; Richard et al. 2015).

Water and nitrate, the two substances most highly acquired by crops, are extremely mobile, leaching into the deeper layers of the soil and reducing availability in the surface strata. Generally, levels of water and nitrate are higher deeper in the soil profile. These levels are further exaggerated throughout the season under terminal drought conditions, where the soil dries progressively from the surface layers as a result of evaporation, drainage and root uptake. Environment modelling of the northern grain-growing region of Australia identified

terminal drought stress as the most common pattern of water-stress for this growing area (Chenu et al. 2011). A deep-rooted phenotype is thought to be optimal for maximum resource capture under water-limited conditions for a number of crop species (Herder et al. 2010; Lynch 2011). Root foraging for resource acquisition is a high metabolic cost for crops (Lynch et al. 2005), thus plants with deep roots in close proximity to resources minimises the need for extensive foraging (Lynch 2013; Dathe et al. 2016). In addition, plants with the deep root phenotype are also believed to adequately access water and nutrients from the top layers of the soil through their shallow lateral roots (Lynch 2013). Thus, theoretically, a deep-rooted phenotype would be optimal for cereal crops grown across the northern grain-growing region of Australia, where in-season rainfall is limited and terminal drought stress is common. In support of this, a narrow root angle, representative of a steep and deep root system architecture, improves deep-soil foraging and water extraction under terminal drought in wheat, sorghum and rice (Uga et al. 2011; Manschadi et al. 2008; Mace et al. 2012; Uga et al. 2013).

Comparative analysis of root system architecture in drought-adapted and non-adapted wheat genotypes, revealed the drought-adapted genotype had a compact root system, allocating more roots at depth with a significantly greater root length (Manschadi et al. 2006). Furthermore, application of a cropping system model found that each additional millimetre of water extracted during grain-filling generated an extra 55 kg of yield per ha^{-1}. In addition, the deep-rooted phenotype provided an average yield benefit of 14.5% under water-limited conditions (Manschadi et al. 2006; Manschadi et al. 2010). This suggests a small modification to the root distribution to improve water access and uptake (i.e. 4-5 mm) can lead to large yield gains. Such increases may be the difference between making a profit in developed countries and survival in developing countries. In sorghum, research suggests that plants with a narrow root system have a greater proportion of roots at depth directly beneath the plant (Singh et al. 2012). Field studies in sorghum also found that 1 mm of additional water transpired during grain-filling can increase grain yield by approximately 50 kg ha^{-1} grown under terminal drought conditions (Borrell et al. 2014). Furthermore, in rice, following identification of a key gene influencing deeper rooting, field studies demonstrated a deep root system significantly improved yield under water-limited conditions (Uga et al. 2013). The latter studies highlight that increased access to water during grain-filling via a narrow and deep root system improves yield under water stress conditions and is thus an important drought adaptation trait. However, it is important to note sorghum and rice are C4 species

whereas wheat and barley are C3 species, which means their methods for fixing carbon during photosynthesis differs. C4 species are more adapted to warm growing conditions and have lower moisture requirements compared to C3 species due to their high water-use efficiencies and the presence of their CO_2 concentrating mechanisms (Edwards et al. 2004).

2.1.2 Roots are associated with above-ground drought adaptation traits

In sorghum, root system architecture is associated with the rate of foliar senescence, specifically a 'stay-green' phenotype (Borrell et al. 2014). Visually, foliar senescence is observed as the gradual yellowing of the leaves and stem of the crop, signifying initiation of the final crop developmental stage. Once initiated, foliar senescence involves the large remobilisation of nutrients from the senescing parts of the plant to the developing grain. A crop's ability to stay-green or delay the senescence process can be classed as either non-functional (or cosmetic) or functional. In cosmetic stay-greens, the early stages of chlorophyll catabolism is disrupted by a lesion resulting in the retention of chlorophyll and thus green leaves despite reduction in photosynthetic capacity typical of normal senescence (Thomas and Howarth 2000; Thomas and Ougham 2014). In contrast, functional stay-greens maintain green leaves and photosynthetic capacity throughout the grain-filling period due to either a delayed onset or a decreased rate of senescence (Thomas & Howarth 2000; Borrell et al. 2001; Thomas and Ougham 2014). Crops with functional stay-green often have increased yield due to a longer period of active photosynthesis during grain-filling (Borrell et al. 2000; Kichey et al. 2007; Gregersen et al. 2008; Gregersen et al. 2013).

The major known genetic controller of senescence onset in wheat is *Gpc-B1* (also referred to as *NAM-B1*) located on chromosome 6BS (Uauy et al. 2006a). Initially identified in tetraploid wheat (*Triticum turgidum* L.) as the first gene influencing grain protein content (Joppa et al. 1997), *Gpc-B1* also increases Fe and Zn concentrations in the grain (Cakamk et al. 2004; Distelfield et al. 2007). *Gpc-B1* has been shown to encode a NAC transcription factor (Uauy et al. 2006b; Distelfield and Fahima 2007), however modern wheat varieties carry the non-functional allele (Uauy et al. 2006b). In an across species analysis, *HvNAM-1* was identified as the orthologue of *Gpc-B1* in barley (Distelfield et al. 2008). Furthermore, both genes in wheat and barley had similar expression patterns, where gene expression was induced around heading and then continued to increase until leaves had completely yellowed (Uauy et al. 2006b). The functional allele of *Gpc-B1*, donated by ancestral wild wheat, and the allele of

HvNAM-1 donated by the cultivar Lewis, both accelerate the rate of senescence and increase grain protein content (Uauy et al. 2006b; Distelfield et al. 2008).

In sorghum, the physiological mechanism allowing crops to stay-green (or delay senescence) throughout the grain-filling period is due to an enhanced balance between supply and demand for water (Borrell et al. 2014). In support of this, Borrell et al. (2014) demonstrated that under terminal drought conditions, sorghum stay-green lines had a significantly improved water uptake during grain-filling. Furthermore, crop water-use during grain-filling was positively correlated with yield. As mentioned above, shared genetic control was identified between roots and foliar senescence in sorghum, whereby all quantitative trait loci (QTL) identified for narrow nodal root angle by Mace et al. (2012) co-located with QTL detected for stay-green (Borrell et al. 2014). Thus, it is likely that a narrow and deep root system contributes to delayed senescence via improving access to deep-stored soil moisture later in the season. Not only has delayed senescence been associated with increased yield, but it has also been correlated with less nitrogen remobilisation and reduced grain protein content (Gregersen et al. 2008). Furthermore, prolonged photosynthesis requires higher retention of nitrogen in the senescing leaves, resulting in increased carbohydrate accumulation in the grain, thus diluting the grain protein content (Gregersen 2011). Generally, reduced nutrient-use efficiency is undesirable, however in barley, low protein content is often targeted to achieve malt barley grades, therefore delayed foliar senescence and the resultant yield gains could be advantageous.

Root traits, such as root dry weight and root volume, have also been associated with flowering time in barley (Arifuzzaman et al. 2016). Adaptation through selection for early flowering is achieved by avoiding the majority of the drought stress, whereby anthesis and grain-filling occurs earlier in the growing season when there is a higher likelihood of sufficient soil moisture for crop-cycle completion (Cattivelli et al. 2008). In Australia, barley with a spring growth habit is predominately grown due to the warmer climate. Cereal cultivars without a vernalisation requirement (spring types) flower earlier than those with a vernalisation requirement (winter types). The spring growth habit is largely due to a loss of function mutation in the first intron of the key flowering gene, *VERNALIZATION1* (*VRN1*) (Fu et al. 2005). In plants with the winter growth habit *VRN1* is inhibited by *VERNALIZATION2* (*VRN2*), a flowering repressor that also prevents *VERNALIZATION3* (*VRN3*; orthologous to Arabidopsis *FLOWERING LOCUS T*) expression under extended photoperiods (Yan et al. 2006; Dubcovsky et al. 2005). Therefore, the three vernalization genes *VRN1, VRN2* and *VRN3,* form a regulatory loop for floral initiation. To relieve

inhibition of *VRN1* by *VRN2*, an extended period of cold exposure (vernalization) is required. Following vernalization, *VRN1* transcription is upregulated, consequently down regulating *VRN2* expression, thereby releasing *VRN3* to assist in promoting flowering once the long day photoperiod is also met (Chen and Dubcovsky 2012). For the spring growth habit, transcription of *VRN1* is independent of vernalization (Trevaskis et al. 2003; Yan et al. 2003), where the mutation in the first intron of *VRN1* alters the recognition site for *VRN2*, thus impeding its inhibitory effects (Yan et al. 2003).

Recently, Arifuzzaman et al. (2016) proposed a broader role for *VRN3* in that it also influences root development in barley. A less fibrous root system, with a reduced root dry weight and total volume, was identified in genotypes that were early flowering with a spring growth habit. In contrast, barley genotypes with a winter growth habit, and thus a longer vegetative growth phase, had a larger more vigorous root system (Arifuzzaman et al. 2016). Putative genetic associations were also identified between root dry weight and *VRN1*, where QTL mapped to the projected location of *VRN1* (Arifuzzaman et al. 2016). These findings suggest that root development could be regulated by key flowering genes in barley, yet further research exploring the relationship with root system architecture would help elucidate whether this is a dual mechanism for drought adaptation.

2.1.3 The environment and crop management contribute to drought adaptation

The genetics driving drought adaptation traits do not function in isolation but are influenced by interactions with the environment and crop management practices. As an example of this, the majority of drought adaptation traits are context dependent, where they are advantageous for yield stability in one drought environment, but not in another. Therefore, it is important to understand the most common drought patterns in a target growth environment. In addition, certain management practices, such as pre-crop fallow management, sowing time and seeding rate, can influence water availability during the growing season. To effectively optimise drought adaption in a specific environment the interactions between genetics, environment and management (G × E × M) need to be considered.

The Australian grain-growing region is vast, covering over 14 million hectares. Due to this large area, the growing conditions vary widely. For example, the soils range from deep clay to shallow sandy, and temperatures from sub-tropical to Mediterranean. Similarly, the rainfall patterns differ across growing regions from winter-dominant to summer-dominant accompanied by wide deviations in inter-annual rainfall (Potgieter et al. 2002; Chenu et al.

2013). Variability in rainfall patterns accompanied by differing soil types consequently results in different patterns of water-stress across Australia. In eastern Australia, where summer rainfall is dominant, water stress is generally long lasting. In contrast, in the western parts of Australia winter rainfall is dominant and water stress occurs in frequent small periods. Across the Australian cropping region, Chenu et al. (2013) described four drought environment types (ETs) that characterise the main water stress patterns occurring in Australia. The four ETs can be summarised as follows: ET1 was characterised as relatively stress-free with only short-term water deficits; ET2 was defined by minor water-stress predominately occurring during grain-filling but was relieved by maturity; ET3 was distinguished by more serious water-deficit occurring during the vegetative period but relieved prior to maturity; ET4 was categorised by the most severe water stress with an early onset and no relief throughout crop development. Each main cropping region experiences each of these ETs to some extent, however in eastern Australia ET3 and ET4 are most common and likely a consequence of the summer-dominant rainfall (Chenu et al. 2013; Lobell et al. 2015). Passioura (2006) also identified that management practices, specifically early season sowing and crop nutrition, can influence the severity of water stress.

Management practices impact crop yield potential in water-limited environments, with practices such as early season sowing, pre-crop fallow management and crop spacing contributing to water availability throughout the season (Radford et al. 1995; Fischer et al. 2014). For instance, in wheat it was demonstrated that osmoprimed wheat planted in narrow row spacings improved crop performance under early season (ET3) and terminal drought (ET4) stress (Hussain et al. 2016; Farooq et al. 2015). Further, soil water storage can be increased through improved agronomic practices during pre-crop fallow periods, for instance conservation tillage and fallow weed control (Radford et al. 1995; Fischer et al. 2014). Crop rotation and the exact crop sequence for rotation also impacts yield and soil nutrient availability, and are therefore important management strategies (Bender and van der Heijden 2015; Fischer et al. 2014). Drought adaptation in crops is complex, influenced by the interaction of the underlying genetics, with the environment and agronomic practices (i.e. G × E × M) and therefore all must be considered for optimal adaptation.

2.2 Root traits: The hidden below-ground and almost forgotten trait in barley

2.2.1 Phenotyping root traits: the past challenges and the bright future

Although root traits are strong candidates for improvement of drought adaptation, roots have been largely overlooked (Fleury et al. 2010). This is predominately due to the challenges faced in attempting to accurately and efficiently phenotype root systems. Traditionally, root trait phenotyping methods have been regarded as labour intensive, potentially unreliable, and un-relatable (Zhu et al. 2011). For instance, the 2-dimensional gel-filled chamber system that enables non-invasive, sequential measurements of root systems preserved in natural orientation, is limited when it comes to evaluating large numbers of lines (Bengough et al. 2004). Further, the artificial anaerobic environment may not reflect soil conditions in the field. Sand- and soil-based 3-dimensional methods, such as soil coring, monoliths or plant excavation, provide high precision root phenotypes yet are limited by their destructive analysis and single point measurement (Hargreaves et al. 2009; Kuchenbuch et al. 2009; Wasson et al. 2014; Chen et al. 2017). To mitigate the destructive nature of monoliths, a large field-based rhizo-lysimeter complex can be constructed, however the complex is expensive and limited by sample size (Eberbach et al. 2013). The shovelomics method based on visual scoring of excavated adult plants in situ (Trachsel et al. 2011) provides field-based accuracy, but is limited by sample size and its time consuming and destructive nature.

As an alternative to excavation, minirhizotrons, transparent tubes acting as a protective casing for cameras, can be deposited into the soil to image the developing root system architecture and measure elongation rate, root number and root length (Hendrick and Pregitzer 1992). Although non-destructive, minirhizotrons are limited by an inability to capture the entire root system in one image, the high cost and the accuracy required when inserting the tubes into the soil. Similarly, electrical capacitance measured between an electrode inserted at the base of a plant and another in the soil space of roots is often used as a non-destructive method to assay root mass (Chloupek 1972). However, the electrical model of which the method was based has been found to be inaccurate, measuring the cross-sectional area of roots at the soil surface rather than the total root mass (Dietrich et al. 2012). An alternate non-destructive method to phenotype roots in the field using a penetrometer was described and validated by Whalley et al. (2017). The penetrometer measures soil strength and is used as a proxy for soil water status, whereby as the soil dries and less soil water is available the soil becomes stronger. The study by Whalley et al. (2017) validated the use of a penetrometer to estimate soil water status by comparing the tool to other methods, such as electrical resistance tomography, electromagnetic inductance and measurements of soil water content, across three growing seasons. As a result, the penetrometer was found to be closely

related to soil matric potential, accurately discriminating between genotypes and reliable across seasons (Whalley et al. 2017). X-ray microtomography imaging and automated two-dimensional imaging, such as that used in the Growscreen-Rhizo system (Nagel et al. 2012), is non-invasive and non-harmful, but is restricted to small sample sizes and is expensive, rendering it inappropriate for screening large numbers (Pierret et al. 2003; Hargreaves et al. 2009). The soil-filled root chamber phenotyping platform described by Joshi et al. (2017) is high-throughput, non-destructive and is able to handle large sample sizes, yet requires a large financial commitment to set up. Recently, a high-throughput phenotyping method was described by Richard et al. (2015) which uses transparent pots and imaging to rapidly evaluate seminal root angle and number in wheat seedlings. This 'clear pot' method allows a large number of seedlings (600 per m^2) to be assayed within a very short period of time (7-10 days), while maintaining high heritability for root angle and root number (0.65 and 0.80, respectively; Richard et al. 2015). The clear pot method presents new opportunities for inexpensive phenotyping of large populations with potential application to commercial breeding programs.

Downstream of root phenotyping platforms is the software packages required to analyse and extract quantitative data from the images captured during phenotyping. There are a large number of image analysis tools dedicated to roots, such as RootScan (Burton et al. 2012), RootNav (Pound et al. 2013), GiARoots (Galkovskyi et al. 2012), IJ Rhizo (Pierret et al. 2013) and RooTrack (Mairhofer et al. 2012). Root phenotyping platforms and the accompanying analysis software are continually advancing, yet there is still scope for more high-throughput soil-based phenotyping platforms that are inexpensive with high repeatability.

2.2.2 An overview of root system architecture in barley

Evaluation of root traits in wild barley (*Hordeum spontaneum* L.) and landraces has highlighted the wide phenotypic variation evident for seminal root number, root length, root fresh weight, root dry weight and root water content in barley (Grando and Ceccarelli 1995; Tyagi et al. 2014). For instance, seminal root length varied significantly from 15 to 188 mm (Tyagi et al. 2014). Variation in root morphology was also reported in the Scarlett × ISR42-8 barley doubled haploid (DH) population, where the wild barley parent (ISR42-8) was reported to have increased root length compared to the modern parent and donated the dominant allele for this trait (Arifuzzaman et al. 2014; Sayed et al. 2017). Similarly, the wild parent, ICB181160, in a cross with the modern parent Cheri, was also found to have an

increased maximum root length (Arifuzzaman et al. 2016). Furthermore, studies comparing root length in commercial cultivars to a Syrian barley found the landrace to have 25% of its total root length below 40cm at anthesis compared to only 5% in the commercial lines (Gregory et al. 1992). In comparison to wild and landrace barley accessions, modern cultivars appear to have a more uniform root system typically comprised of several short seminal roots (Grando and Ceccarelli 1995). Introgression of the wild and landrace alleles driving increased root length into modern barley cultivars could improve root depth and lead to enhanced drought adaptation.

Seminal root angle and root number in barley vary substantially, and again the most beneficial phenotypes for drought adaptation appear to be present in wild and landrace barley. For instance, wild and landrace germplasm tend to have a narrow root angle (Bengough et al. 2004; Hargreaves et al. 2009; Sayed et al. 2017), which is thought to be a consequence of originating in water-limited environments where access to deep-stored soil moisture was critical for survival. In addition, wild and landrace lines appear to produce fewer roots than their modern counterparts. For example, the study by Bengough et al. (2004) reported a mean root angle for wild barley of 40° with an average root number of three. In comparison, modern cultivars were reported to have a wider angular spread of up to 120° and a higher root number of up to seven (Bengough et al., 2004). In addition, the wild parent, ISR42-8, in the Scarlett × ISR42-8 DH population was shown to have a narrower and more vertical root system architecture than the modern parent (Sayed et al. 2017). It is likely that domestication has caused inadvertent selection for root systems beneficial for exploiting topsoil nutrients and high in-season rainfall (i.e. wide root architecture and high root number) common to fertilised and irrigated post-green revolution agricultural soils. Further research in a larger collection of modern and wild barley lines is required to confirm these phenotypic trends detailed in the literature.

As a result of the challenges associated with accurately and efficiently phenotyping roots, the relationship between root traits and yield is still uncertain in barley. Previous research has examined the effect of root system size (RSS) on yield in a DH population grown in the field using electrical capacitance (Chloupek et al. 2006). A weak but significant genetic correlation (0.21) was found between RSS and yield in the DH population (Chloupek et al. 2006). In contrast, variable correlations were observed between RSS and yield in a small number of barley cultivars (Chloupek et al. 2010). Recently, Svačina et al. (2014) observed a significant correlation between RSS and yield, yet the study was limited by a

small sample size and a single environment. The latter research suggests there is likely a relationship between RSS and yield in barley, however, it is important to note that the electrical model used in the phenotyping method adopted by these studies has been shown to have inaccuracies (Dietrich et al. 2012). Seminal root length and weight have also been linked to yield in barley, where correlations were detected between traits and yield (length: r = 0.36–0.71 and weight: r = 0.38–0.61), however, only a small number of lines were examined using an artificial growth environment (Bertholdsson and Brantestam 2009). Previous research provides some preliminary evidence that roots and yield are inter-related, however the small sample sizes accompanied with phenotyping inaccuracies means further research is required to confirm the association.

To date, a number of QTL mapping studies have been performed for root traits in barley (Table 2.1). The first mapping study characterised the Derkado × B83-12/21/5 DH population for RSS using electrical capacitance (Chloupek et al., 2006). As a result, four QTL were identified on chromosomes 1HS, 3HL, 4HL and 7HS (Chloupek et al., 2006). Prior to this PhD project, two other root trait mapping studies were published, both mapping root traits in the Scarlett × ISR42-8 DH population. Naz et al. (2014) mapped QTL for root length, root dry weight and root volume in the DH population, where a number of QTL were identified for each trait across multiple chromosomes (Table 2.1). Similarly, multiple QTL were detected for root length, root dry weight and root-to-shoot ratio across multiple chromosomes in the DH population (Table 2.1; Arifuzzaman et al. 2014). Interestingly, each of the latter studies found different sets of QTL for the same trait even though the methods used appear relatively similar. This suggests there may have been issues with repeatability in one or both of the studies. The study by Naz et al. (2014) reports high to adequate heritabilities for each of the root traits examined, however the study by Arifuzzaman et al. 2014 does not provide any heritabilites (Table 2.1). Throughout the coarse of this PhD project, four more mapping studies for root traits in barley have been published, three of which map QTL in a DH population and one in a panel of global barley cultivars (Table 2.1). The first study to map QTL for seminal root angle and seminal root number in barley was published as part of this thesis (Chapter 3; Robinson et al. 2016). Following this, Sayed et al. (2017) mapped QTL for seminal root angle in the Scarlett × ISR42-8 DH population and detected two QTL on chromosome 3H and 7H. It is clear from the increase in mapping studies published for barley roots over the past four years that research in this area is gaining

in popularity. This is most likely a result of improvements in root trait phenotyping coupled with the decreased costs associated with genotyping.

Table 2.1 Summary of barley root trait QTL reported in literature, including details on the trait phenotyped, population, heritability of the trait, number of QTL identified, chromosomal location, percentage of variation explained by all QTL identified for the trait and reference for original study.

Root trait phenotyped	Population used	Broad-sense heritability	No. significant QTL detected	Chromosome	Total % variation explained by all QTL	Reference
Deep root ratio (>45°)	ISR42-8 × Scarlet	0.93	2	3HL, 7HS	28.70	Sayed et al. 2017
Deep root ratio (>60°)	ISR42-8 × Scarlet	0.75	2	4HL, 5HS	5.66	Sayed et al. 2017
Dry weight	ISR42-8 × Scarlet	Not reported	7	1HS, 2HS, 3HS, 4HS, 5HS, 7HS	43.30	Arifuzzaman et al. 2014
Dry weight	ISR42-8 × Scarlet	0.56	8	1HS, 2HL, 3HS, 4HS, 5HL, 6HL, 7HS, 7HL	Not reported	Naz et al. 2014
Dry weight	Cheri × ICB181160	Not reported	3	1HS, 5HL, 7HS	19.20	Arifuzzaman et al. 2016
Dry weight	Global barley panel	0.62	4	1HL, 2HL, 3HL, 5HL	78.09	Reinert et al. 2016
Length	ISR42-8 × Scarlet	Not reported	3	2HS, 3HS, 5HS	13.30	Arifuzzaman et al. 2014
Length	ISR42-8 × Scarlet	0.72	6	1HS, 1HL, 4HS, 4HL, 5HL	Not reported	Naz et al. 2014
Length	Cheri × ICB181160	Not reported	4	3HL, 4HS, 4HL, 7HS	19.70	Arifuzzaman et al. 2016
Length	Global barley panel	0.48	2	5HL, 7HS	24.41	Reinert et al. 2016
Root-shoot ratio	ISR42-8 × Scarlet	Not reported	5	1HS, 3HS, 5HS, 7HS	31.80	Arifuzzaman et al. 2014
Root-shoot ratio	Global barley panel	0.66	5	2HL, 3HS, 4HS, 5HL, 7HS	86.66	Reinert et al. 2016
Seminal angle	ND24260 × Flagship	0.64	2	3HL, 5HL	13.40	Robinson et al. 2016
Seminal angle	ISR42-8 × Scarlet	0.84	2	3HL, 7HS	33.84	Sayed et al. 2017
Seminal number	ND24260 × Flagship	0.99	5	1HL, 3HL, 4HL, 5HL, 6HL	30.00	Robinson et al. 2016
Shallow root ratio (<30°)	ISR42-8 × Scarlet	0.23	3	4HL, 6HL, 7HL	7.66	Sayed et al. 2017
Size	Derkado × B83-12/21/5	Ranged from 0.07 – 0.38	4	1HS, 3HL, 4HL, 7HS	28.10	Chloupek et al. 2006
Volume	ISR42-8 × Scarlet	0.64	5	1HS, 2HL, 5HL, 6HL, 7HS	Not reported	Naz et al. 2014

Volume	Cheri × ICB181160	Not reported	1	7HS	13.70	Arifuzzaman et al. 2016

2.3 The path of acceleration: from root trait phenotyping to genetic control

2.3.1. The evolution of molecular markers in plant breeding

Molecular markers have revolutionised plant genetic analysis, allowing reliable differentiation between genotypes based on polymorphisms present in DNA sequences. Over the past three decades molecular markers have evolved dramatically due to advances in molecular biology, such as improvements in the techniques of nucleic acid hybridization, polymerase chain reaction and DNA sequencing (Grover et al. 2016). Therefore, molecular marker technologies popular in the 1980's, 90's and early 21st century, such as restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs), have been surpassed by sequence targeted approaches (Mammadov et al. 2012; Grover et al. 2016). Sequence targeted approaches, such as single nucleotide polymorphisms (SNPs), genotype-by-sequencing (GBS) and Diversity Arrays Technology (DArT), are highly polymorphic, require low quantities of DNA, are amendable to high-throughput automation and are highly reliable (Glover et al. 2016).

SNPs (substitutions, deletions or insertions) are highly abundant, yet a large proportion of SNPs are found within non-coding regions of DNA. Despite this, an important collection of SNPs represent mutations in genes corresponding to diseases and other important phenotypes, making them highly useful for genetic studies (Glover et al. 2016). Advances in next generation sequencing (NGS) have contributed to the identification of a large number of SNPs (van Tassell et al. 2008), significantly improving the resolution of genetic studies for diversity and QTL mapping. A high-quality reference genome sequence is used for SNP calling (Ramos et al. 2009) and many SNP array platforms have been developed for a number of crops (Rasheed et al. 2017). All array platforms are based on technologies by Illumina and Affymetrix, where technologies by Illumina are based on BeadArray Infinium chip and technologies by Affymetrix either GeneChip arrays or Axiom technology (Rasheed et al. 2017).

The first-generation DArT platform was a hybridization-based method, producing whole-genome fingerprints by scoring the presence versus absence of DNA fragments originating from reference genomic DNA samples (Akbari et al. 2006). DArT has been

applied in a large number of crops including wheat (Akabari et al. 2006) and barley (Wenzl et al. 2004). GBS was developed based on the concept of reduced complexity in large genomes (Elshire et al. 2011) This is most often achieved by targeting and sequencing genomic regions near recognition sites of restriction enzymes (Glover et al. 2016). Selective targeting of restriction enzymes can avoid sequencing of high-repetitive and low complexity regions. DArT have also developed a GBS platform based on next-generation sequencing, known as DArT-seq. This platform is reasonably high density, routinely returning approximately 40K polymorphic markers across the barley genome and relatively low cost (~50 AUD/sample). A hybridization-based exome capture platform has also been set up in barley for cultivated barley and related species, allowing high specific sequencing of targeted mRNA-exome coding regions that can be used for mapping-by-sequencing and genetic diversity analyses (Mascher et al. 2013). In 2012, the first partly ordered draft barley genomic sequence was published by the International Barley Genome Sequencing Consortium (2012). More recently, in 2017, a map-based reference sequence of the barley genome was reported using chromosome conformation capture mapping to determine the linear order of sequences across the pericentromeric space (Mascher et al. 2017). Advances in molecular biology, genome sequencing and the advent of sequence targeted molecular markers makes high resolution and precision QTL mapping for in-depth genetic dissection of traits an everyday reality.

2.3.2. Strategies for mapping QTL

QTL refers to molecular marker/s that correlate with variation in a phenotype of the measured trait and is typically linked to or contains the gene that is influencing the phenotype (Miles and Wayne, 2008). Conventional QTL mapping, known as linkage analysis, detects QTL in bi-parental populations segregating for the trait of interest. Initially, markers are assigned to linkage groups and ordered based on recombination frequency (Jones et al. 1997). QTL are then detected using the maximum likelihood method, which estimates the likelihood that the measured marker effect is random or due to linkage (Jones et al. 1997). Linkage mapping has been used to successfully identify QTL influencing a number of important traits in many crop species, however this method of mapping does have a number of limitations. One of the key constraints of linkage analysis is the low mapping resolution, often resulting from small population sizes, moderate-low levels of recombination within a population and, as a result, long stretches of the chromosome being in high LD (Flint-Garcia et al. 2005). Using bi-parental populations also means only two alleles can be examined at any one loci, limiting detection of allelic diversity (Flint-Garcia et al. 2005). Furthermore, if both parents carry the

same homozygous allele at any one loci, then these loci will be fixed and undetectable via linkage mapping (Flint-Garcia et al. 2005). In addition, the parental material, and thus the bi-parental population, is often not representative of backgrounds used in elite germplasm for breeding (Jannink et al. 2001). Despite these limitations, the high frequency and relative balance of alleles at each locus means linkage mapping has greater statistical power to detect QTL if they are segregating in the population (Jones et al. 1997).

Association mapping (AM) or linkage disequilibrium (LD) mapping takes advantage of historical LD between a molecular marker and the causative polymorphism influencing the trait phenotype (Jones et al. 2009). Due to historical recombination, the LD within a population has decreased to short chromosomal intervals, which allows more precise mapping of QTL intervals (Jannink and Walsh 2002). A key benefit of AM is no longer needing to create large bi-parental populations, as AM can be performed using any collection of lines with unknown or arbitrary relationships between genotypes, thus allowing identification of more diverse allelic variants (Malosetti et al. 2007). Therefore, more than just two alleles per locus can be examined and mapped in populations, which are also highly relevant for plant breeding (Malosetti et al. 2007). Furthermore, the estimates of the effect size of QTL may be more realistic for plant breeding populations if estimated in plant breeding material. Most importantly, by using LD, and thus tightly linked markers, the resultant QTL will be mapped with higher precision (Malosetti et al. 2007). A key limitation of AM is the increased risk of false positive marker-trait associations as a result of LD from sources other than linkage. The most frequent cause of false positives is population structure (Jannink et al. 2001), therefore AM strategies must first inspect the population for structure and appropriately account for it in the model (Malosetti et al. 2007). To further minimise the occurrence of false positives, a mixed linear model can be applied to the AM approach. This involves accounting for population structure as a fixed effect and also correcting for familial relatedness by including a kinship matrix as a random effect in the model (Yu et al. 2006). Overall, the accuracy of AM is dependent on the ability to separate significant marker-trait associations representative of LD arising from true linkage from LD arising as a result of other causes, such as population structure.

Nested-association mapping (NAM) has been developed as a powerful mapping approach to combine the advantages of linkage analysis and AM in a single population (Yu et al. 2008). NAM uses a unique population structure, where a panel of donor lines are crossed to a reference line and small sub-populations of recombinant inbred lines (RILs) are

generated from each-donor reference combination (Yu et al. 2008). NAM has increased statistical power, as well as more effective use of dense molecular markers, while maintaining high allele frequencies as a result of the unique population structure exploiting both recent and ancient recombinant (Yu et al. 2008; Stich et al. 2009). Furthermore, due to the balanced design of the population in combination with the systematic reorganising of the parental genomes during RIL development, NAM populations have an improved power to detect QTL across the genome (Buckler et al. 2009). A comparative analysis of the NAM approach in relation to linkage analysis and AM is outlined in Table 2.2, adapted from Yu et al. (2008). The NAM approach was first described in maize (*Zea mays* L.; McMullen et al. 2009) and has now been adopted in many crops species, such as sorghum (Jordan et al. 2011), barley (Maurer et al. 2015; Ziems et al. 2015), wheat (Bajgain et al. 2016) and rice (Fragoso et al. 2017).

Table 2.2 Comparison of main characteristics for QTL mapping strategies (adapted from Yu et al. 2008).

Feature	Linkage analysis	AM	NAM
Allelic diversity	Minimal - only 2 alleles at each locus	High – multiple alleles at each locus	High – multiple alleles at each locus
No. markers required for whole-genome scan	Minimal	Large number markers	Minimal (only large number for donor line/s)
Efficiency using sequence information	Inefficient	Efficient	Very efficient
Mapping resolution	Low precision	High precision	High precision
Designed mapping population	Yes or no	No	Yes
Sensitivity to genetic heterogeneity	Insensitive	Sensitive	Insensitive
Statistical power	Intermediate	High	High

In addition to NAM, multi-parent advance generation inter-cross (MAGIC) populations have been developed to address limitations in conventional mapping resources. The MAGIC approach involves inter-crossing as many founder lines as desired and then performing a certain number of inter-crosses based on the number of founder lines divided by two, which ensures an equal distribution of founder lines across the population (Cavanagh et al., 2008). A key constraint of the MAGIC approach is the increasing time and resources required to increase genetic diversity through increasing the number of founder lines (Rakshit et al. 2012). Furthermore, once population development is complete, the genetic diversity

within the population is fixed (Rakshit et al. 2012). MAGIC populations have been developed in wheat (Huang et al. 2012; Mackay et al. 2014), rice (Bandillo et al. 2013) and barley (Sannemann et al. 2015).

2.3.3. Genomic regions influencing root traits in cereals

The identification and subsequent cloning of the *DRO1* gene in rice is an excellent example of the power of linkage analysis to detect genes underpinning traits with simple genetic control. *DRO1* was initially detected in the IR64 × Kinandang Patong bi-parental RIL population, a shallow rooting cultivar crossed to a deep rooting cultivar, via composite interval mapping (Uga et al. 2011). *DRO1* was mapped to chromosome 9, explaining 66.6% of the total phenotypic variation for the ratio of deep rooting, and thus was the major QTL influencing the trait (Uga et al. 2011). It was later demonstrated that *DRO1* controls root growth angle, increasing root growth in a downward direction (Uga et al. 2013). Introgression of *DRO1* into a shallow-rooting rice cultivar to create a near-isogenic line (NIL) was field tested under drought conditions. The increased deep rooting of the *DRO1* NIL improved access to deep soil moisture and had a significantly increased yield compared with the shallow-rooting donor (Uga et al., 2013). The genetics underlying the root angle trait in rice is simple, whereby a major gene controls root growth angle, and thus can be easily identified in a bi-parental population segregating for the root angle trait. In wheat and sorghum, on the other hand, the genetic control is more complicated, with many small effect QTL spread across the chromosomes influencing root angle (Hamada et al. 2012; Mace et al. 2012; Christopher et al. 2013; Atkinson et al. 2015; Maccaferri et al. 2016). Thus, root angle appears to be under complex genetic control in wheat and sorghum, whereby not one QTL has a major influence, but each QTL has an additive yet modest effect on the trait. Recent research in barley has also demonstrated that root growth angle is under complex genetic control, with multiple QTL spread across many chromosomes influencing the trait (Robinson et al. 2016; Sayed et al. 2017). Thus, to further dissect the genetic control of root angle in barley, a QTL mapping strategy with high statistical power to detect QTL across the genome with increased mapping resolution is required. A strategy like NAM has been successfully applied in other crop species to dissect complex traits, such as flowering time, kernel composition and disease resistance (Buckler et al. 2009; Kump et al. 2011; Cook et al. 2012; Mace et al. 2013; Maurer et al. 2015; Bajgain et al. 2016; Fragoso et al. 2017), and thus would be an ideal mapping strategy for examining root traits in barley.

2.4 Root angle validation and introgression for barley breeding

2.4.1. Determining the value of seminal root traits for barley breeding

To successfully integrate selection for root angle in barley breeding programs, the validity of the trait needs to be clear, as well as the value across growing regions (Rebetzke et al. 2013). Barley is grown across several different environment types throughout Australia, with soil composition varying from shallow sandy soil with a low water-holding capacity to deep clay soils with a high water-holding capacity (Potgieter et al. 2002; Rebetzke et al. 2013). The climate across the growing region ranges from temperate Mediterranean climates with frequent in-season rainfall to subtropical climates with limited in-season rainfall, but also characterised by variation in inter-annual rainfall (Chenu et al. 2011; Rebetzke et al. 2013). Thus, to validate root angle as an important trait contributing to drought adaptation in barley, the differing environment types throughout Australia must be taken into consideration.

Unfortunately, controlled environment trials, such as climate-controlled glasshouse experiments, are limited in their ability to provide an accurate representation of the field environment and management practices. For instance, the climate and water availability in a controlled glasshouse is constant, thus not representative of the varying climate conditions and rainfall experienced in the field. The inability to accurately determine yield, due to plant root growth constrained by pot size, is another key limitation to controlled glasshouse trials. Managed environment studies could assist in the validation of narrow and deep root system architecture as a drought adaptive trait in barley by permitting evaluation under controlled stress (Campos et al. 2004; Rebetzke et al. 2013). A successful managed environment trial for drought stress should consider: (1) selecting managed environment trial locations representative of environment types in the target growing regions, (2) monitoring of the climate at each trial, (3) controlling field-based spatial variability, (4) using appropriate germplasm for trait assessment, (5) assessing traits contributing to water-limited adaptation, and (6) phenotyping using standardised measurement protocols (Rebetzke et al. 2013). It is important to note that it is difficult to manage drought stress under field conditions, as it is largely dependent on in-season rainfall or lack thereof. One management option is the use of rain out shelters to shield field trials from the negative effects of in-season rainfall, however these shelters are expensive to build and limited to testing small numbers of genotypes, such as near-isogenic lines (Tuberosa 2012). Alternatively, selection of a location with a high

probability of low in-season rainfall is an option, however unseasonable rainfall is always a risk. Following validation of narrow root system architecture for drought adaptation in barley, the appropriate selection strategy in breeding programs needs to be considered.

2.4.2. Selection strategies for root growth angle in barley

Malt quality is the driving factor of value for most barley grain-growers in Australia and most developed countries, hence the key target trait in combination with yield, for barley breeders in these countries. Breeding for improved drought adaptation in barley is futile without considering the end use of the product, typically malt quality. Once a drought adaptation trait, such as root angle, has been validated in the target environment, and molecular markers identified, selection can begin in breeding programs.

The most obvious form of trait selection in a breeding program is phenotypic selection, which in the case of selection for root growth angle could be applied as an early generation screen to assist in selection decisions for the next generation advancement. The introduction of high-throughput phenotyping methods for root traits, for instance the clear pot method (Richard et al. 2015), makes phenotypic selection for root traits in breeding programs a realistic possibility. However, due to early generation material consisting of large numbers of lines per population (Witcombe and Virk 2001), adopting a phenotyping platform for selection of a new trait is a large financial commitment requiring on-going resources. Therefore, using molecular screening methods for selection maybe more cost-effective if early generation material has been previously genotyped with molecular markers.

Marker-assisted selection (MAS) and marker-assisted backcrossing (MABC) makes use of tightly linked molecular markers, previously identified in QTL mapping, as a diagnostic tool for selection of a specific phenotype (Collard et al. 2005). MAS can be advantageous when phenotypic selection is time consuming, costly, unreliable or not feasible (Collard et al. 2005). Furthermore, it can avoid the transfer of undesirable genomic regions through linkage drag and allow the pyramiding of multiple genes for multiple traits (Eathington et al. 2007). However, for MAS to be successful the markers need to be high resolution, ideally sequence targeted markers, and require validation for the prediction of the target phenotype in an independent population (Collard et al. 2005). Furthermore, MAS is most suited to traits under simple genetic control, where one major gene is responsible for the majority of the phenotypic variation, independent of the background population and environmental effects (Bernardo 2016). MAS has been successfully implemented in barley

breeding for the recessive resistance genes *rym4/rym5* to barley yellow mosaic viruses and *mlo* to barley powdery mildew (Chelkowski et al. 2003; Miedaner and Korzun, 2012). Despite this, MAS is limited for complex polygenic traits with multiple small effect QTL and QTL × environment interactions.

For traits under complex polygenic control, adoption of genomic selection (GS) is often the most effective genomics-based selection strategy. GS is a predictive tool, using dense marker information in combination with prior phenotypic data to predict the performance of new candidate genotypes for quantitative traits (Meuwissen et al. 2001). Although the predicted breeding values provide no information on the underlying genes, they identify the best candidates in a population (Heslot et al. 2012; Bernardo 2016). GS reduces time and resources required for phenotyping and shortens the breeding cycle length while improving the anticipated genetic gain (Crossa et al. 2017). Prediction accuracy of GS models have been found to improve with increased marker density, increased size of the training population, increased heritability of the trait and when the trait is under simple genetic control (Lorenzana and Bernardo 2009; Sallam et al. 2015; Bernardo 2016). Some key considerations to implementing GS in a breeding program is the low prediction accuracies, costs associated with genotyping, as well as unclear guidance as to where GS fits best within a breeding program (Crossa et al. 2017). Fortunately, research is now focusing on how best to incorporate GS into traditional breeding programs and a recent study by Gaynor et al. 2017 demonstrates a two-stage approach provides the greatest genetic gain compared to conventional breeding and standard genomic selection strategies.

2.5 Conclusion

Water stress is one of the greatest limitations of yield globally, and coupled with a growing population, this makes yield stability and cereal crop improvement vital. Our ability to meet the demands of our changing environment will depend on the development of cereal cultivars better adapted to less productive growing conditions. The focus of this PhD project is on barley, the fourth largest cereal crop produced worldwide and a staple food crop for many developing countries in Northern Africa and the Middle East. Despite barley's reputation for high adaptability, barley cultivars in Australia are often susceptible to drought stress due to their origins in European germplasm. Therefore, Australian germplasm will most likely

benefit from improved drought adaptation, especially with the frequency and duration of drought predicted to increase over the coming years.

Drought adaptation as a whole is complex and is made up of many component traits all contributing to improved tolerance. Root system architecture, specifically a narrow and deep root system, is proposed in literature as an important drought adaptation trait, allowing optimum resource capture under water-stress. Research in sorghum, rice and wheat suggest that improved access to water during the grain-filling period via a narrow root system leads to increased yield under water-limited conditions. Root traits in sorghum and barley have also been linked to above-ground drought adaptation traits, suggesting there may be shared genetic control between above- and below-ground drought adaptation. Research also demonstrates that the complexity of drought adaptation extends beyond purely the genetics, with the environment, management practices and the interactions (G × E × M) also playing a role. Therefore, to thoroughly evaluate drought adaptation, all components of G × E × M must be examined.

This PhD project focuses on the genetic components of drought adaptation, in particular those underpinning root system architecture. The advent of high-throughput, repeatable and inexpensive root trait phenotyping platforms provides a unique opportunity to thoroughly dissect the genetics of root system architecture in a number of barley populations. Prior to this project, the genetic control of root system architecture in barley was relatively unknown. However, previous research based on phenotypic observations suggests the genetic drivers of a narrow and deep root systems may have been left behind in wild barley throughout the process of domestication.

Over the past decade, significant improvements in molecular marker technology has made high-density sequence targeted markers an affordable reality for pre-breeding research. As a result, more QTL mapping approaches have been developed, improving mapping precision and statistical power to detect significant marker-trait associations. Alongside these advances, molecular breeding has also progressed and provides more opportunities for introgression and selection for traits under complex polygenic control in breeding programs.

With the advent of new technologies for root-trait phenotyping, affordable genotyping, high precision QTL mapping and selection of genetically complex traits, there is an ideal opportunity to thoroughly investigate the genetics controlling root system architecture in barley. Due to the complexity of drought adaptation, this PhD project aims to

investigate root traits for drought adaptation in a holistic manner, examining roots in combination with above-ground canopy traits measured in the field. Based on this literature review, it is hypothesised that root architecture in barley is under complex polygenetic control and the value of specific root ideotypes in certain environment scenarios are context dependent.

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Chapter 3

GENOMIC REGIONS INFLUENCING SEMINAL ROOT TRAITS IN BARLEY

3.1 Abstract

Water availability is a major limiting factor for growth and production of crops, making drought adaptation, and its many component traits, a desirable attribute of plant cultivars. Previous studies in cereal crops indicate that root traits expressed at early plant developmental stages, such as seminal root angle and root number, are associated with water extraction at different depths. Here, we conducted the first study to map seminal root traits in barley (*Hordeum vulgare* L.), the fourth largest cereal crop worldwide. Using a recently developed high-throughput phenotyping method, a panel of 30 barley genotypes and a doubled haploid (DH) population (i.e. ND24260 × Flagship) comprising 330 lines genotyped with DArT markers, were evaluated for seminal root angle and root number under controlled environmental conditions. A high degree of phenotypic variation was observed in the panel of 30 genotypes; 13.5–82.2° and 3.6–6.9 for root angle and root number, respectively. A similar range was observed in the DH population; 16.4–70.5° and 3.6–6.5, for root angle and number, respectively. Seven QTL for seminal root traits (root angle: two QTL, root number: five QTL) were detected in the DH population. A key QTL influencing both root angle and root number (i.e. *RAQ2*/*RNQ4*) was positioned on chromosome 5HL. Across species analysis identified 10 common genes underlying root trait QTL in barley, wheat and sorghum. Here, we provide insight into seminal root phenotypes and provide a first look at the genetics controlling these traits in barley. Further investigation is required to better understand barley root system architecture and determine the most beneficial root system for growth in water-limited environments.

3.2 Introduction

Barley (*Hordeum vulgare* L.), the fourth largest cereal grain produced (in metric tonnes) worldwide, is an essential raw material for malting and beer production (Food and Agricultural Organisation of the United Nations 2014). It is also an important food source in some countries in Northern Africa and the Middle East (Food and Agricultural Organisation of the United Nations 2014). To date, barley breeding conducted in developed countries has focused on yield and the commercial value of improved malt quality. To ensure farmers get a greater return from barley breeding, more emphasis is needed to assemble new cultivars with increased adaptation to abiotic stress and with improved yield stability.

Actual yields are dependent on seasonal and local environmental factors, but one of the more critical factors in Australia is rainfall. Annual crop yields decline sharply during low rainfall seasons (Lobell et al. 2015) – a result of drought stress and the lack of drought-adapted cultivars. Barley cultivars predominately grown in Australia are largely based on northern European germplasm, which was developed for high rainfall environments. Hence, Australian barley cultivars are relatively susceptible to drought stress.

Drought adaptation is a complex trait, not only interacting with environment and management practices, but also with underlying physiological mechanisms that can be partitioned into many component factors. For example, the drought adaptation trait stay-green alters canopy development, root architecture and leaf anatomy to maintain green stems and upper leaves during grain-filling in water-limiting environments (Borrell et al. 2000ab; Pinheiro and Chaves 2011; Borrell et al. 2014ab). Expression of heat shock proteins, another component trait of abiotic stress tolerance, is switched on during heat stress to improve photosynthesis and water-use efficiency (Wahid et al. 2007). Transpiration efficiency (Sinclair 2012), relative water content, osmotic adjustment capacity (Blum 2005) and canopy temperature (Talebi 2011) are other target traits for the improvement of drought adaptation in small grains such as barley. Quantitative trait loci (QTL) mapping studies have reported QTL for drought-adaptive traits in barley for leaf relative water content (Teulat et al. 2003), osmotic adjustment capacity, and water-soluble carbohydrate concentration (Teulat et al. 2001).

While a number of studies have examined above-ground water-use traits in barley (Diab et al. 2004; Teulat et al. 2001; Teulat et al. 2003; Chen et al. 2010; Siahsar and Narouei 2010), little research has been conducted for root system traits. The root system architecture of a crop can influence the efficiency of water capture and extraction (Kondo et al. 2000; Pennisi 2008). The fibrous root system of cereals is broadly divided into two categories: seminal roots

originating from the primordia in the embryo of the seed and nodal roots developing from the lower tillering and leaf bearing area of the stem (Hochholdinger et al. 2004). Seminal roots emerge first while nodal roots develop once the plant reaches the tillering growth stage. For drought adaptation, the seminal roots are of interest due to their early development and association with root system architecture of mature plants (Richard et al. 2015). For instance, in wheat, a more vertical (narrow) angle of the seminal roots and a higher number of seminal roots in seedlings has been linked to a more compact root system with more roots at depth (Manschadi et al. 2006). Therefore, seminal root traits are considered useful proxy traits for desirable root system architecture within a breeding context (Richard et al. 2015).

Studies in wheat comparing the root architecture of a drought-adapted genotype versus a standard genotype, revealed the drought-adapted genotype to have a compact root system (maximum lateral spread of 45 cm from stem base), where the roots occupied the soil volume uniformly and allocated more root growth to the deepest soil layers, resulting in greater root length (3.8 times more than the standard) in the deepest soil layer (Manschadi et al. 2006). This root system architecture improves the plant's access to stored moisture deep in the soil profile. Furthermore, application of a cropping system model in the study by Manschadi et al. (2006) demonstrated that each additional millimetre of water extracted during grain-filling generated an extra 55 kg of yield per hectare. Field studies have also found that 1 mm of additional water transpired during grain-filling can increase grain yield by about 50 kg ha^{-1} in sorghum grown under post-anthesis drought in a rain-out shelter facility (Borrell et al. 2014a). Studies of this nature are yet to be conducted in barley; therefore, the most beneficial root system architecture for barley, grown under water-limiting conditions, is unknown. Previous research in wheat suggests there may be a relationship between plant height and root length, whereby increased height is associated with longer root length (Subbiah et al. 1968), however research results in this area appear ambiguous with multiple contradictory reports (Wojciechowski et al. 2009). An association between plant height and root length, specifically via the *sdw1*, has also been proposed in barley (Chloupek et al. 2006), however like in wheat further validation is required.

The importance of identifying QTL for root traits in cereal crops for drought adaptation has been established by several recent QTL mapping studies conducted in wheat, rice, sorghum and barley. In wheat, a large number of QTL, each with minor effect on components for root system architecture have been reported, with some 31 QTL identified on chromosomes 2A, 2D, 3A, 3B, 4D, 5A, 5B, and 6A (Hamada et al. 2012; Ren et al. 2012; Bai et al. 2013; Christopher et al. 2013; Liu et al. 2013; Zhang et al. 2013). More specifically for root angle,

four QTL have been identified on 2A, 3D, 6A and 6B and two suggestive QTL on 5D and 6B and for root number two QTL have been detected on 4A and 6A with four suggestive QTL position on 1B, 3A, 3B and 4A (Christopher et al. 2013). The study by Christopher et al. (2013) examined a DH population, therefore further studies on genetically diverse or elite breeding material may reveal additional QTL. Also, QTL regions identified for root angle and root number did not co-locate, suggesting that the gene × gene and gene × environment interactions may not be the only challenging obstacle faced when breeding for root traits. In rice, the ratio of deep rooting has been used to evaluate root architecture and can vary from 5–95% across rice genotypes. Seven QTL have been identified for rice root traits on chromosomes 2, 4, 6, 7 and 9 (Uga et al. 2011, 2012, 2013a, 2013b). Two QTL were reported as major effect QTL for root angle; *qSOR1* on chromosome 7 (Uga et al. 2012) and *DRO1* on chromosome 9 (Uga et al. 2013a, 2013b). The recent cloning and characterisation of *DRO1* demonstrated that this gene improved deep rooting and enhanced drought adaptation by increasing yield in the field under drought conditions (Uga et al. 2013a). In sorghum, Mace et al. (2012) mapped QTL for nodal root angle (ranging from 14.5—32.3°) in 141 recombinant inbred lines and reported two major QTL, both positioned on linkage group SBI-05 and two suggestive QTL on SBI-08 and SBI-10. All four QTL appeared to co-locate with previously identified QTL for stay-green expressed under drought conditions (Mace et al. 2012).

In barley the following root traits have been mapped: root system size (RSS), root dry weight (RDW), root volume (RV), root-to-shoot ratio (RSR) and root length (RL). RSS was measured at three time points throughout the life-span of a field grown barley DH population (Derkado (European cultivar) × B83-12/21/5 (European breeding line)) that was later found to segregate for the trait (Chloupek et al. 2006). Four QTL were identified for total RSS on chromosomes 1H, 3H, 4H and 7H and therefore reported as a polygenic trait (Chloupek et al. 2006). Three studies have mapped the remaining root traits, all with a focus on detecting QTL in the wild barley accession ISR42-8 and the modern cultivar Scarlet (Naz et al. 2012; Arifuzzaman et al. 2014; Naz et al. 2014). As a result, 37 QTL have been identified for root related traits in barley (RDW: 16 QTL across all chromosomes; RV: seven QTL 1H, 2H, 5H, 6H and 7H; RSR: five QTL on 1H, 3H, 5H and 7H; RL: nine QTL on chromosomes 1H—5H). Prior to the mapping of root traits, Linde-Laursen (1977) demonstrated that root number is under genetic control through examination of a low rooting barley mutant. Other than this, the availability of barley mutants affecting seminal root angle and number is limited. A mutant with a highly geotropic root system was identified through a chemically mutagenized barley

population, however the population was reported to be unstable and display inconsistent phenotypes (Bovina et al. 2011). QTL for seminal root angle and number are yet to be reported in barley.

The lack of efficient phenotyping methods for root traits is a reason why such traits have not been the subject of study in the past. Methods have been traditionally labour intensive, potentially unreliable and un-relatable (Zhu et al. 2011). For instance, the 2-dimensional (2D) gel-filled chamber system that enables non-invasive, sequential measurements of root systems preserved in natural orientation, is limited when it comes to evaluating large numbers (Bengough et al. 2004). Further, the artificial anaerobic environment may not reflect soil conditions in the field. Sand- and soil-based 3-dimensional (3D) methods such as soil coring or plant excavation are limited by their destructive analysis and single point measurement (Hargreaves et al. 2009). X-ray microtomography imaging is non-invasive and non-harmful, but is restricted to small sample sizes and expensive, therefore inappropriate for screening large numbers (Pierret et al. 2003; Hargreaves et al. 2009). Recently, a high-throughput phenotyping method was described by Richard et al. (2015) which uses clear (transparent) pots and imaging to rapidly evaluate seminal root angle and number in wheat seedlings. This high-throughput method presents new opportunities for mapping of root traits in other cereals, particularly barley where knowledge is limited.

This study exploits the high-throughput clear pot phenotyping method to characterise a panel of 30 barley genotypes (comprising Australian cultivars and advanced breeding lines) for seminal root angle and number. The panel is included in this study to take a first look at the variation for seminal root traits in Australian cultivars and advanced breeding lines. In addition, the method is employed to characterise a barley DH population (ND24260 × Flagship) which was previously genotyped with Diversity Array Technology Pty. Ltd. (DArT) markers (Hickey et al. 2011) for discovery of QTL controlling root traits; seminal root angle and root number. We aligned root trait QTL with previously reported QTL for abiotic stress tolerance in barley to help identify key genomic regions possibly underpinning drought adaptation.

3.3 Materials and methods

3.3.1 Plant material

Seminal root angle and number were measured for a panel of 30 barley genotypes, comprising a selection of commercial barley cultivars and advanced breeding lines (Table 3.1). Included in this panel are four Australian cultivars (Commander, Compass, Shepherd and La Trobe), two European cultivars (Oxford and Westminster) and 24 advanced breeding lines from the Northern Region Barley (NRB) breeding program, Warwick, Australia.

In addition, seminal root angle and number were examined in 330 DH lines derived from the cross ND24260 × Flagship using the F_1 anther culture method performed by the Cereal Double Haploid Program at the Department of Agriculture and Food, Western Australia (Hickey et al., 2011). ND24260 (ND19869-1//ND17274/ND19119), an advanced breeding line from Barley Breeding Program North Dakota State University, has superior grain quality and displays a stay-green phenotype during water deficit (Gous et al. 2013). Flagship (Chieftain/Barque//Manley/VB9104) is an Australian malting cultivar released by the Barley Program at the Waite Campus University of Adelaide.

Table 3.1 Details for the panel of 30 barley genotypes evaluated in this study. [a] Genotype and pedigree confidential under exclusive licence with third party.

Genotype	Pedigree	Advanced breeding line/ commercial cultivar
C07.276>DH049	ND24260-1/Flagship	Breeding
Commander	Keel/Sloop//Galaxy	Commercial - Malting
Compass	County/W13416//Commander	Commercial – Malting
La Trobe	Reselection from Hindmarsh	Commercial – Malting
NRB090257	Barke/Rawson	Breeding
NRB090885	ND24205-1//Grout/Dash	Breeding
NRB11077	Shepherd/Pinnacle	Breeding
NRB11116	NRB03470/2ND25389	Breeding
NRB11755	ND24260-1/Flagship	Breeding
FND001[a]	Not available[a]	Breeding
NRB120567	NRB091087/NRB091047	Breeding
NRB120579-4	NRB091087/NRB091047	Breeding
NRB120742	CLE 245/NRB090734	Breeding
NRB120834-4	NRB08040-1/2ND25316	Breeding
NRB120850	NRB08040-1/NRB08708	Breeding
NRB120883	NRB090031/NRB090326-3	Breeding
NRB130203	Bowman*5/PI 584760//NRB091087	Breeding
FND002[a]	Not available[a]	Breeding
FND003[a]	Not available[a]	Breeding
NRB130851	NRB091124-405//NRB091087/NRB091047	Breeding
FND006[a]	Not available[a]	Breeding
FND007[a]	Not available[a]	Breeding
FND004[a]	Not available[a]	Breeding
FND008[a]	Not available[a]	Breeding
FND005[a]	Not available[a]	Breeding
FND009[a]	Not available[a]	Breeding
NRB131326	NRB091098/NRB100285-1-1	Breeding

Oxford	Not available[a]	Commercial – Feed
Shepherd	Reselection from Baronesse	Commercial – Feed
Westminster	NSL97-5547/Barke	Commercial - Malting

[a] Genotype and pedigree confidential under exclusive licence with a third party

3.3.2 Characterising seminal root angle and number

The seminal root angle and root number of barley seedlings were measured using the "clear pot" method detailed by Richard et al. (2015). Grains were sown vertically with the embryo pointing towards the base of the pot at a depth of 2 cm with a 2.5 cm space between kernels and 24 grains per pot, against the wall of the transparent ANOVApot® (ANOVApot Pty. Ltd., Brisbane, QLD, Australia) pot (200 mm diameter, 190 mm height, 4 L) in pine bark potting media (pH 6.35, EC = 650 ppm, nitrate = 0, ammonia < 6 ppm and phosphorus = 50 ppm). Post-sowing, the clear ANOVApot® pots were placed in black ANOVApot® pots (i.e. 200 mm diameter, 190 mm height, 4 L) to exclude light from the developing roots. Seedlings were watered once after sowing and no other nutrients were supplied. Seedlings were grown in a climate-controlled growth facility, where a diurnal (12 h) artificial light and temperature setting of 22/17°C (day/night) was adopted.

Five days post-sowing, roots were imaged using a Canon D500 camera and image analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij/). Seminal root angular spread was defined as the deviation angle from the first vertical root to the first pair of seminal roots (Figure 3.1), as outlined by Christopher et al. (2013) and Richard et al. (2015). The first pair of seminal roots was measured at a point 3 cm below the embryo of the grain.
Six days post-sowing, seedlings were manually removed from pots by the initial removal of excess soil in the centre of the pot. Individual seedlings were then carefully removed along with their roots intact. Excess soil was removed by hand and the individual root axes of each seedling were then counted to determine total seminal root number for each seedling.

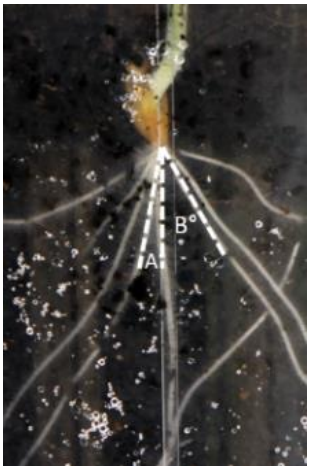

Figure 3.1 Illustration of seminal root angle measurement of the first pair of seminal roots. Angle (A) and angle (B) make up the seminal root measurement, where angle (A) is measured from the vertical root to the first seminal root and angle (B) is measured from the vertical root to the second seminal root. Angle (A) and angle (B) are added combined to give the seminal root angle, defined as the angle between the first pair of seminal roots.

Characterisation of the panel of 30 barley genotypes used eight replicates, corresponding to a design of 10 pots arranged in 10 rows along a single column on one bench, where each pot contained 24 barley seeds. The 30 barley genotypes were allocated to the 24 positions within a pot using a non-resolvable or unbalanced incomplete block design, where pots formed the incomplete blocks and not all genotypes where present within each block.

Characterisation of the 330 DH lines (including the two parent lines) used eight replicate seeds for each DH line and 32 replicates for each parental line. The experiment included 117 pots, 24 genotypes per pot, with genotypes randomised to the positions within the pots using an optimal resolvable design (Butler et al. 2008). Pots were placed across three benches in the growth facility, with 44 pots per bench aligned in a two-dimensional array of 4 columns by 11 rows on benches one and two. Bench three contained the remaining 29 pots arranged with 11 rows for columns one and two, and seven rows in column three. The eight replicates of the 330 DH lines were aligned with benches, where benches one and two each contained three replicates and bench three contained two replicates. The two parent lines were also randomised across the pots and benches, with 13 to 14 replicates of each parent on benches one and two, and five replicates of each on bench three. The variable distribution of the two parents across the benches is a result of less pots on bench three as well as the need for having two complete replicates on this bench, thus limiting the availability of space for more parent

replicates. In this experimental design benches formed complete replicates and pots formed the incomplete blocks.

3.3.3 Analysis of phenotypic data

A linear mixed model was fitted to the data for each experiment, where spatial location was accounted for in the design model allowing for bench, column and row positions in the growth facility. For the panel of 30 barley genotypes, a fixed term for Genotype and a random term for Pot were used. For the DH population, a fixed term was included for Genotype and a random term for Bench, Pot and the positional effect of Pot using rows and columns of the design array. Variance components were estimated using residual maximum likelihood (REML) and best linear unbiased estimators (BLUEs) were formed for the fixed genotype effects. Variance components of the analysis were used to calculate the heritability of each trait (root angle and root number) in the panel of 30 genotypes and the DH population. The model was fitted in ASReml-R (Butler et al. 2008).

Linear regression analysis was performed in Genstat 17 (VSN International 2014) to determine the correlation coefficient between root angle and root number trait means (in each experiment), where a significant correlation was deemed as having a p value < 0.05. Linear regression analysis was also used to determine the correlation coefficient between root traits (root angle and root number) and plant height at four field locations (i.e. Bithramere, Brookstead, Walgett, Warwick). Variance components were used to determine the heritability for plant height at each field location. Summary statistics (population means, standard deviation and 95% confidence intervals) were calculated using Genestat 17 for each experiment.

3.3.4 Linkage map and QTL analysis

The linkage map for the ND24260 × Flagship DH population reported by Hickey et al. (2011) was used for initial marker order and mapping in this study. The map comprises 605 polymorphic (DArT) markers for the 330 DH lines. The distribution of markers across the chromosomes are as follow: 60 markers on 1H, 129 on 2H, 105 on 3H, 44 on 4H, 83 on 5H, 95 on 6H and 89 markers on 7H.

QTL analyses were performed using BLUEs for seminal root angle and root number in Genstat 17. QTL analysis using composite interval mapping (CIM) was performed, where seminal root angle and root number were analysed and mapped individually. $-\log_{10}(P)$ values

greater than 3.6 were considered preliminary candidate QTL. This QTL significance level was calculated based on the Bonferroni-based multiple-test control threshold that corrects the experiment-wide error rate for the number of tests performed (Malosetti et al. 2006). A REML variance components analysis was performed to select the final QTL.

3.3.5 Collation of published QTL studies

Previously reported QTL for traits underpinning drought adaptation in barley were collated from six discovery studies (Teulat et al. 2001; Diab et al. 2004; Chloupek et al. 2006; Chen et al. 2010; Siahsar and Narouei 2010; Arifuzzaman et al. 2014). From each study, information on the population pedigree, population type (i.e. double haploid: DH, recombinant inbred line: RIL), population size, observed traits, marker platform, QTL positioning and the amount of the variation explained by the QTL (R^2) was collected.

Across the six discovery studies, 11 different traits related to drought adaptation were analysed, with a total of 62 QTL reported. The location of individual QTL were projected onto the DArT consensus map (Wenzl et al. 2006) along with the QTL identified in this study using the projection strategy detailed by Mace and Jordan (2011). A confidence interval of 4 centimorgans (cM) (i.e. 2 cM above and below the peak marker location) was implemented for display purposes. DArT consensus marker data and QTL positions were visually displayed using MapChart v2.2 (Voorrips 2002).

3.3.6 Across species analysis of genes underlying root trait QTL

Barley QTL detected in the current study, seminal root angle and number QTL reported in wheat (Christopher et al. 2013) and QTL identified for root traits in sorghum (Mace et al. 2012, Rajkumar et al. 2013, Bekele et al. 2014, Hufnagel et al. 2014, Li et al. 2014, Phuong et al. 2014, Wang et al. 2014) were used for the across species comparison. BLAST analysis was performed to identify the physical location of the QTL using QTL flanking markers against the respective genome (i.e. sequence for barley DArT marker BLAST against barley genome), and results filtered by $E < 0.0005$. The Ensembl genome browser and the genome assembly versions barley 082214v1, wheat IWGSC1.0+popseq and sorghum 2.1 were used to perform BLASTs (http://www.ensembl.org/). Genes underlying each QTL confidence interval were identified using the genome annotations available for each species, and BLASTp was performed to determine the locations of the genes underlying the root CI in wheat and sorghum on the barley genome.

The physical positions of the wheat and sorghum QTL were used to project wheat and sorghum root QTL onto the barley ND24260 × Flagship map. A confidence interval of 10 cM was applied to the projected QTL for display purposes based on the average confidence intervals reported in the original QTL mapping studies. MapChart v2.2 (Voorrips 2002) was used to visually display the marker locations and QTL.

3.4 Results

3.4.1 Root trait expression in the panel of barley genotypes

A high degree of variation in phenotypes for root angle and root number was observed in the panel of 30 barley genotypes (Figure 3.2). Seminal root angle ranged between 13.5° and 82.2° with a mean of 49.4° and SD of 16.6°. Seminal root numbers varied from 3.6 to 6.9 roots with a mean of 5.5 and SD of 0.7. Seminal root angle and seminal root number were not significantly correlated ($R^2 = 0.004$). The heritability of the differences observed for seminal root angle was 0.64 and root number 0.99. NRB130937 displayed the narrowest root angle for the breeding lines (13.5°) and La Trobe displayed the narrowest root angle for the commercial cultivars (37.4°). NRB120834-4 and Shepherd displayed the widest root angles of 82.2° and 71.8°, for breeding lines and commercial cultivars, respectively (Figure 3.2). Breeding lines NRB090885 and NRB11077 had the lowest and highest root numbers, respectively (Figure 3.2).

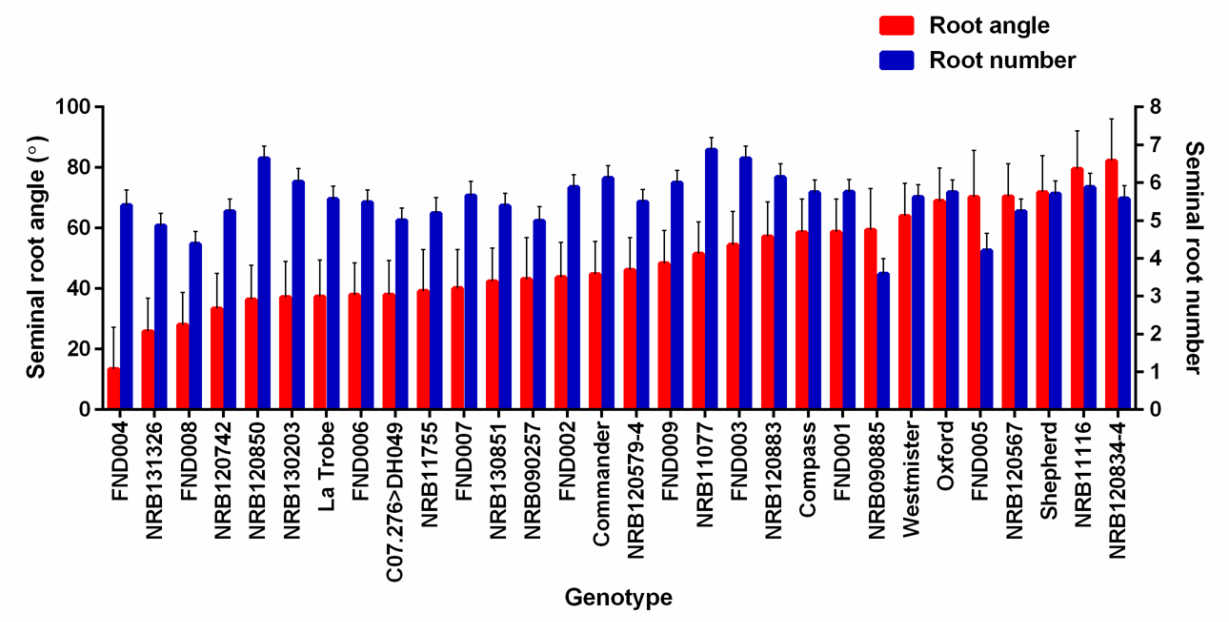

Figure 3.2 Seminal root angle and root number for the panel of 30 barley genotypes characterised in this study. Seminal root angle (°) is displayed on the left y-axis, represented by red columns and root

number is displayed on the right y-axis, represented by blue columns. Best linear unbiased estimators (BLUEs) are displayed for each genotype, along with standard errors. Genotypes are arranged in ascending order of seminal root angle (left to right).

3.4.2 Root trait expression in the DH population

The phenotypic distribution for root traits in the segregating ND24260 × Flagship DH population ranged from narrow to wide root angles (Figure 3.3a) and low to high root numbers (Figure 3.3b). BLUEs for seminal root angle ranged from 16.4°—70.5°, with a population mean of 40.2° and SD 9.2°. Root number varied from 3.6 to 6.5 roots, with a population mean of 5.5 roots and a SD of 0.4. In comparison to means obtained by parental lines, Flagship (seminal root angle 34.8°and 5.7 roots) and ND24260 (seminal root angle 45.6° and 5.1 roots), the DH population displayed transgressive segregation for seminal root traits. Transgressive segregation was depicted by the population exceeding the 95% confidence intervals of both parents (Figure 3.3a, b). Seminal root angle and number were weakly associated in the DH population (R^2 = 0.08, P <0.001). High heritabilities were obtained for both traits in the DH population: 0.63 and 0.95 for root angle and number, respectively. Previous research identified a possible association between root length and plant height in barley, whereby a reduced plant height is associated with reduced root length (Chloupek et al., 2006). In the current study, no significant associations were identified between seminal root traits and plant height, assessed across four field environments (Figure 3.4). The broad-sense heritability for plant height across the four environments ranged from 0.38 to 0.60.

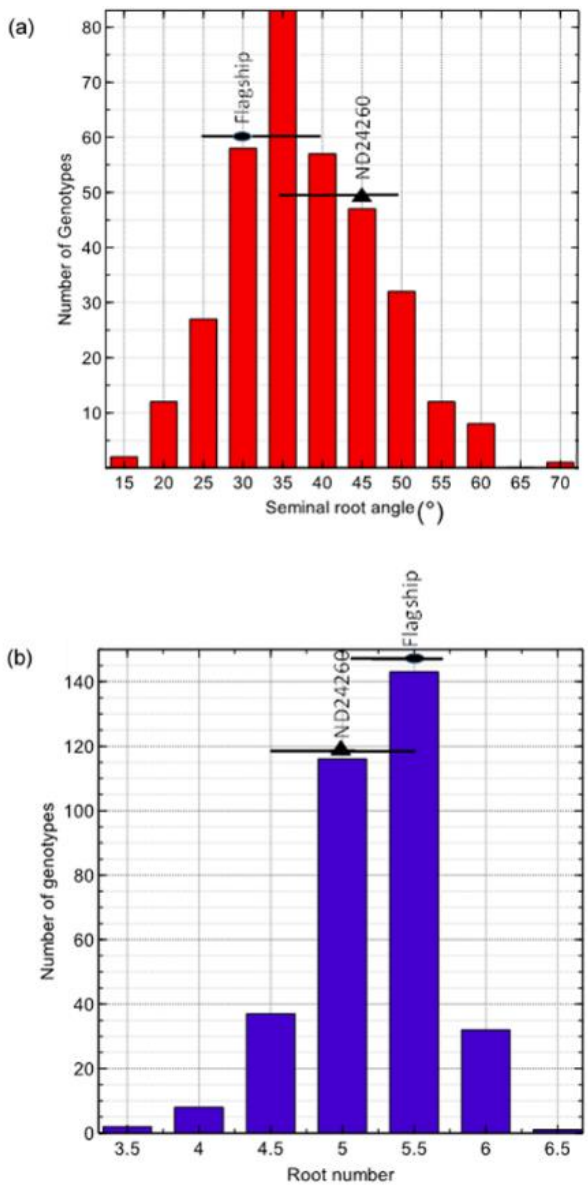

Figure 3.3 Population distributions for root traits in the ND24260 × Flagship DH population: (a) distribution of seminal root growth angle (°), and (b) distribution of root number. The trait means for Flagship and ND24260 are represented by the circle and triangle, respectively. The lines extending from each parental symbol represents the 95% confidence interval for each parental trait mean.

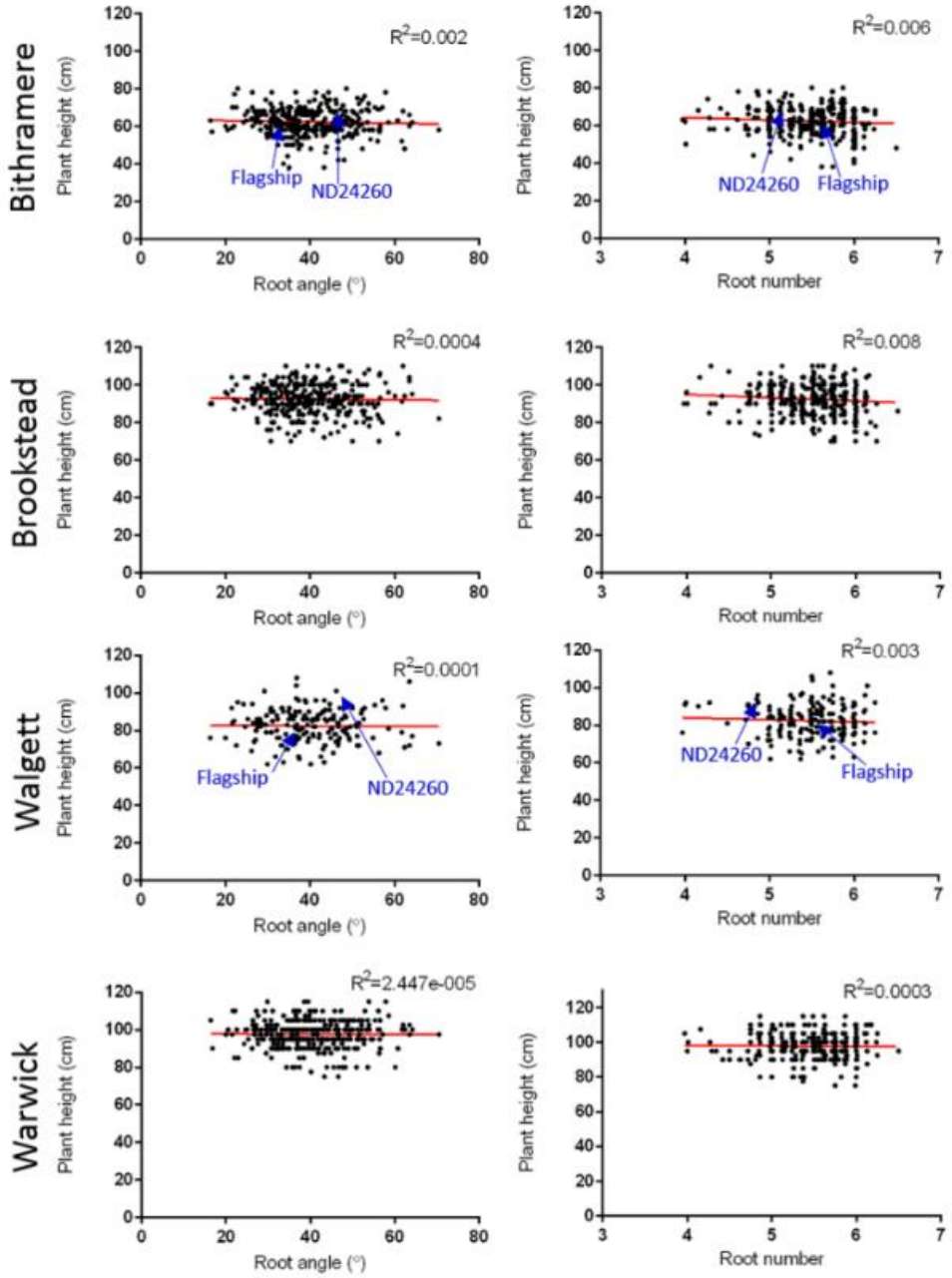

Figure 3.4 Linear regression between root angle and plant height means, and root number and plant height means (across four field environments: Bithramere, Brookstead, Walgett and Warwick) for the ND24260 × Flagship DH population. Plant height measurements were collected at spike emergence, stage 5 on the Zadoks growth scale. Positions of the parental lines (ND24260 and Flagship) are represented by blue arrows in Bithramere and Walgett environments, where plant height data was available. The percentage of variation explained by the regression model is represented by R^2 value for each linear regression.

3.4.3 Mapping genes for seminal root traits

CIM identified a total of seven QTL for seminal root traits in the ND24260 × Flagship DH population: two QTL for root angle (i.e. *RAQ1–2*) and five QTL for root number (i.e. *RNQ1–5*). Of the seven QTL, four were deemed major effect QTL (i.e. $-\log_{10}(P) > 6$), including; *RNQ1* at bPb-8983 on chromosome 1H-1 ($-\log_{10}(P)$ 6.1), *RNQ2* at bPb-9273 on 3H ($-\log_{10}(P)$ 9.6), *RNQ4* at bPb-2689 on 5HL ($-\log_{10}(P)$ 7) and *RAQ2* also on 5HL at bPb-34072 ($-\log_{10}(P)$ 8), (Table 3.2; Figure 3.5). The two QTL mapped to 5HL (i.e. *RAQ2* and *RNQ4*) aligned with the same four DArT markers in the region, spanning 3.5cM. Thus, *RAQ2/RNQ4* appeared to be the same QTL influencing both traits. The effect associated with the ND24260 allele donating wide root angle and high root number in this region accounted for 9.6% of the phenotypic variation for root angle and 6.8% for root number.

Table 3.2 QTL for seminal root traits in the ND24260 × Flagship DH population

QTL	LG	Peak marker[a]	Pos. (cM)	$-\log_{10}(P)$[b]	CI (cM)[d]	Flanking markers	Source[e]	Variation explained[g]
RAQ1	3H	bPb-8021	226.9	3.8	7.6	bPb-0049 bPb-2420	Flagship	3.8%
RAQ2	5H-2	bPb-1217	235.9	8.1	16.2	bPb-5053 bPb-2689	ND24260	9.6%
RNQ1	1H-1	bPb-8983	120.8	6.1	12.2	bPb-5877 bPb-7949	ND24260	5.8%
RNQ2	3H	bPb-9273	79.6	9.6	19.2	bPb-0285 bPb-4645	Flagship	10.1%
RNQ3	4H	bPb-6101	171.1	3.6	7.2	bPb-2677 bPb-5743	Flagship	3.2%
RNQ4	5H-2	bPb-1217	235.9	7	14	bPb-5053 bPb-2689	ND24260	6.8%
RNQ5	6H-1	bPb-0696	117	4.6	9.2	bPb-3184 bPb-6721	Flagship	4.1%

[a] Peak position of QTL region on genetic linkage map of the ND24260 × Flagship DH population

[b] $-\log_{10}(P)$ score for QTL peak position derived from CIM, where a QTL significance threshold - $\log_{10}(P)$ of 3.6 was applied based on the Bonferroni threshold.

[d] Confidence interval of QTL calculated by the two LOD drop method

[e] Parental allele source for wide root angle and high root number for each QTL derived from CIM

[g] Percentage of phenotypic variation for root angle or root number explained by the QTL

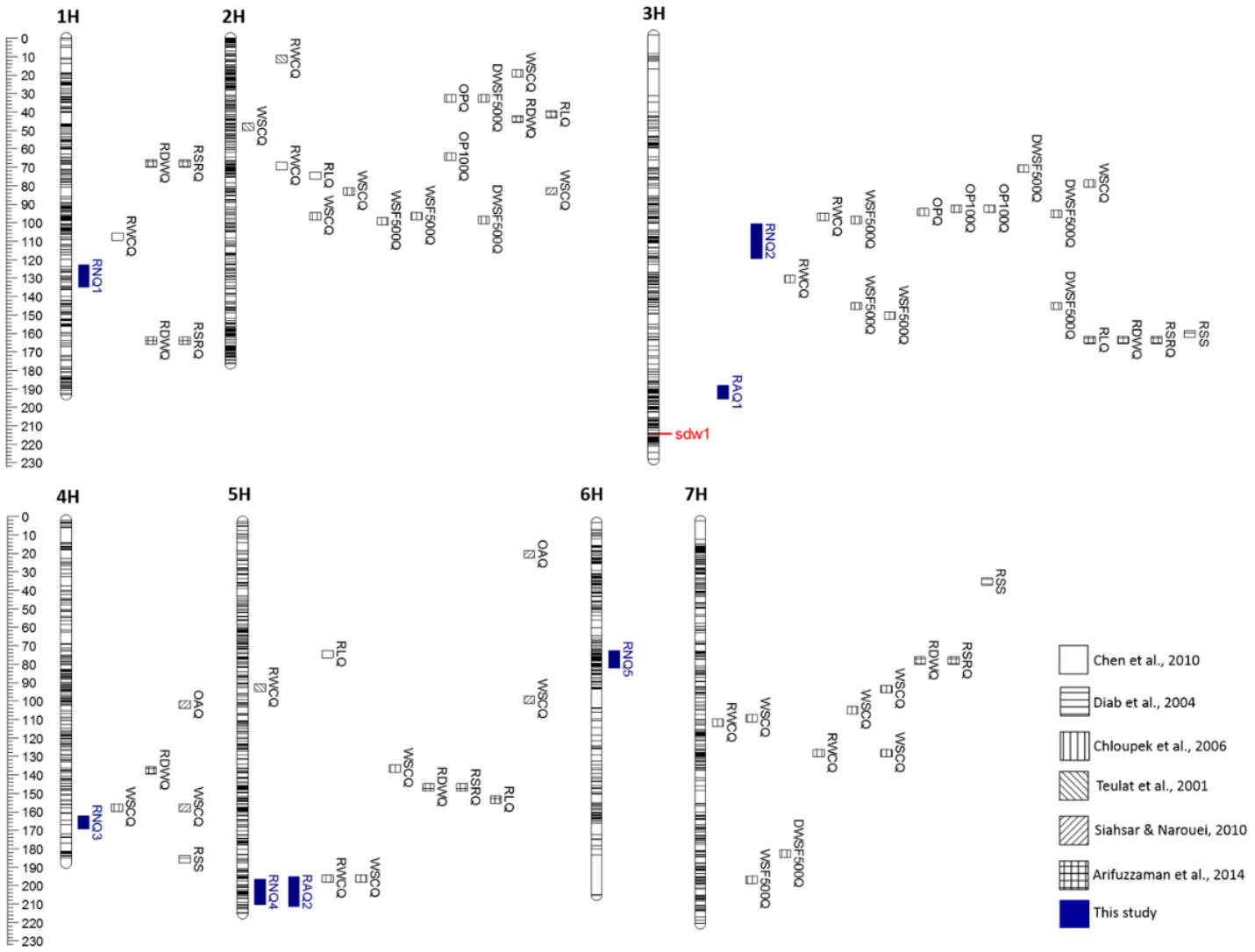

Figure 3.5 Barley drought tolerance QTL projected onto the DArT consensus map. A total of 62 QTL

were sourced from six discovery papers (Teulat et al. 2001; Diab et al. 2004; Chloupek et al. 2006; Chen et al. 2010; Siahsar and Narouei 2010; Arifuzzaman et al. 2014), along with the seven QTL identified for seminal root traits in this study. Twelve traits for drought tolerance are displayed on the map (root angle (*RAQ)*, root number (*RNQ*), root length (*RLQ*), root dry weight (*RDWQ*), root to shoot ratio (*RSRQ*), root system size (*RSSQ*), relative water content (*RWCQ*), accumulation water-soluble carbohydrate at 100% RWC (*DWSC100Q*), osmotic potential (*OPQ*), osmotic potential full turgor (*OP100Q*), water-soluble carbohydrate (*WSCQ*), WSC full turgor (*WSC100Q*), osmotic adjustment (*OAQ*)). Confidence intervals adjusted to 4cM for display purposes for 62 previously published QTL only.

RAQ1, RNQ3 and *RNQ5,* the remaining QTL detected in this study, were mapped to chromosomes 3H, 4H and 6H, respectively (Figure 3.5). Although significant, the effects associated with these QTL were minor, where *RAQ1* explained 3.8% of the phenotypic variation and *RNQ3* and *RNQ5* explained 3.2% and 4.1%, respectively (Table 3.2). All QTL identified for both seminal root traits are relatively small effect QTL (Table 3.2), leaving large proportions of phenotypic variation unexplained by the QTL identified in this DH population. The two parents, ND24260 and Flagship, both contributed positive and negative QTL for seminal root angle and root number.

Of the seven QTL identified in this study, three QTL (*RAQ2, RNQ2, RNQ3, RNQ4*) co-locate with previously reported genomic regions influencing drought adaptation traits in barley (Figure 3.5). *RNQ3* positioned on chromosome 4H co-located with QTL influencing water soluble carbohydrate (WSC; Diab et al. 2004). The QTL on chromosome 5HL (*RAQ2*/*RNQ4*) was in close proximity to two QTL influencing WSC and leaf relative water content (RWC; Diab et al. 2004) (Figure 3.5). The majority of previously identified QTL for drought adaptation traits were located on chromosomes 2H, 3H, 5H and 7H (Appendix 1). This study is the first to map a QTL for a drought adaptation trait to chromosome 6H (Figure 3.5).

3.4.4 Across species analysis of genes underlying root trait QTL

The across species genetic analysis identified 10 putative genes underlying root QTL across barley, wheat and sorghum. The 10 putative genes identified underlie three QTL in barley (*RNQ1, RNQ2, RAQ2/RNQ4*), four QTL in wheat (*QRA.qgw-2A, QRA.qgw-3D, qRN.qgw-3B, qRA.qgw-5D*) and 10 QTL in sorghum (*QRtWgt9.1, QBrcRt6.1, QRtShtR3.2, QRtWgt4.1, QRtShtR3.1, QRtAng10.1, QBrcRt7.1, QRtAng5.1, QRtWgt6.1, QRtShtR4.1*) (Table 3.3). The gene annotations for the 10 common putative genes are inexplicit, except for one gene that belongs to the expansin gene family.

Table 3.3 Common putative genes found underlying root trait QTL across barley (Hv), wheat (Ta) and sorghum (Sb)

Hv gene	Ta gene	Sb gene	Hv root QTL	Ta root QTL	Sb root QTL	Sb gene annotation
MLOC_81957	Traes_3DL_FCBD5B687	Sb09g023440	RNQ2	QRA.qgw-3D	QRtWgt9.1	similar to Expansin-A4 precursor
	Traes_3DL_BF5DFF112	Sb06g003250			QBrcRt6.1	similar to Alpha-expansin 3 precursor
		Sb03g009420			QRtShtR3.2	similar to Expansin-A9 precursor
		Sb04g028090			QRtWgt4.1	similar to Expansin-A5 precursor
		Sb03g005140			QRtShtR3.1	similar to Expansin-A9 precursor
		Sb10g030370			QRtAng10.1	similar to Expansin-A29 precursor
		Sb06g003250			QBrcRt6.1	similar to Alpha-expansin 3 precursor
		Sb09g023440			QRtWgt9.1	similar to Expansin-A4 precursor
		Sb04g028090			QRtWgt4.1	similar to Expansin-A5 precursor
		Sb10g030370			QRtAng10.1	similar to Expansin-A29 precursor
		Sb03g009420			QRtShtR3.2	similar to Expansin-A9 precursor
		Sb03g005140			QRtShtR3.1	similar to Expansin-A9 precursor
MLOC_42209	TRAES3BF057400110CFD_g	Sb07g021930	RNQ2	qRN.qgw-3B	QBrcRt7.1	similar to Putative uncharacterized protein
		Sb05g018060			QRtAng5.1	similar to Transferase family protein, putative, expressed
		Sb10g029610			QRtAng10.1	similar to Os06g0710700 protein
		Sb06g021640			QRtWgt6.1	similar to OSJNBa0029H02.19 protein
		Sb04g025760			QRtShtR4.1	similar to Putative uncharacterized protein
		Sb10g029620			QRtAng10.1	similar to Os06g0710700 protein
MLOC_37551	Traes_2AL_DB4861821	Sb06g001503	RNQ1	QRA.qgw-2A	QBrcRt6.1	Predicted protein
		Sb07g021667			QBrcRt7.1	weakly similar to Putative uncharacterized protein
MLOC_64669	Traes_5DL_538A91D19	Sb10g028360	RAQ2/RNQ4	qRA.qgw-5D	QRtAng10.1	similar to Putative uncharacterized protein
MLOC_78515	Traes_5DL_DDE21C2D8	Sb07g021490	RAQ2/RNQ4	qRA.qgw-5D	QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
	Traes_5DL_D1095CE51	Sb05g007340			QRtAng5.1	Predicted protein
		Sb04g023790			QRtShtR4.1	Predicted protein
MLOC_201	Traes_5DL_0FE07B4F4	Sb04g028940	RAQ2/RNQ4	qRA.qgw-5D	QRtWgt4.1	similar to Putative uncharacterized protein

MLOC_58351	Traes_5DL_DDE21C2D8	Sb07g021490	RAQ2/RNQ4	qRA.qgw-5D	QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
		Sb04g023790			QRtShtR4.1	Predicted protein
		Sb05g007340			QRtAng5.1	Predicted protein
		Sb07g021490			QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
		Sb04g023790			QRtShtR4.1	Predicted protein
		Sb05g007340			QRtAng5.1	Predicted protein
MLOC_31941	Traes_5DL_DDE21C2D8	Sb04g023790	RAQ2/RNQ4	qRA.qgw-5D	QRtShtR4.1	Predicted protein
	Traes_5DL_D1095CE51	Sb07g021490			QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
		Sb04g023790			QRtShtR4.1	Predicted protein
		Sb07g021490			QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
		Sb04g023790			QRtShtR4.1	Predicted protein
		Sb07g021490			QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
		Sb04g023790			QRtShtR4.1	Predicted protein
		Sb07g021490			QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
		Sb04g023790			QRtShtR4.1	Predicted protein
		Sb07g021490			QBrcRt7.1	weakly similar to Putative uncharacterized protein P0022B05.123
MLOC_61963	Traes_5DL_0FE07B4F4	Sb04g028940	RAQ2/RNQ4	qRA.qgw-5D	QRtWgt4.1	similar to Putative uncharacterized protein
MLOC_72666	Traes_5DL_59AAC9844	Sb04g024850	RAQ2/RNQ4	qRA.qgw-5D	QRtShtR4.1	similar to Chloroplast translational elongation factor Tu
	Traes_5DL_B38FE5CB3					

Projection of wheat and sorghum QTL onto the barley ND24260 × Flagship map (Figure 3.6) shows the overlap of root QTL across all three species. Furthermore, chromosome 5H and 6H appear to have key regions where root QTL from all three species co-locate.

3.5 Discussion

This is the first study to measure both seminal root angle and root number in barley. Importantly, this root phenotyping was completed in less than seven days, thus highlighting the high-throughput capacity of this phenotyping system. As a result, seven novel genomic regions influencing barley seminal root traits have been detected in the ND24260 × Flagship DH population. Four major effect QTL were identified; one for root angle (i.e. *RAQ2* located on 5HL) and three for root number (i.e. *RNQ1, RNQ2, RNQ4* located on 1H-1, 3H and 5HL, respectively). The collocation of previously reported QTL for drought adaptation traits (i.e. RWC and WSC; Diab et al. 2004) on the barley consensus map suggests the genomic region identified on chromosome 5HL (bPb-34072, bPb-5053, bPb-1217, bPb-2689) may be an important region for abiotic stress tolerance in barley. Furthermore, this genomic region on 5HL is identical to the major QTL previously detected for grain dormancy (i.e. *qSDND*) in the ND24260 × Flagship DH population (Hickey et al. 2012), with the allele for both QTL (i.e. grain dormancy and *RAQ2*/*RNQ4*) donated by ND24260. It is possible that seed harvested from a plant with a root system that allows increased access to soil moisture may have less abscisic acid (ABA) due to less stress during grain-filling and thus reduced dormancy. It is probable that underlying mechanism(s) for grain dormancy expressed in mature grain may also influence seminal root growth characteristics in barley.

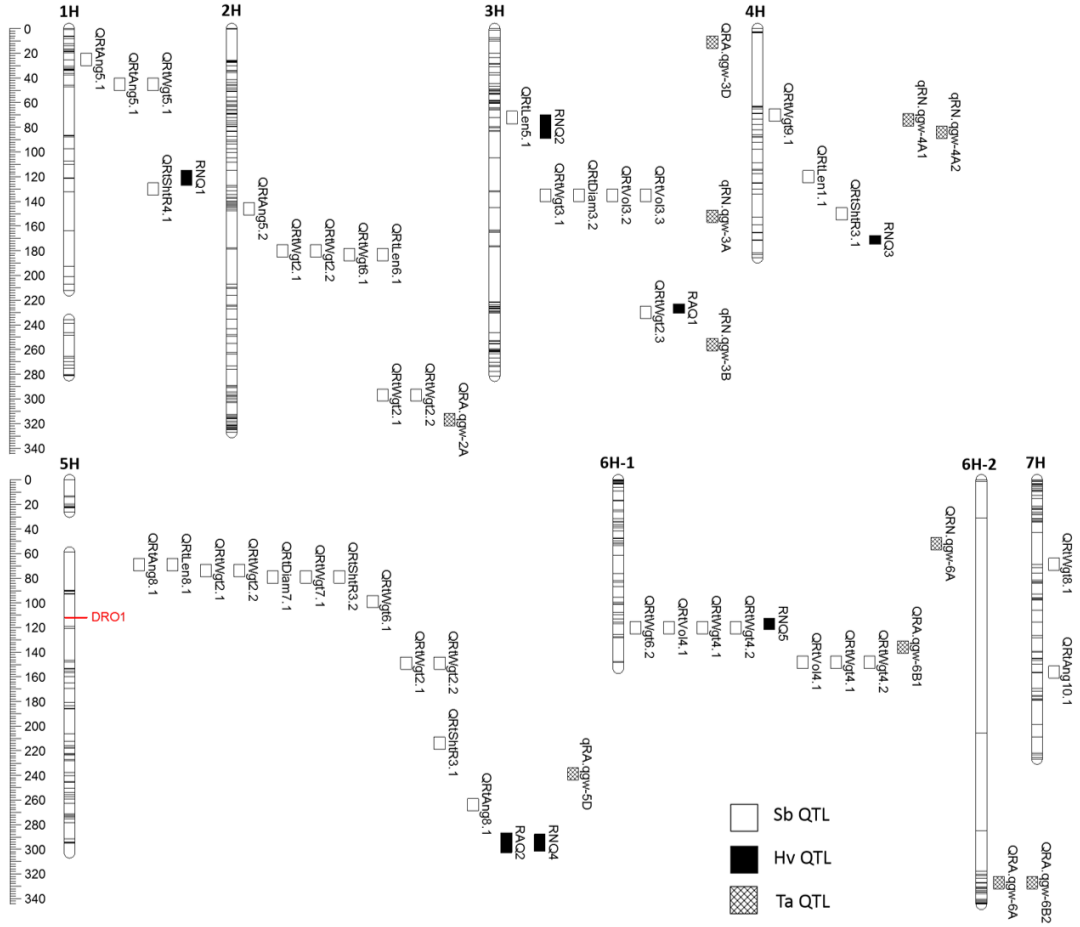

Figure 3.6 Barley ND24260 × Flagship DH map with barley, wheat and sorghum root QTL. Barley seminal root angle and root number QTL reported in the current study (Hv QTL), seminal root angle and number QTL reported in wheat (Christopher et al. 2013; Ta QTL) and QTL identified for root traits in sorghum (Mace et al. 2012; Rajkumar et al. 2013; Bekele et al. 2014; Hufnagel et al. 2014; Li et al. 2014; Phuong et al. 2014; Wang et al. 2014; Sb QTL) were projected onto the ND24260 × Flagship DH map using physical positions of the QTL. A confidence interval of 10 cM was applied to the projected QTL for display purposes based on the average confidence intervals reported in the original QTL mapping studies.

3.5.1 Phenotypic expression of root traits

Previous studies of root traits in wheat, rice and sorghum have identified narrow root angle and high root number (expressed in seedlings) as a precursor for deep rooting and greater branching at depth. These traits were reported to be particularly beneficial under terminal drought conditions with evidence of water stored at depth (Manschadi et al. 2006; Uga et al. 2011; Mace et al. 2012; Christopher et al. 2013). Based on these trends across cereal crops, such root system architecture might be desirable in barley cultivars. However, these root traits are yet to

be validated as beneficial for barley drought adaptation. Field studies are required to better understand the root architecture of barley and how it contributes to drought adaptation. Further evaluation of the ND24260 × Flagship population in water-limited and irrigated field trials should further our understanding of root system architecture in barley and its influence on drought adaptation.

It should be noted that while root traits may influence access to water, there are many physiological traits influencing water-use, and thus yield under water-limited conditions i.e. plant height, maturity, tiller production and early vigour (Gavuzzi et al. 1997; Gonzalez et al. 2010; del Pozo et al. 2012, de Mezer et al. 2014). In rice, no significant difference was observed for shoot traits in the *DRO1*-NIL and its respective standard even though root distribution and drought adaptation differed between the genotypes (Uga et al. 2013a). Similarly, in this study, root angle and root number were not correlated with plant height in the DH population across four field environments (Figure 3.4). Across these four field environments in Queensland and New South Wales, post-anthesis drought stress is commonly observed. Post-anthesis drought stress has less effect on plant height, as plants tend to reach their maximum height at anthesis prior to the stress. Pre-anthesis drought stress on the other hand, is more likely to affect plant height, as the stress occurs during early plant growth. Therefore, in post-anthesis drought environments it is unlikely that root traits and plant height will be correlated, and it is more likely that grain-filling will be affected.

Root length has been associated with some semi-dwarf genes in barley. The semi-dwarfing gene *ari-e*.GP aligns with QTL detected for root length and is thought to be associated with a reduced root length in barley (Chloupek et al. 2006). The Australian cultivar, La Trobe, carries this semi-dwarf gene. Therefore, while La Trobe displays a narrow seminal root angle, the effects associated with the semi-dwarfing gene *ari-e*.GP may hinder root length of mature plants and restrict the depth of roots for this cultivar. Flagship, as with many European-derived cultivars, carries the *sdw1* semi-dwarfing gene, which segregates in the ND24260 × Flagship DH population. The lack of correlation between plant height and root traits across four environments (Figure 3.4) suggests that *sdw1* does not influence seminal root angle and root number. Similarly, the study by Chloupek et al. (2006) also found no relationship between *sdw1* and root length. The *sdw1* gene was also projected onto the DArT consensus map (Figure 3.5) using the 3HL chromosome position reported by Chloupek et al. (2006) to investigate the alignment between *sdw1* and QTL for root traits detected in the current study. Projection of

sdw1 showed no alignment between the gene and the root trait QTL, with *RAQ1* in closest proximity to *sdw1*.

Stay-green is an important trait influencing drought adaptation in cereal grains (Borrell et al. 2000a, b; Christopher et al. 2008; Jordan et al. 2012; Mace et al. 2012; Borrell et al. 2014a, b). In other genetic studies using the ND24260 × Flagship DH population, it has been observed that ND24260 contributes the stay-green phenotype in the cross (Gous et al. 2013). In sorghum, four QTL identified for nodal root angle (Mace et al. 2012) were reported to collocate with stay-green QTL and a putative association was established between nodal root angle and stay-green (Borrell et al. 2014a). Such genetic relationships are yet to be reported for seminal root angle and stay-green in wheat and barley.

In our study, ND24260 displayed a wider seminal root angle than Flagship, and was found to donate the allele for wide root angle and increased root number at the major QTL on chromosome 5HL (i.e. *RAQ2/RNQ4)*. ND24260 is a breeding line that was bred and selected under a short summer season in Fargo, North Dakota. ND24260 has good levels of heat stress tolerance, but has reduced tiller number and yield in some environments. The soil environment in Fargo consists of black silty clays with high water-holding capacity, similar to south-east Queensland (Natural Resources Conservation Service Soils 2015; Queensland Government 2015). When grown in Queensland, ND24260 displays the stay-green drought adaptation phenotype, which is normally a consequence of the improved balance between the supply and demand of water during the grain-filling phase (Borrell et al. 2014a). It is possible the heat-stress tolerance of ND24260 is a key factor in maintaining this water balance to enable the plant to remain green. In wheat, field experiments have shown that root number, length and diameter are reduced under high temperatures, especially during the grain-filling phase (Batts et al., 1998). Thus, the heat-stress tolerance of ND24260 could act as a protective mechanism for root traits, allowing roots to extract more water during heat-stress.

Alternatively, it is possible that the architecture of wide root angle and high root number (displayed by ND24260) enhances the plant's ability to capture water stored in the soil, particularly from mid-upper soil layers, resulting in the expression of stay-green. In sorghum, a narrow root angle was associated with the stay-green phenotype, which is thought to improve access to soil water at depth in the profile (Borrell et al. 2014a). Similarly in wheat, the stay-green genotype, SeriM82, extracted more water from depth after anthesis on vertosol soils in north-eastern Australia (Christopher et al., 2008). On the other hand, wheat genotypes with wider root angles might be better equipped to utilise in-crop rainfall due to their denser, but

shallower, root systems (Liao et al., 2006). It is likely such trends stretch across cereal species; however, field and modelling studies are required to assess the interactions between various barley root architectures, target environments and management strategies (i.e. G × E × M interactions) to identify optimum root angle phenotypes for breeders to target.

3.5.2 Novel QTL for seminal root traits in barley

Seven genomic regions influencing seminal root traits were identified in the ND24260 × Flagship DH population in this study. Two QTL were detected for seminal root angle and five QTL for seminal root number. Based on the collocation of the two major QTL on chromosome 5HL (i.e. *RAQ2* for root angle and *RNQ4* for root number), it is highly possible a single gene could underpin both root traits within this genomic region.

Interestingly, the *RAQ2*/*RNQ4* region on 5HL is identical to the major QTL previously detected for grain dormancy (i.e. *qSDND*) in the ND24260 × Flagship DH population (Hickey et al. 2012), with the allele for both QTL (i.e. grain dormancy and *RAQ2*/*RNQ4*) donated by ND24260. It should be noted, in the study by Hickey et al. (2012), spikes were sampled from the field at the point of physiological maturity, dried, grain threshed by hand and stored at 20˚C to preserve grain dormancy prior to germination testing. On the other hand, grain used in the current study was sourced from long term seed storage and lacked grain dormancy – rapid and synchronous germination was observed for all lines. This suggests the underlying mechanism(s) (e.g. accumulation of hormones in the grain) that are responsible for expression of grain dormancy in harvest-ripe grain may also influence seminal root growth characteristics during the early stages of germination in barley. To further investigate this key genomic region on 5HL and to identify other genomic regions influencing seminal root traits in barley, experimentation on a barley population with a greater allelic diversity would be desirable. It is important to note that QTL identified in the current study are based on phenotypes assessed at early seedling growth stage, which has yet to be correlated with adult root trait phenotypes in barley. Further experimentation is required to validate the assumption that root angle and number phenotypes observed in early seedlings are representative of these traits at adult growth stage. This could be determined by phenotyping adult plants using large root chambers commonly used for nodal root angle phenotyping in sorghum (Singh et al. 2011). These chambers allow the root system of a plant to be visualised throughout its entire lifecycle and therefore comparison between early seedling root phenotypes and adult phenotypes is possible.

3.5.3 Across species analysis of genes underlying root trait QTL

The comparative genomics analysis identified 10 common genes underlying root trait QTL CIs in barley, wheat and sorghum (Table 3.3). This suggests that the genetics influencing root traits, more specifically root angle and number, may be similar across the three cereal crops. Of the 10 genes identified, seven of the genes underlie the key barley root trait QTL identified on 5HL in the current study (*RAQ2/RNQ4*). The same seven genes underlie a minor root angle QTL identified in wheat, positioned on chromosome 5D (*qRA.qgw-5D*). Projection of this wheat QTL onto the barley ND24260 × Flagship map (Figure 3.6) shows that the two barley root QTL (*RAQ2 and RNQ4*) and the wheat root QTL (*qRA.qgw-5D*) are in close proximity. For sorghum, three of the seven common genes identified for root angle QTL in barley and wheat underlie a sorghum root angle QTL position on chromosome 5 (*QRtAng5.1*).

The sorghum gene annotation is the most detailed of the three cereal species, and therefore is the annotation used in Table 3.3. Of the 10 common genes identified, one gene has a descriptive annotation and is most likely a member of the expansin gene family. Expansin genes function as principle regulators of cell wall expansion in plants throughout their growth (Lee et al. 2003; Li et al. 2015). Expansin genes have been correlated with the initiation of root growth and root elongation in soybean (Lee et al. 2003), and reported to enhance root growth and improve water stress tolerance in tobacco (Li et al. 2015). The gene identified as a member of the expansin gene family would be a key root trait gene to target for any gene specific investigations.

3.5.4 QTL for seminal root traits co-locate with QTL for drought adaptation

RNQ3 mapped to chromosome 4H co-located with QTL for water soluble carbohydrate (WSC) reported by Diab et al. (2004). WSC stored in the stems and the leaf sheaths provide essential nutrients required during grain-filling, however the WSC concentration of a plant is under complex genetic control (McIntyre et al. 2012). The collocation of the genetic control for WSC and root number could suggest that improved WSC concentration may be due to improved root number and greater access to nutrients stored in the soil. Interestingly, the key QTL controlling both seminal root angle and number (i.e. *RAQ2*/*RNQ4*) on chromosome 5HL is positioned within only 3 cM of QTL influencing WSC and also relative water content (RWC). RWC is an indicator used to assess the water status of a plant (Saura-Mas and Lloret 2007), whereby a high RWC in the leaf during grain-filling indicates that the plant is accessing sufficient water to keep cells turgid during this developmental phase. Therefore, it is likely that a more efficient root architecture (root angle and number) enhances the plant's ability to access water stored in

the soil and improve the plant's RWC and WSC for vital development. Based on the current literature, this is the first study to map a possible drought adaptation trait (root number) to chromosome 6H. However, the current understanding of genomic regions influencing drought adaptation in barley is far from comprehensive. Future knowledge in these areas should evolve with the increased affordability of genotyping and the development of high-throughput phenotyping methods.

3.6 Conclusion

Barley breeders have focused on above-ground traits and have indirectly selected for drought adaptation via selection for yield *per se* in target environments. Here we present the first study to phenotype root system architecture in barley, in particular, seminal root angle and number, using the high-throughput and inexpensive clear pot method. A high degree of diversity for seminal root traits was observed in the panel of 30 barley genotypes and the DH population evaluated in this study. The genomic regions identified in this study provide a first-look at the genetics of seminal root traits (angle and number) in barley. We have flagged regions on chromosomes 1H, 3H, 4H, 5H and 6H as influencing seminal root traits, with a region influencing both root angle and root number positioned on 5HL. This key genomic region was found to collocate and share seven common genes with a wheat root angle QTL. Alignment of previously reported drought adaptation QTL with regions identified in the current study highlight the co-location of above-ground (WSC and RWC) and below ground traits (seminal root angle and root number) related to drought adaptation in barley, particularly on chromosomes 4H and 5HL. Further QTL mapping studies in a population with increased allelic diversity will also assist in identifying other sources of genetic control for seminal root traits that could not be identified in the ND24260 × Flagship population. Furthermore, the QTL identified in this chapter need to be validated in other genetic backgrounds to determine their significance for molecular breeding irrespective of the population. Finally, the relationship between seminal root traits and yield should be explored to determine the value of these traits for crop improvement.

3.7 References

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Chapter 4

ROOT ARCHITECTURAL TRAITS AND YIELD: EXPLORING THE RELATIONSHIP IN BARLEY BREEDING TRIALS

4.1 Abstract

Root system architecture is fundamental to resource capture and productivity of cereal crops. Understanding the genetics modulating root development will assist plant breeders to design cultivars with optimal root systems for the target environment. In Chapter 3, we performed the first study to map quantitative trait loci (QTL) for seminal root traits in barley (*Hordeum vulgare* L.) using the clear pot method. As a result, we detected multiple QTL, yet we identified that these QTL require validation in an independent population with greater allelic diversity. Furthermore, we highlighted the need for the relationship between seminal root traits and yield to be examined to assist in determining the value of these traits in barley. In this chapter, we investigate the genetic association between seminal root traits and yield in elite barley germplasm and perform association mapping to validate QTL detected in Chapter 3. To do this, a panel of 216 breeding lines from the Northern Region Barley Breeding Program in Australia, genotyped with Diversity Arrays Technology (DArT) markers, were characterised for seminal root angle and number. A high degree of phenotypic variation was evident in the population, ranging from 12.0 to 89.4° and 4.8 to 6.1 for root angle and number, respectively. A QTL for root angle (*qRA*-5) was detected on chromosome 5H and collocated with the previously identified *RAQ2* in Chapter 3. The genetic relationship between seminal root traits and yield was investigated using the panel's root phenotypes and yield data from 20 field trials. Genetic correlations with yield ranged from -0.21 to 0.36 for root angle and from -0.20 to 0.25 for root number. The direction and magnitude of the correlations for both root traits varied across the environments, but overall root angle was more strongly associated with yield. This chapter provides insight into the root phenotypes of

breeding lines and delivers a first look at the genetic relationship between root architectural traits and yield in barley breeding trials.

4.2 Introduction

Roots are critical to many plant functions, taking up water and nutrients, while anchoring the plant in the soil. In cereals, the fibrous root system can be separated into seminal roots, emerging from the primordia in the embryo, and nodal or secondary roots, developing later from the lower nodal regions of the culm during tillering (Forster et al. 2007). A key constraint for plant productivity is inadequate resources in the soil space occupied by the root system (e.g. low water, N, P and K), confounded by the possibility that resources are available elsewhere in the soil, but simply out of reach for some root systems (e.g. mobile nutrients leaching deeper in to the soil). Challenging environmental conditions, such as drought and warming, are predicted to increase with the changing climate, and coupled with an expanding global population, improving crop productivity and yield stability is crucial (Lobell et al. 2011; Tilman et al. 2011; Dai 2013; Asseng et al. 2015; Lobell et al. 2015). The root system architecture of crops is complex and can be defined as the geometric dispersion of roots within the soil space (Lynch 1995; Rich and Watt 2013). Because architecture can vary dramatically among and within species, understanding the genetics that drive this variation can allow selection for root systems that capture more resources and increase productivity in marginal environments (Lynch et al. 2014). Recent findings in maize (*Zea mays* L.; Ali et al. 2015), rice (*Oryza sativa* L.; Uga et al. 2013), sorghum (*Sorghum bicolor* L.; Mace et al. 2012) and wheat (*Triticum aestivum* L.; Manschadi et al. 2010) highlight the relationship between root angle and yield and, in particular, the contribution to yield stability under abiotic stress.

In maize grown under water-stressed conditions, Ali et al. (2015) reported a strong positive phenotypic correlation between narrow seminal root angle and yield ($r = 0.75$, $P \leq 0.001$) and narrow nodal root angle and yield ($r = 0.89$, $P \leq 0.001$). Putative genetic associations between maize root traits and yield, whereby quantitative trait loci (QTL) collocate, have also been identified across multiple populations and studies (Tuberosa et al. 2002; Giuliani et al. 2005; Landi et al. 2007). To highlight this, Landi et al. (2010) demonstrated that the root QTL *root-yield-1.06* had a positive influence on plant vigour and yield under various water regimes and genetic backgrounds.

The relationship between root angle and yield in rice was established through the identification of *DEEPER ROOTING1* (*DRO1*), a major gene influencing narrow root system architecture and consequently deep rooting (Uga et al. 2013). When introgressed into a shallow rooting cultivar, the resulting *DRO1* near-isogenic line (NIL) displayed a significantly higher yield when grown under drought conditions (Uga et al. 2013). Further, narrow root angle in rice was shown to improve yield by up to 10% when water was not limiting (Arai-Sanoh et al. 2014). It has been suggested that a *DRO1* homolog in other monocots may be important for drought adaptation in these traits (Uga et al. 2013). A *DRO1* homolog has been identified in barley and appears to be independent of narrow root system architecture (Voss-fels et al. 2018), however further research is required for validation. Other root traits in rice, such as total root length, root thickness, and maximum root length, are also correlated with yield (Zhou et al. 2016).

Early work in wheat by Richards and Passioura (1989) found that a narrower xylem vessel in seminal roots increased hydraulic resistance, thereby decreasing the rate of water-use from the sub-soil when the top-soil was dry, thus conserving water for grain-filling. This regulation of water-use was quite effective, contributing yield advantages up to 11% in water-limited environments. More recently, research has focussed on seminal root angle, a proxy trait for the overall shape of the mature wheat root system architecture (Manschadi et al. 2008; Manschadi et al. 2010; Christopher et al. 2013). Using the crop modelling system APSIM, Manschadi et al. (2006) showed that a narrow seminal root angle, and consequently a compact root architecture, resulted in wheat yield gains in environments with deep soils and low in-season rainfall. The greatest yield advantages were found in environments with the greatest water deficits, where the mean yield benefit was 14.5%.

Development of the root system in sorghum differs to that of wheat and barley, whereby one primary (vertical) is produced first with nodal roots only developing at the fourth and fifth leaf stage. Interestingly, relationships between root traits and yield have been reported in sorghum (Mace et al. 2012; Fakrudin et al. 2013). A putative association between nodal root angle and yield was identified by Mace et al. (2012), whereby the favourable allele for narrow root angle at three of the four QTL was associated with higher yield. Considering a related trait, stay-green sorghum lines containing the *Stg1* and *Stg3* QTL increased water uptake during grain-filling in a terminal drought by 19 and 10 mm, respectively, compared with the senescent control (Borrell et al. 2014). Crop water use during grain-filling was positively correlated with grain yield in this study. The slope of this relationship (50 kg ha^{-1}

mm^{-1}) was comparable to the 55 kg ha^{-1} mm^{-1} reported by Manschadi et al. (2006) for wheat in the simulation study. There was some evidence in the study by Borrell et al. (2014) that *Stg* QTLs could modify root architecture in sorghum. For example, a *Stg4* fine-mapping population varied in biomass partitioning between root and shoot when harvested at the 5-leaf stage, with a trend for greater allocation to roots in the *Stg4* NIL compared with the senescent control. The four nodal root angle QTL in sorghum reported by Mace et al. (2012) co-located with previously identified QTL for stay-green. Across maize, rice, sorghum and wheat studies, it has been demonstrated that root system traits are important components of yield. More specifically, a narrow root angle appears to increase the depth of rooting and tends to improve yield under water deficit conditions.

The focus of this study is on barley (*Hordeum vulgare* L.); the fourth largest cereal crop produced worldwide, a vital raw material for malting, and the staple food crop for people in some North African and Middle Eastern countries, where water deficit is a frequent production problem. The relationship between root traits and yield is yet to be fully explored in barley, partly due to the challenge of accurately and efficiently phenotyping roots. Despite this difficulty, previous research has examined the relationship between root system size (RSS) and yield in field trials. Initially, this relationship was examined in a doubled haploid (DH) population, where RSS was estimated via electrical capacitance and results were averaged across growth stages and field sites. In this population, a weak but significant genetic correlation (0.21) was found between RSS and yield (Chloupek et al. 2006). Fluctuating phenotypic correlations were also observed between RSS and yield evaluated in a small number of barley cultivars (10-22) across multiple field sites and years, but interestingly, the strongest relationships were observed in the driest environments with the warmest temperatures (Chloupek et al. 2010). Svačina et al. (2014) also observed a significant phenotypic correlation between RSS and yield ($r = 0.23–0.40$), but only used a small number of lines evaluated in a single environment. Seminal root length and weight have also been linked to yield in two-rowed barley, where correlations were detected between traits (length: $r = 0.36–0.71$ and weight: $r = 0.38–0.61$), but again, only a small number of cultivars were examined using an artificial growth environment (Bertholdsson and Brantestam 2009). Previous research in this area has provided preliminary evidence that roots and yield are inter-related, however the small sample sizes accompanied with limited genetic diversity means that there is scope for building on our understanding of the relationship between roots and yield in barley.

In this study, we use the clear pot method (Richard et al. 2015) to characterise seminal root angle and root number for a panel of Australian two-rowed spring breeding lines and commercial barley cultivars. Initially, association analysis is conducted to gain a better understanding of the genomic regions influencing seminal root traits in the breeding population and validate QTL identified in Chapter 3 (Robinson et al. 2016). Then, we explore the genetic relationship between each seminal root trait and yield, using data from 20 environments across the north-eastern grain-growing region of Australia.

4.3 Materials and methods

4.3.1 Plant material

A panel of 216 two-rowed spring barley genotypes, predominately consisting of elite breeding lines, were characterised for seminal root angle and root number. Lines were selected to be part of the panel based on concurrence with breeding lines tested as part of the 30 barley genotypes evaluated for seminal root traits in chapter 3 as well as seed based on seed availability. Within the panel, 13 of the genotypes were commercial cultivars, and of these, 12 were Australian cultivars (Commander, Flagship, Fleet, Gairdner, Grimmett, Grout, Hindmarsh, Kaputar, Mackay, Roe, Shepherd and Tallon), and one was a cultivar from the United Kingdom (Static). Commander, Flagship and Shepherd were previous evaluated for seminal root traits in chapter 3. The remaining genotypes consist of breeding lines from the Northern Region Barley (NRB) Breeding Program based at the Hermitage Research Facility, Queensland, Australia, except for a small selection of accessions from the North Dakota State University (NDSU) and the International Center for Agricultural Research in the Dry Areas (ICARDA) barley breeding programs. All 216 genotypes in the panel were previously genotyped with 1,411 unique Diversity Arrays Technology (DArT) polymorphic markers (Ziems et al. 2014).

4.3.2 Phenotyping seminal root traits

Seminal root angle and root number were examined in the panel of 216 barley genotypes using the clear pot method (Richard et al. 2015). Seminal root angle was defined as the inner growth angle between the first pair of emerging seminal roots, as described by Christopher et al. (2013) and Richard et al. (2015). The first pair of seminal roots were measured at a point 3 cm below the embryo of the grain. Root number was measured by manually removing

individual seedlings with their roots intact and counting the number of roots. Across the experiment, eight replicate seeds for each genotype were examined, where the experiment consisted of 72 pots (containing 24 seeds per pot) spread across two benches using a non-resolvable incomplete block design. Each bench consisted of 36 pots spread out in a two-dimensional array of 4 columns × 9 rows, where each column formed one complete replicate and each pot an incomplete block. A linear mixed model was fitted to the data for each root trait, with spatial location included in the model. For the seminal root angle model, replicate, bench and pot were fitted as random terms. In the model for seminal root number, bench, replicate and pot were fitted as random terms. Variance components were estimated using residual maximum likelihood (REML). The best linear unbiased estimators (BLUEs) and best linear unbiased predictors (BLUPs) were calculated considering genotype as a fixed and random effect, respectively, in each model. A generalised heritability (or repeatability) of each trait was determined based on the estimated variance components of the model (Cullis et al. 2006), where the model was fitted in ASReml-R (Butler et al. 2008).

4.3.3 QTL mapping for seminal root traits

Root trait BLUEs and the DArT marker profiles of the panel were used to investigate genome-wide marker-trait associations using the R package 'Genome Association and Prediction Integrated Tool' (GAPIT; Lipka et al. 2012). Prior to analysis, the DArT marker data was filtered so that all markers had no more than 10% missing data and a minor allele frequency greater than 5%; as a result, a total of 1,027 markers were included in the analysis. The distribution of markers across the chromosomes was as follows, 89 markers on chromosome 1H, 146 markers on 2H, 102 on 3H, 48 on 4H, 93 on 5H, 66 on 6H, 128 on 7H and 332 markers with an unmapped position. Across all chromosomes, marker correlations dropped below $r = 0.2$ at a distance of 12 cM. To further improve the marker data, the R package 'Softimpute' was used to impute missing marker data via singular value decomposition (Hastie et al. 2015). Following imputation, a mixed linear model was fitted with a kinship matrix to account for relatedness between individuals, and the first principal component fitted as a fixed effect to adjust for global population structure within the panel (Price et al. 2006; Yu et al. 2006). To adjust for multiple comparisons, the Bonferroni correction at α level 0.05 was used, where the number of independent tests was defined by the estimator calculation described by Patterson et al. (2006). Association analysis results were validated by repeating the analysis in GenStat 18 (Boer et al. 2015), using the same mixed model parameters, as described for GAPIT. Association analysis was performed

separately for each root trait. QTL detected in this study were visualised on a chromosome map using Map-Chart v2.2 (Voorrips 2002) based on the DArT consensus map (Ziems et al. 2014). For comparison, QTL previously detected for seminal root traits in Chapter 3 were positioned on the consensus map using the projection method outlined by Mace et al. (2009).

To investigate possible relationships between population structure and the main QTL for seminal root angle detected in this study (*qRA-5*), three separate principal component analyses (PCA) were conducted using the marker profiles, and genotypes were colour coded according to whether they carried the 'narrow' or 'wide' allele at the locus on chromosome 5H. The three PCAs investigated structure at three levels: the first used all markers across the whole genome, the second used markers on chromosome 5H only, and the third used markers within the 7cM confidence interval on either side of *qRA-5*. The length of the confidence interval was determined by the average rate of linkage disequilibrium (LD) decay on chromosome 5H. PCA and LD decay analyses were performed in R (R Core Team 2016), with the R package 'sommer' used for LD decay (Covarrubias-Pazaran 2016).

4.3.4 Yield data provided by the NRB breeding program

Yield data from the NRB breeding program obtained from 2007 – 2009 were used in this study, which included 20 trials selected on the presence of some or all of the 216 genotypes characterised for seminal root traits. The trials were conducted throughout the northern grain-growing region of Australia (Figure 4.1), with each field trial encompassing a varying number of the 216 genotypes, as detailed in Table 4.1. Soil moisture data pre-sowing and throughout the season was not available for the 20 yield trials. However, heavy-clay cracking soils with high water holding capacity are typical of the growing region. All Stage 2 trials were randomised with two replicates using a latinised row-column design (John, 1987). The Stage 1 trials were designed as partially replicated trials with approximately 25% replication (Cullis et al., 2006). (Table 4.1).

Figure 4.1 Map of yield trial locations in the northern grain-growing region of Australia. Locations of the 20 trials, spread across the north-eastern coast, are depicted by icons. Multiple trials in one location are represented by multiple icons, where the number of icons represents the number of trials.

Table 4.1 Details for 20 yield trials analysed in this study, including the trial stage, number of lines evaluated, plots and the experimental design

Field trial	Year	Location	Stage	No. genotypes	No. plots	No. columns	No. rows	No. replicates	No. genotypes from elite breeding panel
S1-Breeza-2007	2007	Breeza, NSW[a]	1	394	480	8	60	1	24
S1-Linthorpe-2007	2007	Linthorpe, QLD	1	395	480	8	60	1	24
S1-Clifton-2008	2007	Clifton, QLD	1	922	1152	8	144	2	144
S2-Biloela-2008	2008	Biloela, QLD[b]	2	256	512	8	64	2	48
S2-Bithramere-2008	2008	Bithramere, NSW	2	256	512	8	64	2	48
S2-Breeza-2008	2008	Breeza, NSW	2	256	512	8	64	2	48
S2-Clifton-2008	2008	Clifton, QLD	2	256	512	8	64	2	48
S2-Lundavra-2008	2008	Lundavra, QLD	2	256	512	12	44	2	48
S2-Maules Creek-2008	2008	Maules Creek, NSW	2	256	512	8	64	2	48
S2-Springton-2008	2008	Springton, QLD	2	256	512	12	44	2	48
S2-Tulloona-2008	2008	Tulloona, NSW	2	256	512	8	64	2	48
S2-Biloela-2009	2009	Biloela, QLD[a]	2	397	600	10	60	2	215
S2-Bithramere-2009	2009	Bithramere, NSW	2	397	592	8	74	2	215
S2-Breeza-2009	2009	Breeza, NSW[b]	2	397	600	12	25	2	215
S2-Brookstead-2009	2009	Brookstead, QLD	2	397	592	8	74	2	215
S2-Edgeroi-2009	2009	Edgeroi, NSW	2	397	592	8	74	2	215
S2-Hermitage-2009	2009	Hermitage, QLD	2	397	592	8	74	2	215
S2-Kaimkillenbun-2009	2009	Kaimkillenbun, QLD	2	397	592	8	74	2	215
S2-Macalister-2009	2009	Macalister, QLD	2	397	600	12	50	2	215
S2-Moree-2009	2009	Moree, NSW	2	397	592	8	74	2	215

[a] Flood irrigation applied when needed at this location

[b] Overhead sprinkler irrigation applied when needed at this location

4.3.5 Multi-environment trial (MET) yield analysis

Yield data across the 20 trials was subjected to a MET analysis using a linear mixed model approach, which allows for appropriate modelling of the genotype × environment (G × E) variance and the appropriate error variance structures for individual trials (Smith et al. 2001; Kelly et al. 2007). The yield data was analysed using the following linear mixed model:

$$y = X\tau + Z_0 u_0 + Z_g u_g + e$$

where X and Z are the design matrices associated with the fixed τ and the random u vectors. The fixed effects τ includes environment main effects and trial-specific effects for field variation. More specifically for this MET model, global trends were identified in all but one of the trial sites, thus a fixed linear column term was fitted in 10 sites and a linear row term in 14 sites, with the appropriate fixed term chosen based on the direction of the global trend. For two sites an interaction term for linear row by linear column was also fitted as a fixed effect. In the above equation, u_g incorporates the genotype by trial site effect, and u_0 includes any additional random extraneous effects. For this MET model, a random column term was fitted for 16 trial sites, a random row term at one site, and a cubic spline in the row direction for four sites. A factor analytic (FA) model was fitted for u_g once it was determined that all trial sites did not have equal genetic variance nor equal co-variance between pairs of sites, and thus not an adequate fit to the compound symmetry structure (Smith et al. 2001; Smith et al. 2015). The FA model proposes dependence on a set of random hypothetical factors f and the genotype by trial site effect is modelled as follows:

$$u_g = (\lambda_1 \otimes I_m) f_1 + \ldots + (\lambda_k \otimes I_m) f_k + \delta$$

where the coefficient λ_r are known trial site loadings ($r = 1 \ldots k < p$), f_r is the vector hypothetical scores and δ is the vector of residuals for the model. I_m is the identity matrix. A separable autoregressive process of order one (AR1 × AR1) was fitted as a variance structure for e in the spatial mixed model to account for the local spatial trend (Gilmour et al. 1997). e is the plot error effects from each trial and the errors from different trials are assumed to be independent (Smith et al. 2001). The mixed model was fitted in ASReml-R (Butler et al. 2008) and variance components were estimated using Residual maximum likelihood (REML) estimation (Patterson and Thompson, 1971). To determine the dimensionality of the FA model (best k) the Akaike Information Criteria (AIC; Akaike 1974) (Akaike 1974) was

calculated and the REML log-likelihood assessed. Best linear unbiased predictions (BLUPs) were generated for the genotype effects.

4.3.6 Multi-trait analysis

Raw root trait data (seminal root angle and number) for the panel of 216 genotypes and the spatial models for each trait were incorporated into the MET yield analysis detailed above. The linear mixed model used for the analysis is advantageous in that it can handle unbalanced data (Smith et al. 2001), thus the two root traits can be included as if they were two extra field trials. Additional random terms were added to the MET yield model to account for extraneous variation identified in the spatial models for each root trait. A random column and pot term were added for both traits and a random bench term added for the root angle trait. Again, a FA model was fitted for u_g across all environments and traits and the AIC and REML used to assess the best model fit. Genetic correlations between all traits (20 field sites and two root traits) were calculated from the spatial mixed model.

4.4 Results

4.4.1 Phenotypic variation for seminal root traits

A wide range in seminal root angle and seminal root number phenotypes was evident in the panel of 216 genotypes (Figure 4.2; Figure 4.3). Both traits were distributed relatively normally. Seminal root angle ranged from 12.0° to 89.4° (Figure 4.3A), where the North Dakota breeding line ND20798-12 (PI 643348) displayed the narrowest phenotype and 2ND25459 [Rawson (PI 643149)*2//Zhenongda 7/Bowman (PI 483237)] displayed the widest. Seminal root number varied from 4.8 to 6.1 roots (Figure 4.3B), with the NRB breeding line NRB08351 (Grimmett/Amulet//WADH14613/VB9834) having the lowest number of roots and the breeding line Canela (Maris Canon/Laurel//Aleli) having the highest root number. Within the 13 commercial cultivars, Gairdner (Onslow/TAS83-587) and Shepherd (Baronesse reselection) displayed the narrowest (25.6°) and the widest (64.1°) root angles respectively, while Kaputar (5604/1025/3/Emir/Shabet//CM67/4F3 Bulk HIP) displayed the lowest number of roots (5.0) and Static the highest number of roots (5.9; Table 4.2). The broad-sense heritability (or repeatability) value for the root number experiment was 0.50, while the root angle experiment value was 0.34.

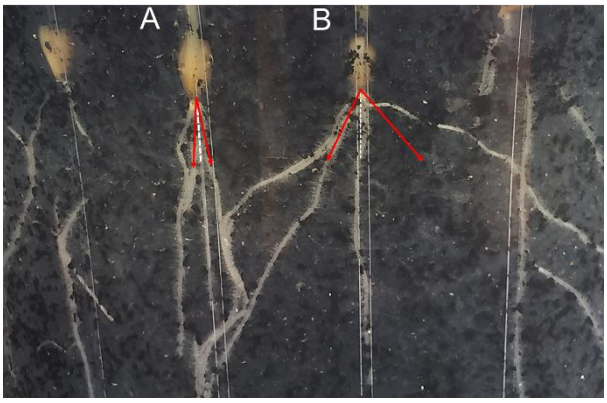

Figure 4.2 An example of extreme root angle phenotypes in the breeding population. **(A)** Narrow seminal root angle, measured as the deviation between the vertical (white dotted line) and first pair of seminal roots (red arrows). **(B)** Wide seminal root angle, where the vertical is highlighted by the white dotted line and the first pair of seminal roots signified by the red arrows.

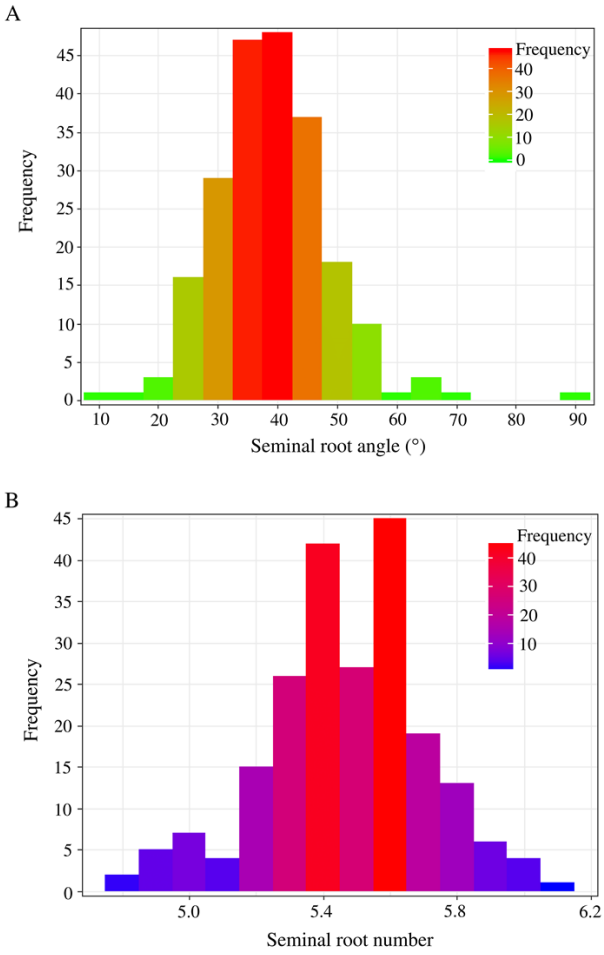

Figure 4.3 Distributions of root traits for the panel of elite breeding lines and commercial cultivars: **(A)** distribution of seminal root angle (°), and **(B)** distribution of root number. Both traits are relatively normally distributed in the panel. Increase in colour brightness (closer to red) resembles an increase in frequency of lines with the root phenotype.

Table 4.2 Seminal root phenotypes and standard error of the mean for 13 barley cultivars

Genotype	Root angle (°)		Root number	
	Mean	Standard error	Mean	Standard error
Gairdner	25.60	10.33	5.34	0.28
Commander	31.36	7.99	5.53	0.21
Kaputar	34.48	9.34	5.03	0.23
Flagship	34.53	7.50	5.81	0.20
Grout	36.49	8.53	5.53	0.21
Grimmett	37.83	7.52	5.02	0.21
Mackay	39.78	7.49	5.57	0.20
Fleet Australia	45.42	8.56	5.40	0.22
Hindmarsh	46.42	8.55	5.58	0.22
Static	47.19	7.50	5.88	0.20
Tallon	55.37	7.51	5.65	0.20
Shepherd	64.05	7.50	5.57	0.20

4.4.2 QTL for seminal root traits

Only one significant marker trait association (bPb-9868) was detected for seminal root angle at 184 cM on chromosome 5H (*qRA-5*; $P < 0.001$; Figure 4.4). On average *qRA-5* reduced root angle by 2.3° for genotypes that carried the "1" allele. Notably, *qRA-5* co-located with a QTL for seminal root angle previously detected in the ND24260 × Flagship DH population in Chapter 3 (Figure 4.4). Although not significant, the strongest marker-trait association for root number was identified on chromosome 7H, positioned at 82 cM (bPb-8956), where the presence of the "1" allele increased root number by 0.2.

Minimal population structure was evident in the panel based on the genome-wide marker profiles, with the first principal component only explaining 13.3% of the variation (Figure 4.5A). Nevertheless, there was no apparent trend in genotypes carrying either allele for *qRA-5*. Similarly, PCA performed for markers specific to chromosome 5H also revealed low population structure, with the first component explaining less than one-fifth of the variation (Figure 4.5B). In contrast, at the 7 cM interval surrounding *qRA-5* (12 markers) a higher degree of structure was apparent, with the first principal component explaining almost 50% of the variation (Figure 4.5C). Based on markers within this region, barley genotypes in the panel clustered into three main groups, however the presence of the 'narrow' or 'wide' allele at *qRA-5* did not match this pattern, as genotypes carrying both alleles were spread across all three groups.

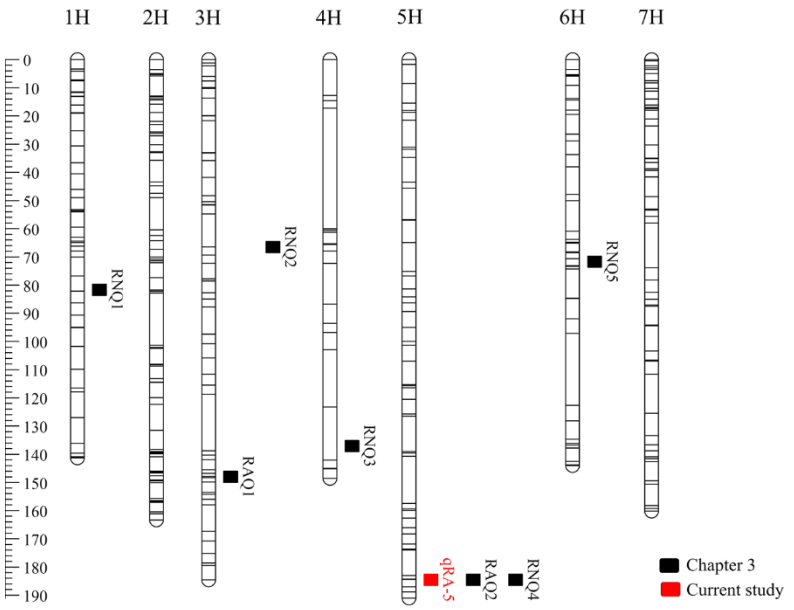

Figure 4.4 QTL for seminal root traits displayed on the DArT barley map. QTL colour coded black were reported in Chapter 3 and QTL coloured red were detected in the current chapter. Two seminal root traits are displayed on the map (RN: root number and RA: root angle). QTL confidence intervals adjusted to 4cM for display purposes only.

4.4.3 Multi-environment trial (MET) FA model goodness of fit

Factor analytic models, FA1 through to FA6, were fitted in increasing order to the MET data until a rule that the total percentage of variance accounted for by the model surpassed 80% (Smith et al. 2015). The final model, FA6, accounted for greater than 75% of the variance in 15 out of the 20 field trials, and had the lowest AIC and highest REML log-likelihood (Table 4.3). All REML log-likelihood comparisons between each order of FA model had a p-value less than 0.001. Higher order models could not be fitted to the MET data due to convergence issues.

Table 4.3 Goodness of fit for the MET yield genetic variance models of increasing order

Models	Parameters	REML	AIC	% Variance accounted by model
DIAG	101	2098.818	-3995.64	N/A
FA1	114	3259.139	-6290.28	58
FA2	138	3450.789	-6625.58	64
FA3	154	3485.783	-6663.57	69
FA4	173	3521.254	-6696.51	74

FA5	186	3546.418	-6720.84	82
FA6	199	3567.962	-6737.92	87

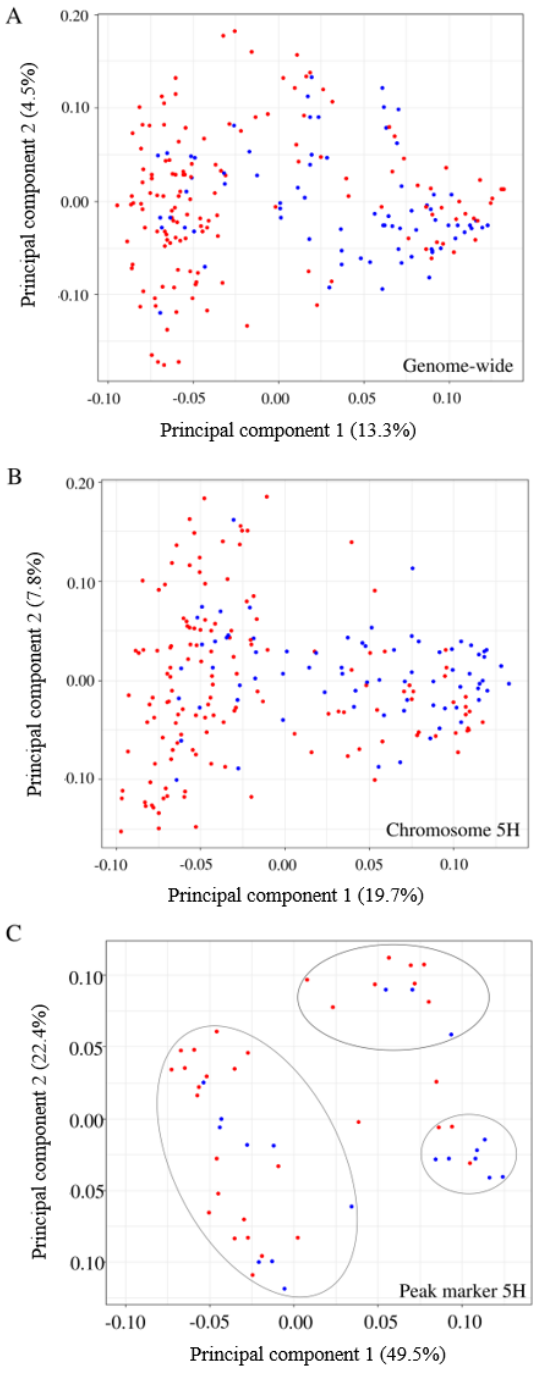

Figure 4.5 Population structure of the barley panel at three levels: **(A)** using all markers genome-wide, **(B)** using only markers on chromosome 5H, and **(C)** using only markers within the confidence interval of *qRA-5*. Biplots display results are from principal component analysis (PCA), where principal component (PC) 1 and PC2 are displayed for each analysis. Red dots represent genotypes with the narrow allele at *qRA-5* and blue dots represent those with the wide allele at *qRA-5*.

4.4.4 MET yield genetic correlations

Wide variation was evident in the genetic correlation matrix for yield across the trial sites, with correlations ranging from -0.19 to 0.95 (Figure 4.6). Using hierarchical agglomerative clustering the field trials were grouped into seven clusters based on dissimilarity of the genotype rankings for yield at each trial site (Figure 4.7). The majority of field trials formed one large cluster (cluster 4), while four sites (cluster 5, 6 and 7) diverged from the other trials with a correlation < 0.4 (Figure 4.7). No obvious trend in correlations was observed across trial year, location or stage.

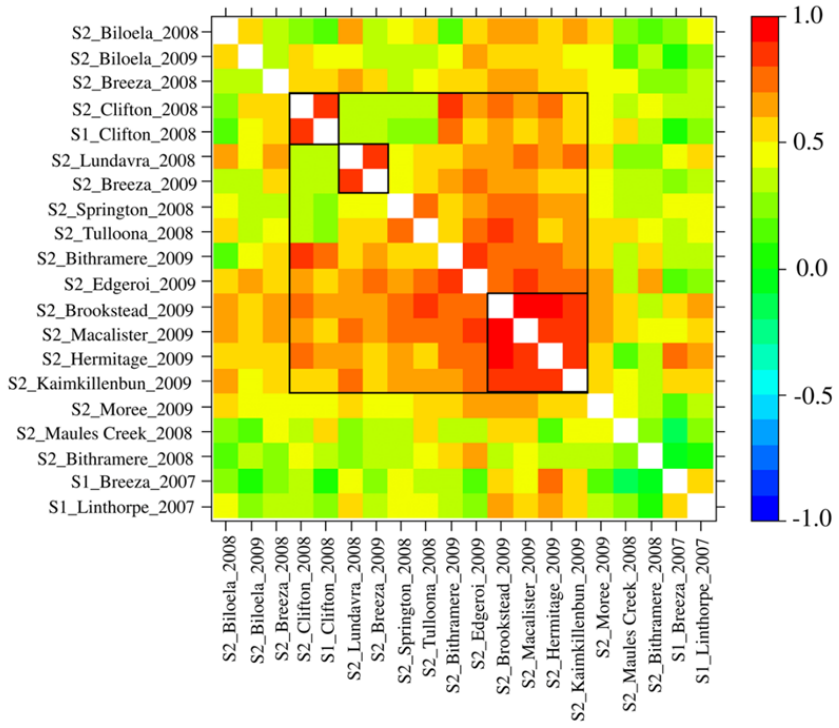

Figure 4.6 Heat map of the MET yield analysis genetic correlation matrix. Positive genetic correlations between trials increase with increasing colour brightness (closer to red) and negative correlations with decreasing brightness (closer to blue). The large outer square highlights a group of trials with high correlations with one or more other field trials. The small inner squares show trials in extremely high genetic correlation with other trials.

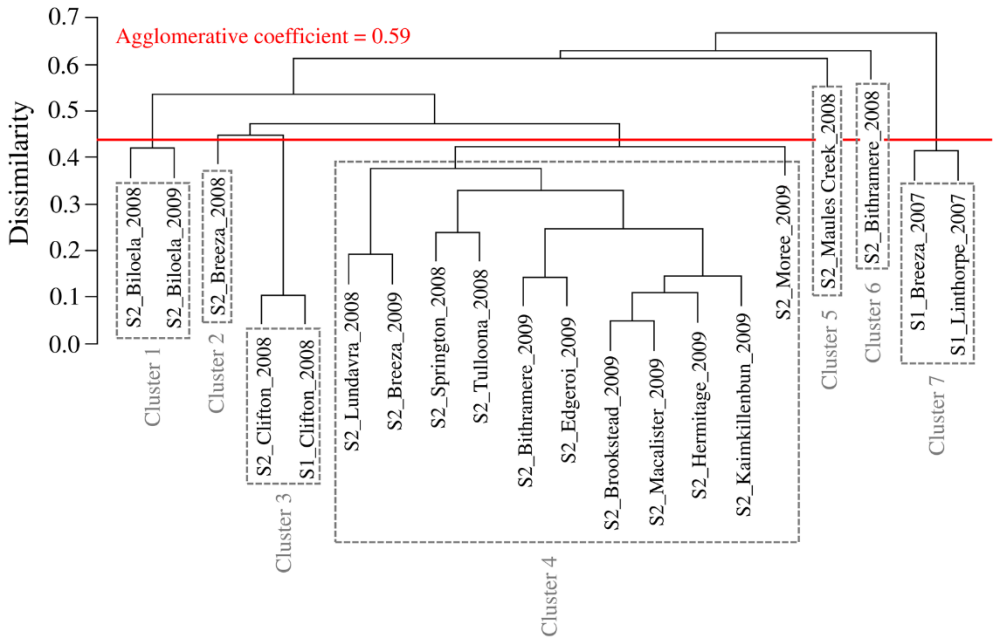

Figure 4.7 Dissimilarity matrix of yield performance for barley breeding lines across the 20 field sites in the northern grain-growing region. The field trials were grouped into 7 clusters based on the similarity of genotype rankings across the trials. A dissimilarity threshold of 0.44 was applied to determine environment clusters (red line).

4.4.5 MET yield predictions

Yield BLUPs were generated from the FA6 model for each genotype within each site, cluster and across all field trials. The average yield across the clusters and the individual sites varied (Figure 4.8A, B). Cluster 2 and 5 had the lowest average yield, while cluster 3 had the highest. More specifically, S2_Maules Creek_2008 had the lowest average yield at 2.18 t/ha and S2_Breeza_2009 had the highest at 5.16 t/ha. The proportion of genetic and error variance varied substantially across field trials (Figure 4.8C, D). Individual yield predictions for each of the 12 Australian cultivars in the panel were examined across field site clusters (Figure 4.9), where some cultivars appeared to perform better in some environments. For example, Fleet Australia exhibited the second and third lowest yield in clusters 7 and 2, respectively, but the highest yield in clusters 5 and cluster 6 (Figure 4.9).

4.4.6 Multi-trait FA model goodness of fit

Increasing orders of FA models (FA1 – FA5) were fitted to the genetic variance structure of the multi-trait-site (yield, seminal root angle and seminal root number) model until the percentage of variance explained by the model reached or exceeded 80% (Table 4.4). All

REML comparisons between each order of fa model had a p-value less than 0.001. An FA6 model was also fitted to the data in attempt to surpass the total percentage of variance explained, however convergence was unsuccessful.

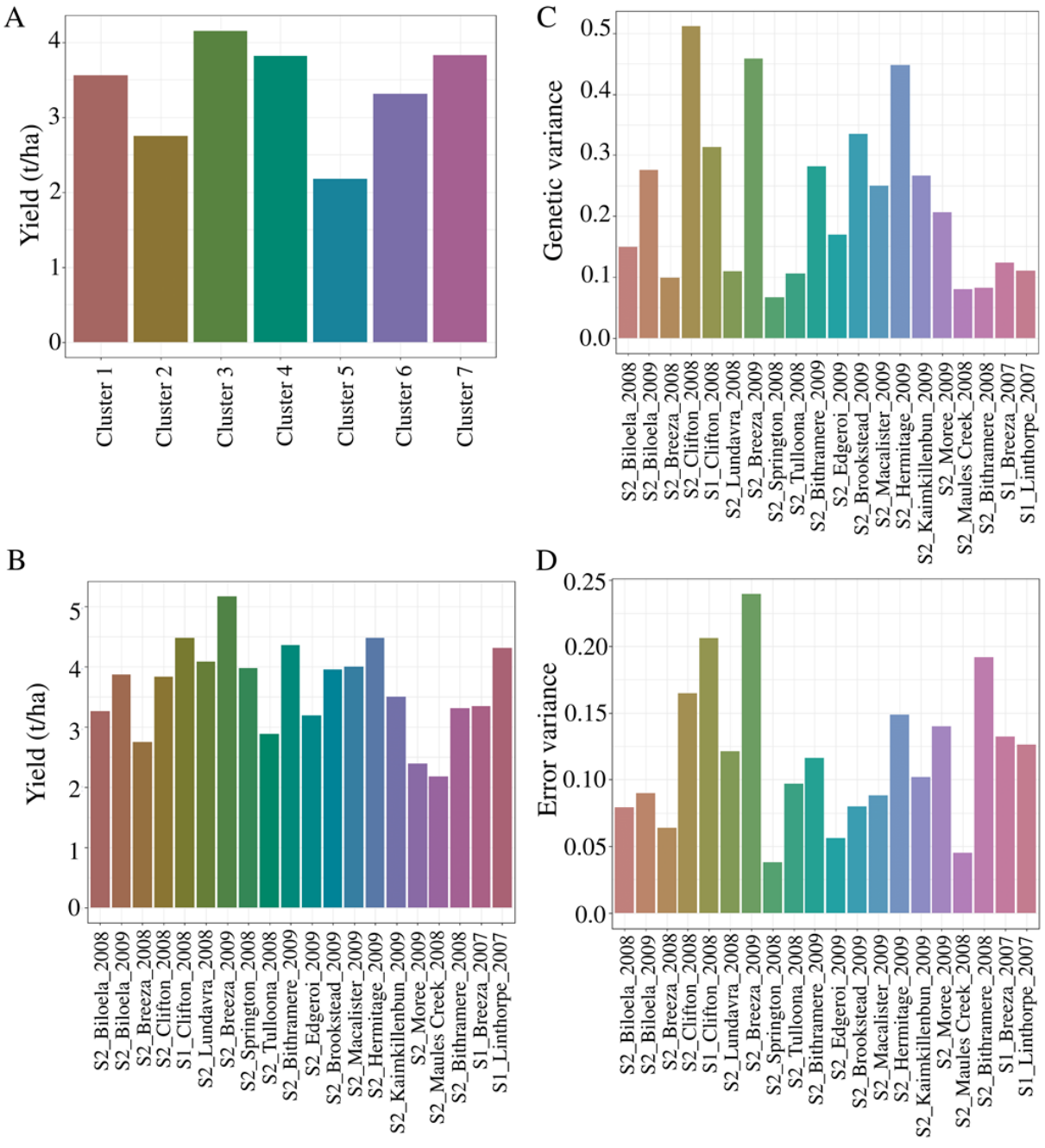

Figure 4.8 Average yield, genetic and error variance across environments for the MET yield analysis. (A) Average yield of each environment cluster, where clusters were previously defined in Figure 6. (B) Average yield of each field trial. (C) Average genetic variance of each of the 20 field trials. (D) Average error variance of each field trial.

Table 4.4 Goodness of fit for the multi-trait genetic variance models of increasing order

Models	Parameters	REML	AIC	% Variance accounted by model
DIAG	112	-3735.47	1979.737	N/A
FA1	134	3260.783	-6253.57	57
FA2	153	3333.833	-6361.67	61
FA3	172	3373.016	-6402.03	66
FA4	190	3414.448	-6448.9	73
FA5	208	3439.375	-6462.75	80

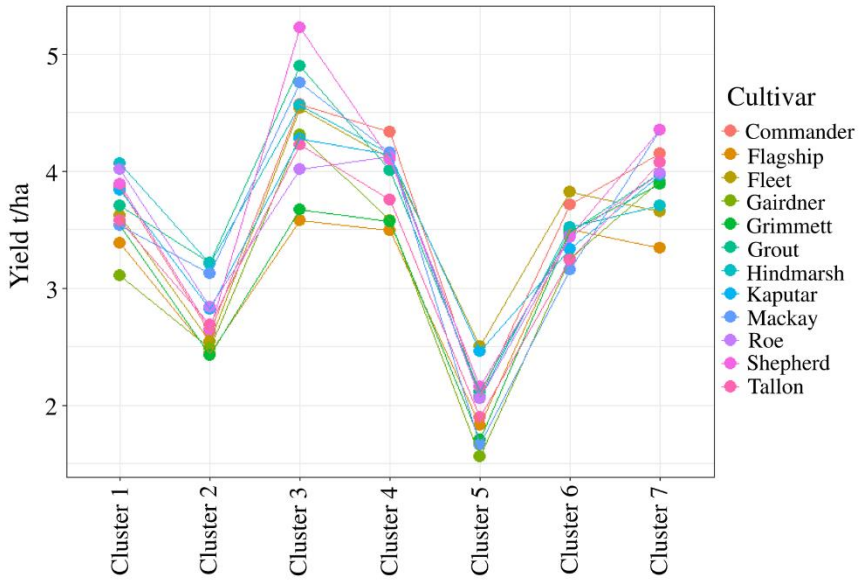

Figure 4.9 Yield BLUPs for the subset of Australian barley cultivars in each of the seven environment clusters generated from the MET yield analysis

4.4.7 Multi-trait genetic correlations

Genetic correlations varied substantially in the multi-trait analysis (Figure 4.10). Seminal root angle and root number displayed the lowest correlations with all other traits. The genetic correlations between seminal root traits and yield were all relatively weak. They were also multi-directional, ranging from -0.21 to 0.36 for root angle and -0.20 to 0.25 for root number (Table 4.5). Within yield clusters the direction of the genetic correlations for each root trait and yield are consistent, except for Cluster 4 (Table 4.5). However, when Cluster 4 is grouped into sub-clusters, based on a dissimilarity cut off of 0.3 in Figure 4.7, the direction of the genetic correlations was consistent for each sub-cluster. No clear trends were observed for

root angle and yield in trials with or without irrigation. However, for root number, all trials grown under irrigation had a positive correlation with yield. For root angle, the strongest correlations with yield were detected at S2_Clifton_2008 (0.36) and S1_Clifton_2008 (0.28), and for root number at S1_Breeza_2007 (0.25) and S2_Maules Creek_2008 (-0.20). Root angle showed a higher frequency of correlations with yield that were ≥ 0.10 compared to root number: 16 out of 21 compared to 10 out of 21 for root number. For 8 of the 10 sites where root number was correlated with yield, root angle was also correlated with yield. A weak genetic correlation was observed between root angle and root number (0.10).

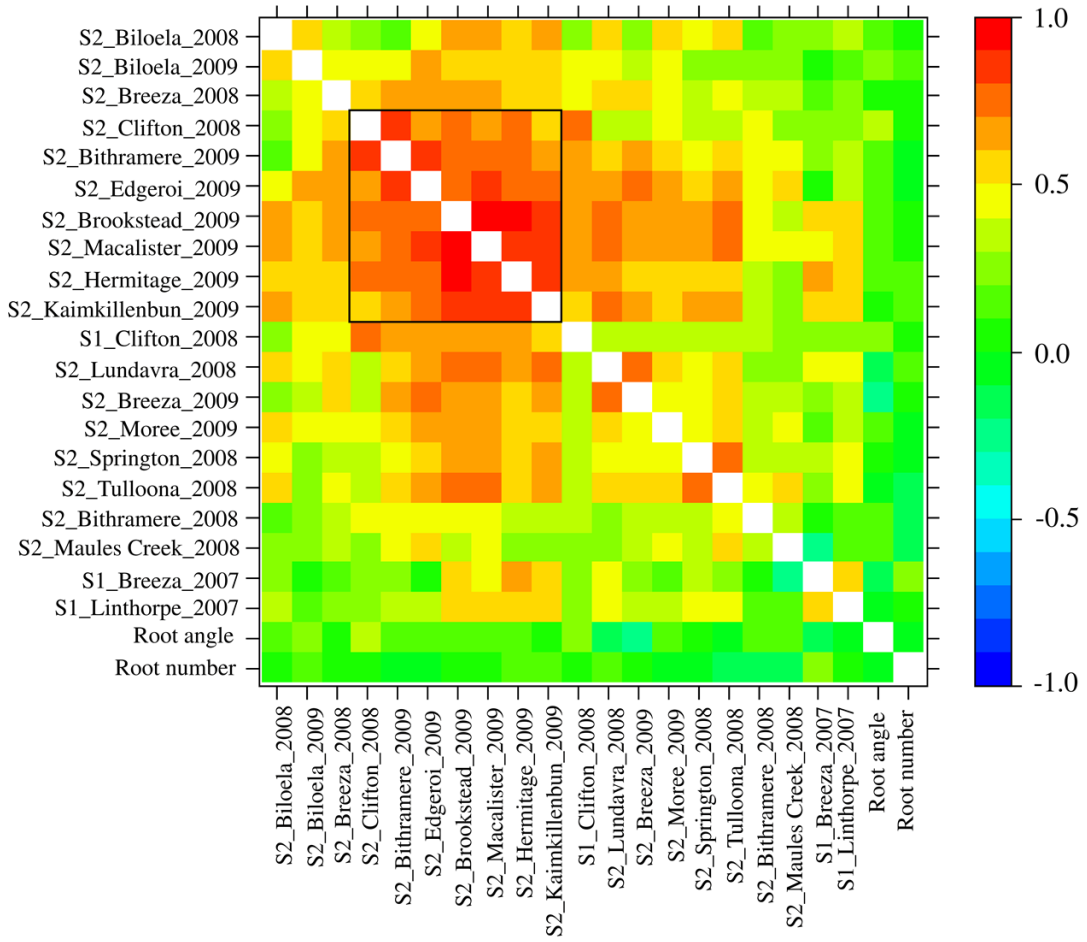

Figure 4.10 Heat map of the multi-trait analysis genetic correlation matrix. Positive genetic correlations between traits increase with increasing colour brightness (closer to red) and negative correlations with decreasing brightness (closer to blue). The black square highlights a group of trials with high correlations with one or more other field trials.

Table 4.5 Genetic correlations between seminal root traits and yield from the multi-trait analysis

Yield cluster	Sub-cluster	Yield trial	Root angle	Root number
1		S2_Biloela_2008[a]	0.11	0.06
		S2_Biloela_2009[a]	0.21	0.10
2		S2_Breeza_2008[a]	0.07	0.06
3		S2_Clifton_2008	0.36	0.02
		S1_Clifton_2008	0.28	0.03
4	4-1	S2_Lundavra_2008	-0.15	0.19
		S2_Breeza_2009[a]	-0.21	0.10
	4-2	S2_Springton_2008	0.02	-0.08
		S2_Tulloona_2008	0	-0.18
	4-3	S2_Bithramere_2009	0.17	0
		S2_Edgeroi_2009	0.18	-0.02
		S2_Brookstead_2009	0.13	0.05
		S2_Macalister_2009	0.13	0.04
		S2_Hermitage_2009	0.15	0.13
		S2_Kaimkillenbun_2009	0.05	0.11
	4-4	S2_Moree_2009	0.12	-0.02
5		S2_Maules Creek_2008	0.12	-0.20
6		S2_Bithramere_2008	0.15	-0.10
7		S1_Breeza_2007[a]	-0.16	0.25
		S1_Linthorpe_2007	-0.07	0.09
		Root number	-0.10	-
		Root angle	-	-0.10

[a]Trials were irrigated as needed throughout the growing season

4.5 Discussion

Here, we present one of the first studies to investigate genetic correlations between seminal root traits and yield, using barley breeding lines, evaluated across a large number of environments. Genotype × environment interactions were observed for all traits and, as a result, genetic correlations varied substantially in both magnitude and direction. Due to the complexity and array of traits contributing to yield in diverse breeding germplasm, it is not surprising the majority of the correlations between seminal root traits and yield were only weak to moderate in strength. But, we have clearly demonstrated there is shared genetic control between seminal root angle and yield, as well as between root number and yield in barley. Overall, seminal root angle was more correlated with yield than seminal root number,

suggesting that root angle and yield traits are more closely related. However, the genetic correlation between root angle and root number was relatively weak, implying each root trait has its own genetic control.

4.5.1 Phenotypic variation for seminal root traits

We observed a wide range of root angle phenotypes in the panel (12.0 to 89.4°). In Chapter 3, in the ND24260 × Flagship population, a narrower range in root angle phenotypes (16.4 to 70.5°) was observed, yet a broader range in root number phenotypes (3.6 to 6.5 roots). The presence of more extreme root angle phenotypes in the current chapter is to be expected, due to the increased genetic diversity in breeding material evaluated here compared to the bi-parental DH population examined in Chapter 3. It is interesting, therefore, that the same phenotypic variation was not observed for root number, since it ranged only from 4.8 to 6.1 roots in the current study. The lower variation for root number may have contributed to the weaker genetic correlations with other traits examined in this study. This may suggest that high seminal root number is under indirect selection in breeding programs. In this case, the population of breeding lines would be enriched for alleles beneficial for root number. The heritability of seminal root traits was moderate (root angle H^2 0.34, root number H^2 0.50), yet considerably lower than those reported in the DH population in Chapter 3 (root angle H^2 0.63, root number H^2 0.95). The lower heritabilities in the current study are most likely a result of increased error variance compared to the previous study. A contributor to this error variance is the heterogeneity of the breeding lines, whereby the lines are F3:F5 lines and not completely homozygous. Therefore, more phenotypic variation among genotype replicates is anticipated due to the heterogeneity of the lines. For root number, a reduction in genetic variance in the breeding population could also contribute to the lower heritability.

4.5.2 QTL mapping for seminal root traits

The marker-trait association for root angle in the panel (*qRA-5*) co-locates with a previously reported QTL for seminal root angle in the ND24260 × Flagship DH population in Chapter 3 (*RAQ2*). Further, the direction of the allelic effects at the peak markers for each QTL are consistent across both studies, where allele "1" is associated with a narrow root angle and "0" is associated with a wide root angle. Consistent with findings here, *RAQ2* only explained a small proportion of the phenotypic variation (<10%) in the DH population. Despite the effect size, the QTL has been consistently detected in a DH population and in a panel of elite

breeding lines, thus validating this QTL as an important genomic region influencing root angle irrespective of population background.

To understand the effect of this QTL, a near-isogenic line (NIL) for the *qRA-5* genomic region could be created in a commercial cultivar with a wide root angle. In addition, a Kompetitive Allele Specific Polymerase Chain Reaction (KASP) marker could be developed for *qRA-5* to assay other populations and then selectively phenotype genotypes with the positive allele at this locus. Yet, evaluating more diverse accessions or structured populations with balanced allele frequencies could identify additional loci that may enable more controlled manipulation of root system architecture. Creation of a NAM population using a commercially relevant cultivar as reference variety, similar to the NAM populations developed in maize (Yu et al. 2008) and sorghum (Jordan et al. 2011), could provide higher resolution and greater statistical power to detect rare QTL for seminal root traits.

4.5.3 MET yield analysis

The diverse range in yield predictions and the variable genetic correlations across trial sites implies that yield evaluated in the northern growing region over a three-year period was subject to considerable G × E interactions and a range of environmental conditions. Interestingly, there were no obvious trends for clustering of trial sites based on year or location. However, one or more abiotic stresses (e.g. high temperature, drought or nutrient deficiency, etc.) and/or contrasting management practices (e.g. sowing date, sowing depth, fertilizer application, irrigation, etc.) likely influenced yield across the trials. In the northern region, drought is often the main yield constraint, and it is a result of the highly variable intra- and inter-seasonal rainfall (Nicholls et al. 1997). Limited water availability throughout crop growth reduces the plant's ability to transpire and photosynthesise, resulting in reduced grain yield (Gilliham et al. 2017). In the future, excessive heat stress as a consequence of climate change, is predicted to result in yield losses similar to drought stress in some cereal crops (Lobell et al. 2015). A key limitation of this study is the lack of environment information for each of the 20 breeder's trials. Detailed environment characterisation, such as the approach adopted by (Chenu et al. 2011), would provide greater insight into the G × E interactions and perhaps identify the key environmental factors resulting in environment clusters.

4.5.4 Relationship between seminal root traits and yield

Our results demonstrated a genetic relationship between seminal root traits and yield in the barley breeding lines, but the relationship was not strong and the size and direction of the association was highly context dependent. Factors driving the similarity of genotype ranking for yield, and thus the environment clusters displayed in Figure 4.7, also appear to be influencing the direction of the genetic correlations between each root trait and yield, whereby the direction of the correlation is consistent across yield clusters and sub-clusters. The context dependency of this relationship is exemplified by the two highest yielding sites S2_Breeza_2009 and S2_Clifton_2008 having very contrasting correlations with root angle (-0.21 for S2_Breeza_2009 and 0.36 for S2_Clifton_2008; Table 3). This circumstantial dependency is likely a result of a number of environmental and/or management factors, such as rainfall, pre-sowing soil moisture, soil moisture throughout season and sowing depth etc.

Interestingly, some sites in close proximity to each other and grown within the same growing season show no similarity in genetic correlations between root angle and yield (i.e. S2_Breeza_2009 (-0.21) and S2_Bithramere_2009 (0.17)). This is unexpected, as the seasonal water availability throughout the northern region varies both spatially and temporally (Potgieter et al. 2002; Chenu et al. 2011). In addition, the timing and the magnitude of the water stress throughout crop development is likely to influence the relationship between root traits and yield, where field sites in close proximity may experience similar timing and intensity of the stress. However, differing management practices between sites, such as time of sowing, may account for the differences in correlations between root angle and yield. For seminal root number, positive correlations with yield were observed in all irrigated environments. This is not unexpected, as an increased water availability coupled with increased extraction, improves the water supply to the crop for use during grain production. For seminal root angle, there were no obvious trends between the irrigated and dryland trials, with positive and negative correlations observed in both trial types. Some consistency was observed across location in the irrigated trials, where positive correlations were found for trials located in Biloela and predominately negative correlations identified for trials at Breeza. It is important to note that irrigation practices varied at each location, which may contribute to such differences.

Our results suggest that root traits interact with environmental factors in addition to water supply that vary across sites and years. In previous research, roots have been shown to interact with other environmental factors such as soil type and strength (Bingham and Bengough 2003; Rich and Watt 2013), nutrient heterogeneity and availability (Drew 1975),

as well as variable management practices that influence crop water use (Richards et al. 2002). In addition, the same set of breeding lines were evaluated within each breeding stage and year (except S1_Clifton_2008; Table 4.1), thus the variability in the genetic correlations of sites within the same stage, year and local area is not a result of changing genetics, but more likely due to interactions between root traits and other environmental factors. Despite MET analyses handling unbalanced data without difficulty (Smith et al. 2001), it is important to note that for 10 out of the 20 breeding trials the genotype concurrence, between yield and root trait data, was relatively low (< 50). The 10 trials were made up of stage 1 trials in 2007 and stage 2 trials in 2008. For these trials, the genetic correlations between root traits and yield are not as reliable as the remaining 10 trials, which contained 214 lines (except S1_Clifton_2008). This highlights the limitation of working with real breeding datasets, yet the research and results are likely more directly applicable for barley breeding in the northern grain-growing region. For instance, previous association mapping studies examining the NRB germplasm for resistance to leaf rust (Ziems et al. 2014; Ziems et al. 2017) and spot form of net blotch (Wang et al. 2015) has enabled rapid adoption of marker-assisted selection for these traits in the breeding program. It is also important to note the limitation of comparing glasshouse generated root trait data to yield data generated from field trial yield plots, likely another contributing factor for the low correlations. Ideally, the measurement of root traits for correlation with yield would be completed under the same environmental conditions as the yield evaluation, however the low-throughput nature of destructive in-field based root assessment methods (i.e. shovelomics) makes this impractical for large population sizes.

Other agronomic traits could have shared genetic control with root traits and may also interact with the environment, and therefore may contribute to the observed variation in root trait correlations with yield. Borrell et al. (2014) detailed a genetic relationship between nodal root angle and delayed foliar senescence in sorghum and proposed that this relationship improved access to water during the grain-filling period. This is just one of the underlying mechanisms contributing to delayed foliar senescence and subsequent improved yield in sorghum. The relationship between delayed foliar senescence and root angle in sorghum is a good example of the balance between water supply and demand contributing to improved yield. While extending photosynthesis by delaying senescence increases crop water demand late in the season, a narrow and deep root architecture allows the crop to access water deep in the soil profile, thus improving supply. In barley, any shared genetic control between root angle and delayed senescence has yet to be elucidated, but future research investigating this

relationship would be beneficial for our understanding of root angle as a drought adaptive trait. Integrated drought adaptation traits, such as delayed senescence, could be contributing to the variability in the correlations between root traits and yield observed in this study.

4.6 Conclusion

Here we demonstrate a genetic relationship between seminal root traits and yield in two-rowed spring barley, although importantly, the relationship appears highly context dependent. Further research is required to thoroughly assess the interactions between seminal root phenotypes, abiotic stresses and management practices to identify the preferred root ideotype for different production scenarios in the northern grain-growing region of Australia. Modelling different genotype × environment × management scenarios will be crucial to understanding the value of root traits for barley breeders. For example, is a narrow and deep root system beneficial for yield improvement under terminal drought stress? Furthermore, does a low sowing density further increase yield in this scenario? *QRA2*, a previously identified genomic region influencing root angle in barley, co-locates with *qRA-5* detected in this study, demonstrating that *qRA*-5/*QRA2* is a key region influencing the direction of root growth. Further investigation into the genetic control of seminal root traits in barley using advanced mapping populations is likely to identify more QTL influencing these traits. In summary, we demonstrate that seminal root traits are associated with yield in barley, thus further exploration into the genetic control and the interaction of root traits with important drought adaptation traits, the environment and management practices should be beneficial for crop improvement.

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Chapter 5

ASSOCIATIONS BETWEEN ROOT TRAITS, CANOPY SENESCENCE AND YIELD IN BARLEY

5.1 Abstract

Water availability is a major limiting factor for crop production worldwide. For some cereal crops, narrow and deep root systems, and delayed foliar senescence have been associated with enhanced yield under water deficit. In Chapter 4, we validated a key genomic region influencing seminal root angle and demonstrated that there is a relationship between seminal root traits and yield. We suggested that further quantitative trait loci (QTL) controlling seminal root traits may be identifiable using advanced mapping approaches, such as nested-association mapping (NAM). Furthermore, we identified the need to explore the relationship between seminal root traits, yield and other drought adaptation traits under water-limited conditions. In this chapter, yield trials were conducted under rainfed and irrigated conditions to explore the relationships between seminal root traits, yield and delayed canopy senescence. To better understand the underlying genetics, QTL for canopy senescence and seminal root traits were mapped using a doubled haploid (DH) and NAM population, both genotyped with DArTseq markers. Canopy senescence and flowering time were phenotyped in five field trials grown across two cropping seasons in north-eastern Australia. A total of 44 QTL were identified for canopy senescence traits, of which 8 QTL were associated with the rate of senescence, 16 for an overall 'stay-green' measure, and 20 QTL for the time taken from anthesis until maturity. Fewer QTL were identified for the root traits: 4 QTL for seminal root angle and 8 QTL for seminal root number. Several leaf senescence QTL mapped to *HvNAM-1* on chromosome 6H and to the semi-dwarfing and gibberellic acid (GA) biosynthesis gene *sdw1/denso* (*Hv20ox2*) on chromosome 3H. Leaf senescence and root QTL were also mapped to *Hv20ox1*, another key GA biosynthesis gene, located on chromosome 5H. Our results suggest that key plant

developmental genes may be associated with above- and below-ground drought adaptation traits in barley.

5.2 Introduction

Environmental events, such as drought and warming, are predicted to increase in duration and intensity (Dai 2013). This will be further compounded by an expanding global population (Tilman et al. 2011), highlighting the need for improved cereal crop productivity and yield stability (Lobell et al. 2011; Asseng et al. 2015; Lobell et al. 2015). In Australia's northern grain-growing region, intra and inter-seasonal rainfall is highly variable and, as a result, water availability is often a key yield and grain quality constraint (Nicholls et al. 1997). Inadequate water availability reduces transpiration and photosynthesis of the crop, resulting in reduced grain yield and altered grain morphology and protein content (Gilliham et al. 2017). Many protective mechanisms to either safe-guard against water loss and/or improve water access have been described previously, for instance transpiration efficiency (Condon et al. 2002), osmotic adjustment (Morgan 1995), reduced tillering (Dabbert et al. 2010), lower canopy temperature (Elsayed et al. 2015), delayed foliar senescence (Borrell et al. 2014b), and deep root systems (Manschadi et al. 2006).

In several crops, a deep root system is predicted to be optimal for maximum resource capture in most water-limited environments (Lynch 2011, 2013). For example, a narrow root angle in sorghum improves access to water stored deep in the soil profile and is associated with increased yield (Mace et al. 2012; Singh et al. 2012). Foliar senescence is the final crop developmental stage, and once initiated results in a large remobilisation of phloem-mobile nutrients from the senescing parts of the plant to the developing grain. Stay-green (or delayed foliar senescence) can be broadly divided into two types: cosmetic stay-green and functional stay-green crops. A cosmetic (or non-functional) stay-green crop remains greener for longer as a result of a lesion interfering with the early stages of chlorophyll catabolism but does not provide any yield advantage (Thomas and Howarth 2000; Thomas and Ougham 2014). Alternatively, in functional stay-green crops, leaf photosynthesis continues throughout grain-filling due to either a delayed onset or a decreased rate of senescence (Thomas & Howarth 2000; Borrell et al., 2001; Thomas and Ougham 2014). The timing of senescence onset, along with the rate, have been shown to influence important agronomic traits such as nutrient-use efficiency and yield, where delayed senescence has been associated with an extended grain-

filling period and increased yield (Harris et al., 2007; Gregersen et al. 2013; Christopher et al., 2014). In sorghum, the mechanism by which crops are able to maintain green stems and upper leaves during the grain-filling period is due to an improved balance between water supply and demand (Borrell et al. 2014a). Borrell et al. (2014a) demonstrated that under terminal drought conditions, sorghum germplasm with the *Stg1* and *Stg3* QTL have a significantly increased water uptake throughout grain-filling compared with the senescent control. Furthermore, crop water-use during grain-filling was positively correlated with grain yield. Interestingly, Borrell et al. (2014a) highlighted shared genetic control between above- and below-ground drought-adaptive traits, whereby all four nodal root angle QTL previously identified by Mace et al. (2012) co-located with QTL previously detected for stay-green. Therefore, root architecture appears to contribute to the stay-green phenotype by improving access to stored soil moisture during the critical grain-filling stage. However, deep rooting can only access water at depth if there is water available. The increased availability of post-anthesis water in stay-green types is due to the conservation of water before anthesis – a result of a smaller canopy size due to reduced tillering and smaller upper leaves (Borrell et al., 2014ab). Early onset of foliar senescence and/or an accelerated rate have also been shown to increase grain protein content (GPC) in wheat (Uauy et al. 2006), while delayed senescence reduces GPC since prolonged carbohydrate accumulation dilutes the protein and increases grain weight and yield (Gregersen 2011). For malting barley, an increased grain weight coupled with reduced protein content (to an extent) is desirable to meet malt quality standards, thus delayed senescence could be advantageous.

Recently, a number of studies have begun to investigate the genetic control of foliar senescence in barley (Emebiri 2013; Gong and McDonald 2017; Gous et al. 2016; Obsa et al. 2016). However, these studies used a range of methodologies for phenotyping and calculating the rate of senescence. The development of stay-green phenotyping methods using normalised difference vegetative index (NDVI; Lopes and Reynolds 2012), commonly measured using a hand-held GreenSeeker (Trimble Inc., Sunnyvale, CA, USA), has greatly surpassed previous methods by increasing objectivity, efficiency and accuracy. In a recent study, 17 QTL were detected for stay-green in barley, where NDVI was measured in the field every two weeks from stem elongation through to maturity and the rate of senescence was estimated as the area under the NDVI curve (Gong and McDonald 2017). A QTL on chromosome 4H was deemed a major effect QTL for stay-green due to the high frequency of detection at the majority of the field sites. Interestingly, QTL for root diameter and stay-green also co-located on chromosome 6H

in one field environment with opposite allelic effects, whereby an allele contributed by the commercial cultivar Commander increased root diameter but decreased stay-green (Gong and McDonald 2017). Two other stay-green QTL mapping studies also detected QTL for the trait on chromosome 4H (Emebiri 2013; Obsa et al. 2016). Measuring stay-green as the relative difference in plot greenness, based on optical sensing between two time points (anthesis and maturity), Emebiri (2013) identified a number of QTL across all chromosomes. Two QTL were strongly associated with the flowering time genes *Ppd-H1* and *VRN-H3*, where a negative association was identified between time to flowering and the rate of senescence. This relationship highlights the importance of flowering time for driving water-use and yield in water-limited environments. For instance, a late flowering time allows the plant to produce a large number of tillers and greater biomass, which creates an increased water demand during grain-filling and under post-anthesis drought stress will likely limit grain-filling and accelerate senescence. Only one QTL, on chromosome 5H, was detected consistently across the two DH populations examined in both dryland and irrigated environments. This 5H QTL was independent of flowering time, but associated with grain plumpness and grain size, yet the direction of the associations differed between the DH populations. Emebiri (2013) proposed the genetics influencing functional stay-greens in barley was under simple genetic control; however, the genetic diversity of this study was limited to only four genotypes. On the other hand, Obsa et al. (2016) detected four QTL for stay-green in three DH populations across six field trials. Stay-green was estimated using a single NDVI reading, on a plot basis, at early milk stage of grain development using a GreenSeeker. Of the four QTL identified, three were located on chromosome 3H and one on chromosome 4H. No consistent QTL were detected across the DH populations and none of the QTL co-located with flowering time genes (Obsa et al. 2016). Interestingly, none of the recent stay-green mapping studies detected *HvNAM-1*, which has been previously described to be associated with foliar senescence and GPC in barley (Christiansen et al. 2011).

There is much scope for further research into decrypting the genetic and physiological control of stay-green in barley, especially in populations with wider genetic diversity than those previously studied. Further, more advanced methods to estimate stay-green from multiple NDVI measures recorded over time, such as fitting linear regressions (Lopes and Reynolds 2012) or logistic functions (Christopher et al. 2014), could improve the accuracy of phenotypes in relation to flowering time and enhance the ability to differentiate senescence patterns in barley. However, the critical thing is to identify the causal mechanisms which drive the

underlying changes in water supply and demand, ultimately resulting in a stay-green phenotype. After all, senescence patterns are only descriptive (emergent consequences) rather than causal. QTL identified for seminal root traits in Chapter 3 and Chapter 4 also provide an opportunity to investigate any shared genetic control between root architectural traits and stay-green, similar to that found in sorghum. In this study we aim to further the understanding of the genetic architecture of canopy senescence in barley by characterising a subset of lines from a nested-association mapping (NAM) population and a DH population across five field environments using a comprehensive stay-green phenotyping method (Christopher et al. 2014). Using multiple QTL mapping strategies, we identify novel genomic regions underpinning senescence and seminal root traits. Herein, we provide new insights genomic regions associated with canopy senescence by mapping QTL to known plant developmental genes for semi-dwarfing habit and regulation of flowering time.

5.3 Materials and methods

5.3.1 Plant Material

Two spring two-rowed barley populations were examined in this study, the ND24260 × Flagship doubled haploid (DH) population consisting of 338 lines (Hickey et al. 2011) and a subset of 165 F4:F5 lines from the multi-parent reference NAM population (Ziems et al. 2015). The subgroup of the NAM population consisted of five families derived from crosses between reference variety Commander and four elite breeding lines from the Northern Region Barley (NRB) breeding program (Warwick, Australia) and one line from the ND24260 × Flagship DH population. The five families were chosen based on two factors, firstly seed availability and secondly presence of the positive allele for narrow root system architecture in the five founder lines at the 5HL locus identified in chapter 3 and 4. All genotypes were previously genotyped using Diversity Arrays Technology (DArTseq) markers.

5.3.2 Phenotyping and statistical analysis of seminal root traits

The ‘clear-pot’ method (Richard et al. 2015) was used to phenotype seminal root angle and root number in the NAM subset. Eight replicate seeds for each genotype were characterised, with the experiment consisting of 76 pots (containing 24 seeds per pot). Using a randomised complete block design, the pots were spread across two benches, where bench one contained 44 pots positioned in a 2D array of four columns by 11 rows and bench two comprised of 32

pots in an array of three columns by 11 rows, with one pot missing. The spatial location of each pot was accounted for in the design model, where a linear mixed model was fitted to the data. Replicate and pot were fitted as random terms in the spatial models for both root traits along with an independent correlation term for pot and position. Variance components of the model were estimated using residual maximum likelihood (REML) and best linear unbiased estimators (BLUEs) were generated for the fixed genotype effects. Using the variance components of the model, a generalised heritability of each trait was calculated (Cullis et al. 2006). A linear mixed model was fitted in ASReml-R (Butler et al. 2008). The DH population was previously phenotyped for root traits in Chapter 3 using the same methodology.

5.3.3 Field trials and treatments

Five yield trials were conducted in north-eastern Australia across the main growing seasons of 2015 and 2016. Four of the field trials were conducted at the Department of Agriculture and Fisheries (DAF) Hermitage Research Facility (Hermitage, QLD) and the remaining trial was located at the DAF Wellcamp Research Station (Wellcamp, QLD). Two of the five trials were regularly irrigated throughout the season (H_15_irri and H_16_irri) and the remaining three trials were rain-fed (H_15_dry, H_16_dry and W_16_dry). Three irrigation events were applied to the H_15_irri trial, one pre-anthesis and two post anthesis. Irrigation was applied using strip tape at a rate of 6.5 litres/metre/hour, where each irrigation was applied for eight hours. For H_16_irri, the trial was irrigated five times, twice just prior to anthesis and three post-anthesis during the mid-grain filling period. Again, irrigation was applied using strip tape at a rate of 6.5 litres/metre/hour for a total of eight hours per irrigation event. Further trial environment details are outlined in Table 5.1, where weather data for the closest weather station was accessed online from the Bureau of Meteorology (http://www.bom.gov.au/). A subset of 198 genotypes from the ND24260 × Flagship DH population and the NAM subset were assessed in the H_16_dry and H_16_irri trials, and only the DH subset was evaluated in the H_15_dry, H_15_irri and W_16_dry trials, due to limited seed availability. All field trials were designed as partial replicated (0.5) row-column designs (Cullis et al. 2006). Plots were sown at a size of 2 m × 6 m with 25 cm row spacing and a target density of 120 plants/m^{-2}. Weeds and diseases were controlled as required throughout the growing season. Heavy clay cracking soil with high water holding capacity predominated at all trial sites and was typical of the Australian northern cereal cropping region. For example, the soil type at HRF was a cracking and self-mulching grey clay with abundant CaCO3 concretions (Elphinstone depositional,

McKeown 1978; Ug 5.16, Northcote 1974). The degree of swelling on wetting indicates a high montmorillonite clay content (McKeown 1978).

Table 5.1 Description of trial environments

Trial	Year	Location	Irrigated[a] (Litres/Meter)	Sowing date	Annual RF[b] (mm)	CSRF[d] (mm)
H_15_dry	2015	Hermitage QLD 28.2° S, 152.1° E	N/A[e]	10.06.2015	653.8	101.8
H_15_irri	2015	Hermitage QLD 28.2° S, 152.1° E	156	10.06.2015	653.8	101.8
H_16_dry	2016	Hermitage QLD 28.2° S, 152.1° E	N/A[e]	22.07.2016	729.4	370.0
H_16_irri	2016	Hermitage QLD 28.2° S, 152.1° E	260	22.07.2016	729.4	370.0
W_16_dry	2016	Wellcamp QLD 27.6° S, 151.9° E	N/A[e]	17.06.2016	618.5	304.8

[a] Total irrigation applied throughout entire season (litres/meter) for irrigated trials
[b] Annual rainfall
[d] Cropping season rainfall
[e] Not applicable as these are non-irrigated dryland trials

5.3.4 Phenotyping and statistical analysis of field traits

For the estimation of stay-green traits, weekly NDVI measurements were recorded using a Trimble® Greenseeker, with 20-30 individual measurements taken across the plot and averaged. NDVI measurements were recorded for all plots from mid-booting through to maturity. Days to anthesis (DTA) and days to maturity were recorded for each plot across all trials. The senescence evaluation tool previously described by Christopher et al. (2014) involves regular NDVI measurements coupled with analysis using a logistic model. As a result, this tool generates improved insight into the variation of senescence patterns between genotypes both within and across environments (Christopher et al. 2014). Using this tool, stay-green traits were estimated by fitting a logistic function (Eqn. 5.1) to the NDVI data centred on the anthesis date of each plot.

Eqn. 5.1
$$NDVI = N_{final} + \left(\frac{N_{green\_max}}{1+\left(\frac{t}{TFN50^{SR}}\right)}\right)$$

where N_{final} is the final NDVI measurement of the senesced plant, N_{green_max} is the difference between the maximum NDVI and the final NDVI measurements, t is the time in days since anthesis for the plot, $TFN50$ is the number of days from anthesis to 50% loss of N_{green_max} and SR is an indicator of senescence rate. The rate of senescence (RS; SR in Eqn. 5.1), the NDVI integral from anthesis to maturity (SGI) and the number of days from anthesis until senescence conclusion (TFN10) were estimated for each plot. The SGI estimate corresponds

to area under the stay-green curve from anthesis until maturity and is an indicator of the overall green-leaf retention of the plot after anthesis (Christopher et al. 2014). In all trials, plots were harvested using a plot harvester and grain was weighed to calculate yield per plot. The spatial location of each plot was accounted for in the design model of each trial, where a linear mixed model was fitted to the data for each stay-green trait. Variance components of the model were estimated using residual maximum likelihood (REML) and best linear unbiased estimators (BLUEs) were generated for the fixed genotype effects of each trait across each trial. Using the variance components of the model, a generalised heritability of each trait was calculated (Cullis et al. 2006). Linear regression was performed in R to calculate phenotypic correlations.

5.3.5 Linkage and composite interval mapping (CIM)

The DArTseq single nucleotide polymorphism (SNP) data for the ND24260 × Flagship DH population was filtered for markers with a significant segregation distortion (deviating from the expected 1:1 for DH populations) using a χ^2 test. Markers with ≥ 10% missing data were also removed, leaving 586 polymorphic markers from the initial 21, 367 SNP calls. A linkage map was created using QTL Inclusive Composite Interval Mapping (ICIM; Meng et al. 2015) using the physical barley map (Mayer et al. 2012) to provide two SNP anchor points per chromosome. A LOD threshold of 3.00 and a cut-off recombination value of 0.35 was used to group SNP markers. The optimum ordering of markers across each chromosome were determined by the nnTwoOpt ordering algorithm and refined by rippling across a window of six markers using the sum of the adjacent LOD scores. Distances between markers were determined by the recombination values using the Kosambi function (Kosambi 1943).

Single environment QTL analysis was performed for all traits measured in the DH population using the BIP ICIM-ADD function in ICIM software (Meng et al. 2015) for composite interval mapping (CIM) with a step size of 1.00 cM. The p-value for step-wise regression was set to 0.001 and the LOD threshold for significant QTL was deemed 3.07 based on the Bonferroni correction for multiple testing, where $\alpha = 0.05$. Multi-environment (MET) QTL analysis, using the MET ICM-ADD, function was run for all traits measured in the DH population, combining trait phenotypes from across all five trials. To account for the five-fold increase in individual tests the LOD threshold for significance was increased to 4.00, based on the Bonferroni correction where $\alpha = 0.05$.

5.3.6 Genome-wide association study (GWAS)

The DArTseq SNP data for the NAM subset was filtered to remove markers containing ≥ 10% missing data, ≥ 30% heterozygote frequency and a minor allele frequency ≤ 2.5%, resulting in 3,375 high quality polymorphic markers. A genome-wide association study (GWAS) was performed in the R package Genome Association and Prediction Integrated Tool (GAPIT; Lipka et al. 2012), using both a compressed mixed linear model (CMLM) and a settlement of MLM under progressively exclusive relationship (SUPER) model. A genomic relationship matrix based on markers thinned to a linkage disequilibrium (LD) of < 0.90 and five principal components, each accounting for greater than 5% of the phenotypic variation, were fitted to both models. In the final QTL analysis, DTA BLUEs for each genotype were also included in the model as a covariate. A false-discovery rate (FDR; Benjamini and Hochberg 1995) adjusted p-value of < 0.05 was applied as a significance threshold and significant markers within 5cM were deemed a single QTL. The QTL detected for all traits across both populations were positioned on the barley physical map (Mayer et al. 2012) and visualised using Map-Chart v2.2 (Voorrips 2002).

5.3.7 Multi-environment trial (MET) yield analysis

Yield was analysed across the five trials in a multi-environment trial (MET) analysis using the following mixed model:

$$y = X\tau + Z_0 u_0 + Z_g u_g + e$$

where X and Z are the design matrices associated with the fixed τ and the random u vectors. The fixed effects τ includes environment main effects and trial-specific effects for field variation. The u_g term incorporates the genotype by trial site effects, and u_0 includes any additional random extraneous. A factor analytic (FA) model was fitted for u_g, as all trials did not have equal genetic variance nor equal co-variance between pairs of sites, therefore the FA model was a superior fit to the compound symmetry structure (Smith et al. 2001; Smith et al. 2015). The FA model proposes reliance on a set of random hypothetical factors, where the genotype by trial site interaction effects are modelled as follows:

$$u_g = (\lambda_1 \otimes l_m) f_1 + \ldots + (\lambda_k \otimes l_m) f_k + \delta$$

where the coefficient λ_r are the estimated trial site loadings ($r = 1 \ldots k < p$), f_r is the vector of genotype scores and δ is the vector of residuals for the model, and I_m is the identity matrix. A separable autoregressive process of order one (AR1 × AR1) was fitted as a variance structure for e in the mixed model to account for the local spatial trend.

5.4 Results

5.4.1 Phenotypic variation

A wide range in root phenotypes were observed in the NAM subset, with angle ranging from 12.50° to 109.70° (h^2 0.66) and root number from 3.00 to 6.19 roots (h^2 0.44; Figure 5.1). A strong positive correlation of 0.82 ($p < 0.01$) was observed between root angle and number. The phenotypic ranges for each stay-green trait within each population across all trials are detailed in Table 5.2. The population distribution of stay-green trait phenotypes and DTA were similar throughout the 2016 trials (Figure 5.2), except for H_16_irri where a slightly wider range in RS and DTA phenotypes were observed. The distributions for the 2015 trials were distinct from the 2016 trials and were marginally different from each other, especially for RS and TFN10 traits (Figure 5.2). The heritability for all field traits was notably lower in both 2015 trials compared to 2016 trials (Table 5.2).

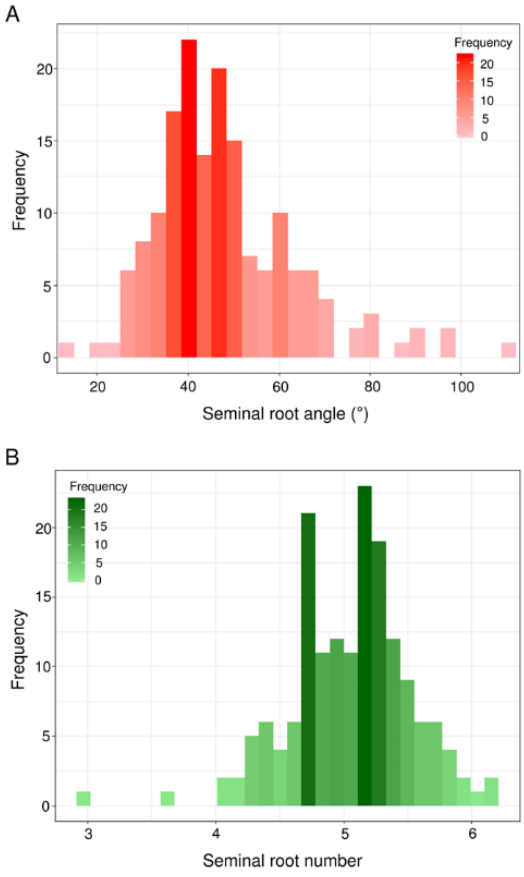

Figure 5.1 Distributions of root traits for the NAM subset. **(A)** distribution of seminal root angle (°), and **(B)** distribution of root number. Increase in colour brightness (closer to red for angle and closer to dark green for number) represents an increase in frequency of lines with the root phenotype.

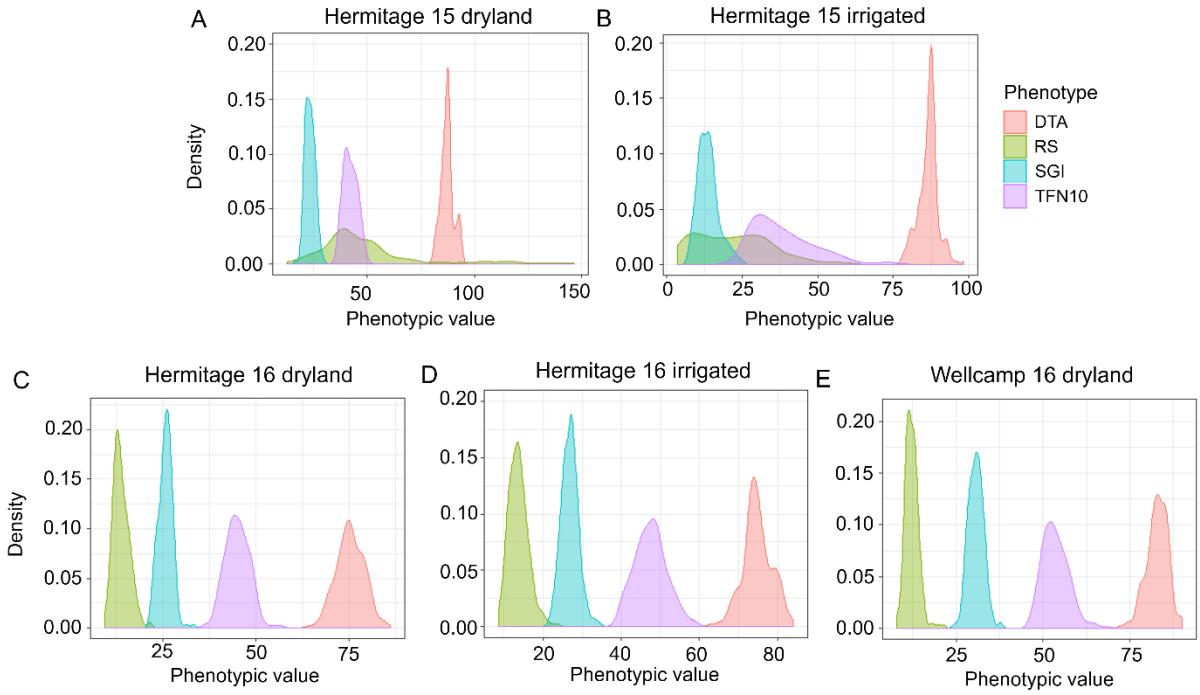

Figure 5.2 Density plots for the phenotypic distribution of days to anthesis (DTA; pink), rate of senescence (RS; green), stay-green integral (SGI; blue) and time from flowering to 10% greenness (TFN10; purple) across all field trials. **(A)** Hermitage 15 dryland, **(B)** Hermitage 15 irrigated, **(C)** Hermitage 16 dryland, **(D)** Hermitage 16 irrigated and **(E)** Wellcamp 16 dryland, where at least 200 genotypes are concurrent across all trials.

Table 5.2 Phenotype ranges and heritabilities (h^2) for senescence traits and DTA in all trials

	Trait	H_15_d	h^2	H_15_i	h^2	W_16_d	h^2	H_16_d	h^2	H_16_i	h^2
DH	RS	12.34 - 146.50	0.26	3.47 - 58.86	0.21	7.80 - 21.50	0.44	9.30 - 21.50	0.62	8.60 - 23.40	0.69
	SGI	16.39 - 29.69	0.00	7.37 - 24.85	0.00	24.30 - 37.90	0.74	21.40 - 33.20	0.84	21.50- 34.30	0.74
	TFN10	34.54 - 50.44	0.21	20.49 - 76.11	0.05	46.60 - 68.60	0.73	36.30 - 56.50	0.85	39.20 - 59.10	0.72
	DTA	80.50 - 95.00	0.17	77.72 - 98.25	0.12	72.24 - 90.04	0.88	64.16 - 86.02	0.93	63.00 - 84.00	0.93
NAM	RS	NA	NA	NA	NA	NA	NA	7.70 - 20.60	0.62	9.30 - 32.00	0.69
	SGI	NA	NA	NA	NA	NA	NA	18.40 - 32.90	0.84	19.60 - 34.20	0.74
	TFN10	NA	NA	NA	NA	NA	NA	30.00 - 56.20	0.85	34.00 - 59.30	0.72

	DTA	NA	NA	NA	NA	NA	NA	62.98 - 88.93	0.93	63.00 - 92.00	0.93

5.4.2 Mapping QTL for stay-green and root angle traits

Preliminary QTL mapping for each trait × environment × population dataset detected a total of 106 QTL for stay-green traits and DTA (Table 5.3). A large proportion of the preliminary QTL collocated across environments (Table 5.3), indicating a MET QTL analysis combining environmental effects may be more informative. Co-location of stay-green QTL with the majority of the QTL detected for DTA was also evident in the preliminary analysis (Table 5.3). Estimates for SGI and TFN10 were modelled based on the DTA of each plot to minimise the influence of time to flowering on the stay-green traits. Strong negative correlations were observed between each of the two stay-green traits (SGI and TFN10) and DTA (Figure 5.3). In addition, SGI and TFN10 had a strong positive correlation with each other in all trials, which was not surprising as both traits relate to the area under the stay-green curve suggesting these traits provide very similar information (Figure 5.3). More specifically, SGI is a measure of the total area under the stay-green curve, whereas TFN10 increases the area under the stay-green curve by delaying the time to senescence conclusion (Christopher et al. 2014). To further minimise the effect of DTA on the stay-green traits, a second GWAS analysis was performed for the NAM subset including DTA phenotypes as a covariate in the mixed model. The final QTL analysis consisted of a MET QTL study for the DH population and a SUPER GWAS including DTA as a covariate for the NAM subset. A total of 69 QTL were detected across all chromosomes for stay-green traits and DTA across the NAM subset and the DH population (Table 5.4). In comparison to the preliminary analysis, there were 19 QTL initially identified that were not significant in the final MET analysis (Table 5.3). In addition, 18 new QTL spread across all traits were identified in the MET analysis that were not previously identified (Table 5.4). Of the 69 QTL detected in the final MET analysis, 25 were identified for DTA, 8 for RS, 16 for SGI and 20 for TFN10. For seminal root angle, 4 QTL were detected on chromosomes 3H and 5H in the DH population and no QTL were statistically significant in the NAM population. A total of 8 QTL were identified for root number with 2 detected on chromosome 5H in the NAM population and the remaining 6 in the DH population on chromosomes 1H, 3H, 5H and 6H (Table 5.4).

Table 5.3 QTL detected for all traits across all environments in preliminary analysis

Environ.	Trait	Pop.	QTL	Marker	Ch.	Pos. (cM)	LOD	FDR p	PVE[a]	Add. Effect	MET QTL[b]
Glasshouse	RA	DH	QRA.DH-3H.1	3257777	3H	15.08	5.79	NA	6.07	3.07	NA
Glasshouse	RA	DH	QRA.DH-3H.3	3260646	3H	78.21	6.53	NA	6.74	-3.22	NA
Glasshouse	RA	DH	QRA.DH-3H.2	3259496	3H	139.59	5.26	NA	7.17	3.32	NA
Glasshouse	RA	DH	QRA.DH-5H	3257021	5H	168.54	11.37	NA	12.18	-4.36	NA
Glasshouse	RN	DH	QRN.DH-1H.1	3666092	1H	61.54	3.35	NA	5.00	-0.10	NA
Glasshouse	RN	DH	QRN.DH-1H.2	7750085	1H	61.54	4.80	NA	14.04	0.16	NA
Glasshouse	RN	DH	QRN.DH-3H	3256610	3H	39.07	3.67	NA	4.01	0.09	NA
Glasshouse	RN	DH	QRN.DH-5H	6273033	5H	168.54	5.54	NA	6.11	-0.11	NA
Glasshouse	RN	DH	QRN.DH-6H.2	3263313	6H	64.09	4.73	NA	6.53	-0.11	NA
Glasshouse	RN	DH	QRN.DH-6H.1	3257613	6H	65.72	5.45	NA	6.22	0.11	NA
Glasshouse	RN	NAM	QRN.NAM-5H.2	3398279	5H	51.88	NA	0.04	6.50	-0.32	NA
Glasshouse	RN	NAM	QRN.NAM-5H.1	5247903	5H	164.72	NA	0.03	4.72	-0.27	NA
H_15_dry	TFN10	DH	QTFN10.DH.dryH15-1H	3926997	1H	118.94	3.54	NA	7.62	-0.93	Yes
H_15_dry	TFN10	DH	QTFN10.DH.dryH15-2H.1	3258331	2H	18.91	3.11	NA	41.05	2.12	Yes
H_15_dry	TFN10	DH	QTFN10.DH.dryH15-2H.2	5239962	2H	149.26	3.82	NA	40.32	2.10	No
H_15_irri	DTA	DH	QDTA.DH.irriH15-2H	3256420	2H	68.40	4.33	NA	9.60	-0.95	Yes
H_15_irri	RS	DH	QRS.DH.irriH15-2H.1	5241797	2H	0.00	4.13	NA	63.14	-0.13	No
H_15_irri	RS	DH	QRS.DH.irriH15-2H.2	3259455	2H	94.87	3.19	NA	62.60	0.13	No
H_15_irri	RS	DH	QRS.DH.irriH15-4H	3432492	4H	53.33	4.09	NA	65.42	0.14	No
H_15_irri	RS	DH	QRS.DH.irriH15-7H.1	3260769	7H	24.06	6.32	NA	62.40	0.13	No
H_15_irri	RS	DH	QRS.DH.irriH15-7H.2	3810620	7H	116.08	4.92	NA	63.66	0.13	No
H_15_irri	TFN10	DH	QTFN10.DH.irriH15-7H	3260769	7H	24.06	3.15	NA	54.26	-0.35	Yes
H_16_dry	DTA	DH	QDTA.DH.dryH16-1H	3926997	1H	118.94	9.00	NA	12.69	1.36	Yes
H_16_dry	DTA	DH	QDTA.DH.dryH16-2H	3271009	2H	17.85	7.58	NA	10.85	-1.25	Yes
H_16_dry	DTA	DH	QDTA.DH.dryH16-7H.2	3273432	7H	20.96	3.53	NA	5.22	-0.85	No
H_16_dry	DTA	DH	QDTA.DH.dryH16-7H.1	3257953	7H	21.88	6.10	NA	8.32	1.09	No
H_16_dry	DTA	DH	QDTA.DH.dryH16-7H.3	3269952	7H	126.13	4.52	NA	6.06	0.93	Yes
H_16_dry	DTA	NAM	QDTA.NAM.dryH16-2H.1	3263305	2H	5.38-29.39	NA	0.00	12.69	3.44	NA
H_16_dry	DTA	NAM	QDTA.NAM.dryH16-2H.2	4792852	2H	72.65-80.59	NA	0.00	5.60	-4.40	NA
H_16_dry	DTA	NAM	QDTA.NAM.dryH16-3H	3254972	3H	108.32-117	NA	0.00	9.26	3.71	NA
H_16_dry	DTA	NAM	QDTA.NAM.dryH16-6H	3256859	6H	114.13	NA	0.02	2.81	-1.80	NA
H_16_dry	DTA	NAM	QDTA.NAM.dryH16-7H.1	3259226	7H	24.15-32.61	NA	0.00	5.81	-3.88	NA
H_16_dry	DTA	NAM	QDTA.NAM.dryH16-7H.2	3258738	7H	41.43-51.49	NA	0.00	5.97	-3.94	NA
H_16_dry	RS	DH	QRS.DH.dryH16-2H.1	3432739	2H	7.63	5.04	NA	8.53	-0.64	Yes
H_16_dry	RS	DH	QRS.DH.dryH16-2H.2	3262925	2H	18.91	10.21	NA	18.45	-0.94	Yes
H_16_dry	RS	DH	QRS.DH.dryH16-6H.1	3666186	6H	49.08	11.51	NA	21.00	0.99	Yes

H_16_dry	RS	DH	QRS.DH.dryH16-6H.2	3273498	6H	49.15	8.43	NA	18.40	-0.93	Yes
H_16_dry	RS	DH	QRS.DH.dryW16-6H	3273498	6H	49.15	5.17	NA	11.40	-0.66	Yes
H_16_dry	RS	NAM	QRS.NAM.dryH16-2H.1	3262115	2H	5.38	NA	0.01	5.00	-0.75	NA
H_16_dry	RS	NAM	QRS.NAM.dryH16-2H.2	3433304	2H	12.11-13.93	NA	0.01	6.03	-0.84	NA
H_16_dry	RS	NAM	QRS.NAM.dryH16-2H.3	3261503	2H	18.91-23.8	NA	0.01	5.42	0.79	NA
H_16_dry	RS	NAM	QRS.NAM.dryH16-3H	3432505	3H	17.85	NA	0.04	3.24	0.63	NA
H_16_dry	SGI	DH	QSGI.DH.dryH16-5H	3256623	5H	85.56	5.11	NA	7.78	0.51	Yes
H_16_dry	SGI	DH	QSGI.DH.dryH16-6H.1	5241039	6H	15.72	3.24	NA	23.24	0.87	No
H_16_dry	SGI	DH	QSGI.DH.dryH16-6H.2	3258496	6H	49.01	5.31	NA	12.11	-0.63	Yes
H_16_dry	SGI	DH	QSGI.DH.dryH16-6H.3	3432742	6H	90.65	4.36	NA	6.60	-0.46	No
H_16_dry	SGI	DH	QSGI.DH.dryH16-7H.1	3260769	7H	24.06	3.12	NA	8.53	0.53	No
H_16_dry	SGI	DH	QSGI.DH.dryH16-7H.2	3271300	7H	34.40	8.66	NA	13.76	-0.68	Yes
H_16_dry	SGI	DH	QSGI.DH.dryH16-7H.3	3271300	7H	34.40	4.10	NA	7.74	0.51	Yes
H_16_dry	SGI	DH	QSGI.DH.dryH16-7H.4	3257523	7H	120.40	4.65	NA	7.20	0.50	No
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-2H.1	3256113	2H	13.53-13.93	NA	0.01	2.40	0.68	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-2H.6	5256412	2H	136.05-140.79	NA	0.01	7.80	-2.33	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-2H.2	3258331	2H	18.91-23.8	NA	0.00	5.90	-1.17	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-2H.3	3255759	2H	56.52-60.84	NA	0.01	5.70	-2.08	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-2H.4	3257880	2H	70.82-80.91	NA	0.01	7.80	-2.60	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-2H.5	3932084	2H	86.76-91.01	NA	0.04	4.70	-1.97	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-3H.1	3432505	3H	17.85	NA	0.03	2.00	0.67	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-3H.2	3257891	3H	114.65-120	NA	0.02	4.70	1.31	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-5H.2	3262195	5H	41.81	NA	0.04	3.70	-1.18	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-5H.1	3265055	5H	151.39-159.79	NA	0.02	2.20	-0.68	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-6H	3256859	6H	114.13	NA	0.04	2.83	0.92	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-7H.1	3259226	7H	23.76-32.61	NA	0.00	11.60	2.86	NA
H_16_dry	SGI	NAM	QSGI.NAM.dryH16-7H.2	5248535	7H	41.43-51.49	NA	0.00	6.91	-1.57	NA
H_16_dry	TFN10	DH	QTFN10.DH.dryH16-5H	3256623	5H	85.56	8.59	NA	11.55	1.10	Yes
H_16_dry	TFN10	DH	QTFN10.DH.dryH16-6H.1	3666186	6H	49.08	17.02	NA	24.32	-1.60	Yes
H_16_dry	TFN10	DH	QTFN10.DH.dryH16-6H.2	3273498	6H	49.15	11.03	NA	18.22	1.40	Yes
H_16_dry	TFN10	DH	QTFN10.DH.dryH16-6H.3	3432742	6H	90.65	5.06	NA	6.24	-0.81	No

H_16_dry	TFN10	DH	QTFN10.DH.dryH16-7H.1	3260769	7H	24.06	4.97	NA	11.41	-1.12	Yes
H_16_dry	TFN10	DH	QTFN10.DH.dryH16-7H.2	3271300	7H	34.40	9.04	NA	11.67	0.98	Yes
H_16_dry	TFN10	DH	QTFN10.DH.dryH16-7H.3	3271300	7H	34.40	5.93	NA	8.92	0.65	Yes
H_16_dry	TFN10	DH	QTFN10.DH.dryH16-7H.4	3265972	7H	131.73	3.11	NA	3.94	0.64	No
H_16_dry	TFN10	NAM	QTFN10.NAM.dryH16-2H.1	3263305	2H	14.38-18.91	NA	0.01	4.71	-1.79	NA
H_16_dry	TFN10	NAM	QTFN10.NAM.dryH16-2H.2	3257880	2H	72.56-20.59	NA	0.03	5.56	-3.69	NA
H_16_dry	TFN10	NAM	QTFN10.NAM.dryH16-3H.1	3257891	3H	108.32-117	NA	0.00	12.59	3.59	NA
H_16_dry	TFN10	NAM	QTFN10.NAM.dryH16-3H.2	4169654	3H	126.7-128.76	NA	0.03	3.64	-2.14	NA
H_16_dry	TFN10	NAM	QTFN10.NAM.dryH16-6H	3273498	6H	49.52	NA	0.04	2.90	1.56	NA
H_16_dry	TFN10	NAM	QTFN10.NAM.dryH16-7H.1	3259226	7H	23.76-32.61	NA	0.00	12.07	4.84	NA
H_16_dry	TFN10	NAM	QTFN10.NAM.dryH16-7H.2	5248535	7H	41.43-51.49	NA	0.00	6.76	-2.61	NA
H_16_irri	DTA	DH	QDTA.DH.irriH16-1H	3926997	1H	118.94	7.76	NA	12.81	1.35	Yes
H_16_irri	DTA	DH	QDTA.DH.irriH16-2H	3271009	2H	17.85	7.72	NA	13.11	-1.35	Yes
H_16_irri	DTA	DH	QDTA.DH.irriH16-7H	3260483	7H	30.56	4.89	NA	7.81	-1.05	Yes
H_16_irri	DTA	NAM	QDTA.NAM.irriH16-2H.2	3256573	2H	56.52	NA	0.02	3.66	2.27	NA
H_16_irri	DTA	NAM	QDTA.NAM.irriH16-2H.3	5256412	2H	136.05	NA	0.01	5.85	3.76	NA
H_16_irri	DTA	NAM	QDTA.NAM.irriH16-2H.1	3263305	2H	5.38-33.5	NA	0.00	10.24	3.07	NA
H_16_irri	DTA	NAM	QDTA.NAM.irriH16-3H	3254972	3H	108.32-117	NA	0.00	10.70	3.95	NA
H_16_irri	DTA	NAM	QDTA.NAM.irriH16-6H	3256859	6H	114.13	NA	0.02	3.00	-1.84	NA
H_16_irri	DTA	NAM	QDTA.NAM.irriH16-7H.1	3259226	7H	23.8-32.61	NA	0.00	6.20	-3.96	NA
H_16_irri	DTA	NAM	QDTA.NAM.irriH16-7H.2	3258738	7H	46.39-50.71	NA	0.00	6.14	-3.95	NA
H_16_irri	RS	DH	QRS.DH.irriH16-6H	3666186	6H	49.08	3.50	NA	18.13	0.08	Yes
H_16_irri	RS	NAM	QRS.NAM.irriH16-3H	3254972	3H	108.89	NA	0.03	9.80	0.21	NA
H_16_irri	SGI	DH	QSGI.DH.irriH16-1H	3926997	1H	118.94	3.50	NA	4.72	-0.48	Yes
H_16_irri	SGI	DH	QSGI.DH.irriH16-5H.3	3398320	5H	96.25	5.89	NA	8.55	-0.64	No
H_16_irri	SGI	DH	QSGI.DH.irriH16-5H.2	4171535	5H	111.32	5.86	NA	8.66	0.64	No
H_16_irri	SGI	DH	QSGI.DH.irriH16-5H.1	3430617	5H	139.10	3.97	NA	9.72	0.67	Yes
H_16_irri	SGI	DH	QSGI.DH.irriH16-6H	3265876	6H	49.52	5.43	NA	7.57	0.59	Yes
H_16_irri	SGI	DH	QSGI.DH.irriH16-7H	3260483	7H	30.56	5.33	NA	7.61	0.60	Yes
H_16_irri	SGI	NAM	QSGI.NAM.irriH16-2H.3	5256412	2H	136.05-140.79	NA	0.00	7.71	-2.39	NA
H_16_irri	SGI	NAM	QSGI.NAM.irriH16-2H.1	3263305	2H	5.38-33.5	NA	0.00	6.36	-1.22	NA

H_16_irri	SGI	NAM	QSGI.NAM.irriH16-2H.2	3261257	2H	54.25-60.84	NA	0.01	5.01	-1.36	NA
H_16_irri	SGI	NAM	QSGI.NAM.irriH16-7H.1	3259226	7H	29.89-32.61	NA	0.00	8.55	2.50	NA
H_16_irri	SGI	NAM	QSGI.NAM.irriH16-7H.2	3258738	7H	46.29-52.27	NA	0.01	6.59	2.17	NA
H_16_irri	TFN10	DH	QTFN10.DH.irriH16-5H.3	3398320	5H	96.25	8.24	NA	11.69	-1.38	No
H_16_irri	TFN10	DH	QTFN10.DH.irriH16-5H.2	4171535	5H	111.32	7.17	NA	10.46	1.30	Yes
H_16_irri	TFN10	DH	QTFN10.DH.irriH16-5H.1	3430617	5H	139.10	3.39	NA	8.12	1.14	Yes
H_16_irri	TFN10	DH	QTFN10.DH.irriH16-6H	3265876	6H	49.52	11.94	NA	17.11	1.66	Yes
H_16_irri	TFN10	DH	QTFN10.DH.irriH16-7H	3263743	7H	119.33	4.71	NA	11.27	-1.36	Yes
H_16_irri	TFN10	NAM	QTFN10.NAM.irriH16-2H.1	3263305	2H	18.91	NA	0.00	6.30	-2.09	NA
H_16_irri	TFN10	NAM	QTFN10.NAM.irriH16-2H.2	5256412	2H	136.05	NA	0.01	7.09	-4.06	NA
H_16_irri	TFN10	NAM	QTFN10.NAM.irriH16-3H	3254972	3H	108.89-117	NA	0.00	9.38	-3.44	NA
W_16_dry	DTA	DH	QDTA.DH.dryW16-1H	3926997	1H	118.94	9.10	NA	16.51	1.26	Yes
W_16_dry	DTA	DH	QDTA.DH.dryW16-2H	3255437	2H	99.26	4.91	NA	12.15	-1.05	Yes
W_16_dry	DTA	DH	QDTA.DH.dryW16-3H	3432405	3H	36.98	3.24	NA	5.51	0.72	Yes
W_16_dry	SGI	DH	QSGI.DH.dryW16-5H	4171535	5H	111.32	3.14	NA	12.10	0.77	No
W_16_dry	SGI	DH	QSGI.DH.dryW16-7H	3260483	7H	30.56	6.61	NA	13.46	0.82	Yes
W_16_dry	TFN10	DH	QTFN10.DH.dryW16-2H	3432460	2H	2.27	3.70	NA	8.20	1.10	No
W_16_dry	TFN10	DH	QTFN10.DH.dryW16-5H	3262488	5H	95.57	4.74	NA	8.08	1.10	No
W_16_dry	TFN10	DH	QTFN10.DH.dryW16-6H	3272925	6H	49.22	8.53	NA	14.85	1.50	Yes
W_16_dry	TFN10	DH	QTFN10.DH.dryW16-7H	3260769	7H	24.06	3.64	NA	13.82	-1.40	Yes

[a]Percentage explained variation

[b]QTL significant is MET QTL analysis

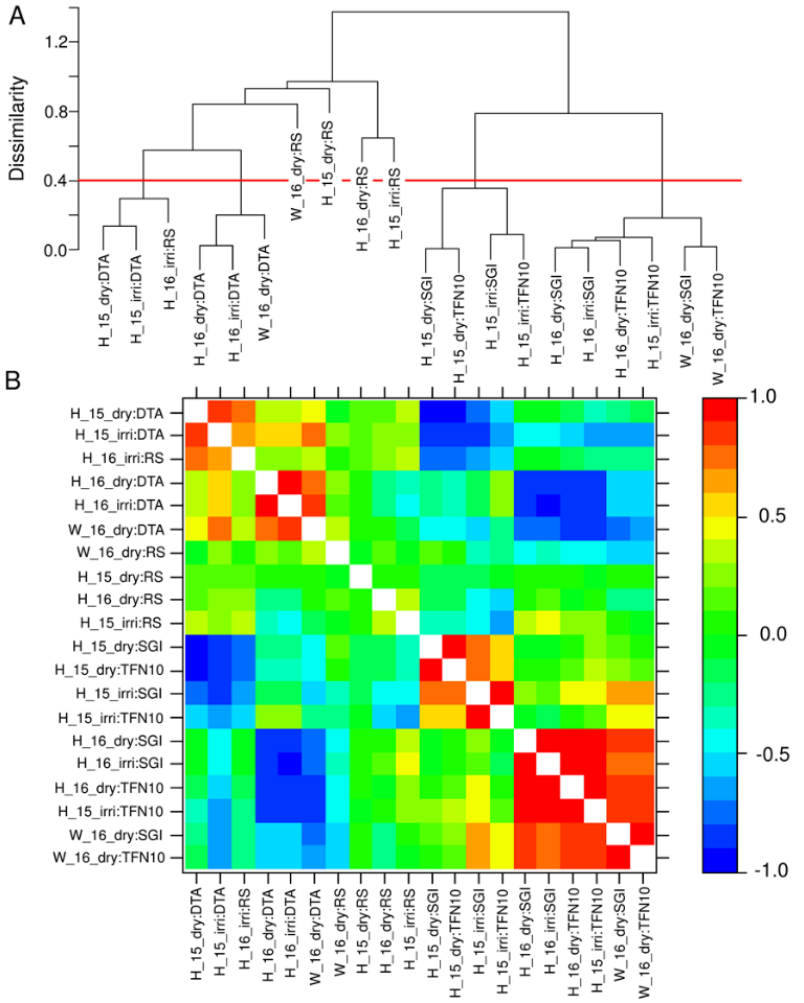

Figure 5.3 Dendrogram and heat map from MET analysis of all field traits (DTA, RS, SGI and TFN10). **(A)** Dissimilarity matrix of field traits across the five field trials. A dissimilarity of 0.40 was used as a cut off for the clustering and is represented by the red line. **(B)** Heat map of the MET field trait genetic correlation matrix. Positive genetic correlations between traits increase with increasing colour brightness (closer to red) and negative correlations with decreasing brightness (closer to blue).

Table 5.4 QTL for all traits detected in final analysis

Trait	Pop.	QTL	Marker	Ch.	Pos. (cM)	LOD	FDR p	PVE[a]	Add. effect	Prelim. QTL[b]
RA	DH	QRA.DH-3H.1	3257777	3H	15.08	5.79	NA	6.07	3.07	NA
RA	DH	QRA.DH-3H.3	3260646	3H	78.21	6.53	NA	6.74	-3.22	NA
RA	DH	QRA.DH-3H.2	3259496	3H	139.59	5.26	NA	7.17	3.32	NA
RA	DH	QRA.DH-5H	3257021	5H	168.54	11.37	NA	12.18	-4.36	NA
RN	DH	QRN.DH-1H.1	3666092	1H	61.54	3.35	NA	5.00	-0.10	NA
RN	DH	QRN.DH-1H.2	7750085	1H	61.54	4.80	NA	14.04	0.16	NA
RN	DH	QRN.DH-3H	3256610	3H	39.07	3.67	NA	4.01	0.09	NA
RN	DH	QRN.DH-5H	6273033	5H	168.54	5.54	NA	6.11	-0.11	NA
RN	DH	QRN.DH-6H.2	3263313	6H	64.09	4.73	NA	6.53	-0.11	NA
RN	DH	QRN.DH-6H.1	3257613	6H	65.72	5.45	NA	6.22	0.11	NA

RN	NAM	QRN.NAM-5H.2	3398279	5H	51.88	NA	0.04	6.50	-0.32	NA
RN	NAM	QRN.NAM-5H.1	5247903	5H	164.72	NA	0.03	4.72	-0.27	NA
RS	DH	QRS.DH-2H.1	3432739	2H	7.63	4.63	NA	1.44	-0.08	Yes
RS	DH	QRS.DH-2H.2	3262925	2H	18.91	12.86	NA	4.21	-0.13	Yes
RS	DH	QRS.DH-2H.3	5249711	2H	107.15	4.49	NA	1.25	-0.09	No
RS	DH	QRS.DH-3H.1	4198294	3H	128.75	5.21	NA	0.90	0.08	No
RS	DH	QRS.DH-3H.2	4198294	3H	128.75	5.90	NA	1.09	-0.09	No
RS	DH	QRS.DH-6H.1	3666186	6H	49.08	14.29	NA	3.62	0.11	Yes
RS	DH	QRS.DH-6H.2	3273498	6H	49.15	15.98	NA	3.80	-0.16	Yes
RS	NAM	QRS.NAM.dry-3H	3254972	3H	108.89	NA	0.00	11.94	1.84	NA
SGI	DH	QSGI.DH-1H	3926997	1H	118.94	10.75	NA	1.41	-0.20	Yes
SGI	DH	QSGI.DH-2H.1	3255954	2H	18.13	5.07	NA	0.63	-0.01	No
SGI	DH	QSGI.DH-2H.2	6429426	2H	91.10	6.27	NA	0.86	0.14	No
SGI	DH	QSGI.DH-2H.3	5249711	2H	107.50	4.59	NA	0.78	-0.12	No
SGI	DH	QSGI.DH-5H.4	3256623	5H	85.56	5.35	NA	0.63	0.12	Yes
SGI	DH	QSGI.DH-5H.2	3264185	5H	137.08	6.12	NA	0.64	-0.10	No
SGI	DH	QSGI.DH-5H.1	3430617	5H	139.10	8.64	NA	0.87	0.11	Yes
SGI	DH	QSGI.DH-5H.3	3262718	5H	167.29	11.31	NA	1.54	0.15	No
SGI	DH	QSGI.DH-6H	3265876	6H	49.52	8.33	NA	1.13	0.15	Yes
SGI	DH	QSGI.DH-7H.2	3260483	7H	30.56	11.70	NA	1.88	0.16	Yes
SGI	DH	QSGI.DH-7H.1	3271300	7H	34.40	7.28	NA	0.93	-0.10	Yes
SGI	NAM	QSGI.NAM.dry-5H.1	4170092	5H	144.65	NA	0.04	1.85	0.99	NA
SGI	NAM	QSGI.NAM.dry-5H.2	3273752	5H	159.79	NA	0.04	1.21	0.47	NA
SGI	NAM	QSGI.NAM.irri-6H.1	3262236	6H	10.46	NA	0.01	1.43	-0.52	NA
SGI	NAM	QSGI.NAM.irri-6H.2	3264121	6H	15.86	NA	0.00	1.83	0.66	NA
SGI	NAM	QSGI.NAM.dry-7H.1	3432762	7H	29.82	NA	0.04	2.15	1.37	NA
TFN10	DH	QTFN10.DH-1H	3926997	1H	118.94	5.71	NA	0.89	-0.25	Yes
TFN10	DH	QTFN10.DH-2H.1	3262925	2H	18.91	5.13	NA	0.70	0.11	Yes
TFN10	DH	QTFN10.DH-2H.2	3256260	2H	81.94	4.03	NA	0.33	0.17	No
TFN10	DH	QTFN10.DH-5H.5	3256623	5H	85.56	5.22	NA	0.56	0.20	Yes
TFN10	DH	QTFN10.DH-5H.4	4171535	5H	111.32	5.56	NA	0.82	0.25	Yes
TFN10	DH	QTFN10.DH-5H.2	3264185	5H	137.08	5.23	NA	0.66	-0.20	No
TFN10	DH	QTFN10.DH-5H.1	3430617	5H	139.10	8.16	NA	1.04	0.23	Yes
TFN10	DH	QTFN10.DH-5H.3	3262718	5H	167.29	11.88	NA	2.02	0.28	No
TFN10	DH	QTFN10.DH-6H.1	3258791	6H	1.42	4.56	NA	0.65	-0.18	No
TFN10	DH	QTFN10.DH-6H.2	3666186	6H	49.07	14.92	NA	1.86	-0.21	Yes
TFN10	DH	QTFN10.DH-6H.3	3265876	6H	49.52	11.60	NA	2.08	0.23	Yes
TFN10	DH	QTFN10.DH-6H.4	3257358	6H	73.68	4.46	NA	0.53	0.11	No
TFN10	DH	QTFN.DH-7H.1	3260769	7H	24.06	4.64	NA	0.45	0.10	Yes
TFN10	DH	QTFN10.DH-7H.2	3271300	7H	34.40	10.28	NA	1.42	-0.21	Yes
TFN10	DH	QTFN10.DH-7H.3	3257523	7H	120.40	5.71	NA	0.98	0.22	Yes
TFN10	NAM	QTFN10.NAM.dry-2H.2	3433304	2H	12.11	NA	0.05	1.16	0.83	NA
TFN10	NAM	QTFN10.NAM.dry-2H.1	3261503	2H	23.8	NA	0.00	2.07	-1.12	NA
TFN10	NAM	QTFN10.NAM.dry-3H	3434122	3H	114.65	NA	0.03	1.97	1.46	NA
TFN10	NAM	QTFN10.NAM.dry-6H	3273498	6H	49.15	NA	0.00	2.41	1.38	NA
TFN10	NAM	QTFN10.NAM.dry-7H	3432762	7H	29.82	NA	0.03	2.32	2.36	NA
DTA	DH	QDTA.DH-1H	3926997	1H	118.94	25.45	NA	3.43	0.55	Yes
DTA	DH	QDTA.DH-2H.1	3432505	2H	17.85	17.30	NA	2.50	-0.21	Yes
DTA	DH	QDTA.DH-2H.2	3256260	2H	81.94	5.41	NA	0.59	-0.20	Yes
DTA	DH	QDTA.DH-2H.3	3258017	2H	91.01	4.89	NA	0.62	0.24	No
DTA	DH	QDTA.DH-2H.4	3255437	2H	99.26	9.22	NA	1.11	-0.33	Yes
DTA	DH	QDTA.DH-3H.1	3432405	3H	36.98	5.78	NA	0.64	0.24	Yes
DTA	DH	QDTA.DH-3H.2	3256610	3H	39.07	6.47	NA	0.73	-0.26	No
DTA	DH	QDTA.DH-5H.1	5243153	5H	139.10	10.15	NA	1.43	0.28	No
DTA	DH	QDTA.DH-5H.2	3262718	5H	167.29	6.31	NA	0.91	-0.23	No
DTA	DH	QDTA.DH-7H.2	3260483	7H	30.56	6.72	NA	0.90	-0.17	Yes
DTA	DH	QDTA.DH-7H.1	3271300	7H	34.40	6.04	NA	0.79	0.11	No
DTA	DH	QDTA.DH-7H.3	3432643	7H	126.12	7.45	NA	0.91	0.23	Yes

DTA	NAM	QDTA.NAM.dry-2H.1	3263305	2H	18.91	NA	0.00	12.69	3.44	NA
DTA	NAM	QDTA.NAM.irri-2H.1	3263305	2H	18.91	NA	0.00	10.24	3.07	NA
DTA	NAM	QDTA.NAM.irri-2H.2	3256573	2H	56.52	NA	0.02	3.66	2.27	NA
DTA	NAM	QDTA.NAM.dry-2H.2	4792852	2H	80.06	NA	0.00	5.60	-4.40	NA
DTA	NAM	QDTA.NAM.irri-2H.3	5256412	2H	136.05	NA	0.01	5.85	3.76	NA
DTA	NAM	QDTA.NAM.dry-3H	3254972	3H	108.89	NA	0.00	9.26	3.71	NA
DTA	NAM	QDTA.NAM.irri-3H	3254972	3H	108.89	NA	0.00	10.70	3.95	NA
DTA	NAM	QDTA.NAM.dry-6H	3256859	6H	114.13	NA	0.02	2.81	-1.80	NA
DTA	NAM	QDTA.NAM.irri-6H	3256859	6H	114.13	NA	0.02	3.00	-1.84	NA
DTA	NAM	QDTA.NAM.dry-7H.1	3259226	7H	32.08	NA	0.00	5.81	-3.88	NA
DTA	NAM	QDTA.NAM.irri-7H.1	3259226	7H	32.08	NA	0.00	6.20	-3.96	NA
DTA	NAM	QDTA.NAM.dry-7H.2	3258738	7H	46.39	NA	0.00	5.97	-3.94	NA
DTA	NAM	QDTA.NAM.irri-7H.2	3258738	7H	46.39	NA	0.00	6.14	-3.95	NA

[a] Percent explained variation across all field trials for QTL identified in DH population (MET QTL) and across individual trials in the NAM population

[b] QTL significant in preliminary QTL analysis (Table 5.3)

Consistent with the preliminary results, the majority of the stay-green QTL collocated with QTL for DTA in the DH population in the final analysis. More specifically, QTL collocated on chromosome 1H at 119cM, on 2H at 19cM (*PpdH1*), 82cM and 91cM, on 5H near *sdw8* at 86cM, 139cM and 168cM, on 6H at 49cM (*HvCMF3*) and on 7H near *VRN-H3* and *HVCO6* at 37cM and 121cM, respectively (Figure 5.4). Many QTL mapped to known plant development genes for semi-dwarfing and flowering regulation. For example, NAM QTL for DTA, RS and TFN10 on 3H mapped to the *sdw1*/*denso* gene, with all QTL having positive allelic effects (Table 5.4). Stay-green QTL detected in the DH and NAM populations also mapped to *HvNAM-1* on chromosome 6HS, a gene coding for NAC transcription factors (Uauy et al. 2006). Three stay-green QTL, located on the short-arm of chromosome 6H, appear to be independent of DTA QTL and known flowering time loci. Other stay-green QTL could be independent, for example the RS and SGI QTL at 107 cM on 2H, however they are still in close proximity to QTL for DTA. Interestingly, the root number QTL, *QRN.DH-3H*, collocated with *QDTA.DH-3H.2* a flowering time QTL on chromosome 3H. Furthermore, QTL for both root traits in the DH population mapped to the *HvGA20ox1* gene on 5HL along with QTL for DTA, SGI and TFN10. Another association between root traits and developmental genes was also identified with two root number QTL on chromosome 6H co-locating with the maturity genes *HvCO11* and *HvCO2*.

5.4.3 Multi-environment trial (MET) analysis

FA1 and FA2 models were fitted sequentially to the yield data from the five field sites. FA2, the final model, explained 63.55% of the variance across all trials and greater than 75% of the

variance in two out of the five trials. Higher order FA models were unsuitable for this analysis due to the number of environments in the study.

Average yield varied across the trials (Table 5.5), with the highest yield observed at H_16_dry and the lowest at H_15_dry, while the two irrigated trials had a relatively similar yield. A substantial amount of lodging was observed in the H_16_irri trial and is most likely the reason for the trial yielding less than the H_16_dry. Notably, both 2015 trials had very low genetic variance compared to the 2016 trials, demonstrating that genetics had a minimal impact on yield at these environments. The error variance was relatively consistent across all trials, except W_16_dry where the error was slightly higher (Table 5.5).

Table 5.5 Mean yield, genetic variances and error variances for each field trial

Site	Yield (t/ha)	Genetic Variance	Error Variance
H_15_dry	2.73	0.03	0.13
H_15_irri	4.52	0.04	0.15
H_16_dry	5.03	0.20	0.15
H_16_irri	4.58	0.22	0.15
W_16_dry	3.67	0.14	0.27

Genetic correlations for yield between trials were derived from the FA model (Figure 5.5), where at least 200 concurrent genotypes were tested across all environments. Correlations varied from 0.03 to 0.77, with the strongest correlation observed between H_16_dry and W_16_dry and the weakest between H_15_dry and H_16_dry. All 2016 trials were reasonably well correlated and clustered together using the correlation matrix (Figure 5.5A), while the 2015 trials were somewhat dissimilar from each other with a correlation of 0.12. Therefore, across the five trials three distinct environment clusters were identified.

Genetic correlations for seminal root traits and yield across trials were estimated from an FA3 model. Correlations between root angle and yield ranged from -0.02 to 0.42, with the highest correlation identified in H_16_dry (Figure 5.6). Genetic correlations between root number and yield varied from -0.18 to 0.05 (Figure 5.6). To investigate the relationship between seminal root traits and stay green, genetic correlations were estimated between root traits and SGI across trials using an FA3 model. The genetic relationship between each root

trait and SGI appeared to be environment dependent (Table 5.6). Genetic correlations between root angle and SGI ranged from -0.02 to 0.43 and correlations between root number and SGI varied from -0.11 to 0.32 (Table 5.6).

Table 5.6 Genetic correlations between seminal root traits and SGI for all trials

	H_15_dry SGI	H_15_irri SGI	H_16_dry SGI	H_16_irri SGI	W_16_dry SGI	Root angle	Root number
Root angle	-0.02	0.43	0.25	0.05	0.13	NA	0.41
Root number	-0.01	0.32	0.06	-0.11	-0.03	0.41	NA

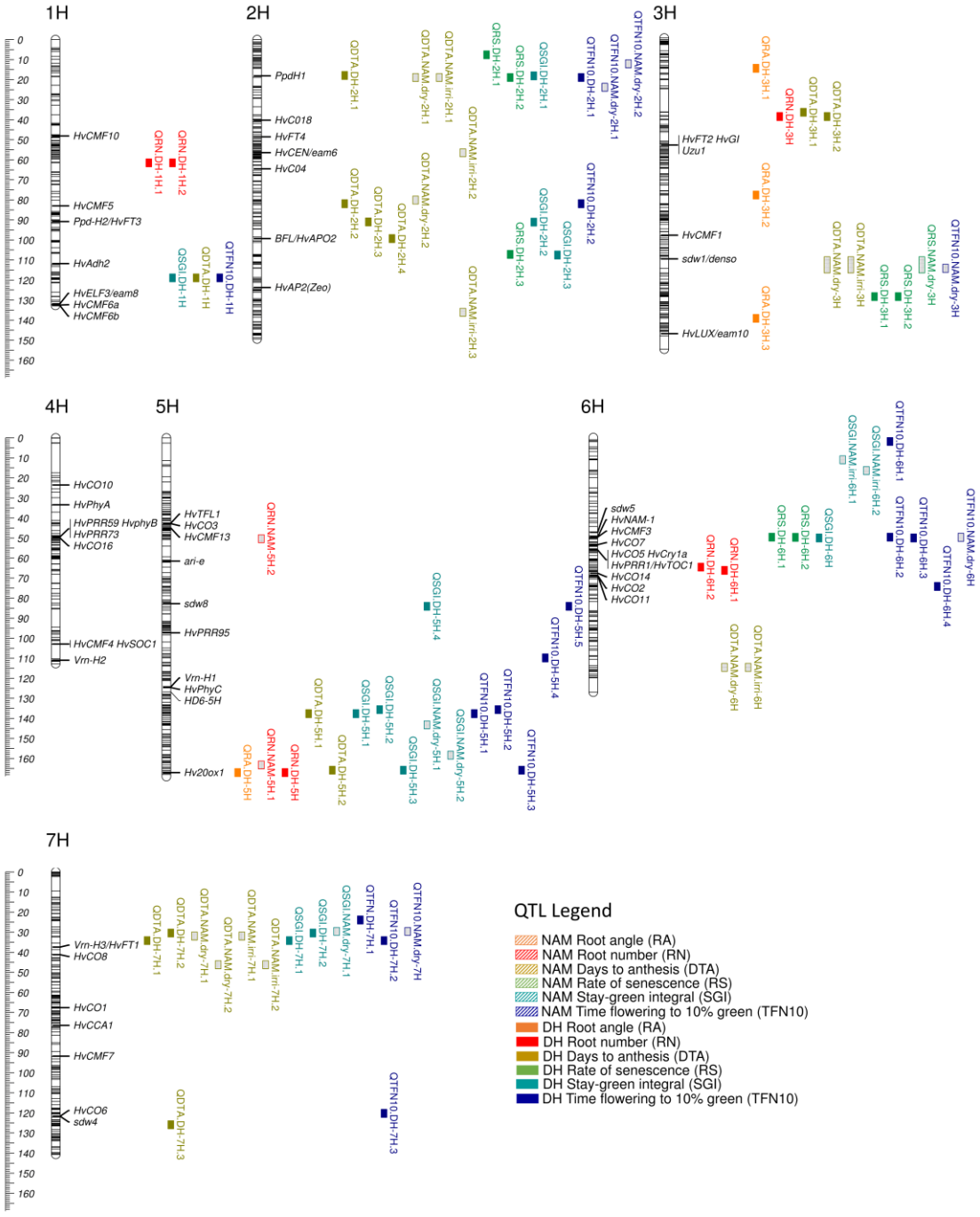

Figure 5.4 Physical barley map with QTL for leaf senescence traits, seminal root traits and DTA detected in the DH population and the NAM subset. QTL with a thatched icon were identified in the NAM subset, while QTL with a solid icon were found in the DH population. The colours of the QTL icon are trait specific, where orange is root angle (RA), red is root number (RN), yellow is days to anthesis (DTA), green is rate of senescence (RS), teal is stay-green integral (SGI) and blue is time from flowering to 10% greenness (TFN10). A selection of plant developmental genes were projected onto the map. A confidence interval of 4 cM was applied to all QTL at their peak marker for display purposes only, unless the original confidence interval was already greater than 4 cM.

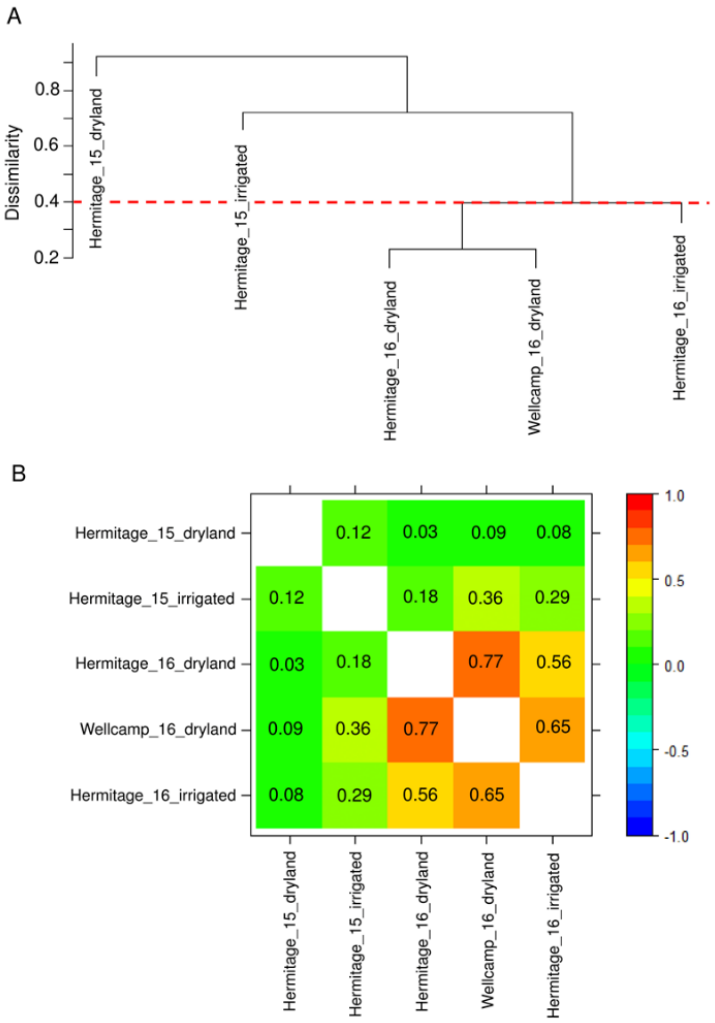

Figure 5.5 Dendrogram and heat map from the MET yield analysis. **(A)** Dissimilarity matrix of yield performance across the five field trials, where three distinct clusters were detected based on the similarity of genotype rankings across the trials. A dissimilarity of 0.40 was used as a cut off for the clustering and is represented by the red line. **(B)** Heat map of the MET yield analysis genetic correlation matrix. Positive genetic correlations between trials increase with increasing colour

brightness (closer to red) and negative correlations with decreasing brightness (closer to blue). Exact correlations are specified within each square.

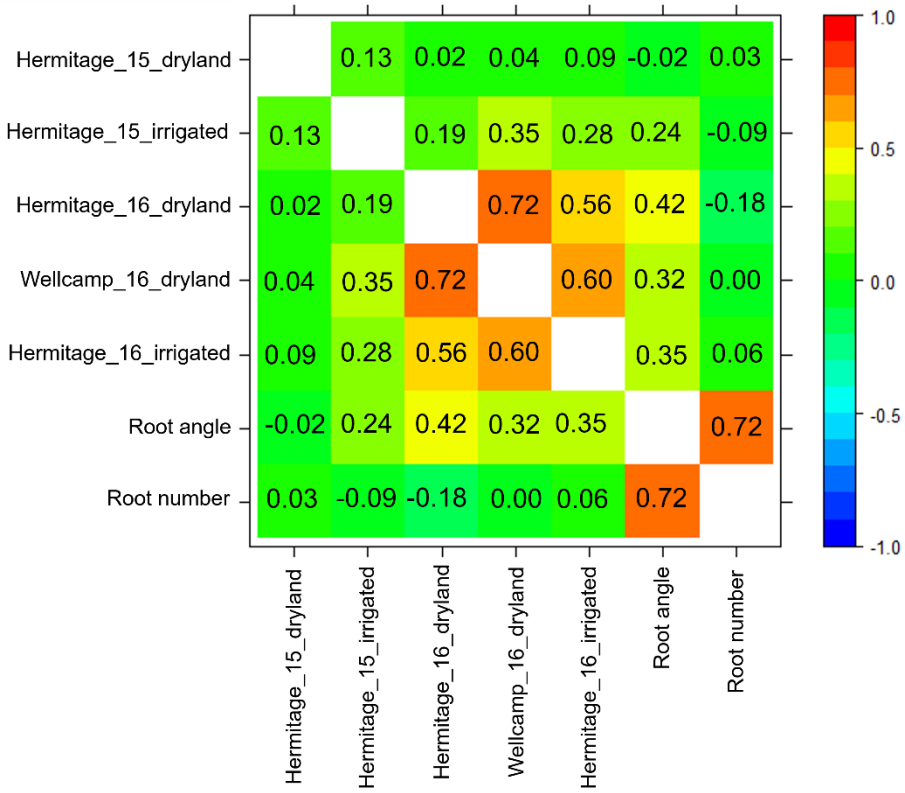

Figure 5.6. Heat map from the MET yield and root traits analysis genetic correlation matrix. Positive genetic correlations between trials increase with increasing colour brightness (closer to red) and negative correlations with decreasing brightness (closer to blue). Exact correlations are specific within each square.

5.5 Discussion

5.5.1 The relationship between root traits, stay-green and yield

One of the overarching aims of this study was to investigate the possibility of shared genetic control between root architectural traits and stay-green in barley. In this study, one genomic region on chromosome 5HL was identified as influencing root traits, DTA, SGI and TFN10 in the DH population. This suggests there is some shared genetic control between root architecture, flowering time and stay-green in barley. However, the genetic correlations between root traits and SGI are weak in this study (Table 5.6), except for in the H_15_irri trial where a wide root angle and high root number is associated with increased stay-green. Overall, our results suggest the level of shared genetic control between root architectural

traits and stay-green is not as high as that observed in sorghum, where all root angle QTL co-located with stay-green QTL (Borrell et al. 2014a). This suggests the mechanism by which barley crops delay canopy senescence may differ to that described for sorghum. It is important to note that the type of stay-green assessed in this study was not explicitly ascertained and detailed measurements of photosynthetic activity would have been required to determine whether the stay-green was cosmetic or functional. However, a recent study also proposes the mechanism for stay-green in wheat may also deviate to that previously detailed in sorghum (Christopher et al. 2018). Taken together, this highlights the need for detailed physiological dissection of stay-green in wheat and barley.

In this study, it appears the genetic relationship between root angle and yield was environment dependent, consistent with the findings of Chapter 4. In the H_15_dry trial there was no relationship with yield, while in all other trials a wide root angle was strongly associated with yield. The strongest correlations between wide root angle and increased yield were observed in the 2016 trials that clustered together in one environment grouping, characterised by frequent in-season rainfall. Further research, in a larger number of dry environments, is required to better examine the relationship between narrow root angle and yield in barley, as the current study only examined one dry environment that had limited gentic diversity.

5.5.2 Most QTL were detected in one environment cluster

This study aimed to not only identify genomic regions influencing canopy senescence and root architecture but also any gene × environment interactions for senescence in barley. Despite the DH population being tested in five environments across two years, it was evident from the MET yield analysis that there were only three distinctly different environment clusters (Figure 5.5). Furthermore, two of the three environments (H_15_dry and H_15_irri) had very low genetic variance for yield (Table 5.5) and low heritabilities (Table 5.2), which is common in low-yielding and high stress environments, indicating that factors besides genetics (most likely environment) are predominately driving yield in these trials. This was consistent with the low number of QTL identified in the H_15_dry and H_15_irri trials in the single environment analysis, where only three and seven QTL were detected, respectively (Table 5.3). Due to the low heritabilities for all traits assessed in the H_15_dry and H_15_irri trials, it is important to interpret the results of these trials with caution. Although the final MET analysis detects QTL significant across all five trials, the limited genetic variance in H_15_dry and H_15_irri allows for greater influence of the 2016 trials, which had high levels of genetic variance. Therefore,

the QTL identified in this study are most representative of the three 2016 trials that evidentially group together in a single environment cluster. Although two of the three 2016 trials were dryland, their similarity to the irrigated trial was likely a result of the uncharacteristically high in-season rainfall during the 2016 cropping season, where within-cropping season rainfall was tripled in comparison to the 2015 trials (Table 5.1). Despite our attempt to dissect the genetics of canopy senescence across water-limited and well-watered environments, the MET yield results indicate our genetic analysis was most representative of one environment cluster characterised by high in-season rainfall. In terms of rainfall, this environment cluster is similar to the field trial performed by Obsa et al. (2016) to also investigate the genetic control of stay-green in barley. In the study by Emebiri (2013) expression of the stay-green phenotype was also much higher under irrigated conditions compared to water-limited conditions. This suggests the physiological mechanism of stay-green may differ between barley and sorghum, whereby in sorghum the greatest discrimination among genotypes for delayed foliar senescence occurs under post-anthesis drought. However, further experimentation in a larger number of dry environments and implementing high experimental precision to drive up heritability would provide more understanding into the physiological basis and genetic control of stay-green in barley. Inter- and intra-seasonal environmental conditions appear to have large impact on the genetic variance of stay-green, thus large-scale studies across multiple environment types and multiple years would provide the best data set to thoroughly dissect the physiological and genetic control of this trait. In addition, a measurement of root water-uptake could be coupled with stay-green results to better understand the physiological mechanisms driving the relationship between root architecture and delayed foliar senescence. To date, research investigating the relationship between root water uptake (i.e. hydraulic conductance) and the rates of senescence is yet to be published in barley.

5.5.3 HvNAM-1 is potentially associated with canopy senescence

All three stay-green QTL in the DH population and a TFN10 QTL in the NAM subset mapped to the projected location of *HvNAM-1* on chromosome 6H, thus suggesting it is a putative candidate gene underlying these stay-green QTL. Homologous to *NAM-B1* in tetraploid wheat, *HvNAM-1* encodes for NAC transcription factors previously described for their influence on age-related foliar senescence under well-watered growing conditions and grain protein content (Distelfeld et al. 2008; Uauy et al. 2006). For *HvNAM-1* specifically, RNA expression was significantly upregulated in senescing leaf tissue (Christiansen et al. 2011), consistent with the previous association with senescence identified for *NAM-B1* in wheat. In the well described

cross between Karl (a low grain protein, six-row malting cultivar) and Lewis (a high protein, two-row feed and malting cultivar), a major grain protein content QTL was detected on chromosome 6HS explaining approximately 45% of the phenotypic variation (See et al. 2002). *HvNAM-1* has previously been shown to be responsible for the peak at this QTL (Uauy et al. 2006). Furthermore, comparison of functional versus non-functional alleles in *Hordeum* species identified that the functionality of *HvNAM-1* is associated with increased GPC (Jamar et al. 2010). In addition, polymorphic deviations between functional alleles from wild versus cultivated *Hordeum* were also related to GPC (Jamar et al. 2010).

In the current study, the parents of the DH population segregate for *HvNAM-1* alleles along with two out of the five parents in the NAM subset, therefore it is not surprising that stay-green QTL in both populations mapped to the projected location of this gene. Within this 6HS region, two additional QTL were identified for RS and for TFN10 in the DH population and were within close proximity (< 1 cM) to QTL mapped to the projected location of *HvNAM-1* but with opposite allelic effects (Table 5.4). These extra QTL are likely representative of additional genes within this region influencing senescence and GPC, where previous research has shown strong up-regulation of many genes associated with this locus (Jukanti et al. 2008). For example, the *HvGR-RBP1* gene, coding for a glycine-rich RNA-binding protein, is located within this 6HS GPC region and was upregulated > 45 fold in lines with high GPC allele and early senescence (Parrott et al. 2012). This 6HS region appears to be the most influential region controlling stay-green in the current study with the highest collection of QTL LODs, where no QTL had a LOD < 8 (LOD range 8.3 -16.0; Table 5.4). In the 2016 trials, the time from anthesis till 10% greenness (TFN10) was extended by 1.5 days on average for genotypes carrying the favourable allele at this locus. Further, the region was consistently detected for all stay-green traits in the DH population across all 2016 trials (Table 5.3) yet was not detected in the drier environments (H_15_Dry and H_15_irri). This suggests the region is associated with age-related senescence under well-watered conditions, however further research is required to confirm the importance of the QTL during water-stress. From a breeding perspective, this is an important genomic region to select for GPC and yield in environments with high in-season rainfall, whereby delayed senescence dilutes GPC by increasing carbohydrate accumulation and improving yield (Gregersen 2011).

Interestingly, root trait QTL did not map to the 6HS GPC region, demonstrating that a key genomic region influencing foliar senescence is most likely not associated with root system architecture. This is consistent with previous research, where *HvNAM-1* expression was not

present in root tissue sampled at the three leaf stage, thus speculated to have minimal influence on initial root development (Christiansen et al. 2011), but does not rule out their importance at other plant developmental stages. In sorghum, delayed foliar senescence is a result of improved supply and demand of water, where generally root traits influence the supply and canopy development effects the demand (Borrell et al. 2014a). The lack of association between the major QTL influencing stay-green and seminal root traits in this study suggests the physiological mechanisms for stay-green in barley under well-watered conditions may differ to those previously described for sorghum. However, further research characterising delayed foliar senescence in barley under water-limited conditions is required.

5.5.4 Genes promoting flowering are associated with root development

Interestingly, two root number QTL mapping to chromosome 6HL were in close proximity to the projected locations of two CONSTANS (CO) genes: *HvCO2* and *HvCO11* (Figure 5.4). The function of CO genes is thought to be conserved between *Arabidopsis* and barley (Greenup et al. 2009), where CO genes promote flowering under long days via the photoperiod pathway. CO transcription is regulated by diurnal rhythm, peaking approximately 16 hours after dawn, where a period of light is required for CO proteins to stabilise. Under long days CO expression occurs during daylight, where stabilised proteins induce transcription of *FLOWERING LOCUS T1* (*FT1*), resulting in promotion of floral development. This process is restricted to long days, as under short days CO expression peaks during night hours with the proteins unable to stabilise and upregulate *FT1* (Searle and Coupland 2004; Suarez-Lopez et al. 2001; Valverde et al. 2004).

To date, limited connections have been established between CO genes and root traits in cereal crops. However, in *Arabidopsis* the CO gene, *CO3*, has been shown to regulate light-dependent lateral root formation and development, as well as regulation of shoot elongation and branching (Datta et al. 2006). The conserved function of CO genes in barley makes it possible that CO genes may also have a function in regulating root development in barley. Our results suggest that there may be some association between root growth and CO genes in barley, however further research is required to investigate whether these genes, like in *Arabidopsis*, have an extended function below ground. Nevertheless, QTL influencing root traits in barley have been mapped in close vicinity to another key gene in the flowering time pathway, *VERNALIZATION1* (*VRN1*; Arifuzzaman et al. 2014; Arifuzzaman et al. 2016).

5.5.5 GA-20 oxidase genes may influence leaf senescence and seminal root development

Stay-green and DTA QTL mapped to the projected locations of semi-dwarfing genes *sdw1/denso*, *sdw8* and *sdw4* on chromosome 3H, 5H and 7H, respectively. The QTL detected at the projected location of *sdw1/denso* were identified in the NAM subset for DTA, RS and TFN10, while QTL detected at the projected location of *sdw8* and *sdw4* were discovered in the DH population for SGI, TFN10 and DTA (Figure 5. 4). The introduction of semi-dwarfing genes in cereal crops revolutionised plant breeding, with the use of dwarfing genes now an essential component of modern cultivar development. The semi-dwarf phenotype consists of short and strong stalks with an increased resistance to lodging, which increases yield and protects grain quality. Previous QTL mapping studies have mapped QTL for a number of different traits to *sdw1/denso* in barley, for example DTA, maturity, productive tiller number and yield, (Kuczyńska et al. 2013). Plant height influences the source-sink relationship in a plant and stay-green in other cereal crops is thought to be an emergent consequence of a balance in this relationship, therefore plant height may affect stay-green in barley. Theoretically, assuming grain nitrogen demand, nitrogen uptake and leaf area are all equal in a tall and a short plant, then the rate of senescence might be higher in the tall plant due to an increased nitrogen requirement for the additional stem, forcing the plant to access more nitrogen from its leaves, resulting in accelerated senescence. Thus, it is not surprising that QTL detected in the current study for stay-green mapped to the projected locations of a key semi-dwarfing gene.

Hv20ox2 has been identified as a functional gene controlling *sdw1/denso* in barley encoding the GA 20-oxidase2 enzyme, an essential component of GA biosynthesis (Jia et al. 2009). In terms of the semi-dwarfing phenotype, Jia et al. (2009) postulated that decreased expression of *Hv20ox2* results in low levels of GA in the apical meristem inhibiting apical growth, thus reducing internode length, plant height and, in turn, promoting the development of tillers. Interestingly, *Hv20ox1*, located on the long-arm of chromosome 5H, was also projected in close proximity to QTL for root number, DTA, SGI and TFN10 in the current study (Figure 5.4). Like *Hv20ox2*, *Hv20ox1* is one of the three *Hv20ox* genes in barley that are essential for biosynthesis of the active form of the plant development hormone GA (Spielmeyer et al. 2004).

In barley, GA has been recently shown to play a role in promoting flowering, independent of FT1, through transcription regulation by the circadian clock gene *Early Flowering3* (*ELF3*) (Boden et al. 2014). Expressed during night hours, *ELF3* encodes for proteins that control the input of light signals to the circadian clock, repressing the activity of core clock genes during darkness and maintaining correct diurnal expression (Dixon et al.

2011; Nusinow et al. 2011; Thines and Harmon 2010). *Hv20ox2* was shown to be one of the input genes repressed by *ELF3* during night hours and functional mutations resulted in increased GA biosynthesis and an early flowering phenotype under short days. Therefore, Boden et al. (2014) demonstrated that GA promotes flowering in barley through the photoperiod pathway, similar to *Arabidopsis*. In the current study, the identification of QTL for flowering time at the projected locus for *Hv20ox2* and for other GA biosynthesis genes is consistent with the previously described role of GA for floral initiation in barley.

Interestingly, our mapping results also suggest that GA biosynthesis genes influence leaf senescence and seminal root traits in barley. For instance, stay-green QTL, independent of flowering time, mapped to the projected location of *Hv20ox2*, and indicate a potential autonomous role for GA in leaf senescence. As an aside to the previously discussed indirect effect of GA biosynthesis on leaf senescence via changes in plant height, here we propose the possibility of a direct effect of GA on senescence. Recent findings in *Arabidopsis* are consistent with this theory, whereby GA biosynthesis directly influences the onset of leaf senescence. When DELLA repression of the GA signalling pathway was blocked and GA biosynthesis upregulated, leaf senescence was accelerated and occurred earlier in *Arabidopsis*, while senescence was significantly delayed when GA biosynthesis was inhibited (Chen et al. 2014). WRKY DNA-binding protein 45 (WRKY45) has also been shown to interact with DELLA proteins in this pathway and positively regulate leaf senescence in *Arabidopsis* (Chen et al. 2017). It is possible that this function of GA may be conserved between *Arabidopsis* and barley, but further research is required to confirm the direct influence of GA on senescence in barley, yet our results suggest *Hv20ox2* does play a role.

Also in *Arabidopsis*, GA has been well described as influencing root growth through signalling from auxin. This provides a potential explanation for root angle and root number QTL mapping to the *Hv20ox1* locus on 5HL. In *Arabidopsis*, auxin derived from the shoot apex regulates root growth through control of GA on the DELLA protein REPRESSOR of GA_1 (RGA; Fu and Harberd 2003). For example, when auxin signalling or transport is blocked, the GA-induced breakdown of RGA is delayed, inhibiting root growth (Fu and Harberd 2003). Thus, following auxin signalling, GA promotes root growth through the destabilisation of RGA and potentially other DELLA proteins. It is conceivable that GA may function similarly in barley, especially since research in other cereal crops have described a role for auxin in regulating root system architecture (Uga et al. 2013). It is also possible that *Hv20ox1* is influencing seminal root traits indirectly through the role of GA in promoting flowering

initiation. This is consistent with the association also identified between root number QTL and CO genes, which like GA, are promotors of flowering via the photoperiod pathway. Furthermore, a root number QTL collocated with a DTA QTL on chromosome 3H. Taken together, these results indicate that key genes controlling flowering may also be influencing root traits in barley. Specifically, our results suggest there may be a relationship between *Hv20ox1* and seminal root traits. However further research is required to elucidate whether GA influences roots directly, like in *Arabidopsis*, or whether the relationship is an indirect result of GA promoting flowering.

5.6 Conclusion

This study provides a detailed genetic dissection of delayed-canopy senescence in barley, by analysing both NAM and DH mapping populations. Despite attempts to investigate genotype × environment interactions for senescence, the genetic associations described here are mostly representative for age-related senescence in barley grown under minimal water-stress conditions. Further research is required to better understand the physiological basis and genetic control of stress-induced canopy senescence under water-limited conditions in barley. Furthermore, this study could be extended to measure NDVI from early in plant development (i.e. 2–3 leaf stage) as an assessment of crop establishment and early vigour, whereby early vigour is another component trait of drought adaptation. In this study, a large number of QTL were identified for canopy senescence with several co-locating with the projected locations of key plant developmental genes. We re-affirmed the importance of the GPC locus on chromosome 6HS (*HvNAM-1)*, which is potentially a major factor contributing to the rate of senescence in this study. In addition, we outlined a potential novel association between GA biosynthesis genes and leaf senescence at the well descried *sdw1/denso* locus on chromosome 3H. Further, we identified QTL co-locating for seminal root angle, root number, DTA and stay-green traits on chromosome 5HL at the projected location of the *Hv20ox1* locus. We postulate that GA may play a central role in regulating root growth either via a pleiotropic effect of *Hv20ox1* or as a consequence of the role of GA as a floral promoter in the photoperiod pathway. Thus, further research is required to understand the direct and indirect effects of GA on root development. We also hypothesise that CO genes may influence root number and thus root development may be associated with key flowering time genes in barley. Characterisation of near-isogenic lines of major flowering time genes for root traits would provide insight into the

association between root traits and flowering time identified in this study. Here we demonstrate canopy senescence in barley is under complex genetic control and reveal novel associations between canopy senescence, seminal root traits and GA biosynthesis genes.

5.7 References

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Chapter 6

VERNALIZATION1 MODULATES ROOT SYSTEM ARCHITECTURE IN BARLEY

6.1 Abstract

Roots assume critical functions in water uptake, nutrient acquisition and anchorage, an essential characteristic to maintain plant stability under increased grain load. Despite their fundamental importance, knowledge about genetic control of root growth in major grain crops is limited and very little is known about interactions between below-ground and above-ground plant development. In Chapter 5, we provide evidence for shared genetic control between above- and below-ground drought adaptive traits in barley. Furthermore, we suggest that root development may be associated with key flowering time genes in barley. In this chapter, we demonstrate that *VERNALIZATION1* (*VRN1*), a key regulator of flowering behavior in cereals, also modulates root architecture in barley. Using *VRN-H1* near-isogenic lines varying for spring alleles, we demonstrate that spring alleles had a divergent effect on root system architecture. The common spring allele, Morex, had the most pronounced phenotype, with the narrowest root system and the greatest proportion of roots at depth. Functional characterization in transgenic barley confirmed that *VRN-H1* influences root growth directly, via gravitropism. These discoveries provide unexpected insight into underground functions of a major player in the well-characterized flowering pathway, revealing the intersection of above-ground gene regulation with the largely unexplored genetic architecture of plant root development. Understanding the pleiotropic involvement of this key developmental gene in overall plant architecture will help to breed cereal cultivars better adapted to changing environmental conditions.

6.2 Introduction

The dramatic gains in crop productivity generated during the green revolution were largely a result of high-input systems, targeting above-ground plant components with the ability to capitalize on unlimited nutrient access (Khush 1999). As water stress becomes one of the greatest yield constraints globally, the sustainability of these intensively managed systems becomes increasingly uncertain (Bishopp and Lynch 2015). Numerous authors propose that roots will pave the way to a second green revolution in low-input systems, whereby root systems are developed to capture more water and nutrients for minimal metabolic costs (Bishopp and Lynch 2015; Herder et al. 2010; Lynch 2007, 2013, 2015; Topp et al. 2016; Waines and Ehdaie 2007). Despite the fundamental importance of the root system in plant productivity, the functional and genetic basis of root system architecture in crops remains relatively unknown. This is largely a symptom of the challenges arising from phenotyping an underground plant component.

Root system architecture, describing the spatial configuration of roots within the soil, is a complex trait exhibiting both diversity and plasticity within species (Lynch 1995; Sandhu et al. 2016). Root curving, elongation and branching are the dominant processes underlying root architecture (Rich and Watt 2013). Many studies have demonstrated that seminal root angle, defined as the inner growth angle between the first emerging pair of seminal roots, is correlated with aspects of the mature root system architecture in wheat (Christopher et al. 2013; Manschadi et al. 2010; Manschadi et al. 2008). Water is a mobile resource essential for crop survival, filtering through to deeper soil strata over time, and diminishing moisture availability in the surface soil strata if not replenished. To maximise resource capture in most water-limited environments, a deep-rooted phenotype is expected to be advantageous for many crop species (Lynch 2011, 2013). For example, a narrow root angle in sorghum improves the plant's ability to access deep-stored water and is associated with increased yield (Mace et al. 2012; Singh et al. 2012). To date, our understanding of the genes influencing root system architecture stems mainly from research in the dicot taproot system of *Arabidopsis thaliana* (Jung and McCouch 2013). Among major crops, *DEEP ROOTING 1* (*DRO1*) in rice is the only cloned gene to date shown to influence root architecture and subsequent water uptake (Uga et al. 2013). To improve the environmental resilience of future crop cultivars, further studies are required that elucidate the molecular basis of root growth and also provide diagnostic molecular markers for selection (Ren et al. 2012).

Here we demonstrate that *VERNALIZATION1* (*VRN1*), a key regulator of flowering behaviour in cereals (Trevaskis 2010; Yan et al. 2003), also modulates root architecture in barley. *VRN1* encodes a MADS box transcription factor with high similarity to the *APETALA/FRUITFUL*-like class of genes in *Arabidopsis* (Preston and Kellogg 2006; Yan et al. 2003). *VRN1* is well-known for its regulation of flowering (Danyluk et al. 2003; Trevaskis et al. 2003; Yan et al. 2003), and also categorizes barley into either 'winter' or 'spring' growth habit types based on its allelic state (Fu et al. 2005). For plants with the *VRN1* winter allele (*v*), *VRN1* levels are low prior to vernalization, inhibited by *VRN2*, a repressor of flowering, that downregulates *VRN3* (an orthologue of *FLOWERING LOCUS T* in *Arabidopsis*) under extended photoperiods (Dubcovsky et al. 2005; Yan et al. 2006). Hence, the three genes *VRN1*, *VRN2* and *VRN3*, form a regulatory loop for the floral induction. Following an extended cold exposure, *VRN1* transcription is upregulated, consequently binding to the promotor of *VRN2* and downregulating expression, thereby releasing *VRN3* (*FT*) to aid in promoting flowering once the long day photoperiod is also met (Chen and Dubcovsky 2012). Whereas for the spring growth habit, transcription of *VRN1* is independent of vernalization (Trevaskis et al. 2003; Yan et al. 2003), whereby a mutation in the first intron of *VRN1* alters the recognition site for *VRN2*, thus impeding its inhibitory effects (Yan et al. 2003).

Recently, Arifuzzaman et al. (2016) proposed a broader role for *VRN-H3* in which it also influences root development in barley. In this study, the authors identified an association between root mass traits and *VRN-H3* through QTL mapping of an F2 population, developed from a spring barley cultivar crossed with a wild barley accession. Using a gene specific marker, they identified genotypes with a spring growth habit (thus early flowering) to have a less fibrous root system, with a reduced root dry weight and total root volume. While, genotypes with a winter growth habit, and thus a longer vegetative growth phase, had a larger more vigorous root system (Arifuzzaman et al. 2016). Putative genetic associations were also identified between root dry weight and *VRN1*, where QTL for this root trait mapped to the projected location of *VRN-H1* (Arifuzzaman et al. 2016). These findings provide initial evidence that roots are, in part, controlled by key flowering genes in barley.

Here, we demonstrate that *VRN1* is a major gene influencing root system architecture in barley. Using *VRN-H1* near-isogenic lines, we demonstrate that the common *VRN-H1* spring allele, Morex, has a significantly narrower root system architecture and a greater proportion of roots at depth compared to the *VRN-H1* winter allele. Functional characterization in transgenic barley confirmed that *VRN1* influences root growth directly, via gravitropism, rather than

through linkage. This discovery provides unexpected insight into underground functions of a major player in the flowering pathway. Understanding the pleiotropic involvement of this key developmental gene in overall plant architecture may help to breed barley cultivars better adapted to water-stressed environments. It furthermore provides a starting point to reveal the conjunction of well-characterized above-ground gene pathways with the largely unexplored genetic architecture of barley root development.

6.3 Materials and methods

6.3.1 Plant material

Five previously developed barley *VRN-H1* NILs (Oliver et al. 2013) were characterised for root traits in this study. The NILs were generated by backcrossing the desired *VRN-H1* spring allele into the recurrent parent WI4441 (Oliver et al. 2013), which normally carries the *VRN-H1* wildtype winter allele. Spring allele donors used to develop the NILs were: AUS40413 (*HvVRN1-1*; Morex), AUS405184 (*HvVRN1-3*; Triumph), AUS403647 (*HvVRN1-4*; C-sib), according to the allele notation described previously (Hemming et al. 2009). For the remaining NILs, *HvVRN2* has the *VRN-H1* wildtype winter allele with a deletion in *VRN-H2*, and Winter WT has the *VRN-H1* wildtype winter allele. All NILs (except Winter WT) have a deletion at *VRN-H2*, ensuring non-expression of *VERNALIZATION2*. Three Golden Promise barley lines transformed with a *VRN-HA* construct by Deng et al. 2015 using *Agrobacterium* transformation (here designated GP[*VRN1-HA*]-6, GP[*VRN1-HA*]-14 and GP[*VRN1-HA*]-22), where homozygous plants were selected from the BC3F2 generation, were also examined in this study. The barley cultivar, Golden Promise, already has the *VRN-H1* Morex allele, and thus following transformation with the *VRN-HA* construct had an additional copy of the allele.

6.3.2 Phenotyping barley NILs differing in common VRN-H1 spring alleles and VRN-H2

The five NILs were phenotyped for seminal root angle using the clear-pot method (Richard et al. 2015), where thirty-two replicate seeds for each line were characterised. Adopting a randomised complete block design, the experiment consisted of eight pots spread across one single bench in a 2D array of three columns by three rows. The experiment was grown under controlled conditions, with 12 h light and 12 h dark at 20°C. A linear mixed model was fitted to the data, where the spatial location of each pot was accounted for in the model. Replicate, column and pot were fitted as random terms in the spatial model with an independent

correlation term for pot and position. Variance components of the model were estimated using residual maximum likelihood (REML) and best linear unbiased estimators (BLUEs) were generated for the fixed genotype effects. Using the variance components of the model, a generalised heritability of each trait was calculated (Cullis et al. 2006). The linear mixed model was fitted in ASReml-R (Butler et al. 2008).

To investigate whether root angle measures at the seedling stage under controlled conditions correspond with the root growth angle in the field, barley NILs were evaluated using a 'shovelomics' approach (Trachsel et al. 2011). The trial was planted on the 15th July 2016 at The University of Queensland (UQ) Research Station, Gatton, Queensland, Australia. Genotypes were sown in 2 m x 3 m plots with a 40 cm row spacing and a target density of 70 plants m^{-2}. Plots were replicated using a completely randomized design. The trial was subjected to natural rain-fed conditions. Once the majority of plots had reached anthesis, root phenotyping was carried out on the 27th September, when 16 individual plants from each plot (32 plants per NIL) were excavated using a standard shovel and excess soil removed by briefly shaking, all the while taking care not to interfere too much with the nodal root structure of the plants. The excavated roots were placed onto a black backing board and individually photographed using a camera. The root images were analysed using ImageJ software (http://rsb.info.nih.gov/ij/). Root growth angle was defined as the outer angle of nodal roots capturing the overall direction of root growth. An ANOVA, and where appropriate, a Tukey's honest significant difference (HSD) test was performed where statistical significance was $p \leq 0.05$.

To investigate the effect of *VRN-1* alleles on the mature root system architecture, the distribution of root biomass at different depths was examined for the NILs in two experiments: one sampled at anthesis and a second sampled at the mid grain-filling stage. These growth stages were selected for sampling because the continued root elongation at depth post-anthesis was considered a key feature in historical root architecture and drought studies of wheat (Manschadi et al. 2006; Manschadi et al. 2010). Plants were grown in 2 m PVC pipes (9 cm diameter) outdoors under natural conditions during the winter and spring months at UQ, St Lucia campus, Queensland, Australia. PVC pipes were filled with UQ23 pine bark potting media (pH 6.35, EC = 650 mg kg^{-1}, nitrate = 0, ammonia < 6 mg kg^{-1}, and P = 50 mg kg^{-1}) with slow-release fertilizer (Osmocote®; N:P:K 11:1:3) pre-mixed at a rate of 2 g/L. Each experiment contained three replicates of each NIL and used a randomized complete block design. Seeds were germinated in petri plates lined with moist filter paper and once the

emerging radicle was visible, a single germinating seed was transplanted into each pipe. Plants were monitored daily and sampled when an individual plant reached the required developmental stage for each experiment. For the experiment sampled at anthesis, the days to anthesis was recorded for each individual plant, along with the number of tillers. When sampling roots, the pipes were cut at 20 cm intervals using a standard hacksaw. The roots extracted from each interval were washed to remove soil and dried for one week at 60°C in a dehydrating oven. The dry root samples were weighed and the percentage of the total root biomass (up to a depth of 80 cm) was calculated for each 20 cm interval. The 80 cm threshold was applied due to a high number of missing values below this depth. BLUEs were calculated and Fisher's LSD 0.05 test was used to determine statistical significance. The root-to-shoot ratio (R/S) for the NILs was also evaluated using the same experimental set up and design as the distribution of root biomass experiment, but NILs were sampled at flag-leaf emergence. Root and shoot samples were dried down at 60°C for seven days before measurement of total dry biomass of the samples. R/S was calculated by dividing shoot by root biomass. BLUEs were calculated and Fisher's LSD 0.05 test was used to determine statistical significance.

6.3.3 Phenotyping barley transformed with VRN-HA construct

The three transformed barley lines (Deng et al. 2015) were phenotyped for seminal root angle in comparison to non-transformed control plants. Root phenotyping was again performed using the clear-pot method, adopting a randomized complete block design where 30 seeds of each genotype were randomized across 20 pots on a single bench. Following assessment of root angle, all plants were further cultivated until anthesis. Environmental conditions were held constant with 24 h light and a 12 h cycling temperature regime of 22/17 °C. Days to anthesis (DTA) and spike length of the primary tiller were recorded. For each genotype, BLUEs for seminal root angle, DTA and spike length were calculated and Fisher's LSD 0.05 test was used to determine statistical significance.

To quantify differences in the mature root system, GP[*VRN1-HA*]-14 and the control line GP-[Control]-14 were evaluated using purpose built root observation chambers (Singh et al. 2010). The root chambers (60 cm high, 40 cm wide and 3 cm thick) were constructed with transparent perspex (8 mm thick) sides to allow viewing and scanning of the root system. Chambers were filled with Searles® Premium Potting Mix and aluminium foil was wrapped around each chamber to ensure the developing roots were not exposed to light and to reflect sunlight to minimize effects on soil temperature. The chambers were randomized with three

replicates of each line and only one seed per chamber and grown under controlled temperature conditions (22/17°C day/night) and diurnal light (12 h photoperiod) in a PC2 facility at UQ, St Lucia, Queensland, Australia. Five weeks after sowing, when GP[*VRN1-HA*]-14 reached anthesis, the chambers were imaged. Prior to analysis, whole-chamber images were scaled and cropped into four equal sections representing differing soil depths: 0-15 cm, 15-30 cm, 30-45 cm and 45-60 cm. The automated root phenotyping software GIA Roots (Galkovskyi et al. 2012) was then used to analyse each section, focusing on six root architectural traits, including: total root system area, convex area, volume, median number of roots, length and width. For each image a grey scale version was generated and adaptive threshold imaging with pre-set parameters was applied. Following this, white roots were separated from the dark soil background, and the pixel values calculated were transformed to centimetres. Means and standard deviation were calculated and a t-test was used to determine statistical significance ($P \leq 0.05$) between the transformed and control line for each of the various root architectural traits at each soil depth.

The three transformed and respective control lines were also assayed for R/S. Implementing a randomized complete block design and three replicates per genotype, plants were cultivated in 4 L pots with a density of 3 plants per pot, and grown under controlled conditions, with diurnal light (16 h photoperiod) and cycling temperature 22/17°C. At 35 DAS, leaf and root tissue was harvested and dried using a dehydrating oven at 60°C. Dry leaf and root tissue samples were weighed and R/S calculated, as above. BLUEs were calculated and Fisher's LSD 0.05 was used to determine statistical significance.

6.3.4 Quantifying expression of VRN-H1 in transformed barley

To quantify expression of *VRN1* at the early growth stage, plant tissue representing the entire seedling (bulk of seed, seminal roots and coleoptile) was sampled from the transformed line GP[*VRN1-HA*]-14 and GP[Control]-14 five DAS in clear pots. Tissue samples were collected and ground in liquid nitrogen and RNA was extracted, as described previously (Wang et al. 2012). RT-PCR was carried out using a SensiFAST SYBR No-ROX One-Step kit (Bioline, London, UK), with 200 ng total RNA and previously-described primers (Greenup et al. 2010), and cycling was done using a Lightcycler 96 (Roche Diagnostics, Mannheim, Germany). *VRN1* transcript was quantified relative to *ACTIN*, as described previously (Ramakers et al. 2003).

6.3.5 Quantifying expression of VRN-H1, DRO1 and OsGH3-2 in roots of barley NILs

The expression of *VRN-H1*, the barley homologue of *DRO1* (Genbank accession EX599993.1), and the barley homologue of the root-expressed and strongly auxin-induced *OsGH3-2* (Jain et al. 2006; Genbank accession DK814019) in root tissue of the *HvVRN1-1* and winter wild type NILs was analysed using qRT-PCR. *OsGH3-2* was chosen as an indicator of auxin expression due to being strongly induced by auxin (Jain et al. 2006). To enable sampling of root tissue at the same adult growth stage, at the same point in time, the NILs were vernalized to ensure plants reached anthesis simultaneously. Seeds were sown in chambers and germinating plants were vernalized for a duration of 9 weeks at 4ºC, then grown-on under controlled conditions of 22/17ºC (day/night) using a 16 h photoperiod. At anthesis, nodal roots were sampled and briefly rinsed in water to remove excess soil before being frozen in liquid nitrogen. RNA was extracted using the Isolate II RNA Plant Kit (Bioline, London, UK), and cDNA synthesis was done using the Superscript IV First Strand Synthesis System (Invitrogen, Carlsbad, California) primed with oligo dT. PCR was done using the FastStart Essential DNA Green Master kit (Roche, Mannheim, Germany) and a Lightcycler 96 (Roche Diagnostics, Mannheim, Germany), with previously-described primers for *VRN-H1* and ACTIN (Greenup et al. 2010), primers HvDRO1F1 (GCTCAATTCCAGGGTGCTCT) and HvDRO1R1 (GCTCAATTCCAGGGTGCTCT) for barley *DRO1*, and primers HvGH3_2F (ATGCTAGCTGCTGAATGCCA) and HvGH3_2R (GCTCAATTCCAGGGTGCTCT) for barley *GH3-2*. Expression levels were calculated relative to ACTIN, as described previously (Ramakers et al. 2003).

6.3.6 Testing gravitropism response of barley NILs

To investigate the mechanism underlying the narrow root angle conferred by the Morex allele, the *HvVRN1-1* and winter wild type NILs were evaluated for gravitropism response using a method adapted from Uga et al. (2013). Seeds were germinated in on 0.7% water agar, and incubated vertically in the dark at room temperature. After 48 hours the position of root tips was marked, and the plates were rotated 90°. After a further 10 hours, the plates were photographed to record the gravitropism response. The change in root angle in response to gravity was measured for all roots that were growing within 30° of vertical prior to rotating the plate. In total, 19 and 27 plants of the *VRN1-1* and winter WT NILs, respectively, were tested. A t-test was used to determine statistical significance ($P \leq 0.05$).

6.4 Results

6.4.1 Phenotypic variation for root traits in VRN-H1 NILs

Intriguingly, each *VRN-H1* spring allele influenced root phenotype in a unique manner, suggesting divergent selection of functional allelic variants. At the seedling stage, the barley spring allele *VRN1-3*, characterised by a variation in the first intron (Cockram et al. 2007), displayed a significantly wider seminal root angle (+13°; Figure 6.1). The NIL carrying spring allele *VRN1-4* and *VRN2* deletion showed no significant difference in root phenotype compared to the winter wild-type allele. In contrast, the spring allele *VRN1-1* was associated with significantly narrower seminal root angle (-12°) compared to the winter wild-type allele. Root angle patterns observed at the seedling stage were similar to those identified in the field for the mature root system architecture. Under field conditions, *VRN1-1* exhibited a significantly narrower nodal root growth angle (-40°) than the winter WT (Figure 6.2). In the seedling assay *VRN1-4* was slightly narrower than the wild type, yet under field conditions the NIL's root angle was significantly reduced from that of the wild type. Similar to the seedling assay, *VRN1-3* had a wider mature root system, yet not statistically different from the control. The nodal root angles of the *VRN2* deletion NIL and the wild type were consistent with the seminal root angles observed in the seedlings.

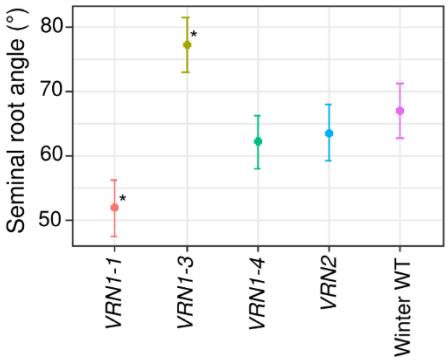

Figure 6.1 Phenotypic variation in seminal root angle among NILs of the barley genotype WI4441, differing in composition of spring alleles at *VRN-H1* (*VRN1-1*, *VRN1-3* and *VRN1-4*) and in deletion of *VRN-H2* (*VRN2*). *VRN1-1* is the common Morex spring allele, *VRN1-3* is the Triumph allele, *VRN1-4* is the C-Sib allele, *VRN2* is the wild-type winter allele along with a deletion in *VRN-H2*, and Winter WT is the wild-type winter allele. BLUEs standard errors are displayed. * Indicates statistical significance, calculated by Fisher's LSD 0.05, of each NIL compare to Winter WT.

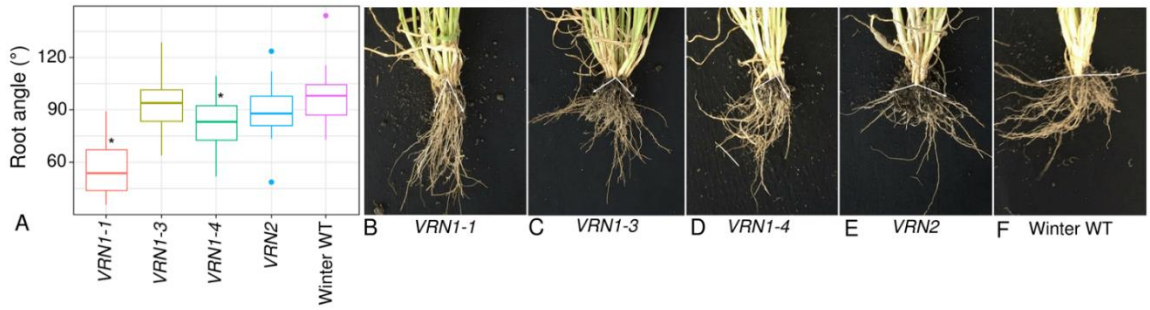

Figure 6.2 Phenotypic variation for nodal root growth angle among NILs of the barley genotype WI4441grown and excavated from field plots. **(A)** Root angle, measured as the outer angle capturing the overall direction of nodal root growth, illustrated by the white arrows in **(B)** *VRN1-1*, **(C)** *VRN1*-3, **(D)** *VRN1*-4, **(E)** *VRN2*, and **(F)** Winter WT. * Indicates statistical significance calculated by Tukey's HSD test $P < 0.05$, where each NIL is compared to the Winter WT.

As expected, *VRN-H1* spring alleles significantly influenced above-ground development and architecture, including days to anthesis and tiller number (Figure 6.3). All three spring NILs and the *VRN2* deletion NIL had significantly reduced tiller number and time to flowering, consist with the *VRN1* spring growth habit. All NILs carrying spring alleles also produced a higher proportion of roots at moderate soil depths (20-60cm; Figure 6.4). This was particularly evident during the grain-filling stage (Figure 6.4B). The *VRN1-1* NIL was the only spring allele with a significantly increased proportion of roots at depth from 40 cm through to 80 cm, during the grain-filling stage. For the first time in the root phenotyping, the *VRN-H2* deletion NIL diverged from the wild-type with significantly different percentage of roots at 40 cm at anthesis and for 20 and 40 cm during the grain-filling period (Figure 6.4A, B). All spring alleles and the *VRN-H2* deletion NIL had a significantly reduced root to shoot ratio compared to the wild-type (Figure 6.5C). As anticipated, the root dry mass of the spring alleles was also diminished (Figure 6.5B).

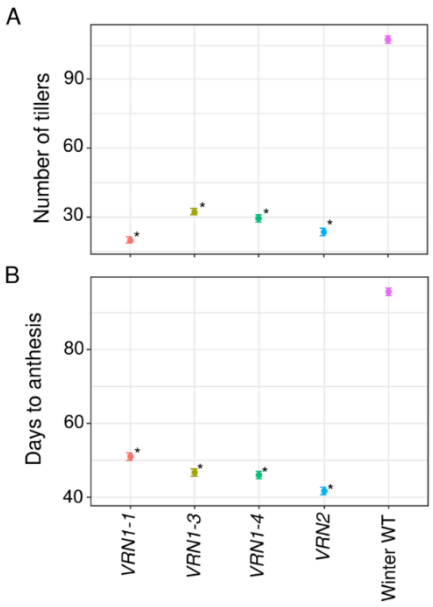

Figure 6.3. Phenotypic variation among NILs, differing in composition of spring alleles at *VRN-H1* (*VRN1-1*, *VRN1-3* and *VRN1-4*) and in deletion of *VRN-H2* (*VRN2*) at anthesis. **(A)** Number of tillers, and **(B)** days to anthesis. BLUEs are displayed for each NIL along with standard errors. Fisher's LSD 0.05 test was used to determine significant differences between Winter WT and each NIL. * Indicates statistical significance.

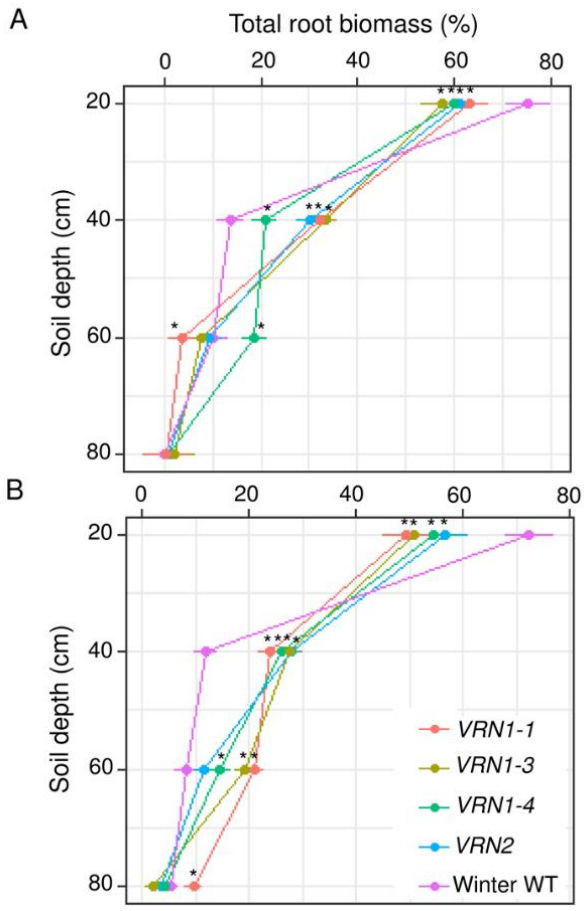

Figure 6.4 Phenotypic variation for distribution of root biomass at different soil depths among NILs differing in composition of spring alleles at *VRN-H1* and in deletion of *VRN-H2*. **(A)** Percentage of total root biomass at anthesis at differing soil depths (0-20, 20-40, 40-60 and 60-80 cm), **(B)** percentage of total root biomass three weeks post anthesis at differing soil depths (0-20, 20-40, 40-60 and 60-80 cm). BLUEs are displayed for each NIL along with standard errors. Fishers' LSD 0.05 test was used to determine significant differences between Winter WT and each NIL. * Indicates statistical significance.

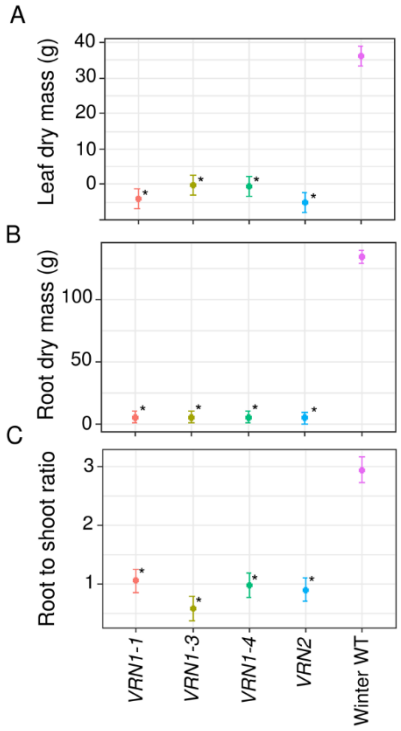

Figure 6.5 Phenotypic variation among NILs at 5 weeks post sowing. **(A)** Leaf dry mass, **(B)** root dry mass, and **(C)** root-to-shoot ratio. BLUEs are displayed for each NIL along with standard errors. Fisher's LSD 0.05 test was used to determine significant differences between Winter WT and each NIL. * Indicates statistical significance.

6.4.2 Phenotypic variation in root architecture for barley transformed with VRN-HA

Three transgenic lines with an additional copy of *VRN1-HA* construct were selected, based on their transgene expression level (Deng et al. 2015). Seminal root angle phenotypes were compared between *VRN1-HA* over- expressing plants and non-transformed controls. Coordination of above-ground and below-ground architecture in barley by *VRN-H1* was highlighted by the striking reflection of shoot and root architecture in transformed line GP[*VRN1-HA*]-14, in which a significant increase in overall *VRN-H1* expression was detected compared to the non-transformed control (Figure 6.6). The additional *VRN1-HA* allele reduced seminal root angle by 26° compared to control plants, while line GP[*VRN1-HA*]-22 showed 9° reduction in root angle (Figure 6.6A and Figure 6.7A). At adult stage, GP[*VRN1- HA*]-14 displayed significant differences for all root traits measured at four different soil depths (Figure 6.6 E-J). In particular, the percentage of total root system area and root volume were significantly increased (26.4 and 25.9%) at the deepest level (60cm) compared to GP[Control]-14. Root mass was significantly reduced for all three transformed lines compared to their

respective control, consistent with the spring growth habit of the lines. The transformed lines also displayed a significant reduction in spike length and DTA, consistent with previous knowledge of *VRN1* function (Deng et al. 2015) (Figure 6.7 B-C). A significant reduction in R/S was only observed in transformed lines GP[*VRN1-HA*]-6 and GP[*VRN1-HA*]-22 (Figure 6.8).

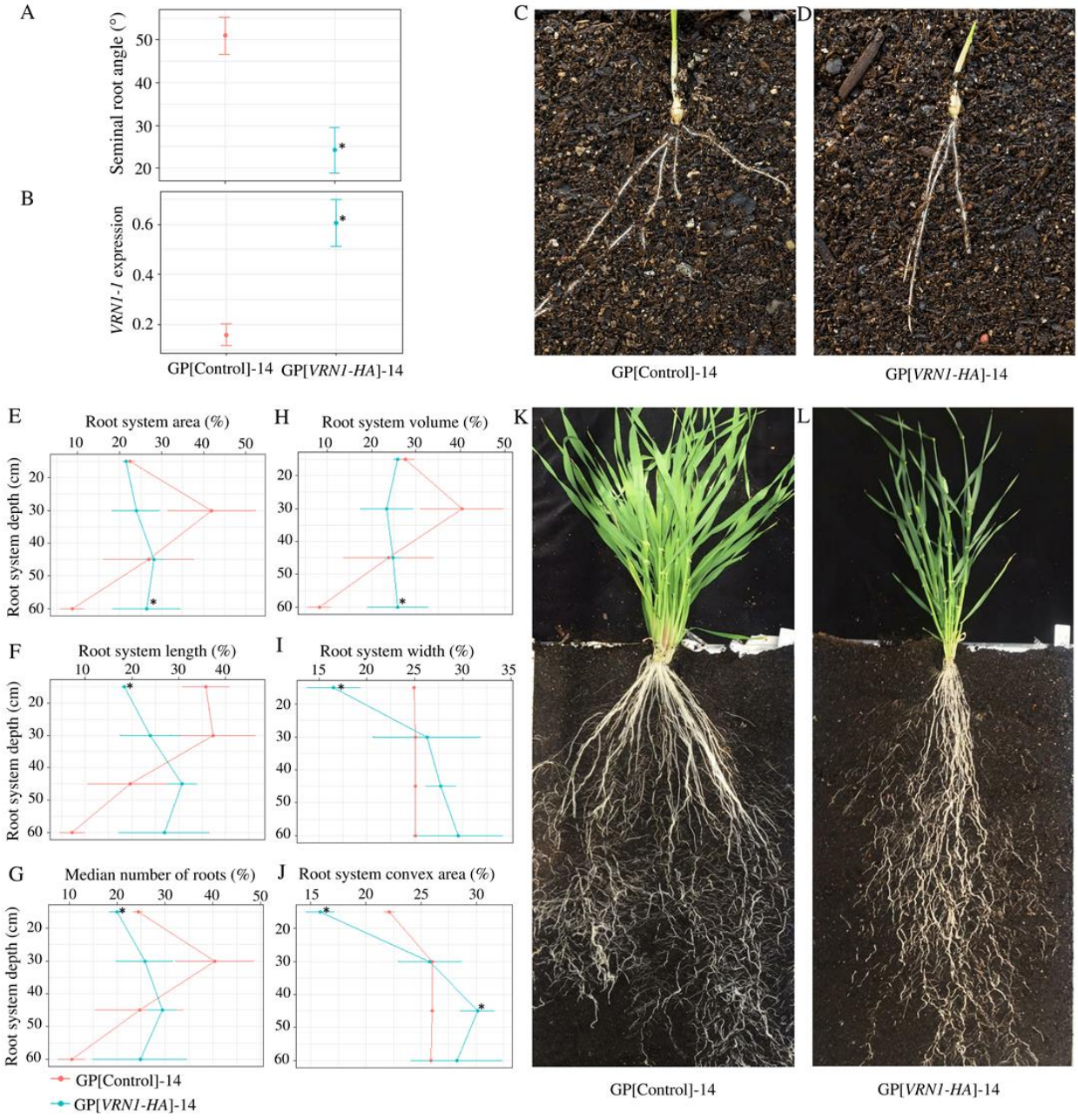

Figure 6.6. Comparisons of root phenotypes and gene expression between the *VRN-1H* transformed line GP[*VRN1-HA*]-14 and barley cultivar Golden Promise control line GP[Control]-14. **(A)** Seminal root angle; **(B)** *VRN1-1* expression in bulked seedling tissue; **(C)** GP[Control]-14 root system at 5 days post sowing; **(D)** GP[*VRN1-HA*]-14 root system at 5 days post sowing. **(E, F, G, H, I, J)** Variation in root system parameters at varying soil depths: **(E)** total root system area; **(F)** total root system length,

(G) median number of roots, **(H)** total root system volume, **(I)** root system width; **(J)** root system convex area. **(K)** GP[Control]-14 root system at 5 weeks post sowing, and **(L)** GP[*VRN1-HA*]-14 root system at 5 weeks post sowing. BLUEs and standard errors are displayed for each line (GP[Control]-14: pink, GP[*VRN1-HA*]-14: blue), except in (A) where means and standard errors are displayed. T-tests were used to determine statistical significance in (A, B), $P \leq 0.05$. Fisher's LSD 0.05 test was used to determine significant differences in (D, E, F, G, H, I, J). * Indicates statistical significance.

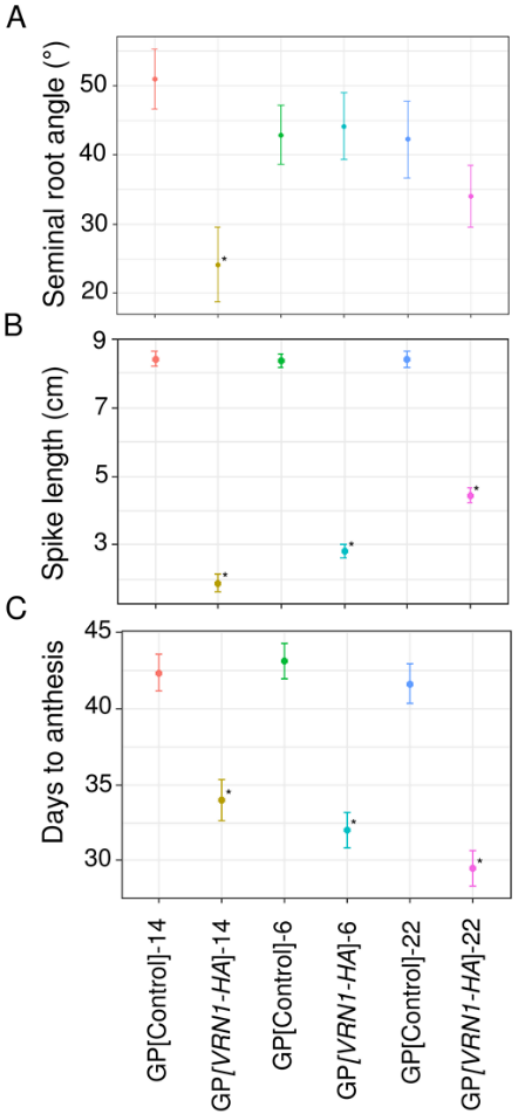

Figure 6.7. Phenotypic variation among *VRN-1H* transformed lines of the barley cultivar Golden Promise. **(A)** Seminal root angle, **(B)** spike length, and **(C)** days to anthesis. BLUEs are displayed for each NIL along with standard errors. Fisher's LSD 0.05 test was used to determine significant difference between each *VRN-1H* transformed line and their respective control. * Indicates statistical significance.

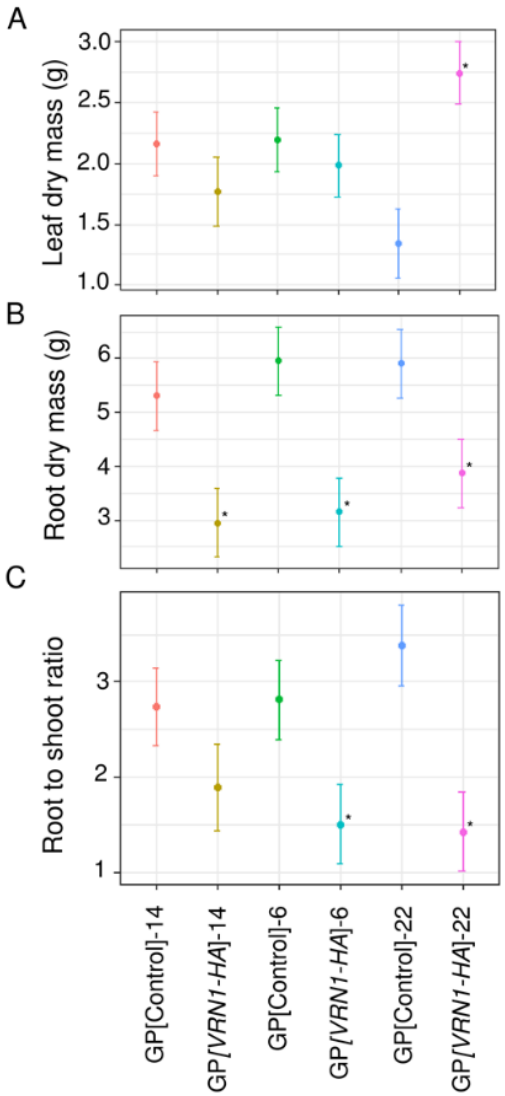

Figure 6.8 Phenotypic variation among *VRN-1H* transformed lines of the barley cultivar Golden Promise at 5 weeks post sowing. **(A)** Leaf dry mass, **(B)** root dry mass, and **(C)** root-to-shoot ratio. BLUEs are displayed for each line along with standard errors. Fisher's LSD 0.05 test was used to determine significant differences between each *VRN-1HI* transformed line and their respective control. * Indicates statistical significance.

6.4.3 Gravitropic response and expression analysis in VRN1-1 spring allele

To gain first insights into the biological mechanism with which *VRN1* influences root architecture, we investigated gene expression and used time-lapse imaging to compare root gravitropic responses between barley NILs carrying different alleles (Figure 6.9). Strong differences in gravitropic response were observed depending on the *VRN-H1* allelic state, with *VRN1-1* causing the strongest response to 90° horizontal rotation. High *VRN-H1* expression levels were observed in mature root system tissues of barley NILs carrying *VRN1-1* and the

winter wild type allele (Figure 6.9B). Expression of the barley homologue for *DRO1* (Uga et al. 2013) was similar in both *VRN1-1* and the winter wild-type barley NILs (Figure 6.9C). Expression of the barley homologue of the auxin-induced *GH3-2* gene was increased in the *VRN1-1* allele compared to the wild-type, although not statistically significant (Figure 6.9D).

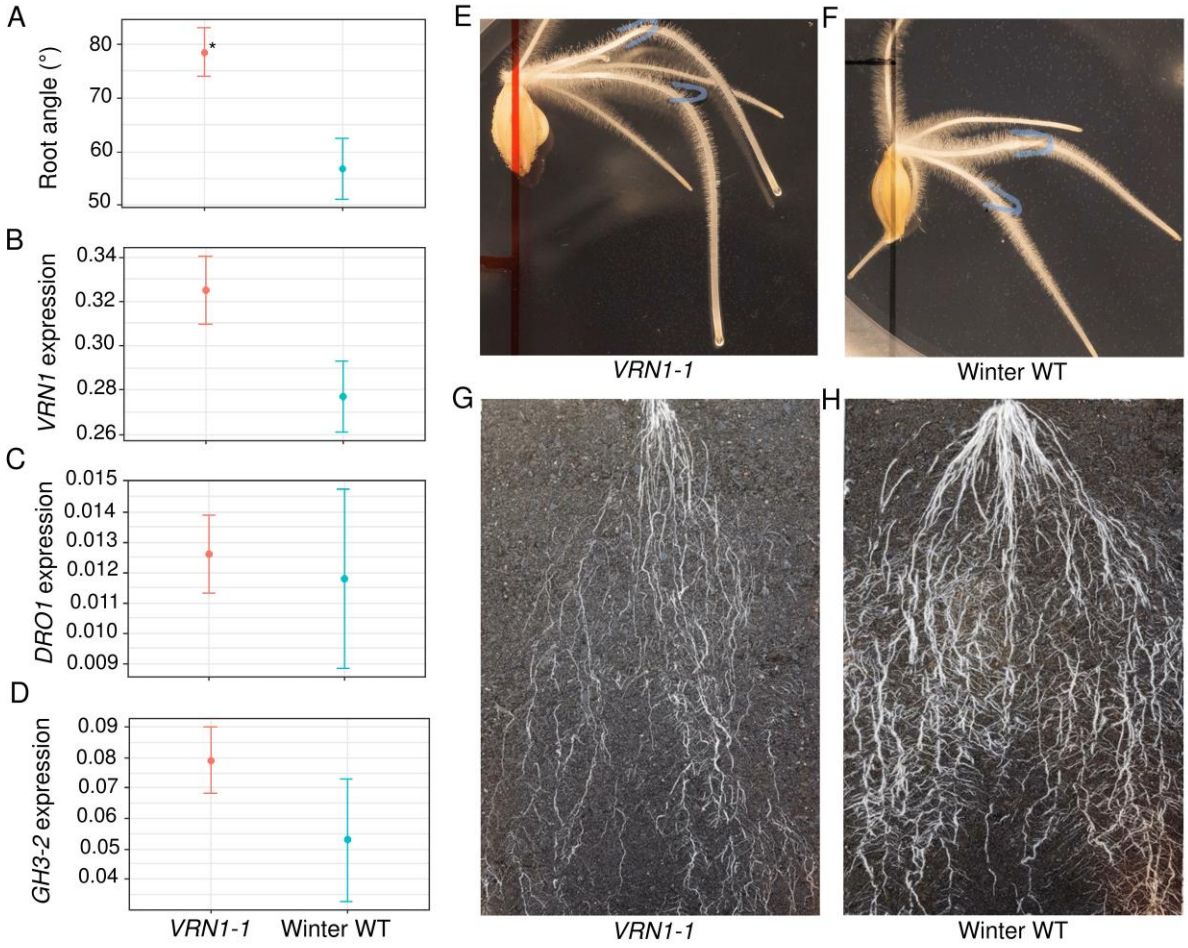

Figure 6.9 Expression of *VRN-H1*, *DRO1* and *OsGH3-2* in roots, gravitropism response and mature root system architecture among NILs, differing in composition of spring allele (*VRN1-1*) and the winter wild type allele (Winter WT) at the barley locus *VRN-H1*. **(A)** Mean change in root angle after gravitropism testing of *VRN1-1* and the Winter WT NIL. **(B, C, D)** Expression levels determined by qRT-PCR, in root tissue of vernalized barley NILs sampled at anthesis, for **(B)** *VRN-H1*, **(C)** *DRO1*, and **(D)** *OsGH3-2*. **(E, F)** Response to gravity 10 hours after rotating agar plates 90˚ for **(E)** *VRN1-1* and **(F)** the WT Winter NIL, respectively. **(G, H)** Mature root system of **(G)** *VRN1-1* and **(H)** the Winter WT NIL prior to sampling root tissue for gene expression studies. Whisker plots indicate means and standard errors.

6.5 Discussion

6.5.1 VRN-H1 spring alleles display allelic divergence for root architecture phenotypes

Each *VRN-H1* spring allele characterised had a unique root architecture deviating from that of the winter wild-type allele. Most similar was *VRN1-1* and *VRN1-4*, both with a generally narrower architecture, yet in *VRN1-1* the phenotype was a lot more pronounced and consistent. The *VRN1-1* spring allele not only conferred narrow root growth behaviour, but also prolonged root growth at the deepest level (60-80 cm) during grain-filling. This narrow and deep root system is consistent with the general root ideotype identified in other cereal crops as being advantageous under water-limited conditions (Lynch 2013; Manschadi et al. 2006) Notably, the *VRN1-1* allele is common in Australian barley varieties grown in regions with highly variable rainfall and severe seasonal drought. This suggests selection for *VRN1-1* variants that simultaneously induce early flowering and deep roots provides a dual mechanism imparting flowering-mediated drought escape coupled with improved water acquisition (Foulkes et al. 2007; Manschadi et al. 2006; Manschadi et al. 2010). 'Steep, cheap and deep' root systems (Lynch 2013), like that observed for *VRN1-1*, allow efficient access to stored water or leached nitrates deep in the soil, yet offer savings in underground carbon deposition in favour of above-ground biomass and grain production, which is critical during water-stress. Therefore, of the spring alleles, the above- and below-ground phenotype of *VRN1-1* is thought to be the most advantageous for growth under water-limited conditions, especially terminal drought stress. To confirm this, yield trials under water stress conditions across a range of environments are required. Ideally, the germplasm used in the previous chapters 3 – 5 could have been assayed with the *VRN1-1* marker to determine the allelic frequency, however resource restrictions made this unachievable. To further investigate the *VRN-H1* spring allelic diversity and the relationship between *VRN-H1* alleles and environmental adaption in Australia, the frequency of the alleles in Australian cultivars could be explored and analysed in relation to the environmental conditions of the typical production areas of each cultivar. This would provide information on the distribution of the 12 *VRN-H1* spring alleles across Australian germplasm as well as identify any role of indirect selection on yield in particular environment.

In contrast to *VRN1-1 and VRN1-4*, the root architecture of *VRN1-3* is wider than the winter allele, nevertheless during the grain-filling period the allele had a greater proportion of roots at 40 and 60 cm compared to the winter allele. In addition, the total root mass of the *VRN1-3* root system is similar to the other spring alleles and significantly leaner (reduced)

compared to the wild-type. This ideotype, wide and lean root system with relatively deep roots, has not been well described in previous literature. Although the *VRN1-3* root system does not reach the same depths of *VRN1-1*, it may still be advantageous in water-limited environments, especially when water-stress is intermittently relieved throughout season, where more water can be captured in the top layer.

Interestingly, deletion of *VRN-H2*, an important plant developmental gene and the signalling target of *VRN-H1* in the vernalization response, had no significant influence on seminal and nodal root growth angle. Consistent with previous research, the *VRN-H2* deletion NIL flowered earlier and had significantly less tillers, as a result of the reduced vegetative period compared to the control. However, the line exhibited a unique root system distribution, whereby it had a greater proportion of roots in the mid-point of the soil profile (40 cm) at anthesis and mid grain-filling compared to the wild type. Furthermore, the *VRN-H2* deletion had a relatively lean root system, similar to those observed in the spring alleles. This reduced root mass and R/S is most likely a result of early flowering and thus less time to develop roots and shoots during the vegetative period. The unique root system distribution observed in the *VRN-H2* deletion line suggests that *VRN1-H2* may also influences root development, but with independent regulation.

6.5.2 VRN-H1 influences root system architecture via pleiotropy and not linkage

All three transformed lines had a consistent above-ground plant architecture, similar to that previously described (Deng et al. 2015), with a significantly reduced time to flowering and a more compact spike length. Characterisation of the transformed lines, specifically GP[*VRN-HA*]-14, revealed that *VRN-H1* has a pleiotropic effect on root architecture. *VRN1* encodes a MADS box transcription factor with high similarity to the *APETALA/FRUITFUL*-like class of genes in *Arabidopsis* (Preston and Kellogg 2006; Yan et al. 2003). Many related genes from the MADS-box transcription factor family are highly expressed in roots of Arabidopsis (Burgeff et al. 2002; de Folter et al. 2005; Melzer et al. 2008; Yu et al. 2014), rice (Guo et al. 2013; Yu et al. 2015) and soybean (Liu et al. 2015), where they induce and modulate underground plant development. Investigations in Arabidopsis confirmed roles of MADS-box genes in local auxin accumulation in root primordia or root cap tissue (Tapia-López et al. 2008; Yu et al. 2014), or in meristem cell determinacy (Melzer et al. 2008).

6.5.3 VRN-H1 influences roots via gravitropism

The *VRN1-1* NILs had a more prominent curvature response to the rotation from the normal vertical axis to the horizontal axis than the wild-type NILs. Thus, the *VRN1-1* allele significantly increases the gravitropic response in the line. This result is not unexpected, as gravitropism is one of the most crucial factors defining root growth angle (Morita and Tasaka 2004) and has been shown to influence root growth angle in rice (Uga et al. 2013). The hormone auxin plays a key role in root gravitropism, where it accumulates in roots following gravitstimulation and consequently inhibits elongation and promotes downward bending of the roots (Vanneste and Friml 2009). Despite not being statistically significant, the auxin induced *GH3-2* had increased expression in the *VRN1-1* allele, consistent with the gravitropic response in this NIL. The accumulation of auxin in the root, following gravistimulation, is thought to occur in the root tip (Vanneste and Friml 2009), thus a more targeted approach of analysing *GH3-2* expression in the root tip of *VRN1-1* NILs may be more informative. Furthermore, biochemical assays could also be used to precisely quantify the concentration of auxin in the root tips of *VRN1-1* and compared to the then wild-type NIL.

The barley homologue of *DRO1*, the major deep-rooting gene in rice, showed no differences in expression between *VRN1-1* and wild-type NILs. Despite similarities between the influences of *DRO1* and *VRN1-1* on root architecture, our results suggest that *DRO1* does not influence root architecture in barley. However, in the rice *DRO1*-NIL the highest expression of *DRO1* was observed in the root tips, while the lowest expression was observed in the middle portion of the root (Uga et al. 2013). Therefore, expression analysis of *DRO1* in the root tips of the *VRN-H1* alleles may provide more accurate representation of the influence of *DRO1* on root architecture in barley.

6.5.4 Minimal trade-offs for the pleiotropic effect of VRN-H1

The *VRN-H1* allele analysis revealed minimal trait trade-offs for *VRN-H1* pleiotropy, with complementary root architecture and flowering-time phenotypes associated with different allelic states. For example, the *VRN1-1* spring allele had a narrow yet deep root system coupled with early flowering and a conservative tiller number. As detailed above, this phenotypic combination has the potential to maximize yield in water-limited environments by optimizing pre-anthesis growth and water use so that sufficient post-anthesis water is available to meet yield potential (Fischer 1979). On the other hand, the winter allele, which is associated with a wide root architecture, elevated biomass, and delayed flowering, appears better adapted both above and below ground to temperate environments with frequent in-season rainfall. The

complementary nature of these traits suggests *VRN-H1* allele selection can be harnessed for improved target growth environment adaptation. The variant root architectures observed for the *VRN-H1* spring allele NILs suggests the relationship between maturity and root architecture can be de-coupled, enabling differing combinations of phenology and root architecture to be breed for depending on selection of the *VRN-H1* spring allele. De-coupling this relationship allows breeders to combine differing root architecture and phenology for adaptation to varying environmental conditions. For example, an early maturing variety with a wide root system architecture is potentially better adapted to growing environments with frequent in-season rainfall pre-anthesis, soils with low water-holding capacity and warm temperatures during grain-fill. It is also important to note that only five out of the 12 *VRN-H1* spring alleles were evaluated for root system architecture in this study, therefore further research should be conducted to characterise the remaining alleles.

6.6 Conclusion

In summary, we conclude that *VRN1* variants modulate root morphology in barley. Previously, in barley, QTL for root traits were detected in the vicinity of *VRN-H1* (Arifuzzaman et al. 2016; Arifuzzaman et al. 2014), a well-known flowering regulator that was previously shown to influence development of key traits besides flowering (Deng et al. 2015). Previous research in wheat confirmed that *VRN1* is involved in canopy development (Steinfort et al. 2017) and overall plant growth (Eagles et al. 2011), however its direct involvement in cereal root system architecture was to date unknown. Our findings reveal pleiotropic action of a major flowering gene that simultaneously shapes growth habit both above-ground and below-ground. A trade-off in the dual regulation of root and shoot architecture is not necessarily evident, since mutual positive effects on environmental adaptation appear to be associated with *VRN-H1* alleles. For example, the *VRN1-1* spring allele in barley should decrease water demand via early flowering and a conservative tiller number, while simultaneously increasing water supply via a narrow yet deep root system. It thereby potentially enhances the balance between water supply and demand under drought. In addition, the divergent root architectures identified for the *VRN-H1* spring NILs suggests that this relationship between root architecture and phenology can be de-coupled to improve environmental adaptation. First investigations of the underlying biological mechanism in our study revealed differences in gravitropic responses associated with the *VRN1* allelic state. Identification of the downstream targets of *VRN1* will elucidate further crucial elements of root system development, connecting well-characterised above-ground and

unexplored below-ground expression networks. Ultimately this will help to design modern cereal cultivars with root systems tailored for adaptation to different target growth environments. Further studies will improve our understanding of how well-characterized above-ground developmental pathways interact with the largely unknown genetic architecture of plant root development.

6.7 References

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Chapter 7

GENERAL DISCUSSION

7.1 Fulfilment of objectives

The over-arching objective of this thesis was to investigate the genetics influencing root system architecture in barley and their association with above-ground drought adaptation traits. This was achieved by integrating high-throughput phenotyping techniques with genome-wide marker datasets to identify QTL in three divergent barley populations. To investigate the genetics controlling root architecture in barley, genomic regions for seminal root angle and root number were identified in the ND24260 × Flagship doubled haploid (DH) population, a panel of elite breeding lines from the Northern Region Barley (NRB) Breeding Program, and a subset of the multi-reference nested-association mapping (NAM) population. For the NRB breeding and NAM populations, the contribution of root traits for yield improvement was evaluated for Australia's northern grain-growing region. To investigate the relationship between above-ground drought adaptation traits and root architecture, genomic regions influencing delayed canopy senescence (or stay-green) and days to anthesis were identified in the DH population and the NAM population across five field trials. Finally, a more in-depth evaluation of the relationship between flowering time and root system architecture was undertaken by investigating the effects of *VERNALIZATION1* on root growth in barley. The overall thesis objective was accomplished through the research outcomes described in Chapters 3 – 6.

In Chapter 3, seminal root angle and number were phenotyped in the ND24260 × Flagship DH population using the high-throughput clear-pot method. The root trait phenotype data was then combined with the Diversity Arrays Technology (DArT) molecular marker profiles for each line to position QTL using composite interval mapping. Two QTL were identified for root angle on chromosome 3H and 5H, while five QTL were detected for root

number on chromosomes 1H, 3H, 4H, 5H and 6H. Interestingly, the largest effect QTL for each root trait was mapped to the same marker on the long arm of chromosome 5H. Furthermore, this key QTL was found to collocate and share seven genes underlying a previously reported QTL influencing seminal root angle in wheat. This chapter provided first insight into the genetic control of seminal root traits in barley, however, further research in populations with greater allelic diversity is required.

Chapter 4 also described the mapping of QTL for seminal root angle and number, but this time using a panel of elite breeding lines from the NRB breeding program. A genome-wide association mapping approach facilitated QTL detection in the breeding population. Only a single QTL was identified for root angle and no significant associations were detected for root number. Interestingly, the root angle QTL mapped to the same genomic region previously detected for root angle and number in Chapter 3 on chromosome 5H. This suggests that the region on 5HL is a key QTL influencing seminal root traits, especially root angle, in elite barley germplasm. Also, in this chapter, the relationship between seminal root traits and yield was investigated by combining the root phenotype data with yield data from 20 trials across Australia's northern grain-growing region. Both seminal root traits were found to be related to yield across the environments evaluated, yet of the two traits, root angle was more strongly associated with yield. The direction and magnitude of the association for both root traits with yield was found to be highly context dependent. Further research is required to determine the value of each root ideotype in specific environment and management scenarios.

The potential for shared genetic control between above- and below-ground drought adaptation traits was explored in Chapter 5. A subset of the ND24260 × Flagship population and a subset of the multi-reference NAM population were evaluated in this study in field trials. QTL were mapped in the NAM subset for seminal root angle and root number using a genome-wide association mapping approach. Two QTL were identified for root number, both on chromosome 5H, but no significant associations were identified for root angle. Of the root number QTL identified, one collocated with the QTL on 5HL previously detected for root angle and root number in Chapters 3 and 4. Root trait phenotypes previously identified in Chapter 3 were used to map QTL in the DH subset. Four QTL were identified for root angle on chromosome 3H and 5H, while six QTL were detected for root number across chromosomes 1H, 3H, 5H and 6H. Also, in this chapter, the DH subset was phenotyped for canopy senescence and days to flowering in five field trials run across two years. These

drought-adaptation traits were also phenotyped in the NAM subset but only in two field trials. QTL were mapped for senescence traits and flowering time in both populations and projected, along with root trait QTL, onto a consensus map to identify collocated QTL. Notably, QTL for seminal root traits, days to flowering and senescence traits collocated together and mapped to the genomic region on 5HL consistently detected throughout this thesis. The gibberellic acid (GA) biosynthesis gene, *Hv20ox1*, is located within this 5HL region and may be the gene underlying this QTL. The results of this chapter suggest that seminal root traits, flowering time and senescence traits may be under shared genetic control, however further research, possibly using GA signalling mutants, is required to determine whether *Hv20ox1* is influencing these above- and below-ground drought adaptation traits.

The relationship between root system architecture and flowering time was evaluated further in Chapter 6, with a specific focus on *VRN1*, a key regulator of flowering time in barley. Near-isogenic lines (NILs) for variants of the *VRN1* spring allele and a *VRN2* deletion NIL were evaluated in comparison to the NIL for the *VRN1* winter allele. The NILs were characterised for root system architecture in the glasshouse and field. The distribution of root biomass at differing soil depths was also examined in the NILs. The variants of the *VRN1* spring allele all displayed divergent root phenotypes, which suggested that *VRN1* influences root system architecture in barley. To determine whether this was a result of linkage drag or a pleiotropic effect of *VRN1*, Golden Promise, a spring barley cultivar, transformed with an additional copy of the spring allele *VRN1-1* was evaluated. The additional copy of *VRN1-1* significantly reduced the angular spread of the root system architecture at both the seedling and adult stages. Furthermore, a greater proportion of root area and volume was identified deeper in the soil profile for the transgenic line compared to the control. Thus, the results of this chapter confirmed that *VRN1*, a key regulator of flowering, directly influences root system architecture in barley. Future research to understand the pleiotropic involvement of *VRN1* in overall plant architecture will help to breed barley cultivars adapted to water stress environments.

The major findings of these four research chapters are summarised in this final chapter. The implications for barley breeding are discussed, along with the limitations of the research. In addition, strategies for future research expanding on the thesis outcomes are outlined.

7.2 The polygenic control of root system architecture in barley

Three genetically and structurally divergent populations were used to investigate the genetic control of seminal root angle and seminal root number in barley. The two most common QTL mapping approaches, linkage mapping and association mapping, were used to complete this analysis. A total of nine QTL were detected for both traits across all three populations (Figure 7.1). Six QTL were detected in the ND24260 × Flagship population, one QTL was identified in the NRB breeding panel and two QTL were detected in the NAM subset. Linkage mapping was used for analysis of the DH population and has increased statistical power to detect significant marker-trait associations but relatively low mapping resolution (Jones et al. 1997; Jones et al. 2009). The highest number of QTL were detected in the DH population and is likely a result of the greater statistical power of linkage mapping to detect significant marker-trait associations due to the high frequency and thus balance of alleles at each locus. The main constraint of linkage mapping is limited allelic diversity associated with bi-parental populations, where there are only two possible alleles at each locus. In comparison, association mapping allows incorporation of much wider allelic diversity and a greater level of recombination, as any panel of lines can be assessed using the mapping technique (Flint-Garcia et al. 2003; Jones et al. 2009). This mapping approach was implemented for the NRB breeding panel and the NAM subset. Association mapping is, however, limited in statistical power and the ability to detect alleles present at a low frequency in the population (Flint-Garcia et al. 2003; Jones et al. 2009). This multi-pronged mapping approach, incorporating linkage mapping and association mapping, was complimentary and provided a thorough genetic dissection of seminal root traits.

Despite differences in QTL mapping techniques and populations examined in this thesis, a QTL on chromosome 5HL was consistently detected (Figure 7.1). The region appears to influence both root angle and number, where QTL for either and/or both traits were detected across the populations. Identification of this genomic region in combination with the other eight QTL demonstrate that both seminal root traits are under polygenic control, whereby many QTL with smalls effect influence the traits. Yet, through characterisation of *VRN1* NILs and transgenic material, *VRN1* was found to have a relatively large effect on root system architecture, adding to the complexity of genetic control for seminal root traits. The spring allele for *VRN1* was fixed in the DH population and the NAM subset and therefore was not detectable via our QTL mapping studies. For the NRB breeding panel, it is likely the *VRN1* spring allele was at a high frequency and the winter allele at a

very low frequency due to all the lines having a spring growth habit, thus *VRN1* would have been undetectable via association mapping. Therefore, all QTL identified in this study are operating in the presence of *VRN1* spring alleles, of which there are multiple.

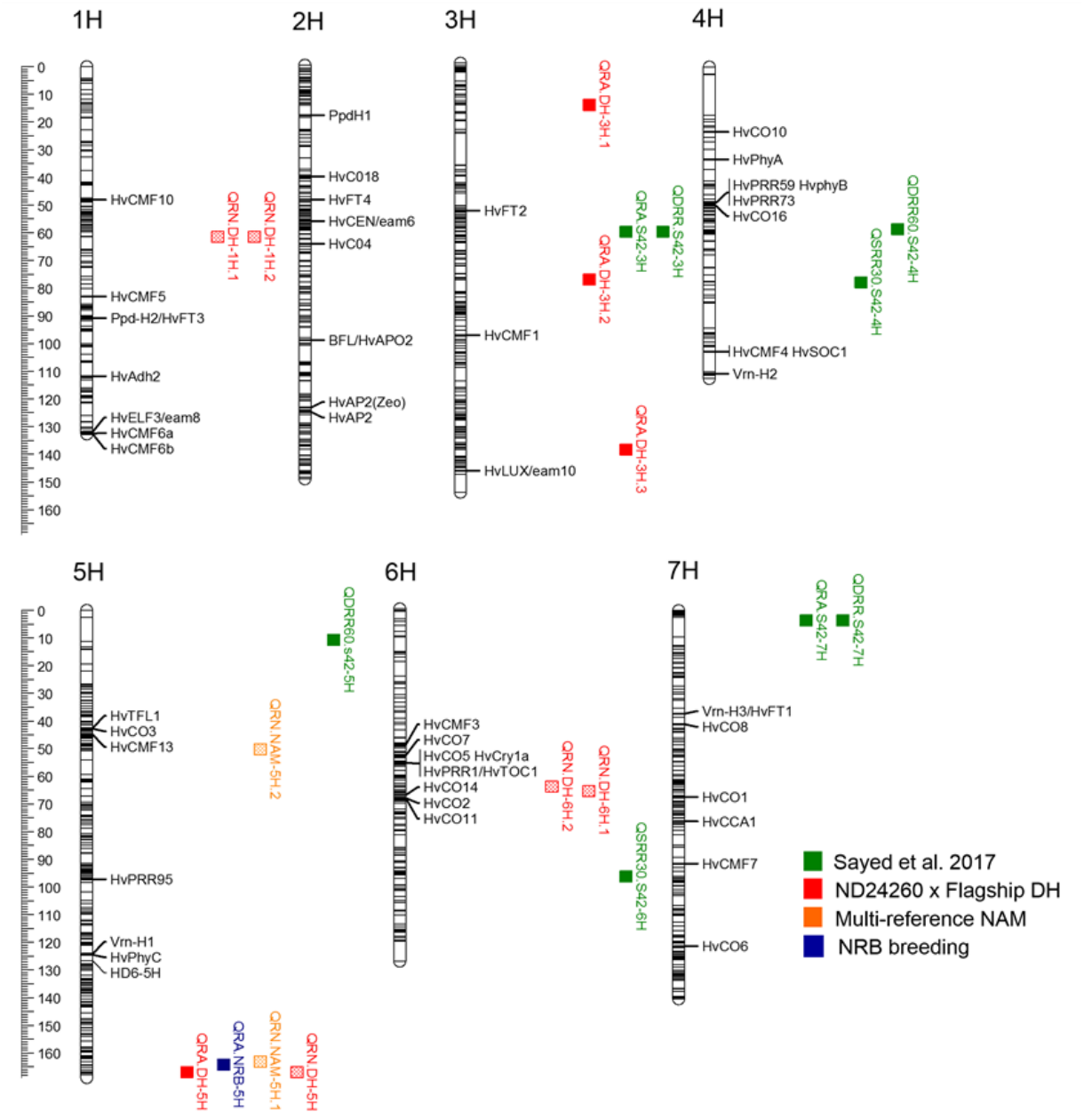

Figure 7.1 Barley consensus map with QTL for seminal root traits. QTL for seminal root angle and root number identified in the three populations evaluated in this thesis (ND24260 × Flagship DH, multi-reference NAM and NRB breeding population) and the study by Sayed et al. 2017. Solid squares represent QTL for seminal root angle and thatched squares symbolize QTL for seminal root number.

Aside from the research described in this thesis, there is one recent study mapping QTL for seminal root angle in barley (Sayed et al. 2017). In this study, the DH population of a cross between the spring cultivar Scarlett and the wild accession ISR42-8 was evaluated and nine QTL were detected for root angle (Sayed et al. 2017). When these QTL were projected onto the barley consensus map used in Chapter 5, none were deemed to collocate with root trait QTL detected in this thesis (Figure 7.1). Furthermore, unlike the root trait QTL described in Chapter 5, the root angle QTL detected by Sayed et al. (2017) did not map to any key plant developmental genes (Figure 7.1). Contribution of exotic alleles from the wild barley parent in the population evaluated by Sayed et al. (2017) is likely the main reason that no common QTL were detected between the studies. This suggests, through domestication, there may have been inadvertent selection for root mechanisms that are in sync with flowering time, thus mechanisms that are out of sync with flowering were left behind in wild barley. These genetic mechanisms, present in wild barley, may be beneficial for breaking the link between root architecture and flowering time for specific adaptation scenarios.

Despite the complexity of the genetic control identified for seminal root traits, it is clear from the additional QTL detected by Sayed et al. (2017) that the intricacy expands beyond that which is detailed in this thesis. To efficiently identify other genomic regions influencing seminal root traits, more advanced methods of QTL detection can be employed. Nested-association mapping (NAM) combines the advantages of both linkage mapping and association mapping due to the unique structure of NAM populations. This structure allows both detection of rare alleles through the multiple recombinant inbred line (RIL) populations, plus an increased allelic diversity and high resolution for mapping of QTL (Yu et al. 2008). In this thesis, 5 of the 23 RIL families belonging to the multi-reference barley NAM population (Ziems et al. 2015) were subjected to phenotyping and mapping of root traits. However, by only using a subset, the full potential of such NAM analyses was not realised. As a next step, the entire NAM population should be analysed for root traits.

The first barley NAM population, HEB-25, was described by Maurer et al. (2015) and consists of 25 wild accessions crossed to one reference, the spring cultivar Barke. This NAM is extremely diverse, as each wild barley founder line was selected based on its unique genetic diversity relative to the other 24 founder lines (Maurer et al. 2015). The multi-reference NAM used in this thesis was designed to be highly relevant for the northern grain-growing region of Australia via the purposeful use of elite breeding lines from the NRB breeding program as founder lines. However, these lines are related with greater levels of

genetic similarity (Ziems et al. 2015). In contrast, HEB-25 was designed to enhance biodiversity in elite breeding populations by enabling detection of favourable exotic alleles that can be introgressed into modern cultivars (Maurer et al. 2015). Genomic dissection of key plant developmental traits in the HEB-25 population has been successful (Maurer et al. 2015; Maurer et al. 2016) and provides much promise for a similar genetic dissection of root architectural traits in the multi-reference NAM and HEB-25 populations. Detection of additional QTL from the two NAM populations will provide further insight into the genetic mechanisms of seminal root traits and deliver potential targets for marker assisted selection in barley breeding programs.

7.3 The relationship between key genomic regions influencing root architecture in barley

Outcomes from this thesis demonstrate that root architectural traits are under polygenic control in barley. Yet overall, the QTL on chromosome 5HL and *VRN1* appeared to be the major players. It was speculated that the causal gene underlying the genomic region on 5HL was likely *Hv20ox1*, a gibberellic acid (GA) biosynthesis gene (Spielmeyer et al. 2004). However, further research is required to confirm this. Using genome editing technology, such as the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated Protein9 (Cas9) system (Cong et al. 2013), a *Hv20ox1* null mutant line could be created and characterised for root architecture to validate *Hv20ox1* as the causal gene of the QTL on 5HL. Recent research in barley has identified *Hv20ox* genes, thus GA, as regulators of flowering time through the photoperiod pathway (Boden et al. 2014). However, GA cannot promote flowering in isolation and requires coordination with *FLOWERING LOCUS T (FT1)* and *VRN1* to complete inflorescence (Boden et al. 2014). Furthermore, *VRN1* expression in spring barley does not appear to be influenced by increased GA biosynthesis resulting from a functional mutation that promotes early flowering under short days (Boden et al. 2014). Despite this preliminary investigation, the relationship between *Hv20ox* genes and *VRN1* is yet to be completely elucidated in barley.

To investigate the effects on root system architecture for the differing combinations of allelic variants for *Hv20ox1* and *VRN1*, reciprocal QTL-specific near-isogenic lines (NILs) could be created. Ideally, the NILs would be generated for divergent alleles of *Hv20ox1* in differing *VRN1* allelic backgrounds. In Chapter 6, divergent root phenotypes were identified

for the panel of *VRN1* spring alleles, the *VRN2* deletion and the *VRN1* winter allele. Therefore, to thoroughly investigate the relationship between *Hv20ox1* and *VRN1*, NILs should ideally be created in all *VRN1* allelic backgrounds. Alternatively, the CRISPR/Cas9 system could be used to generate *Hv20ox1* mutant lines, as this technology is more efficient, however there is a risk of off-target mutations (Cong et al. 2013). A similar phenotyping strategy to that presented in Chapter 6 for the *VRN1* NILs could also be applied to these mutant/NILs, where root system architecture is characterised throughout plant development under controlled conditions and also at maturity under field conditions.

To identify the value of specific ideotypes detected in the *Hv20ox1* NILs for adaptation, the lines should be yield tested in environments that have been specifically characterised for water stress according to the environment types outlined by Chenu et al. (2013). A major limitation to the NILs approach is the time required to create NILs, whereby repeated cycles of backcrossing is required to avoid undesirable linkage drag between loci (Kooke et al. 2012). NIL development can be accelerated by using 'speed breeding', a new method for rapid generation advance where up to six generations of barley can be produced in a single year (Watson et al. 2017). By adopting speed breeding and the same crossing approach to create the *VRN-H1* NILs (Oliver et al. 2013), involving five rounds of backcrossing, the *Hv20ox1* NILs could be generated within a year. As an alternative to developing NILs or mutants with CRISPR/Cas9, previously developed GA mutants (Chandler and Robertson 1999) could be phenotyped for root traits.

7.4 Investigating the direct and indirect effect of GA on canopy senescence in barley

In Chapter 5, stay-green QTL were mapped to GA biosynthesis genes *Hv20ox2* on chromosome 3H and *Hv20ox1* on chromosome 1H. *Hv20ox2* is the causal gene underlying the semi-dwarfing gene *sdw1/denso* and encodes the GA 20-oxidase2 enzyme, which is an essential component of GA biosynthesis (Jia et al. 2009). Stay-green QTL also mapped to the projected locations of other semi-dwarfing genes, *sdw8* and *sdw4*, on chromosomes 5H and 7H, respectively. The results of Chapter 5 suggest GA is associated with canopy senescence in barley, however it is unclear whether this a consequence of plant height or a direct result of GA biosynthesis. To better understand the effect of GA on canopy senescence and determine whether it is a direct or indirect effect, or a combination of both, further research is required.

In sorghum, the stay-green phenotype is a consequence of an improved balance between supply and demand of water (Borrell et al. 2014). Therefore, deviations in plant height can influence the demand for water and thus influence this balance and the plant's ability to stay green. If the physiological basis of stay-green is similar in barley, then GA may indirectly influence stay-green as a consequence of plant height. As a first step to investigate the relationship between plant height and canopy senescence, the DH and NAM subset should be re-evaluated in the field for plant height and delayed canopy senescence. Correlations between plant height and senescence traits should then be examined to provide insight into the relationship between the two traits. If a significant relationship does exist then plant height phenotypic data could be included as a co-variate in QTL mapping studies to minimise the effect of plant height in the genetic analysis. The outcomes of this further research would not only provide insight into the potential indirect effect of GA on canopy senescence but would also reveal more information about the physiological mechanisms of stay-green in barley.

In *Arabidopsis*, GA biosynthesis has recently been shown to directly affect the onset of leaf senescence, whereby increased GA biosynthesis accelerates the rate and the onset of senescence (Chen et al. 2014). The association between stay-green QTL and the two GA biosynthesis genes, *Hv20ox1* and *Hv20ox2*, described in Chapter 5 suggests this direct function of GA maybe conserved between *Arabidopsis* and barley, yet further research is required to confirm this. Developing CRISPR/Cas9 lines or NILs for differing allelic combinations of *Hv20ox1* and *Hv20ox2* would provide the germplasm required to validate the effect of GA on canopy senescence. These lines could then be phenotyped for stay-green traits under well-watered and water-limited field conditions to not only provide insight into the effect of GA on canopy senescence, but also validate that *Hv20ox2* and *Hv20ox1* are the casual genes underlying the stay-green QTL identified on chromosome 3H and 5H, respectively.

7.5 The underlying mechanism of *VERNALIZATION1* on root system architecture in barley

In Chapter 6, *VRN1* was shown to have a pleiotropic effect on root system architecture in barley, influencing root development via gravitropism. Recently, associations been flowering time and root system architecture traits have also been identified in maize (Zhang et al. 2018)

and pea (Desgroux et al. 2017). In barley, the mechanism by which *VRN1* promotes flowering time has been well described in literature. Following a period of low temperatures, *VRN1* expression is induced and, as a consequence, *VRN2* expression is down regulated (Deng et al. 2015; Trevaskis et al. 2006). *VRN2* is a repressor of flowering and downregulation is required to promote *VRN3* (also known as *FLOWERING LOCUS T-like 1*; *FT1*) expression, subsequently promoting flowering at the apical meristem following warm temperatures and long days (Yan et al. 2006). *VRN1* expression is vital for floral transition, however, through domestication barley has evolved to adapt to differing environment scenarios, a result of mutations in the promoter of *VRN1* making *VRN2* unable to bind and repress *VRN1* expression (Fu et al. 2005). Typically, early flowering (or spring growth habit) barley have this *VRN1* mutation, which has been classified as the *VRN1* spring allele. There are at least 10 *VRN1* spring alleles identified in barley and the level of *VRN-H1* expression and flowering behaviour differs for each allele (Hemming et al. 2009). Therefore, it is not unexpected that in Chapter 6, divergent root system architectures were identified for each of the *VRN-H1* spring alleles. Insight into the mechanism by which *VRN-H1* influences root system architecture is essential to identify the value of each *VRN-H1* allele for adaptation of root systems to different environment scenarios.

In Chapter 6, the root system architecture of NILs for only three of the 10 *VRN-H1* spring alleles were evaluated, which is a key limitation of the study. The next step in this research is to thoroughly phenotype NILs for each of the 10 *VRN-H1* spring alleles for root system architecture, flowering time, plant height and spike length, as *VRN1* also influences above-ground traits in barley (Deng et al. 2015). A detailed characterisation of *VRN-H1* expression in the shoots and different sections of the roots should also be undertaken in the panel of NILs to investigate any links between expression levels and root phenotypes. Currently NILs have only been generated for five of the 10 *VRN-H1* spring alleles (Oliver et al. 2013), yet the remaining five could be developed relatively efficiently using the speed breeding system, as mentioned above.

VRN1 encodes a MADS box transcription factor with high similarity to the *APETALA/FRUITFUL*-like class of genes in *Arabidopsis* (Preston and Kellogg 2006; Yan et al. 2003). Genes controlling the vernalization responses in barley and *Arabidopsis* differ, yet there are some parallels between each pathway (Trevaskis 2010). For instance, in both pathways *VRN1* expression under long days is required to induce the floral promoters *FLOWERING LOCUS T* (*FT*) in *Arabidopsis* and *FT1* in barley (Hemming et al. 2008;

Michaels et al. 2005; Yan et al. 2006). Also, both *FT* and *FT1* in each of their respective pathways are downregulated by floral repressors, such as *VRN2* (Helliwell et al. 2006; Hemming et al. 2008; Yan et al. 2006). In *Arabidopsis*, many related genes from the MADS-box transcription factor family are highly expressed in roots (Burgeff et al. 2002; de Folter et al. 2005; Melzer et al. 2008; Yu et al. 2014). Investigations in *Arabidopsis* confirmed roles of MADS-box genes in local auxin accumulation in root primordia or root cap tissue (García-Cruz et al. 2016; Tapia-López et al. 2008; Yu et al. 2014). One example of these MADS-box transcription factors is *XAL1*, which has important roles in root development and floral transition. Two potential models have been described for the role of *XAL1* in *Arabidopsis* root and shoot development. The first model proposes *XALI* has the same role in roots and shoots, as a mediator of auxin signalling, consequently regulating cell behaviour in root and shoot meristems, aiding in root elongation and the shoot meristem transition to flowering (Tapia-López et al. 2008). Hence, auxin response factors have been described to have a role in the regulation of flowering in *Arabidopsis* (Nagpal et al. 2005). Alternatively, *XAL1* may have independent roles in root and shoot development resulting from different complexes with other MADS-Box proteins (Tapia-López et al. 2008). In addition, MADS-Box transcription factor AGL21 regulates lateral root development and positively regulates auxin accumulation by increasing local auxin biosynthesis, consequently speeding up lateral root growth (Yu et al. 2014). MADS-Box transcription factors with a role in root development appear to do so through regulation of auxin, an important plant hormone that promotes cell elongation and root growth.

VRN1 encoding a MADS-Box transcription factor similar to that in *Arabidopsis* suggests *VRN1* may influence roots via a similar mechanism to the *Arabidopsis* genes with a role in auxin regulation. Similarly in rice, one of the major genes influencing root system architecture, *DRO1*, is negatively controlled by auxin and is thought to be an early-auxin-response gene, potentially regulated by auxin response factors in the signalling pathway (Uga et al. 2013). *DRO1* has also been associated with cell elongation in the root tip, resulting in disproportionate root growth and increased root bending following gravistimulation (Uga et al. 2013). In barley, there has been minimal investigation into the homologue of *DRO1* and its influence on root system architecture. Comparison of the *DRO1* homologue's expression in allelic variants of *VRN-H1* in Chapter 6 found no differences in expression levels. However, further research is required to completely exclude the *DRO1* homologue from the genetic control of root system architecture in barley. Also, in Chapter 6, preliminary expression

analysis for the barley homologue of the auxin-induced *GH3-2* gene in the *VRN1-1* NIL was not significantly different from the control, however a more targeted approach focusing on expression in the root tips is recommended for more conclusive results. Based on previous research in *Arabidopsis* and rice, the most obvious initial target for elucidating the mechanism by which *VRN1* influences roots is auxin. Expression analysis in the root tip of the *VRN1* transformed barley lines would provide initial evidence for an association between *VRN1* and auxin. This would be a good starting point for further exposition of the role for *VRN1* in modulating root system architecture in barley.

7.6 The value of root traits to improve adaptation of barley to the northern grain-growing region of Australia

The value of roots has been previously discussed in maize (Ali et al. 2015), rice (Uga et al. 2013), sorghum (Mace et al. 2012) and wheat (Manschadi et al. 2010), where a narrow root angle generally increases the depth of rooting and likely improves yield under water deficit. For barley, there has been minimal investigation into the relationship between root architectural traits and yield, thus the value of root traits for improved adaptation is unknown. The research outcomes of Chapters 4 and 5 provide a first look at the association between seminal root traits and yield in barley across the northern grain-growing region of Australia.

The research outcomes of Chapter 4 demonstrate that both seminal root angle and root number influence yield, however the extent to which each trait contributes to yield improvement is highly context dependent. Similarly, the specific root ideotype (i.e. narrow root angle and low root number) contributing to yield is dependent on the environment and management practices. Again, in Chapter 5, root architectural traits contributed to yield but were also highly context dependent. A more consistent trend was evident in Chapter 5, with a wide root angle and low root number contributing to yield in the wettest environment clusters, however there was no relationship between narrow root angle and yield. Despite the general trend identified for wide root angle in Chapter 5, the specific combination of root ideotype for a certain environment and management practice scenario was not elucidated in this thesis.

Future research determining the value of specific root ideotypes for environments and management scenarios most relevant to the growing regions of Australia will be invaluable. Currently, there is a wide gap between research developed in pre-breeding programs and

delivery to growers through commercial breeding companies. Financial constraints on commercial breeding means that the value of research in a commercial context must be unambiguously clear and must deliver germplasm with a broad range of traits. In addition, to be relevant, the pre-breeding research must consider the type of water stress common in the target environment, as well as using germplasm and phenotyping methods applicable to field environments.

To validate pre-breeding research, management environment facilities (MEFs) can be used to thoroughly investigate the interactions between genetics, the environment and management practices (Rebetzke et al. 2012; Reynolds et al. 2016). Three nationally coordinated MEFs have been developed in Australia targeting assessment of germplasm and traits for increased yield productivity under water and heat stress (Rebetzke et al. 2012). Each MEF is representative of environment types within the cropping zone, such that one MEF is located within each main grain-growing region of Australia. To provide the most accurate phenotypes, the MEFs use remote monitoring of the climate, control field-based spatial variation through statistical analysis, and use standardised, repeatable phenotyping techniques (Rebetzke et al. 2012; Reynolds et al. 2016). MEFs can have a limited capacity to phenotype a large number of lines, therefore the facilities should be used as a validation tool in pre-breeding research. The value of specific root ideotypes and their key genetic drivers for the main cropping regions of Australia could be validated by using MEFs. This would provide the evidence required by breeders to determine the benefit of selecting for root traits in their breeding programs.

7.7 Selection for root architectural traits in barley breeding programs

Progress in crop improvement is limited by the ability to identify favourable combinations of genotypes (G) and management practices (M) for relevant target environments (E), given that the resources available to search among the multitude of possible combinations are limited (Hammer et al. 2016). Phenotypic performance of the many possible combinations forms what can be described as an adaptation landscape (Cooper and Hammer 1996), and crop improvement then becomes a search strategy on that complex G × M × E landscape (Hammer et al. 2016). Therefore, crop simulation modelling could be used to a) better assess the value of candidate genes (e.g. *Hv20ox1* and *Hv20ox2*) in a range of

management and environment scenarios, and b) enhance molecular breeding by adding value to genetic prediction approaches.

Following validation of the ideal G × M × E scenarios for a trait, introducing selection for the trait into breeding programs needs to be considered. There are a number of genomic approaches that can be used to select for a trait in elite germplasm, but ultimately the genetic control of the trait will determine the most appropriate method. Following identification of tightly linked markers through QTL mapping, maker-assisted selection (MAS) can be employed to select for specific phenotypes (Collard et al. 2005). The success of MAS hinges on the quality of the markers being used. For instance, the markers need to be have been validated in an independent population, they need to be high resolution and ideally sequence targeting (Collard et al. 2005). MAS is most advantageous when traditional phenotypic selection is unfeasible due to time, cost, reliability or other factors. In addition, MAS can limit linkage drag of undesirable genomic regions as well as provide a platform to pyramid multiple genes for multiple traits (Eathington et al. 2007). However, MAS is suited to traits under simple genetic control, where one major gene is responsible for the majority of the phenotypic variation, independent of the background population and environmental effects (Bernardo 2016). Therefore, MAS is inefficient for quantitative traits, like root architectural traits, that are often under complex polygenetic control. Furthermore, in past research there has been difficulties with the consistency of QTL detection across environments and genetic backgrounds (Bernardo 2016).

The genomic selection approach is more effective for traits under polygenic control, as it combines the effects of all markers across the genome to explain the total genetic variance and sums these effects to predict the breeding value of individuals (Meuwissen et al. 2001). The principal practice of genomic selection is the prediction of the breeding values (genomic estimated breeding values; GEBVs) for individuals that only have genotype data and no phenotype data. This is made possible by creating a prediction model that is trained by a small group of representative individuals with both genotype and phenotype data (Meuwissen et al. 2001). GEBVs are then used to select the individuals for advancement in the breeding cycle.

The research outcomes of this thesis demonstrate that seminal root angle and number are under polygenic control in barley. Furthermore, based on the additional QTL detected by Sayed et al. (2017), it is likely that the complexity of the genetic control for these traits expands beyond that which is detailed in this thesis. Therefore, genomic selection would be

the most efficient selection strategy for these two complex traits. As all markers are used to estimate the breeding values in genomic selection, individuals need to be genotyped with a large number of markers to maximise the number of QTL in linkage disequilibrium (LD) with at least one marker. The target marker density will depend on the rate of LD decay across the genome of the population evaluated (Heffner et al. 2009). The genotyping cost is one of the main limitations of genomic selection, however advances in molecular marker technology have made it more affordable to densely genotype (using tens of thousands of markers) a large number of individuals. The key advantages of genomic selection are the increased speed of variety development and the reduced cost per selection cycle (Heffner et al. 2009). Despite genomic selection incorporating all marker information, QTL mapping for significant-marker trait associations is not completely redundant. Incorporating markers for QTL as fixed effects in the model have been found to improve the prediction accuracy of the model (Rutkoski et al. 2014). Thus, the marker for the major QTL on 5HL and *VRN1* could be included as fixed effects in a prediction model for seminal root angle, however the QTL would first need to be detected in the breeding population under selection. The prediction models for genomic selection are being further advanced by combining multi-trait multi-environment genomic selection with high-throughput phenotyping to improve genetic gain via increased selection intensity (Crossa et al. 2017). Therefore, based on the genetic correlations identified in Chapter 5, a prediction model could be created to select for root angle, root number and the stay-green integral trait simultaneously. Ultimately, genomic selection and the future advances of this approach should be the most efficient selection strategy for root architectural and stay-green traits in barley breeding programs.

7.8 Conclusion

The outcomes of this thesis demonstrate root angle, root number and stay-green are under complex genetic control in barley. In total, nine QTL were identified as influencing seminal root traits in this thesis, however future research in advanced mapping populations will likely identify new sources of genetic control. The two main genetic components identified in this thesis are both essential genes involved in the transition to flowering. Further investigation is required to understand the effect of divergent allelic combinations of these flowering time genes on root system architecture and canopy senescence. Furthermore, throughout this thesis auxin has been speculated as a key hormone involved in the mechanism by which *VRN1*

influences roots in barley and additional investigation should be undertaken to validate the role of auxin. For the research outcomes of this thesis to be of benefit to the Australian barley industry, the value of root architectural traits and stay-green traits in specific environment and management situations needs to be ascertained. Following this validation, the most appropriate selection strategy for breeding programs would likely be implementation of multi-trait genomic selection, due to the polygenic nature of these traits. Overall, the outcomes of this thesis demonstrate that root architectural traits are under complex polygenic control and the specific value of ideotypes in specific environment scenarios is highly context dependent. The results presented throughout this thesis in combination with the proposed future research directions should assist breeders in developing barley crops better adapted to the variability of the future climate.

7.9 References

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Appendix

Chapter 3: QTL for drought tolerance traits collated from six barley studies

Population	Type[a]	Size[b]	Trait[d]	Growth Stage	Ch[e]	Marker	Peak[g]	R^2	Reference
Scarlet/ISR 42-8	BC_2DH	301	Root Length (RL)[w]	Post-harvest	2H	PpdH1	41.4	6.1	Arifuzzaman et. al. 2014
			RL[w]		3H	bPb-9110	166.8	5.5	
			RL[w]		5H	VrnH1	150.7	1.7	
			Root dry weight (RDW)[w]	Post-harvest	1H	GBM1042	67.9	6.5	
			RDW[w]		1H	bPb-2240	164.0	7.9	
			RDW[w]		2H	bPb-4261	43.9	6.5	
			RDW[w]		4H	EBmac635	135.7	3.4	
			RDW[w]		5H	bPb-0071	144.1	4.2	
			RDW[w]		7H	VrnH3	75.6	6.9	
			Root-shoot ratio (RSR)[w]	Post-harvest	1H	GBM1042	75.6	6.3	
			RSR[w]		1H	bPb-2240	67.9	8.3	
			RSR[w]		3H	bPb-9110	164.0	7.4	
			RSR[w]		5H	bPb-0071	166.8	3.2	
			RSR[w]		7H	VrnH3	144.1	6.6	
WQ2338/MA10-30	DH	134	Relative water content (RWC)[w]	Fourth leave	1H	Bmag770	107.5	n/a	Chen et. al. 2010
			RWC[w]		2H	M4249-346	69.3	n/a	
			Root lenth (RL)[w]	n/a	2H	M3549-138	74.5	n/a	
			RL[w]		5H	EBmac684	71.9	n/a	
Derkado/B83-12/21/5	DH	157	Total root system size (RSS)	n/a	3H	Bmag0606	163.6	n/a	Chloupek et al., 2006
			RSS	n/a	4H	mlo	184.0	n/a	
			RSS	n/a	7H	P25M42c	32.8	n/a	
Tadmor/Er/Apm	RIL	167	Water-soluble carbohydrate at 100% relative water content (DWSC100) $_i$	n/a	2H	HVM36	32.7	0.05	Diab et. al. 2004
			DWSC100 $_i$	n/a	2H	Bmag0125	98.5	0.08	
			DWSC100 $_i$	n/a	3H	CDO395	73.1	0.08	
			DWSC100 $_i$	n/a	3H	BM816463	97.8	0.11	
			DWSC100 $_i$	n/a	3H	BM817178	148.3	0.08	
			DWSC100 $_i$	n/a	7H	CaaaccQ	180.1	0.08	
			Osomotic potential (OP) $_i$	n/a	2H	HVM36	32.7	0.16	
			OP_i		3H	BM816463	96.9	0.08	
			Osmotic potential at full turgor (OP100) $_i$	n/a	2H	HVM36	64.	0.15	
			$OP100_i$		3H	MWG595	95.0	0.07	
			$OP100_i$		3H	Bmag0013	95.0	0.08	
			RWC[w]	n/a	3H	CDO1396A	133.5	0.05	
			RWC_i		3H	BM816463	99.5	0.09	
			RWC_i		5H	CDO484	193.5	0.08	
			RWC_i		7H	Acl3	109.2	0.05	

			RWC_i		7H	Acl3	125.7	0.05	
			Water-soluble carbohydrate (WSC)^w	n/a	2H	Bmag0125	96.5	0.15	
			WSC_i		2H	RZ828	83.0	0.08	
			WSC_i		3H	WG516	19.1	0.07	
			WSC_i		4H	MWG584	81.3	0.07	
			WSC_i		5H	CDO669	156.0	0.06	
			WSC_i		5H	CDO400	133.8	0.06	
			WSC_i		7H	CDO484	193.5	0.08	
			WSC_i		7H	WG940Bs	106.8	0.05	
			Water-soluble carbohydrate at full tugor (WSC100) ^{w}	n/a	2H	BCD1069	99.2	0.05	
			$WSC100^w$		2H	Bmag0125	96.5	0.11	
			$WSC100^w$		3H	BM816463	101.3	0.26	
			$WSC100^w$		3H	BM817178	148.3	0.09	
			$WSC100^w$		3H	BM817178	153.6	0.11	
			$WSC100^w$		7H	CaaaagB	194.2	0.04	
			$WSC100^w$		7H	HVCMA	102.5	0.05	
			$WSC100^w$		7H	BCD351B	91.1	0.06	
			$WSC100^w$		7H	Acl3	125.7	0.06	
Steptoe/Morex	DH	72	RWC_i	n/a	5H	ABC302	90.1	n/a	Siahsar & Narouei, 2010
			WSC_i	n/a	2H	ABC165	167.1	n/a	
Tadmor/Er/Apm	RIL	167	Osmotic adjustment (OA) ^{w}	n/a	4H	CDO541	100.0	n/a	Teulut et. al. 2000
			OA^w		5H	MWG502	17.9	n/a	
			WSC^w	n/a	2H	CDO588	82.9	n/a	
			WSC_i		5H	WG564	96.6	n/a	
			WSC_i		4H	CDO669	156.0	n/a	

[a] Type of population

[b] Number of lines within the population

[d] Trait of interest, root length (RL), root dry weight (RDW), root to shoot ratio (RSR), total root system size (RSS), leaf relative water content (RWC), accumulation of water-soluble carbohydrate at 100% relative water content (DWS100), leaf osmotic potential (OP), osmotic potential at full turgor (OP100), water-soluble carbohydrate (WSC), water-soluble carbohydrate concentration at full turgor (WSC100) and leaf osmotic adjustment (OA). ^{w} denotes that the plants have been grown under water-stress and $_i$ that the plants have been grown under irrigated conditions.

[c] Chromosomal location of markers based on projection onto DArT consensus map

[g] Peak chromosomal position of markers based on projection onto DArT consensus map

n/a in any field of the table denotes information not available