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Genetic mapping and physiological characterization of water-use efficiency in barley (*Hordeum vulgare* L.) on the Canadian Prairies

by

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Abstract

Temporal or seasonal water deficit is one of the major factors limiting crop yield on the Canadian Prairies. Empirical knowledge suggests that carbon isotope discrimination ($\Delta^{13}C$), through its negative relationship with water-use efficiency (WUE), is a good index for selecting crop varieties with stable yield in some rain-fed environments. Identification of quantitative trait loci (QTL) and linked markers for leaf $\Delta^{13}C$ will help select genotypes with improved WUE in breeding programs. This thesis research investigated the genetic and physiological determinants of $\Delta^{13}C$ variation in Canadian spring barley (*Hordeum vulgare* L.) and used two recombinant inbred line (RIL) mapping populations, including 200 RILs of W89001002003 × I60049 (six-row type) and 127 RILs of Merit × H93174006 (two-row type) to identify QTLs and their linked molecular markers for the trait.

The parental lines used to produce the mapping populations and several of the RILs maintained consistent ranking of leaf $\Delta^{13}C$ across years and in different experiments. The broad-sense heritability of leaf $\Delta^{13}C$ was 0.8, suggesting stability of this trait under the environments studied. Leaf $\Delta^{13}C$ was positively correlated with stomatal conductance (g_s) in both greenhouse and field experiments, suggesting that g_s caused most of the variation in leaf $\Delta^{13}C$. Low leaf $\Delta^{13}C$ genotypes such as 'CDC Cowboy' and RIL '147' achieved high WUE and yield by maintaining a high photosynthesis rate at a low g_s, which suggests that it is possible to select low $\Delta^{13}C$ genotypes that can maintain high yield under low moisture conditions.

Using two mapping populations and phenotypic data for leaf $\Delta^{13}C$ and agronomic traits collected from 4 different field environments, a total of 12 (six-row population) and 5 (two-row population) QTLs for leaf $\Delta^{13}C$ were detected. A transgressive segregation pattern for leaf $\Delta^{13}C$ was observed among RILs. For the six-row RILs, a major QTL for leaf $\Delta^{13}C$ co-located with several agronomic traits on chromosome 3H near SSR marker Bmag606 (9.3, 9.4 and 10.7 cM interval) was identified across environments. This marker when validated may be useful in breeding programs for improving WUE and yield stability of barley on the Canadian Prairies.

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List of Symbols and Abbreviations

A: assimilation rate

g_s: stomatal conductance

H^2: broad-sense heritability

$\Delta^{13}C$: carbon isotope discrimination

$\delta^{13}C$: carbon isotope composition

DArT: Diversity Arrays Technology

HI: harvest index

LAI: leaf area index

MAB: marker-assisted breeding

MAS: marker-assisted selection

PCR: polymerase chain reaction

QTL: quantitative trait loci

RILs: recombinant inbred lines

SD: segregation distortion

SLA: specific leaf area

SSD: single seed descent

SSR: simple sequence repeats

WUE: water-use efficiency

Chapter 1 General introduction

Introduction

Water deficit continues to be one of the major factors limiting crop production and productivity in many regions and a threat to food security in the 21st century (Tuberosa et al. 2002). As the world population continues to grow with future global climate change, how to make good use of limited water resources for crop production has become a worldwide concern (Barnabás et al. 2008; Polley 2002). Moreover, the frequency, duration and severity of drought are variable and unpredictable across seasons and locations, especially in arid and semi-arid regions, causing highly unstable crop yields between years and locations. To ensure sustainable food supply, breeding efforts should not only focus on maintaining yield, but more importantly on developing high yielding varieties suited for water-limited environments, i.e., to achieve more production per unit of available water, in other words, "more crop per drop" (Passioura 2006).

Breeding for drought-resistant and water-use efficient crop varieties has been a critical area of agricultural research worldwide. Substantial efforts have been devoted to identifying and selecting for morpho-physiological traits (such as early vigor, specific leaf area, relative water content, osmotic adjustment, etc.) that increase water-use efficiency (WUE) and yield under rain-fed conditions (Blum 1996; Richards 1996; Richards et al. 2002). Nevertheless, the complex underlying mechanisms of drought resistance and our lack of knowledge of the genetic and physiological bases of yield have hindered the breeding process in drought environments (Passioura 2002; Tuberosa and Salvi 2006). In the last decade, plant breeding has been greatly advanced by progress in stress physiology and genomics (Araus et al. 2003; Habash et al. 2009; Ishitani et al. 2004; Morgante and Salamini 2003). For example, the biomass and WUE of transgenic wheat were improved under water deficit conditions by introducing group 3 late embryogenic abundant (LEA) proteins encoded by the barely *HVA1* gene

(Sivamani et al. 2000). A gene (*HARDY*) identified from *Arabidopsis* improved WUE in rice by enhancing photosynthetic assimilation and reducing transpiration (Karaba et al. 2007). Another example is represented by the studies in WUE using carbon isotope discrimination ($\Delta^{13}C$). The $\Delta^{13}C$ has been demonstrated to be a simple but reliable measure of WUE, and the negative correlation between them has been used as an indirect method for the selection of C_3 crops with improved WUE under selected environments (Cattivelli et al. 2008). However, screening for $\Delta^{13}C$ by mass spectrometry remains costly (Teulat et al. 2002).

Drought resistance is a complex trait that is governed by quantitative trait loci (QTL), and QTLs underlying traits such as flowering time, plant height, and $\Delta^{13}C$ may play important roles in plant adaptation to water-limited environments (Forster et al. 2004; Tuberosa and Salvi 2004b). The advancement of DNA-based molecular markers and computational methods in the late 1980s and 1990s has revolutionized the dissection of quantitative trait inheritance and genetic improvement of yield in dry environments (Baum et al. 2007). The QTL mapping and analysis provides unprecedented opportunities to identify and locate chromosome regions controlling adaptive traits such as $\Delta^{13}C$ during plant growth under drought-prone conditions (Cattivelli et al. 2008), and selection efficiency of these traits would be enhanced with a better understanding of their genetic controls. QTLs for $\Delta^{13}C$ have been reported in *Arabidopsis thaliana* (Hausmann et al. 2005; Juenger et al. 2005), barley (*Hordeum vulgare* L.) (Diab et al. 2004; Ellis et al. 2002; Ellis et al. 1997; Teulat et al. 2002), cotton (*Gossypium hirsutum* and *Gossypium barbadense*) (Saranga et al. 2001), rice (*Oryza sativa* L.) (Laza et al. 2006; Takai et al. 2006; This et al. 2010; Xu et al. 2009), soybean (*Glycine max* L.) (Specht et al. 2001), tomato (*Lycopersicon esculentum* and *L. pennellii*) (Martin and Nienhuis 1989; Xu et al. 2008), bread wheat (*Triticum aestivum* L.) (Rebetzke et al. 2008) and durum wheat (*Triticum turgidum* ssp. *dicoccoides*) (Peleg et al. 2009). The application of marker-QTL-trait association in marker-assisted selection (MAS) and marker-assisted breeding (MAB) has been regarded as a promising way to develop cultivars with improved tolerance to abiotic stress

(Gupta and Varshney 2004; Tuberosa and Salvi 2004b). MAS for a target trait such as leaf $\Delta^{13}C$ in barley at the vegetative stage will speed up the breeding process and reduce the cost for measurements of $\Delta^{13}C$ as compared with the conventional plant breeding methods.

However, QTL mapping only represents the first step towards detecting genes affecting traits of interest. The ultimate goal is to assign functions to genes and manipulate either a single gene or pyramiding beneficial QTL alleles through MAS, which will speed up plant breeding in water-limited environments aimed at yield improvement. To further unravel the molecular basis of QTL for a target trait, two approaches are usually deployed: positional cloning (also termed map-based cloning or recombinational mapping) and association mapping (Tuberosa and Salvi 2004a). Xu et al. (2008) fine-mapped a QTL for $\delta^{13}C$ (designated QWUE5.1) in tomato to an interval about 2.2 cM long, which is valuable for cloning the genes underlying QWUE5.1 and eventually MAS of QWUE5.1 in the tomato breeding programs. Comparative analysis of QTL results also provides valuable opportunities for positional cloning and to identify candidate genes for target traits such as WUE in cereals through exploration of related species using sequence co-linearity from small (such as rice) to large genomes (such as wheat) (Sorrells et al. 2000). The most promising breeding strategy of improving WUE in cereals lies in the integration of QTL mapping, genetic engineering, and conventional breeding.

Thesis research background

Barley currently ranks fourth among cereals in worldwide production after maize (*Zea mays* L.), rice and wheat (FAOSTAT 2008). Almost two-thirds of the global barley production is used for livestock feed, and the remaining plays important roles in producing malt beverages and providing human diet (Schulte et al. 2009). According to the Foreign Agricultural Service (2009), Canada is the fourth largest producer of barley in the world, after the European Union, Russia, and Ukraine,

with an average of 12.3 million tonnes produced annually during 1986 – 2006 (FAOSTAT 2008). However, barley production in Canada has remained relatively stagnant since the 1980s (Rosario 2009). Agriculture production in Canada is mostly concentrated on the Western Prairies (Canadian International Grains Institute 2004). The production of barley is mainly in the three Prairie Provinces (Alberta, Saskatchewan and Manitoba), among which Alberta accounts for about 50% of annual production (Statistics Canada 2007). Barley can be classified into either two-row or six-row types, with the type controlled by a major recessive gene *vrs1* on chromosome 2H (Gottwald et al. 2009). Both two- and six-row ear types of barley are commonly grown under rain-fed conditions for malting, livestock feed and food in Canada. Sixty to seventy percent of the barley growing area in Western Canada is seeded for malting barley, and two-row barley accounts for two-thirds of the malting barley production (Canadian International Grains Institute 2004).

The growth condition on the Canadian Prairies is characterized by short and dry growing seasons, sometimes with terminal heat stress (Anyia et al. 2008). The average precipitation during the growing season ranges from over 300 mm in West-Central Alberta to less than 200 mm in Southern Alberta (Bonsal et al. 1999). Barley growth relies on stored soil moisture and limited within season rainfall, and the unpredictable occurrence of drought causes highly unstable barley yield across years. For example, severe soil moisture deficits across Alberta reduced barley yield to 2.6 million tonnes in 2002 compared to an average of 5.6 million tonnes in a normal year (Statistics Canada 2007). Therefore breeding for drought tolerant and water-use efficient varieties has been a critical area of barley research in Canada.

As proposed by Passioura (1977), crop yield is a function of WU (water use through evapotranspiration), WUE (biomass growth per unit water transpired), and HI (harvest index, i.e., the ratio of grain yield to aboveground biomass) under water-limited environments. Accordingly, barley yield on the Canadian Prairies can be improved by increasing: 1) the capacity to capture more stored soil

moisture, either through improving soil water extraction ability or decreasing soil evaporation; 2) the ability to produce more dry matter per unit of water used; and 3) the ability to transfer more assimilates into economic yield (Araus et al. 2002; Turner 2001). Jedel and Helm (1994a; 1994b) evaluated twenty spring barley cultivars released in Western Canada from 1910 to 1987 and concluded that grain yield of barley increased by 12.7 to 41.4 kg ha^{-1} yr^{-1}, with increased HI of 0.08 to 0.17% per year and decreased plant height by 0.14 to 0.29 cm yr^{-1}.

Cereals have achieved great yield improvement through increasing HI by reducing plant height, but the ceiling for genetic increases in crop yields based on HI is likely being approached (Richards et al. 2002). Most of the research that has been conducted on the Canada Prairies has focused on improving crop WUE through soil management practices such as no tillage systems (Cutforth and McConkey 1997; Cutforth et al. 2002; Gan et al. 2000). However, the most economic and effective way to increase grain yield under water deficit environments is to develop varieties with improved WUE. So far, there are few ongoing research on barley WUE in Canada as summarized by Brophy (2011), and represented by Dr. Anyia's project at Alberta Innovates - Technology Futures and Dr. Nyachiro's project at Field Crop Development Centre (Lacombe).

The measurements of WUE (i.e., the ratio of dry matter production to the amount of water transpired) are labor intensive, time-consuming and expensive, which limits its use in breeding programs especially under field conditions. Using $\Delta^{13}C$ as a selection index for WUE, two high-yielding wheat genotypes have been released in Australia (Condon et al. 2004; Rebetzke et al. 2002). Canadian breeders have to explore new breeding strategies such as using $\Delta^{13}C$ as a selection criterion for improving WUE to develop barley lines with improved adaptation to drought conditions on the Canadian Prairies. Selection efficiency of $\Delta^{13}C$ would be enhanced with a better understanding of its genetic controls. The development of molecular markers diagnostic of QTLs controlling physiological traits related to yield potential can improve selection efficiency of yield through MAB (Anyia et al. 2008).

Barley is a diploid organism with seven chromosomes containing a large size genome (5,300 Mbp), but it still is an excellent model for genomic mapping and genetic study in cereal crop development since it has a less complex structure as compared with other species in the tribe Triticeae that includes wheat, rye (*Secale cereale* L.), and Triticale (× *Triticosecale*) (Schulte et al. 2009; Sorrells et al. 2000). The first linkage map in barley was constructed using restriction fragment length polymorphism (RFLP) markers (Graner et al. 1991). Simple sequence repeats (SSR) markers or microsatellites have been used in many studies for identifying QTLs. SSR markers have been proved to be useful in barley genetic studies as they are abundant with good genome coverage, greatly polymorphic, co-dominantly inherited, multi-allelic, easy to be amplified by polymerase chain reaction (PCR), and are amenable to later high-throughput analysis (Becker and Heun 1995; Li et al. 2003; Liu et al. 1996; Saghai Maroof et al. 1994; Struss and Plieske 1998; Varshney et al. 2007). Another promising type of markers commonly used are DArT (Diversity Arrays Technology) which can enable whole-genome profiling without relying on sequence information (Wenzl et al. 2004). This thesis research used SSRs and DArT markers to identify QTLs and linked markers for leaf $\Delta^{13}C$ of barley. The research will provide new tools to barley breeding programs in Canada, and beyond that, new tools from this study when validated can be used in MAB of new barley varieties for improved WUE.

The thesis research is part of Dr. Anyia's project towards the breeding of new barley lines with improved WUE and yield stability under conditions of low moisture availability on the Canadian Prairies. Dr. Anyia's project was initiated in 2005, with 106 diverse barley genotypes screened for variation in leaf $\Delta^{13}C$ at two field locations in Vegreville and Lacombe. Genotypes showing contrasting levels of leaf $\Delta^{13}C$ (low versus high) in the greenhouse experiments and field trials were selected as potential parental materials for mapping population development. Two recombinant inbred line (RIL) mapping populations derived from bi-parental crosses (W89001002003 × I60049, 6-row type and Merit ×

H93174006, 2-row type) have been developed using the single seed descent (SSD) approach since 2006. Anyia et al. (2007) suggested the stability of leaf $\Delta^{13}C$ across environments based on previous evaluations, but the genetic control and physiological characterization of leaf $\Delta^{13}C$ need to be further investigated.

Research objectives

The overall goal of this thesis research was to study the genetic and physiological determinants of $\Delta^{13}C$ variation in barley towards identifying the QTL and their linked molecular markers for the trait that can be used in a marker-assisted selection approach for the breeding of elite barley varieties with improved WUE.

The specific objectives were to determine: 1) how would the leaf $\Delta^{13}C$ of barley change across ear types (two-row versus six-row) under multiple locations and years (the combinations of those two factors constitute different envieonments); 2) how would the physiological (leaf gas exchange parameters, leaf $\Delta^{13}C$ and WUE) and agronomic performance (aboveground biomass, grain yield and HI) of selected barley genotypes with contrasting levels of $\Delta^{13}C$ differ under well-watered and water-deficit conditions in greenhouse experiments; 3) what are the relationships among aboveground biomass, grain yield, HI and leaf $\Delta^{13}C$ under field conditions; 4) how many QTLs are involved in leaf $\Delta^{13}C$ variations and what are the magnitudes of their effects; and 5) whether the common QTL regions are stable across environments and populations.

Hypotheses

1) The rankings for leaf $\Delta^{13}C$ among barely genotypes are expected to be stable across years and locations.
2) The value of $\Delta^{13}C$ is higher for six-row than for two-row barley.
3) A low value of leaf $\Delta^{13}C$ due to reduced stomatal conductance (g_s) or increased assimilation rate (A) is expected, and the difference between the selected barley genotypes with contrasting levels of $\Delta^{13}C$ is intrinsic.

4) A negative correlation between $\Delta^{13}C$ and grain yield is expected on the Canadian Prairies.
5) Leaf $\Delta^{13}C$ in barley is controlled by a few QTLs with major effects.
6) The location and effects of QTLs for leaf $\Delta^{13}C$ are stable across locations, years and populations.

Thesis structure

This thesis includes six chapters. Chapter 1 provides general information about WUE, $\Delta^{13}C$ and related QTL research, and introduces the thesis research background. Chapter 2 reviews the research progress in WUE, $\Delta^{13}C$, and gene discovery through QTL and expression analysis in WUE in cereals. Chapter 3 evaluates the stability of leaf $\Delta^{13}C$ as a measure of WUE in barley under rain-fed conditions on the Canadian Prairies, and examines the physiological responses of barley genotypes to water treatments under greenhouse conditions. Chapter 4 studies the genetics of $\Delta^{13}C$ and identified the number, location and effect of QTLs for leaf $\Delta^{13}C$ under drought prone environments on the Canadian Prairies. Chapter 5 evaluates the physiology and $\Delta^{13}C$ of selected barley lines with contrasting levels of leaf $\Delta^{13}C$ (high versus low) under well-watered and water-deficit conditions in a greenhouse study. The last chapter (Chapter 6) provides a synthesis of WUE research and makes general conclusions and recommendations for future research.

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Chapter 2 Literature review: Gene discovery in cereals through QTL and expression analysis in water-use efficiency measured by carbon isotope discrimination

Physiological traits related to drought adaptation

Periods of soil and/or atmospheric water deficits pose critical constraints on plant survival and productivity (Boyer 1982), especially under future climate-change scenarios (Petit et al. 1999). Generally, for coping with water scarcity plants have evolved different mechanisms, such as escape, avoidance and tolerance, to ensure their survival and reproduction (Chaves et al. 2003; Turner 1986). The escape strategy could be attained by changing the phenology and growth rate before the onset of water deficit, such as shorter life cycles (Barnabás et al. 2008; Chaves et al. 2003). Dehydration avoidance is achieved by maintaining relatively high tissue water potential during drought stress, either by minimizing water loss (e.g., stomatal closure, reduced leaf area, leaf rolling, senescence of older leaves, etc.) or maximizing water uptake (e.g., increased root growth, increased hydraulic conductance, etc.) (Jackson et al. 2000; Mitra 2001). Dehydration tolerance is the plant's ability to withstand severe water deficit, maintain function at low water potential, recover water status and resume full function once water becomes available; this mechanism involves osmotic adjustment (increased solute concentration), increased cell elasticity or decreased cell size (Mitra 2001). Associated with those strategies, plants exhibit multiple adaptations or changes at developmental, phenological, morphological, biochemical, and physiological levels. Physiological traits that have been intensively investigated include osmotic adjustment, osmotic potential, water soluble carbohydrates, stomatal conductance, canopy temperature, relative water content, transpiration efficiency,

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WUE, $\Delta^{13}C$, early vigor, leaf area index, stomatal density, flowering time, root characteristics and so on (Baum et al. 2007).

Genetic gain is limited when directly selecting for increased grain yield under water-limited environments due to a large interaction between genotype and environment and a low heritability of yield components. However, the encouraging heritability of physiological or secondary traits that are highly correlated with yield presents a good opportunity for plant breeding in drought-prone regions (Stiller et al. 2005). WUE is a trait that has been proposed as a criterion for yield improvement under drought conditions (Condon and Richards 1992; Condon et al. 2002; Rebetzke et al. 2002; Richards et al. 2002).

Water-use efficiency in crops

The term WUE may be defined in different ways (Table 2.1). At the photosynthetic scale (leaf-level), instantaneous WUE ($WUE_{instantaneous}$ or WUE_{is}), also referred to as transpiration efficiency (TE), is generally measured as the net amount of carbon assimilated (A) per unit water transpired (transpiration rate, E) during the same period (Bacon 2004; Condon et al. 2002; Farquhar et al. 1989; Farquhar and Richards 1984; Polley 2002). A similar parameter, intrinsic WUE ($WUE_{intrinsic}$ or WUE_{ic}) is defined as the ratio between A and stomatal conductance (g_s) (Choi et al. 2007; Hall et al. 1992), which is thought to be more closely associated with physiological responses due to independency of specific environmental conditions. The following equation gives the relationship between A/E and A/g_s (Farquhar and Richards 1984).

$$\text{WUE}_{is} \text{ or TE} = \frac{A}{E} = \frac{g_c(c_a - c_i)}{g_w(w_i - w_a)} = \frac{1}{\Delta W} \times \frac{A}{g} = \frac{c_a(1 - \frac{c_i}{c_a})}{1.6\Delta W} \qquad (1)$$

Where

c_a and c_i are atmospheric and leaf intercellular CO_2 concentrations,

g_c and g_w are stomatal conductance to diffusion of CO_2 and water vapor, and

($w_i - w_a$) or $_{\Delta}w$ and ($c_a - c_i$) refer to the concentration gradients of water vapor and CO_2 between the outside (w_a, c_a) and the inside (w_i, c_i) of leaf, and 1.6 is the relative diffusivities of water vapor to CO_2 in air.

Equation (1) indicates that WUE_{is} or TE and WUE_{ic} are negatively correlated with the ratio of c_i to c_a. A greater WUE at the leaf-level can be achieved through a lower value of c_i/c_a either by decreasing g_s, or increasing A, or a combination of both (Condon et al. 2002; Polley 2002).

For agronomists and plant breeders, WUE is typically calculated as the accumulated dry matter produced divided by the amount of water consumed by the crop during the whole growth cycle ($WUE_{integrative}$ or WUE_{ie}) (Condon et al. 2004; Tuberosa et al. 2007).

$$WUE_{ie} = \frac{TE}{1+E_S/T} \qquad (2)$$

Where

TE is the transpiration efficiency (aboveground dry matter/transpiration),

E_s is the water lost by evaporation from the soil surface, and

T is water lost through transpiration by the crop.

In the above equation, the denominator could include water loss from run-off during heavy rainfall events and deep drainage below the root zone, depending on the cropping system (Condon et al. 1993; Gregory et al. 1997). From Equation (2), WUE_{ie} could be improved either by increasing TE or maximizing T by reducing E_s through agronomic management practices (Gregory et al. 1997; Passioura 2006; Richards et al. 2002).

Usually the real-time leaf parameters of WUE are measured by gas exchange methods, i.e., infrared gas analyzers and porometers (Long et al. 1996). Estimations of WUE_{ie} might be performed in greenhouse and growth chamber pot studies by minimizing soil evaporation (Lambrides et al. 2004; Turner et al. 2007). Accurate water budget and crop biomass measurements required for WUE estimation is labor intensive, time-consuming and expensive and therefore unattractive to plant breeders, especially under field conditions for large

populations. The WUE calculated as grain yield divided by water supply (kg ha^{-1} mm^{-1} or kg m^{-3}) may be underestimated because it assumes that the same amount of water is transpired by each genotype.

Carbon isotope discrimination as a measure of water-use efficiency

While it is widely recognized that improved WUE can enhance yield in certain environments, the use of the WUE trait in breeding programs has largely been limited due to the lack of a suitable screening method in large populations. A promising screening method for WUE came into the picture in the 1980s when carbon isotopic techniques were introduced (Farquhar et al. 1982; O'Leary 1981).

Approximately 1.1% of the carbon in the biosphere naturally occurs in the form of the stable isotope ^{13}C (Condon et al. 2002; Farquhar et al. 1989; O'Leary 1981). However, the molar abundance ratio of $^{13}C/^{12}C$ (R) in plant tissues usually is less than that in atmospheric CO_2 due to discrimination against the "heavier" ^{13}C (lower reactivity) during photosynthesis (Farquhar et al. 1989; Farquhar et al. 1982; Farquhar and Richards 1984; O'Leary 1981). Plants with different metabolic pathways of carbon assimilation exhibit characteristically different discrimination against ^{13}C when incorporating CO_2 into plant tissues. The isotope discrimination commonly involves diffusion of CO_2 across atmospheric boundary layer and stomata into leaf mesophyll, its inter-conversion of dissolved CO_2 and HCO_3^-, and enzymatic incorporation of CO_2 into carbohydrates by ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco) in plants with conventional (C_3) pathway (Farquhar et al. 1982; Hattersley 1982; O'Leary 1981) .

Isotope compositions are usually determined by an online continuous-flow stable isotope ratio mass spectrometer (IRMS). The ratio of $^{13}C/^{12}C$ in a sample of plant is converted to $\delta^{13}C$ (carbon isotope composition) commonly compared with a reference material, the belemnite carbonate standard (PDB) from the Pee Dee Formation in South Carolina (Craig 1953; O'Leary 1981; Ober et al. 2005). The stable carbon isotope composition of plant samples is calculated as:

$$\delta^{13}C\ (‰) = [\frac{R_{sample}}{R_{standard}} - 1] \times 1000 \qquad (3)$$

Where

R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratios measured in the plant material and the standard.

On the PDB scale, the $\delta^{13}C$ value for free atmospheric CO_2 currently is approximately -8‰ (Farquhar et al. 1989), and this value is becoming slightly more negative over time due to the increasing combustion of fossil fuels (O'Leary 1981). According to Feng (1998), $\delta^{13}C_{air}$ has decreased from -7.4‰ to -8.2‰ from 1976 to 2003. The $\delta^{13}C$ of plants is a negative value because the $^{13}C/^{12}C$ ratio in the atmosphere is less than that in PDB, and also there is a net discrimination against $^{13}CO_2$ during diffusion and carboxylation by plants (Condon et al. 2002; Farquhar et al. 1982). The amount of carbon isotope discrimination is generally expressed as:

$$\Delta^{13}C\ (‰) = \frac{R_{air}}{R_{plant}} - 1 = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}/1000} \qquad (4)$$

Where

R_{air} and R_{plant} refer to $^{13}C/^{12}C$ ratios of the atmosphere and plant samples.

Plants show positive values of $\Delta^{13}C$ and typically C_3 plants have a discrimination rate around 20‰ during photosynthesis (Farquhar et al. 1989; Farquhar et al. 1982; O'Leary 1981).

Ignoring dark respiration and photorespiration, assuming that the major components contributing to the final discrimination are gaseous diffusivities across the boundary layer and stomata (*a*) and the fractionation by Rubisco during the carboxylation (*b*), an approximate expression for the overall $\Delta^{13}C$ in leaves for C_3 plants during photosynthesis has been developed and described as (Farquhar et al. 1989):

$$\Delta^{13}C = a\frac{C_a - C_i}{C_a} + b\frac{C_i}{C_a} = a + (b - a)\frac{C_i}{C_a} \qquad (5)$$

Where

a and *b* in Equation (5) are as mentioned above.

Equation (5) shows that $\Delta^{13}C$ is positively related to the c_i/c_a ratio, which was therefore negatively correlated with WUE_{is} or TE as expected from Equation (1) (Farquhar et al. 1989; Farquhar et al. 1982; Farquhar and Richards 1984; Hubick and Farquhar 1989; O'Leary 1981). Farquhar and Richards (1984) first reported that the extent to which plants discriminate against the ^{13}C during gas exchange was negatively correlated to WUE in a greenhouse experiment with wheat grown in large pots.

Currently $\Delta^{13}C$ is widely used as an indirect assessment of WUE in C_3 crops under water-limited conditions. Extensive studies in C_3 species have been reported and have confirmed the negative relationship between $\Delta^{13}C$ and WUE (Condon et al. 1990; Hall et al. 1992; Hubick and Farquhar 1989; Hubick et al. 1986; Ismail and Hall 1992; Khan et al. 2007; Lambrides et al. 2004; Rebetzke et al. 2002; Rytter 2005). This relationship in C_3 plants has opened up the prospect of utilizing differences in ^{13}C discrimination for selecting crops that have high WUE under specific environments.

Sufficient genotypic variation, stability across environments, and a high broad-sense heritability (H^2) in $\Delta^{13}C$ indicate that it is a promising surrogate for WUE that can be applied in breeding programs (Araus et al. 1998; Condon and Richards 1992; Johnson and Rumbaugh 1995; Merah et al. 2001; Rebetzke et al. 2008; Schuster et al. 1992). The high $H^2$of $\Delta^{13}C$ has been previously reported in barley (*Hordeum vulgare* L.) (Çağirgan et al. 2005; Voltas et al. 1998), cotton (*Gossypium hirsutum* L.) (Stiller et al. 2005), cowpea (*Vigna unguiculata* L. Walp.) (Hall et al. 1990), peanut (*Arachis hypogaea* L.)(Hubick et al. 1988), soybean (*Glycine max* L.) (Specht et al. 2001), bread wheat (Condon and Richards 1992; Ehdaie et al. 1991; Rebetzke et al. 2008) and durum wheat (*Triticum durum* Desf.) (Araus et al. 1998). Non-significant interactions between genotype and environment for $\Delta^{13}C$ have been reported for peanut (Hubick et al. 1988), sugar beet (*Beta vulgaris* L.) (Rajabi et al. 2009) and wheat (*Triticum turgidum* ssp. *dicoccoides*) (Peleg et al. 2009).

However, $\Delta^{13}C$ is not widely applicable to species with C_4 and crassulacean acid metabolism (CAM). CAM plants exhibit either C_3 or time-separated C_4 fixations, showing large variations in isotopic compositions (O'Leary 1981). The mechanisms for $\Delta^{13}C$ changes in C_4 plants are complex, involving HCO_3^- fixation in oxaloacetate by the phosphoenolpyruvate carboxylase enzyme (PEPC), CO_2 release in the bundle-sheath cells and refixation by Rubisco (Farquhar *et al*. 1989). The major difference in $^{13}C/^{12}C$ ratio between C_3 and C_4 plants is the isotopic fractionation activity between Rubisco and PEPC. PEPC fixes HCO_3^- which is ^{13}C-enriched compared to CO_2 and a proportion of carbon fixed by PEP carboxylation that subsequently leaks out of the bundle sheath which reduces the Rubisco discrimination against ^{13}C. Average $\Delta^{13}C$ is around 4‰ in C_4 plants (Delúens et al. 1983). Monneveux et al. (2007) explored the possibility of using $\Delta^{13}C$ as a selection criterion for yield under drought in maize (*Zea mays* L.), with $\Delta^{13}C$ analyzed in different organs at flowering stage under both drought and irrigated conditions. However, they didn't find any correlation between $\Delta^{13}C$ and grain yield within tolerant hybrids, probably because variation of $\Delta^{13}C$ is less affected because c_i/c_a is more stable under drought conditions. Cabrera-Bosquet et al. (2009b) reported that there was no significant relationship between either leaf or kernel $\Delta^{13}C$ and grain yield. Further research is needed for understanding the relationship between $\Delta^{13}C$ and WUE in species with C_4 metabolism.

Although $\Delta^{13}C$ has been intensively exploited as an integrated criterion for screening improved WUE and thus greater productivity in C_3 crops under water-limited environments, there are some challenges associated with the application of $\Delta^{13}C$ in plant breeding programs.

(1) $\Delta^{13}C$ provides a long-term average estimate of cumulative WUE integrating in time and space without giving any information about the change in WUE as a result of altered A or g_s or both (Condon et al. 2002).

(2) The relationship between $\Delta^{13}C$ and grain yield or biomass is either positive, negative or neutral, depending on the season, location and species (Anyia et

al. 2007; Chen et al. 2011; Condon et al. 2004; Jiang et al. 2006; Tambussi et al. 2007). Positive or neutral relationships between $\Delta^{13}C$ and grain yield or biomass are often reported in environments characterized with plentiful within-season rainfall or supplemental irrigation, such as wheat and barley grown under Mediterranean climates (Araus et al. 2003; Condon et al. 1993; Jiang et al. 2006; Merah et al. 1999; Teulat et al. 2002; Teulat et al. 2001; Voltas et al. 1999), while negative relationships are found in environments where crop relies heavily on stored soil moisture (Anyia et al. 2007; Condon et al. 1993; Rebetzke et al. 2002). However, $\Delta^{13}C$ values were not a reliable predictor under severe stress (Jiang et al. 2006).

(3) Timing for sampling can be difficult to determine. Plant materials could be collected during different developmental stages, such as vegetative phase or maturity. Age and stress can affect $\Delta^{13}C$ variation in plants at different times (Francey et al. 1985). For example, $\Delta^{13}C$ of rice was reduced more at tillering than at flowering and maturity when subjected to water stress (Zhao et al. 2004). Condon and Richards (1992) proposed that it would be most effective to assess $\Delta^{13}C$ at early stages in plant development under well-watered conditions.

(4) Which part of the plant should be collected for $\Delta^{13}C$ analysis? Plant samples can be collected from root, leaf, sheath, awn or grain, each characterized with its own $\Delta^{13}C$ value. Jiang et al. (2006) reported that $\Delta^{13}C$ were the highest in flag leaf, intermediate in awn, and lowest in grain in barley. Zhao et al. (2004) also found that in rice root and grain had the lowest $\Delta^{13}C$ values, and stem the highest. Different parts have their own potential advantages. Leaves sampled for $\Delta^{13}C$ at the stem elongation stage, when there is usually little drought stress and low vapour pressure deficit, could reflect the integrated WUE during vegetative development and formation of yield potential (Anyia et al. 2008; Chen et al. 2011; Condon and Richards 1992). In addition, leaf $\Delta^{13}C$ measured before maturity can enable selection and crosses to be made within the same season thereby speeding up the breeding

process. However, grain $\Delta^{13}C$ was preferred in many studies under Mediterranean-type environments due to its positive relationship with yield (Condon et al. 2004).

(5) Location conditions can have a marked effect on $\Delta^{13}C$. The $\delta^{13}C$ values can be more negative in greenhouse than in field studies due to the contribution of respired CO_2 (O'Leary 1981).

(6) Genotypic difference introduces another level of complexity in the use of $\Delta^{13}C$ for evaluating WUE. Most of the studies carried out in cereals showed substantial $\Delta^{13}C$ variation in genotypes that differed in flowering time and plant height, and those two characteristics strongly affected yield and generated complex associations between $\Delta^{13}C$ and productivity (Condon et al. 2004).

(7) Large amounts of carbon lost by photorespiration in C_3 plants could affect the final $\Delta^{13}C$ value in plant tissues. Leaf WUE usually was underestimated without including respiration rates (Tambussi et al. 2007). Water isotopes such as oxygen and hydrogen isotope compositions (expressed as $\delta^{18}O$ and δ D) in plant tissues might be alternative indicators of TE (Farquhar et al. 2007). The complementary measurement "packages" of stable isotopes such as hydrogen, carbon and oxygen in plant substrates and material could provide more insights to the physiological and biochemical responses of plants to water deficit, such as combining $\Delta^{18}O$ and $\Delta^{13}C$ to assess plant growth and total transpiration (Cabrera-Bosquet et al. 2009a).

Breeding for improved water-use efficiency

There is an increasing urgency in plant breeding for improved crop yield potential and better adaptation to current and future prolonged aridity (Araus et al. 2002). Great progress in major cereals has been made through empirical (also termed conventional or traditional) breeding programs during the last 50 years by directly selecting a primary trait (such as grain yield), however, progress in traditional

breeding has been slow due to the variable nature of drought and the complexity of drought resistance mechanisms (Araus et al. 2008). Selection for a secondary trait which is putatively related to a higher yield potential or a limiting yield factor is called analytical, physiological or indirect breeding, which has been very popular (Baum et al. 2007).

A framework based on maximizing grain yield instead of survival was proposed by Passioura (1977). Under water-limited environments, crop yield (CY) is a function of water use (WU, evapotranspiration), WUE (water truly transpired for biomass growth), and harvest index (HI, i.e., the ratio of grain yield to aboveground biomass):

$$CY = WU \times WUE \times HI$$

Water use or evapotranspiration includes crop transpiration and soil evaporation. Accordingly, crop yield in dry environments can be improved by increasing: 1) the capacity to capture more water, either through improving soil water uptake ability or decreasing soil evaporation; 2) the ability to produce more dry matter per unit of water used; and 3) the ability to deliver more assimilates into economic yield (Araus et al. 2002; Turner 2001). None of these components is completely independent, and improvement in any of the components could potentially increase crop yield (Araus et al. 2002; Condon et al. 2004; Richards et al. 2002; Tambussi et al. 2007).

Richards et al. (2002) pointed out that water use is a function of evaporative demand and leaf area. Mediterranean environments are typically characterized by frequent rainfall during vegetative growth and terminal drought during grain filling, and therefore reducing soil evaporation can provide benefit. Early germination, rapid seedling establishment, and good canopy cover (i.e., early or seedling vigor) together with higher specific leaf area (SLA) have been suggested to play an important role in reducing soil evaporation under Mediterranean-type environments (Tambussi et al. 2007). Genotypes with good early vigor tend to have deep rooting systems and exhibit great soil water extraction capacity (Turner and Asseng 2005), which is an attractive trait for

effective use of water under most drought conditions (Blum 2009). For winter-grown crops that rely on stored soil water, restricted leaf area reduces transpiration and conserves soil moisture thereby contributes more water for late grain filling (Rebetzke et al. 2002).

Basically a high HI for a given genotype sets its genetic potential for high yield (Cattivelli et al. 2008). According to Richards et al. (2002), the achievement of a high HI depends on the balance between pre-anthesis and post-anthesis water use under rain-fed conditions. Cereals have achieved great yield improvement through increasing HI by reducing plant height, a process primarily due to the introgression of semi-dwarfing genes since the second half of the last century (Richards et al. 2002; Slafer et al. 1994; Zhang 2007). However, the ceiling for genetic increases in crop yields based on HI is likely being approached (Mann 1999; Richards et al. 1993; Richards et al. 2002).

Target at genetic improvement of WUE has long been attractive because it is potentially related to the other two components (Condon et al. 2004; Tambussi et al. 2007). Richards et al. (2002) also pointed out that selection for specific physiological and morphological traits in water-limited environments, such as a high WUE, could increase the rate of yield improvement. For example, two wheat cultivars, "Drysdale" and "Rees", have been commercially released in Australia during 2002 and 2003 that were selected for improved WUE based on their low $\Delta^{13}C$, and they have demonstrated a yield advantage compared with high $\Delta^{13}C$ lines in environments with lower rainfalls (Rebetzke et al. 2002; Richards 2006). Breeding of hybrids in sunflower (*Helianthus annuus* L.) for high-yielding cultivars using $\Delta^{13}C$ is currently in progress (Lambrides et al. 2004; Richards 2006). Studies with sunflower by Lambrides et al. (2004) suggest that there is a good potential for breeders to develop sunflower germplasm with improved WUE using $\Delta^{13}C$ as a selection tool. In field evaluations, low-$\Delta^{13}C$ hybrids of sunflower significantly out-yielded high-$\Delta^{13}C$ hybrids in three of the four environments studied by Condon et al. (2004).

Although improved WUE and drought resistance without yield penalty offers a promising way to sustainable agricultural production and land use (Karaba et al. 2007), the application of WUE in plant breeding has been a subject of controversy. Blum (2005) has argued that selection for high WUE will result in small or early flowering plants, which achieved high WUE mainly through reducing water use without increasing yield. However, WUE is a ratio of yield to water use, and the different rates of reduction in these two components provide chances for manipulation under drought conditions (Blum 2005). A proportional change in both A and g_s might have no effect on WUE_{ic}, while a comparable change in A with g_s remaining constant would cause a substantial variation in WUE_{ic}, and vice versa. As proposed by Flexas et al. (2010), improved WUE could potentially be achieved through two possible approaches: 1) to increase CO_2 diffusion to the carboxylation sites by maintaining g_s, which could be attained by increasing mesophyll conductance to CO_2 (g_m); and 2) to improve the Rubisco carboxylation efficiency, which could be realized by introducing carboxylase enzyme from other species. Centritto et al. (2009) reported that rice genotypes with inherently higher g_m were capable of maintaining higher A under water-deficit conditions. Galmés et al. (2011) reported that a tomato cultivar 'Tomàtiga de Ramellet' with drought resistance displayed higher WUE_{ic} under water-deficit conditions, which was positively correlated with g_m/g_s. So far, there are no reports about increasing WUE by genetically improving the biochemistry of photosynthesis, which is still possible given the rapid development of biotechnology and genetic engineering tools.

Although the relationship between $\Delta^{13}C$ and grain yield is not consistent across seasons, sites and species (Anyia et al. 2007; Chen et al. 2011; Condon et al. 2004; Jiang et al. 2006; Tambussi et al. 2007), there are still plenty of opportunities for WUE selection. High $\Delta^{13}C$ or low WUE cereal genotypes could be beneficial in Mediterranean, terminal-drought environments (Condon et al. 2004). In these types of environments, plants rely on current rainfall and wide stomatal opening is needed to transpire as much water as possible and to maintain

growth when there is abundant rainfall. For stored-moisture environments such as eastern Australia and the Canadian Prairies, yield improvements through a combination of high WUE and greater early vigor are suggested (Anyia et al. 2008; Condon et al. 2002). Under these environments, genotypes achieve high WUE mainly by reductions in stomatal conductance, and thus conserve soil moisture during the vegetative growth stage for use in post-anthesis growth (Turner and Asseng 2005).

QTL analysis for water-use efficiency

In general, continuous genetic variation underlying quantitative traits such as yield, plant height, flowering time, WUE and so on that are generally under considerable environmental influence, is governed by quantitative trait loci (Austin and Lee 1996; Hall et al. 1994; Juenger et al. 2005; Li et al. 1995). QTL mapping usually provides a starting point for statistically exploiting and identifying the chromosomal regions contributing to genetic variation in agronomically important traits in breeding programs (Zhang 2007).

Understanding the genetic basis of WUE is important for crop improvement under water-limited environments. The first QTL identified for $\Delta^{13}C$ was reported in tomato (*Lycopersicon esculentum* and *L. pennellii*) by Martin and Nienhuis (1989) and subsequently QTL for $\Delta^{13}C$ have been reported in *Arabidopsis thaliana* (Hausmann et al. 2005; Juenger et al. 2005), barley (Diab et al. 2004; Ellis et al. 2002; Ellis et al. 1997; Teulat et al. 2002), cotton (*Gossypium hirsutum* and *G. barbadense*) (Saranga et al. 2001), rice (Laza et al. 2006; Takai et al. 2006; This et al. 2010; Xu et al. 2009), soybean (Specht et al. 2001), tomato (Xu et al. 2008), and wheat (Peleg et al. 2009; Rebetzke et al. 2008). Five QTL affecting $\delta^{13}C$ were mapped in *Arabidopsis* using 162 recombinant inbred lines (RILs), and two QTL were co-located with QTL controlling flowering time, which suggested a potential pleiotropic relationship, and QTL interactions for the above two traits were also observed (Juenger et al.

2005). Teulat et al. (2002) identified ten QTL associated with grain $\Delta^{13}C$ using 167 barley RILs grown in three Mediterranean environments in a field study. Among the ten QTL, one was specific to one environment, two exhibited interaction with the environment, six showed main effects across two or three environments and one presented both main effect and QTL by environment interaction. Results also showed that eight QTL for $\Delta^{13}C$ were co-located with QTL for several physiological traits related to plant water status and/or osmotic adjustment, and/or for agronomic traits previously measured on the same population, and heading date did not contribute to the effects of environment and interaction between genotype and environment on $\Delta^{13}C$ (Teulat et al. 2002). Takai et al. (2009) found that a QTL controlling leaf $\Delta^{13}C$ on the long arm of chromosome 3 in rice was associated with g_s. Diab et al. (2008) reported that QTL for $\Delta^{13}C$ and transpiration were on the same locus (gwm389). However, no single QTL for $\Delta^{13}C$ with large effect have been identified in cereals, and most QTL identified for $\Delta^{13}C$ have small effects. Table 2.2 summarizes the efforts to locate QTL for WUE measured as $\Delta^{13}C$ in cereals.

The marker-QTL-trait association has been regarded as a promising way to develop improved cultivars, i.e., MAS and marker-assisted breeding (Thomas 2000). Although the QTL analysis has provided unprecedented opportunities to identify chromosome regions harbouring genes/QTL controlling WUE in cereals, three major issues must be clarified towards efficient and effective implementation of MAS. First, the linkage between random molecular markers and the target gene or QTL can be broken by recombination unless the markers are completely linked to the target allele or generated from gene sequence data (Araus et al. 2008). Second, the intrinsic nature of polygene underlying WUE, small size of individual QTL for $\Delta^{13}C$, and the interaction with the environment make MAS for WUE or $\Delta^{13}C$ extremely difficult. The ultimate goal of QTL mapping is to transfer QTL for WUE into elite breeding lines to improve their performance when drought happens. Generally, that polygene controlling $\Delta^{13}C$ are multiple genes each with small effects, implies that several QTL must be

manipulated simultaneously to obtain a major impact (Cattivelli et al. 2008). However, MAS will not be effective when more than three QTL are considered (Araus et al. 2008). Theoretically, it is preferable to target QTL with a major effect that is consistent across environments and populations and also independent of the genetic background. So far, most QTL research on $\Delta^{13}C$ were conducted in a single population, and common or repeatable QTL for $\Delta^{13}C$ across environments and genetic pools only have been reported in wheat (Rebetzke et al. 2008). Third, most QTL for $\Delta^{13}C$ have been reported to be co-located with QTL for yield components and heading date and/or plant height (Forster et al. 2004; Juenger et al. 2005). Favorable alleles for $\Delta^{13}C$ and yield components could stem from the two contrasting parents (Lanceras et al. 2004), which may lead to yield penalty by selecting for reduced $\Delta^{13}C$. Furthermore, genotypic variation in $\Delta^{13}C$ is usually associated with heading date and/or plant height; for example, QTL for shoot $\delta^{13}C$ and grain yield in barley were associated with a semi-dwarf gene *ari-e.*GP on chromosome 5H near marker Bmac113 (Ellis et al. 2002), which further confound and compromise the relationship between $\Delta^{13}C$ and grain yield (Rebetzke et al. 2008). It is suggested that $\Delta^{13}C$ effect on yield from plant height and development effects should be separated through covariance analysis (Rebetzke et al. 2008).

Routine QTL analysis comprises four basic components: a segregating population, sufficient segregation markers, accurate phenotypic data for target trait(s), and a sound statistical approach.

Mapping population

Based on the specific project goal, population size, and generations, several types of mapping population can be used for QTL mapping, such as F_2 population, backcross population (BC), RILs, and doubled haploids (DHs) (Gupta and Varshney 2004; Varshney et al. 2004). The F_2 population or BC population are derived by selfing or backcrossing F_1 individuals to one of the parents which

show divergence in target traits. These two types of populations are easy to develop with minimum investment and display tremendous diversity due to representing all the possible combination of parental alleles (Paterson 1996), but the major drawback associated with their application is that they are ephemeral, that is, lines are not fixed and impossible to replicate individuals (Varshney et al. 2004). In contrast, high homozygosity ensures RILs and DHs are permanent, and they can be maintained indefinitely, assessed in repeated experiments across years and locations, and shared with colleagues. RILs are developed through repeated selfing of individual line until F_5 generation or further generations are reached. Single seed descent approach is usually used to obtain RILs. However, generation of RILs is laborious and time-consuming compared with the development of other mapping populations. DH populations have the advantage of taking less time to develop via anther or microspore culture, but several disadvantages also exist, such as the whole plants have to be regenerated by tissue culture if specific varieties are needed under certain circumstance, sometimes resulting in allele frequency skewness during this process. If DHs are derived from F_1 anthers, recombination will be limited to a generation of meiosis, providing low resolution for mapping (Iyer-Pascuzzi et al. 2007).

Phenotyping of water-use efficiency

Accurate and precise phenotyping is a prerequisite for QTL mapping. For cost and labor considerations, the number of replicates and sites is often limited for phenotypic screening, hence reducing the sensitivity of the detection and analysis of QTL. If the evaluation of a target trait is biased, the subsequent QTL mapping steps will be worthless. A good understanding of the ecological and physiological basis of a trait under investigation, a proper and consistent measurement, and a careful experimental design are crucial to the detection of valid QTL. However, fast and accurate measurements of WUE remain a major bottleneck. Especially for the leaf-level WUE, the phenotyping process of gas exchange measurements

using a portable photosynthesis system (e.g., Li-Cor 6400) requires relatively stable and consistent environmental conditions across the populations (such as during the period of maximum rates of net photosynthesis at similar humidity, temperature and daylight conditions), which limits its use in breeding programs for large segregating populations, especially under field conditions.

Although $\Delta^{13}C$ has been demonstrated to be a simple and reliable measure of WUE, and it is easy to sample and store plant materials for carbon isotopic analysis, the screening of large breeding populations for $\Delta^{13}C$ by IRMS remains costly (typically over US$10 per sample) (Araus et al. 2008; Lopes et al. 2011). As a genetically complex trait, the expression of $\Delta^{13}C$ in different plant tissues and organs varies with water-supply (Rebetzke et al. 2008). To maximize genetic variance and heritability and improve QTL detection for $\Delta^{13}C$, screening of populations is suggested to be conducted under favorable and well-watered conditions (Rebetzke et al. 2008).

Genotyping of mapping population

Inheritance patterns regarding the allelic status of individual plants in a population can be monitored or tracked by segregating markers. Conventional markers such as morphological markers and biochemical markers (e.g., isozyme) have disadvantages such as a limited number of distinct markers, uneven distribution on genetic maps, expression affected by environment, unreliable assessment and time- and labor-consuming (Lörz and Wenzel 2004). For example, two morphological markers (rachilla hair length and leaf blade pubescence) and five isozyme markers were used in a barley map (Kleinhofs et al. 1993). The development and application of DNA-based molecular markers in the past two decades have greatly accelerated research of quantitative trait inheritance on major cereals. Molecular markers can be broadly classified into three groups: hybridization-based (such as restriction fragment length polymorphisms, RFLPs; Diversity Arrays Technology, DArT), PCR-based (such as randomly amplified

polymorphic DNA, RAPDs, simple sequence repeats, SSRs, amplified fragment length polymorphisms, AFLPs) and Microarray-based (such as single nucleotide polymorphisms, SNPs) (Somers 2004; Wenzl et al. 2004). All these marker types have been utilized to detect both sequence polymorphisms and length polymorphisms, and to construct molecular maps in all major cereals (Varshney et al. 2004). For example a consensus map of barley combining SSR, RFLP and AFLP markers has been constructed by combining five barley linkage maps (Karakousis et al. 2003), and a high density barley microsatellite consensus map containing 775 unique SSR loci was constructed by joining six independent genetic maps (Varshney et al. 2007).

Statistical tools for QTL mapping

The simplest approach for QTL analysis is to use a 't-test' or 'One-way ANOVA' to test if the differences between the marker means are significant for the target phenotype at specified levels (Kearsey 1998; Tuberosa et al. 2002). However, less information such as the distance between QTL and associated marker can be provided by this method. To overcome these limitations, interval mapping approaches (such as simple interval mapping, SIM, composite interval mapping, CIM) which explore the interval between pairs of markers for the presence of QTL are now widely used (Jansen 1993; Zeng 1994). The mathematical methods behind interval mapping such as maximum likelihood and regression are commonly used (Haley and Knott 1992; Handley et al. 1994; Lander and Botstein 1989). The logarithm of the odds ratio (LOD) score is commonly used for searching a QTL by computing the likelihood of observed data assuming a QTL is present or absent at a position (Lander and Botstein 1989).

Computer programs such as MapMaker/QTL (Lander et al. 1987; Lincoln et al. 1992), JoinMap (Stam 1993), QTL Cartographer (Basten et al. 1997), MapQTL (Van Ooijen and Maliepaard 1996), MQTL (Tinker and Mather 1995) and PLABQTL (Utz and Melchinger 1996) have been used to construct genetic

maps. MapMaker/QTL is freely available and is widely used in UNIX or DOS operating systems. A comprehensive list of QTL analysis and linkage map software has been reviewed by Manly and Olson (1999).

Validation and fine-mapping of detected QTL

One of the major shortcomings of QTL studies is that the number, location and estimated effects of identified QTL are often inconsistent in different genetic background of the mapping population (Bernardo 2008). The estimated effects of detected QTL are actually overestimated due to limited segregating progenies, a phenomenon called the "Beavis effect" (Xu 2003). Beavis (1994) suggested that when population size is less than 150 - 200, only modest fractions of QTL are identified and the effect of each single QTL are usually overestimated. In order to provide a stable and reliable prediction of QTL positions and effects, reasonable population size, and replicated field trials, from multi-sites and across seasons are usually required. A QTL validation approach has also been suggested (Tuberosa and Salvi 2004). Association mapping has been recently proposed for QTL discovery and candidate gene validation in plants (Flint-Garcia et al. 2003; Salvi and Tuberosa 2005), which examines a collection of diverse accessions (e.g., varieties, landraces and breeding lines) without generating large mapping populations. Germplasm collections of diverse genetic backgrounds and with different selection histories likely differ in their QTL alleles (Bernardo 2008), and the same QTL would be expected to be present in different populations, assuming that the particular QTL is stable or consistent. Maccaferri et al. (2011) conducted association mapping to indentify QTL controlling the agronomic performance in durum wheat across a broad range of water availability regimes. In their study, the presence of major QTL at key chromosome regions (such as *Ppd-A1* alleles, photoperiod-responsive gene) previously identified with bi-parental mapping were validated, and highly heritable traits (such as heading date and kernel weight) were found to be less affected by environmental conditions as compared with low heritability traits such as yield. Therefore, association mapping may be suitable

for validating $\Delta^{13}C$ as a high heritability trait. Moreover, QTL can be confirmed at a low density of markers (coarse mapping) by choosing a population with high level of linkage disequilibrium (Abdurakhmonov and Abdukarimov 2008; Yan et al. 2011), which describes the non-random association of alleles or alleles and markers at different loci (Yu and Buckler 2006).

As a valuable complementary tool in detecting marker-trait associations, association mapping has been extensively utilized in cereals (Breseghello and Sorrells 2006; Cockram et al. 2008; Garris et al. 2003; Horvath et al. 2009; Kraakman et al. 2004; Neumann et al. 2011; Ravel et al. 2006; Skøt et al. 2005; Stracke et al. 2009; Waugh et al. 2009; Yan et al. 2011; Yu and Buckler 2006). By utilizing all the historic recombination events from germplasm development, association mapping can provide a high resolution genetic map (fine mapping) and provide more precise locations of individual QTL (Maccaferri et al. 2011; Neumann et al. 2011; Oraguzie et al. 2007), or a step towards positional cloning (Rafalski 2010), which is more challenging but more rewarding for quantitative traits (Sorkheh et al. 2008). Major factors that affect association mapping include the level of linkage disequilibrium, population structure and stratification, familial relatedness and complexity of target traits (Abdurakhmonov and Abdukarimov 2008; Rafalski 2010).

Sufficient genotypic variation, stability across environments, and a high H^2 in $\Delta^{13}C$ (Araus et al. 1998; Condon and Richards 1992; Johnson and Rumbaugh 1995; Schuster et al. 1992) indicate that $\Delta^{13}C$ is a promising phenotype which can be tracked by molecular markers through QTL mapping, and eventually may improve the selection efficiency of WUE through marker-assisted breeding. Knowledge of loci underlying natural variation in WUE and $\Delta^{13}C$ would be valuable and beneficial for breeding programs. QTL mapping is an initial step towards unraveling the molecular basis of WUE. The next step towards the application of molecular markers in breeding for high WUE is to fine-map the candidate gene regions to reduce QTL size and clone DNA sequences underlying QTL. Many efforts have been dedicated to understanding the genetic

basis of $\Delta^{13}C$. According to Sanchez et al. (2002), the average marker interval of 0.5 cM or 350 kb is appropriate for fine mapping genes and QTL. Xu et al. (2008) fine-mapped a dominant QTL for $\delta^{13}C$ (designated QWUE5.1) in tomato to an interval about 2.2 cM long, and located markers SSR 49 and SSR 590 less than 2.2 cM from QWUE5.1, which were valuable for cloning the genes underlying QWUE5.1 and can be effectively used in MAS of QWUE5.1 in tomato breeding programs. To date, map-based cloning is still more challenging to large genome species such as wheat and barley as compared with small genome species such as *Arabidopsis*, rice or tomato (Diab et al. 2008). However, the high heritability of flowering time has allowed a major QTL *Vgt1* isolated via map-based cloning in maize, which has a moderate genome size (Salvi et al. 2007). There are high hopes for cloning major QTL for $\Delta^{13}C$ in rice with its available resource such as the genome sequence.

Gene discovery in drought- and water-use efficiency studies

In the past ten years tremendous progress has been made in identifying the genetic determinants of physiological responses to abiotic and biotic stress. Candidate genes can be defined as all genes involved in the expression of a given trait by physiologists, or only polymorphic genes putatively engaged in the trait variation by geneticists (Pflieger et al. 2001). Candidate genes can be approached either through functional or positional approach (Christiane et al. 2007; Pflieger et al. 2001; Varshney et al. 2005; Zhu and Zhao 2007). Functional candidate genes are shown or suspected to play a functional role in the phenotype under investigation, while positional candidate genes are closely linked to QTL or co-localized with a QTL. Positional candidate genes can be reached through QTL mapping, map-based cloning approaches, and information from closely related species (Li et al. 2010). For cereals, the full genome sequence of rice has been available to researchers (International Rice Genome Sequencing Project 2005), which provides a valuable shortcut for identifying candidate genes in related species

currently lacking high density maps, such as wheat and barley. Functional genomic approaches, such as insertional mutagenesis, Tilling (Targeting Induced Local Lesions in Genomes), RNAi (RNA interference), VIGS (Virus Induced Gene Silencing), transcriptomics and expression genetics, hold great promise for identifying functional candidate genes. Plant responses to stress to a large extent are under transcriptional control (Cattivelli et al. 2008). Theoretically, a difference at the mRNA level could potentially contribute to genotypic variation in the target trait. The microarray technique, also called gene expression profiling or transcriptional profiling, has greatly contributed to gene function analysis in plant species since 1995 (Schena and Shalon 1995), which can monitor in parallel the expression of thousands of transcripts simultaneously. With careful experimental design and appropriate data analysis, the expression profiling of large-scale genes can detect certain up- or down-regulated genes which would help to identify candidate genes, reveal important clues about gene function, provide detailed insights into physiological processes underlying targets of interest, and eventually clone and manipulate candidate genes through genetic engineering (Schulze and Downward 2001; Stéphanie et al. 2001; Varshney et al. 2005; Xue et al. 2006; Zhu and Zhao 2007).

So far, many drought-related genes have been isolated and characterized (Cattivelli et al. 2002; Hazen et al. 2005; Jain et al. 2001; Masle et al. 2005; Ramanjulu and Bartels 2002). Differential gene expression of wheat progeny with contrasting levels of TE conducted in a controlled growth room revealed that 11 genes were positively correlated with high TE trait measured as $\Delta^{13}C$, which was confirmed by quantitative real-time polymerase chain reaction (RT-PCR) analysis (Xue et al. 2006). Results from above experiments suggested that those differentially expressed genes can serve as candidates for further investigation or be used as expression quantitative trait loci (eQTL) for mapping the TE traits. However, the major shortcoming associated with expression profiling is that it does not necessarily provide any information related to post-transcriptional and post-translational modifications (Tuberosa et al. 2002). Diab et al. (2008)

reported that QTL for $\Delta^{13}C$ co-segregated with two differentially expressed sequence tags (dESTs) through combining positional and functional candidate gene approaches, but the role of these dESTs have not yet been elucidated.

Most research have indicated that plants tend to increase drought resistance and WUE through decreasing transpiration via stomatal closure, reducing stomatal pore size or number (density) rather than increasing CO_2 assimilation. *ERECTA*, the first TE gene that has been isolated in the extensively-studied model plant *Arabidopsis thaliana*, influences the coordination between transpiration and photosynthesis through several ways (e.g., stomatal density and epidermal cell expansion), and similar genes have been found in rice, sorghum, and wheat (Masle et al. 2005). The expression of *Arabidopsis HARDY* (HRD) gene in rice improves WUE by enhancing photosynthetic assimilation and reducing transpiration (Karaba et al. 2007). The GT-2 LIKE 1 (GTL1) in *Arabidopsis thaliana* functions as a transcription factor of drought tolerance and WUE by regulating stomatal density and transpiration, and gtl1 plants had high WUE and low transpiration without reduction in CO_2 assimilation (Yoo et al. 2010). A transcription factor *DREB1A* (the Dehydration Responsive Element Binding protein) driven by a *rd*29A promoter from *Arabidopsis thaliana* was transferred into a peanut cultivar, which achieved higher TE at a lower g_s than the untransformed control under well-watered conditions (Bhatnagar-Mathur et al. 2007). The GPA1 in *Arabidopsis thaliana* has also been found as a regulator of TE through control of stomatal density (epidermal cell size) and stomatal development (Nilson and Assmann 2010).

However, there are few reports on improving WUE through increasing photosynthesis. Jeanneau et al. (2002) examined the effect of photosynthesis regulation via the ectopic expression of the *Sorghum bicolor* C_4- PEPC gene in transgenic maize. The C_4- PEPC over-expressed lines increased WUE_{ic} and dry weight by 30 and 20% respectively under moderate drought conditions. The observed improvement in WUE and biomass production in over-expressing transgenic lines was achieved by decreased g_s (reduced stomatal aperture and

density) and improved ability of CO_2 fixation (a higher C_4- PEPC activity). Eventually, these genes will lead to improved TE in both dry and well-watered conditions through the so called strategy of "breeding by design" (Masle et al. 2005; Peleman and van der Voort 2003).

After minimizing some factors in the population structure such as flowering time and plant height which may confound the phenotypic expression of target traits, association mapping can increase the probability of novel gene discovery (Reynolds et al. 2009). Mohan (2010) conducted association mapping for drought tolerant traits using a set of 151 rice germplasm accessions. Twenty-six out of 113 simple sequence repeat (SSR) markers were found to be associated with root traits and $\Delta^{13}C$, among those, marker 'RM 224' on chromosome 11 co-segregated with carbon isotope ratios and root volume, explaining 73.7 and 73.1% of the phenotypic variance, respectively, indicating that there was a potential major gene around this marker.

Several kinds of tools and approaches are available for the introgression of genes and genomic regions into elite crop varieties, which are susceptible to water scarcity (Bajaj et al. 1999; Karaba et al. 2007; Varshney et al. 2005). Various strategies have been used to produce transgenic plants, including overproduction of enzymes responsible for osmolytes biosynthesis, late embryogenic abundant (LEA) proteins, and detoxification enzymes (Bajaj et al. 1999). For example, the biomass and WUE of transgenic wheat were improved under water deficit conditions by introducing a Group 3 LEA proteins encoded by the barely *HVA1* gene (Sivamani et al. 2000). However, caution needs to be taken so that the over-expression of transcription factors or constitutive expression of selected genes may activate adverse or deleterious effects on target traits thus having a negative effect on yield (Araus et al. 2008; Bajaj et al. 1999; Ito et al. 2006; Liu et al. 1998; Wang et al. 2003). Although a large number of molecular markers or candidate genes for WUE or $\Delta^{13}C$ have been reported, few are effective in MAS and the engineering of improved WUE with single genes in major crops has not been achieved so far (Karaba et al. 2007).

Conclusions and Perspectives

It's not easy to achieve large genetic gains in WUE and yield concurrently in dry-land environments, even though genetic improvement holds opportunities in increasing crop yield especially in drought-prone regions. Research from plant physiology, genomics, to molecular biology on drought in the past several decades has produced good techniques and accumulated experiences for future studies. However, routine cloning of genes underlying the QTL for WUE has a long way to go.

In addition to relying on markers from a highly dense map, candidate genes from the outcome of microarray can also be used as markers. Polymorphic markers from functional genes identified in microarrays experiments or physically close to these could be used to identify candidate genes for positional cloning and enrich linkage maps. Association mapping is an alternative way to identify new useful alleles through exploration in wild germplasm (Cattivelli et al. 2008), which is becoming popular with the development of high-throughput genotyping and genome sequencing technologies as well as advanced statistical approaches. The ultimate goal is to tag and isolate genes or pyramid beneficial QTL alleles controlling WUE. With the application of MAS, favorable alleles can be introduced into elite germplasm to derive improved cultivars and speed up plant breeding process in water-limited environments.

However, water deficit combined with other unfavorable environments (such as diseases, insects, weeds, infertile soils etc.) make genetic selection more complicated. Although breeding for high WUE varieties is a promising strategy via the use of $\Delta^{13}C$, particularly in C_3 cereals, the greatest gains still come from the integrated knowledge of physiology and genomics, and the combined strategies of traditional breeding, MAS and appropriate management practices (e.g., reducing water loss from soil surface evaporation, deep drainage and surface runoff) to achieve the yield potential under targeted environments.

Table 2. 1 Several common definitions of water-use efficiency

Level	Time scale	Numerator	Denominator	Equation
Leaf (photosynthetic scale)	Minutes or hours	Net assimilation rate (A) μmol CO_2 m^{-2} s^{-1}	Transpiration (E) mmol H_2O m^{-2} s^{-1}	$WUE_{Instantaneous} = A/E$
		A μmol CO_2 m^{-2} s^{-1}	Stomatal conductance (g_s) mol H_2O m^{-2} s^{-1}	$WUE_{Intrinsic} = A/g_s$
Crop (agronomic scale)	Weeks to months or growing season	Aboveground biomass	Seasonal evapotranspiration	$WUE_{integrative}$= Dry matter/transpired water
		Grain yield	Seasonal evapotranspiration	Water productivity or $WUE_{economic}$= Dry matter/transpired water

Table 2. 2 QTL for WUE measured as carbon isotope discrimination ($\Delta^{13}C$) and/or carbon isotope ratio ($\delta^{13}C$)

Species	Population		Number of QTL detected	Environment	R^2 (%)[1]	References
	Type	Size				
Barley	DHs	156	9	Hydroponic tanks	14 and 15	Ellis et al. 2002
	RILs	167	10	Field (rain-fed and irrigated)	8.9 and 9.6	Teulat et al. 2002
	RILs	167	4	Field (rain-fed and irrigated)	4~17	Diab et al. 2004
	DHs	57	1 and 9	Glasshouse (hydroponic)	14~80	Ellis et al. 1997
Rice	BILs	98	6	Field (rain-fed)	8.5~13.2	Ishimaru et al. 2001
	RILs	205	9	Field (irrigated)	N.A.	Price et al. 2002
	RILs	101	2~4	Field (irrigated)	7.6~19	Laza et al. 2006
	RILs	126	5 and 7	Field (rain-fed)	5.9~14.3	Takai et al. 2006
	BILs	98	7	Glasshouse	7.6~22.2	Xu et al. 2009
	DHs	91	11	Glasshouse	8-19	This et al. 2010
	RILs	165				
Wheat	DHs	161~190	9~13	Field (rain-fed and irrigation)	2~10	Rebetzke et al. 2008
	RILs	152	12	Field (well-watered and water-limited)	0.8~30	Peleg et al. 2009
	RILs	110	29	Field (rain-fed)	N.A.	Diab et al. 2008

[1] R^2: proportion of the phenotypic variance explained by each QTL

RILs: recombinant inbred lines; BILs: backcross inbred lines; DHs: doubled haploids

N.A.: not available

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Chapter 3 The physiology and stability of leaf carbon isotope discrimination as a measure of water-use efficiency in barley on the Canadian Prairies

Introduction

Major concerns have been raised regarding the food security due to current booming population, shrinking arable land area, increasing water scarcity, and future potential changes in precipitation and temperature regimes imposed by global climate change, which require more efficient water productivity in both irrigated and rain-fed cropping systems (Condon et al. 2004; Hamdy et al. 2003).

The production of barley in Canada is mostly concentrated on the Prairies in Western Canada, which is characterized by short and dry growing seasons with anticipated heat stress later in the season (Anyia et al. 2008), and crops rely on stored-moisture from snow melt and low rainfall within the growing season. Therefore, water deficit is a major constraint on barley productivity on the Prairies across locations and years. Even though breeding efforts have been made towards developing high-yielding varieties, barley production in Canada has remained stagnant in the past decade (FAOSTAT 2008). To meet the increasing market demand for barley supply (for malt, food and feed), Canadian breeders must exploit new breeding strategies to increase barley yield per unit area as well as develop varieties with improved adaptation to drought, both targeted at improving barley water productivity or water use efficiency under rain-fed conditions (Barnabás et al. 2008).

Water-use efficiency (WUE) at the photosynthetic scale (leaf-level) refers to the net amount of carbon assimilated (*A*) per unit water transpired (transpiration rate, *E*) during the same period ($WUE_{instantaneous}$ or WUE_{is}) (Farquhar et al. 1989). A similar parameter called intrinsic water-use efficiency ($WUE_{intrinsic}$ or WUE_{ic}) is defined as the ratio between *A* and stomatal conductance (*g*) (Choi et al. 2007;

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Hall et al. 1992), which is thought to be more closely associated with physiological responses. At the agronomic scale (crop-level), WUE is considered as the accumulated dry matter divided by water consumed by the crop during the whole growth cycle ($WUE_{integrative}$ or WUE_{ie}) (Condon et al. 2004; Monclus et al. 2006; Tambussi et al. 2007).

Breeding for improved WUE has largely been limited by lack of effective screening methodology for direct measurements at either leaf-level or crop-level in large populations under field conditions. A promising screening method was developed in the 1980s when carbon isotopic techniques were introduced (Farquhar et al. 1982; Farquhar and Richards 1984; O'Leary 1981). For C_3 species, the extent to which plants discriminate against the heavy carbon isotope (^{13}C) during carbon assimilation was reported to be negatively correlated to their WUE through the ratio of leaf intercellular to atmospheric CO_2 concentrations (C_i/C_a), a parameter reflecting the balance between consumption and supply rates of CO_2 by photosynthetic capacity and stomatal diffusion (Ehleringer 1990; Farquhar et al. 1982; Farquhar and Richards 1984). Extensive studies in C_3 species have confirmed the negative correlation between carbon isotope discrimination ($\Delta^{13}C$) and WUE (Anyia et al. 2007; Condon et al. 1990; Hall et al. 1992; Hubick and Farquhar 1989; Hubick et al. 1986; Ismail and Hall 1992; Khan et al. 2007; Lambrides et al. 2004; Rebetzke et al. 2002; Rytter 2005).

Although $\Delta^{13}C$ has been intensively exploited as an integrated criterion for screening improved WUE and great productivity in C_3 crops under water-limited environments, there are still challenges associated with the application of $\Delta^{13}C$ in plant breeding programs. For example, the relationship between $\Delta^{13}C$ and grain yield or biomass has been found to be either positive, negative or neutral, depending on target environments and species (Anyia et al. 2007; Condon et al. 2004; Jiang et al. 2006; Tambussi et al. 2007). The $\Delta^{13}C$ provides a long-term average estimate of cumulative WUE integrated in time and space (Condon et al. 2002). Different plant parts such as root, leaf, sheath, awn or grain are characterized with their own $\Delta^{13}C$ value and potential advantages to be used to

evaluate WUE. Leaf $\Delta^{13}C$ during the stem elongation stage, which is regarded as the beginning of yield potential (Anyia et al. 2008), could reflect the integrated WUE and the vegetative establishment. In addition, leaf $\Delta^{13}C$ measured before maturity can enable selection and crosses to be made within the same season thereby speeding up the breeding process. In our study penultimate leaf was sampled for $\Delta^{13}C$ analysis to avoid harming the flag leaf, because flag leaf is one of the primary sources in determining grain yield in cereal crops (Sicher 1993; Thorne 1965; Xue et al. 2008).

The objectives of this study were: 1) to validate the stability of leaf $\Delta^{13}C$ as a measure of WUE across years and locations in Alberta, Canada, based on selected barley genotypes; and 2) to characterize the physiological and agronomic performance of four barley genotypes with contrasting levels of $\Delta^{13}C$ under well-watered and water deficit conditions in a greenhouse experiment and to compare the drought recovery performance of the four barley genotypes after re-watering.

Materials and methods

Plant materials

Twelve barley genotypes comprising cultivars registered in Canada and breeding lines including six six-row and six two-row barley types (Table 3.1) were used in this study. These genotypes were also previously surveyed for $\Delta^{13}C$ in 2005 at three field locations (Anyia et al. 2007). The six-row genotypes were 'I60049, '170011, 'H97097006', 'Kasota', 'M92081001', and 'W89001002003' while the two-row genotypes were 'AC Metcalf', 'CDC Cowboy', 'H93174006', 'Merit', 'Niobe', and 'Xena'.

Four of the genotypes 'CDC Cowboy'(2-row, feed type) , 'Niobe' (2-row, feed type), '170101'(6-row), 'Kasota' (6-row, semi-dwarf type) were selected and

characterized for a range of physiological and agronomic traits due to their consistent performance of $\Delta^{13}C$ in previous field experiments (Anyia et al. 2007).

Field experiment

The experiment was conducted at Vegreville (53 °31' N, 112 °6' W, 639.3 m altitude) in a well-drained soil developed on fluvial-lacustrine material over till (Malmo series of an Eluviated Black Chernozem) and Castor (52 °8' N, 111 °54' W, 807.7 m altitude, hard clay Dark Brown Chernozem) in Alberta, Canada, during the 2006 and 2007 growing seasons (from May to September) under rain-fed conditions. The two sites were characterized by distinct soil moisture conditions with Castor as the drier site. The average annual precipitation and within season rainfall (June to August) from 1977 to 2007 in Castor was 340±89 mm and 172±67 mm, respectively compared with Vegreville, which had 382±62 mm and 193±52 mm, respectively (Environment Canada 2009). The experiments were conducted at both locations as a completely randomized block design with six replicates per genotype. Seeds of each genotype were planted in 6 rows of 2 m wide by 6 m long plot with 20 cm row spacing and a seeding rate of 220 seeds per square meter. Fertilizer was applied during the seeding by adding 128 kg N ha^{-1}, 40 kg P_2O_5 ha^{-1}. Weed control was done by hand weeding 4 weeks after seeding.

Stomatal conductance: Stomatal conductance was assessed in the field using a portable Decagon SC-1 leaf porometer (Decagon Devices, Pullman, WA, USA) between 9 am and noon on a sunny day. The intact penultimate leaf blade was inserted into the holder of the porometer (abaxial surface), and readings from four randomly selected plants per plot were taken and averaged for each genotype.

Carbon isotope discrimination analysis: Fully expanded penultimate leaves were sampled for carbon isotope composition ($\delta^{13}C$) at the stem elongation stage. Samples were randomly collected from 5 to 8 plants per plot and bulked. All samples were oven dried at 70 °C for 48 hours. Dried leaf samples were ground

to fine powder with a ball mill (Spex SamplePrep 8000D Mixer) and analyzed for $\delta^{13}C$ using a continuous-flow stable isotope ratio mass spectrometer (IsoPrime, GV Instruments Ltd, Manchester, UK). Carbon isotope composition was calculated as $\delta^{13}C$ (‰) = $(R_{sample}/R_{reference} - 1) \times 1000$, where R is the ratio of $^{13}C/^{12}C$, and the reference material is the belemnite carbonate standard (PDB) from the Pee Dee Formation. The $\Delta^{13}C$ was calculated according to Farquhar et al. (1989) as $\Delta^{13}C$ (‰) = $[(\delta_a - \delta_p)/(1 + \delta_p/1000)] \times 1000$, where δ_a and δ_p refer to the C isotope ratios of atmospheric CO_2 (-8‰) and plant, respectively.

Aerial biomass and grain yield: Biomass and grain yield were determined from hand harvested 1 m^2 sub-plots at Vegreville in 2006 after oven drying at 70 ℃ for 48 hours. Harvest index (HI) was calculated as HI = grain weight/ total aboveground biomass.

Greenhouse experiment

Based on the leaf $\Delta^{13}C$ results from the 2006 and 2007 field experiments, four genotypes ('CDC Cowboy', 'Niobe', '170011' and 'Kasota') were selected. An experiment was conducted in a greenhouse at Alberta Research Council in Vegreville, Alberta, Canada, with a target photoperiod of 16 hours using natural illumination supplemented with sodium halide light bulbs. Day and night temperatures in the greenhouse were programmed at 22 and 16 ℃, respectively, while the recorded temperatures averaged 25±2 ℃ (day) and 18±2 ℃ (night) and the relative humidity was 55%/ 70% (day/night). The maximum photosynthetic photon flux density (PPFD) was ~1700 μmol m^{-2} s^{-1} at the canopy level. Two vertical wall fans were installed in the greenhouse to improve air movement and keep conditions uniform during the entire experiment. The experiment was started on June 15 and plants were harvested on September 17 in 2007.

Plants were grown in plastic pots (30 cm tall by 27 cm diameter) filled with 7.3 kg soil mix containing field soil and peatmoss (Promix "BX") in a 1:3

(v:v) ratio. All pots were flushed with 4 L tap water and drained overnight to determine field capacity before seeding. Eight seeds were sown at 3 cm depth in each pot and later thinned to four seedlings per pot two weeks after emergence. After thinning, soil surface of each pot was covered with a 2 cm layer of perlite to reduce evaporation. All pots were then weighed and kept well-watered until the beginning of the drought treatment.

Each genotype was subjected to well-watered (ww) and water-deficit (wd) treatments with six replicates using a randomized complete block design. The well-watered pots were maintained at field capacity during the whole growth cycle. Water-deficit treatment was imposed for 10 days from the stem elongation stage (BBCH31-32) according to the Zadoks scale (1974), followed by re-watering to restore the pots to well-watered conditions until grain maturity.

Tensiometers and soil moisture sensors (Irrometer Company, Riverside, CA, USA) were installed in the selected reference pots and Time Domain Reflectometry (TDR, Soil Moisture Equipment Corp. Santa Barbara, CA) probes (20-cm long stainless steel 3-rod configuration) were used to monitor soil moisture regularly and water was applied on alternate days. Evapo-transpiration from each pot was determined before the recovery treatment application by weighing the pots. Controls were maintained at field capacity (ca. -10 kPa soil water potential at 25 cm pot depth) by adjusting the water amount lost through evapo-transpiration on a daily basis. Three control pots containing similar amount of soil and perlite but without plants were maintained to determine soil surface evaporation by calculating the successive weight differences.

At the end of the 10-day water-deficit treatment (average -80 kPa soil water potential), two plants per pot were harvested from which fully expanded top leaves were sampled for $\delta^{13}C$ analysis as described above for the field experiment. Similar leaves to those used for $\delta^{13}C$ were used to measure specific leaf area (SLA), the ratio of leaf area to leaf dry weight. Leaf area was measured as leaf length $\times$ leaf width $\times 0.8$, where 0.8 is an empirical coefficient (Rebetzke and Richards 1999).

At maturity, the remaining two plants per pot were harvested, oven dried at 70 ℃ for 2 days, and aerial biomass and grain yield recorded. Water use efficiency was calculated as aerial biomass/total water use less water lost due to evaporation.

Gas exchange measurements: The top most fully expanded and sun-exposed leaf on the main stem was selected for gas exchange measurements (one leaf per replicate) during water deficit and four days after re-watering of pots, using a portable photosynthesis system (Li-Cor 6400; Li-Cor Inc, Lincoln, NE, USA). All gas exchange measurements were recorded between 9 am and noon on sunny days. Air temperature inside the leaf chamber was maintained at ambient levels. The Li-Cor 6400's LED light source was used to set PPFD at 1000 μmol m^{-2} s^{-1} for all measurements while reference CO_2 was set at 400 ppm and flow rate was kept constant at 500 μmol s^{-1}. Measurements were made after the leaf had acclimated to the chamber environment. Net CO_2 assimilation, transpiration rate, stomatal conductance, intercellular CO_2 concentration, $WUE_{intrinsic}$ was calculated from the gas exchange measurements.

Statistical analysis

Data were analysed with SAS, version 9.1 (SAS Institute, Inc., Cary, NC). Homogeneity of variance and normality of distribution were tested before analysis of variance (ANOVA). Differences among genotypes and between water treatments were examined using the GLM procedure. When ANOVA showed significant effects, means were separated by least significant difference (LSD) test. Correlation analysis was performed to detect the relationship between traits using the CORR procedure. An α value of 0.05 was chosen to indicate statistical significance.

Results

Leaf $\Delta^{13}C$ across locations and years

Significant variability in leaf $\Delta^{13}C$ of the twelve genotypes was observed in the field experiment across years and locations ($p < 0.01$). In 2006, leaf $\Delta^{13}C$ of the genotypes ranged from 16.52 to 18.68‰ (Vegreville), while in 2007 it varied from 19.61 to 21.34‰ in Vegreville and 20.07 to 21.05‰ in Castor. In 2006, the extreme genotypes differed by 2.15‰ in Vegreville, while in 2007, they only differed by 0.98 and 1.73‰ in Vegreville and Castor, respectively. The leaf $\Delta^{13}C$ of the 2-row and 6-row genotypes did not show any significant difference ($p = 0.8$).

The leaf $\Delta^{13}C$ ranking of the genotypes was fairly stable across years and locations with genotypes '170011', 'CDC Cowboy' and 'W89001002003' having consistently low and 'Kasota', 'I60049' and 'H93174006' having consistently high leaf $\Delta^{13}C$ (Table 3.2). The leaf $\Delta^{13}C$ values of the genotypes measured in Vegreville showed significant correlations between years (in 2006 and 2007) (Fig. 3.1, $r = 0.72$, $n = 12$, $p = 0.01$). Similarly, leaf $\Delta^{13}C$ measured in 2007 were highly correlated between locations (Vegreville and Castor) (Fig. 3.1, $r = 0.82$, $n = 12$, $p = 0.002$). The average leaf $\Delta^{13}C$ of twelve genotypes grown in 2007 (20.6‰) was significantly higher than that in 2006 (17.8‰; $p < 0.01$), due to higher rainfall in the 2007 growing season (June to August rainfall was 117 mm in 2006 and 161 mm in 2007). There was no significant difference of mean genotypic leaf $\Delta^{13}C$ between Vegreville and Castor in 2007 ($p = 0.9$; Table 3.2).

Barley genotype performance under field conditions

In 2006 (Vegreville), biomass of the twelve genotypes ranged from 458.4 to 634.6 g m^{-2}, grain yield from 133.5 to 308.6 g m^{-2}, harvest index from 0.29 to 0.55, specific leaf area from 204 to 287 cm^2 g^{-1} and g_s from 143.4 to 260.1 mmol m^{-2} s^{-1}

(Table 3.2). Among the genotypes, '170011' and 'W89001002003' consistently had low biomass, grain yield, harvest index and g_s (Table 3.2). No significant correlation was observed between biomass and leaf $\Delta^{13}C$, or between leaf $\Delta^{13}C$ and grain in the 2006 field trial. Data from the 2006 field trial showed significant positive correlation between g_s and leaf $\Delta^{13}C$ among the genotypes (Fig. 3.2, r = 0.76, n = 12, p = 0.005). However, there were no significant differences between the two-row and six-row genotypes in biomass, grain yield, HI, g_s and SLA ($p > 0.05$) in the 2006 field trial.

Physiology of barley genotypes with contrasting leaf $\Delta^{13}C$ levels under drought stress

Leaf $\Delta^{13}C$: The ranking of leaf $\Delta^{13}C$ of the four genotypes measured under well-watered conditions was similar to that from field studies in Vegreville during the 2007 growing season ('Kasota' > 'Niobe' > 'CDC Cowboy' > '170011'), while ranking of leaf $\Delta^{13}C$ measured under water-deficit conditions matched results from field studies in Vegreville during the 2006 growing season ('Kasota' > 'Niobe' > '170011' >'CDC Cowboy') (Tables 3.2 and 3.3). The mean value of leaf $\Delta^{13}C$ measured in greenhouse (23.31 and 22.42‰ for drought and well-watered plants, respectively) were substantially higher than those obtained under field conditions (20.52 and 17.43‰ for the 2007 and 2006 field trials, respectively, in Vegreville) ($p < 0.05$). The range of leaf $\Delta^{13}C$ from the greenhouse (0.92 and 0.71‰ under well-watered and water deficit, respectively) were smaller than that from the field trial (1.89 and 1.73‰ in 2006 and 2007, respectively, in Vegreville).

After re-watering, biomass and grain yield of three genotypes increased significantly, especially the biomass of 'CDC Cowboy' and 'Niobe' (Table 3.3).

Gas exchange parameters: Water deficit significantly lowered the A and g_s of all genotypes. On day 10 of withholding irrigation, A and g_s decreased by 72 and 90% ('CDC Cowboy'), 91 and 97% ('Niobe'), 85 and 94% ('170011'), and 77

and 95% ('Kasota'), respectively, as compared with the respective controls. The *A* was similar between 'CDC Cowboy' and 'Kasota', whether under well-watered, water-deficit and water recovery conditions (Table 3.4). The g_s on the contrary, was remarkably different between the two genotypes with 'Kasota' having the highest value under the well-watered condition. The g_s of all genotypes declined to similar values under water deficit with each recovering to their pre-deficit level after re-watering (Table 3.4). Data from the 2006 field trial and greenhouse well-watered condition had the same genotypic ranking for g_s: 'Kasota' > 'Niobe' > 'CDC Cowboy' > '170011' (Tables 3.2 and 3.5). On the last day of the water deficit treatment, transpiration rate (*E*) and internal CO_2 concentration (C_i) of all genotypes were significantly reduced ($p < 0.05$). 'CDC Cowboy' had the lowest C_i under both well-watered and water-deficit conditions ($p < 0.05$) and lowest *E* under well-watered condition (Table 3.4, $p > 0.05$), while 'Kasota' had the highest *E* under well-watered and water-deficit conditions and highest C_i under well-watered condition (Table 3.4, $p > 0.05$). The C_i showed a 57% decline in 'CDC Cowboy', 50% in 'Kasota', 46% in '170011', and 42% in 'Niobe'. Upon re-watering, all gas exchange parameters gradually recovered to the well-watered values in all genotypes (Table 3.4).

Significant positive associations among *A*, g_s and *E* were discovered under water-deficit conditions (Table 3.5). Significant positive correlation between g_s and leaf $\Delta^{13}C$ was observed under well-watered conditions (Table 3.5), and no significant relationships were found among leaf $\Delta^{13}C$ and any gas exchange parameter under water-deficit conditions.

Specific leaf area: Water deficit significantly reduced SLA ($p < 0.05$) for all genotypes, with the lowest and highest SLA in 'CDC Cowboy' and '170011' under both water treatments, respectively. The SLA ranking of genotypes under field conditions in 2006 ('CDC Cowboy' < 'Kasota' < 'Niobe' < '170011', Table 3.2) was maintained under well-watered conditions in the greenhouse.

WUE_{ie} and WUE_{ic}: Water deficit significantly increased WUE_{ie} of the genotypes by 46% on average (Table 3.3, $p < 0.05$). Under well-watered conditions there was no significant genotypic variation in WUE_{ie} (Table 3.3, $p > 0.05$), but WUE_{ie} differed significantly among the genotypes under water-deficit conditions ($p < 0.05$). 'CDC Cowboy' showed lowest WUE_{ie} under well-watered conditions ($p > 0.05$), but highest WUE_{ie} under water-deficit conditions (Table 3.3). Under water-deficit conditions, WUE_{ic} of the genotypes were significantly increased, but was restored to pre-deficit levels four days after re-watering. The WUE_{ic} of 'Kasota' was the lowest among the genotypes under well-watered conditions, while 'CDC Cowboy' exhibited the highest WUE_{ic} under water deficit conditions (Fig. 3.3).

Discussion

Stability of leaf $\Delta^{13}C$ as a measure of WUE in barley

Leaf $\Delta^{13}C$ ranking among the genotypes evaluated in the field study was consistent across years and locations. The 2-row ('CDC Cowboy' and 'H93174006') and 6-row ('Kasota', '170011', 'I60049', and 'W89001002003') genotypes maintained their top and bottom rankings in leaf $\Delta^{13}C$ across locations and years. These results are in agreement with Anyia et al. (2007) who reported positive rank correlation between leaf $\Delta^{13}C$ measured across two field locations and that genotypic ranking of leaf $\Delta^{13}C$ under field conditions was maintained in a greenhouse experiment. These results suggest that leaf $\Delta^{13}C$ is stable to be reliably used as a measure of WUE under field conditions and are consistent with the literature about the stability and low genotype by environment interaction associated with the $\Delta^{13}C$ trait (Acevedo 1993; Teulat et al. 2001). The present study confirms that ranking of leaf $\Delta^{13}C$ genotypes is stable between field and greenhouse grown plants. The consistency in the ranking of leaf $\Delta^{13}C$ of the four genotypes under greenhouse vs. under field conditions indicate that genotypic variation in leaf $\Delta^{13}C$ under field conditions could be predicted from leaf $\Delta^{13}C$ of well-watered plants in the greenhouse. However, the range of difference in leaf

$\Delta^{13}C$ between the extreme genotypes was narrower under the greenhouse conditions compared with field conditions, and the mean value of leaf $\Delta^{13}C$ measured in greenhouse were substantially higher than those obtained under field conditions. The significantly higher value of leaf $\Delta^{13}C$ in the greenhouse was attributed to respired CO_2 (O'Leary 1981). The use of greenhouse grown plants to predict leaf $\Delta^{13}C$ ranking should be done with care to avoid re-ranking of genotypes which can occur in poorly planned experiments.

Although leaf $\Delta^{13}C$ ranking among the studied genotypes was highly stable across locations and years in this study, it is also known that the use of this trait to improve yield can be challenging. The relationship between $\Delta^{13}C$ and aerial biomass or grain yield has been reported to be positive, negative or neutral, depending on the target environment and species (Anyia et al. 2007; Condon et al. 2004; Jiang et al. 2006; Tambussi et al. 2007). This is not surprising since low $\Delta^{13}C$ or high WUE under dry conditions can occur at the expense of absolute yield performance (Bloch et al. 2006). In this study, for example, leaf $\Delta^{13}C$ was not associated with grain yield under field conditions in 2006 ($r = 0.36$, $n = 12$, $p = 0.24$).

Significant positive correlations existed between g_s and leaf $\Delta^{13}C$ in the 2006 field experiment (Fig. 3.2, $r = 0.76$, $n = 12$, $p = 0.005$), and in the well-watered greenhouse experiment (Table 3.5, $r = 0.99$, $n = 4$, $p = 0.003$). Ehleringer (1990) also reported similar positive relationship between g_s and leaf $\Delta^{13}C$ of common bean (*Phaseolus vulgaris* L.) under field conditions. Previous studies have indicated that variation in $\Delta^{13}C$ of cereals may arise from variation in photosynthetic capacity as well as g_s (Condon et al. 1990; Morgan and LeCain 1991). Roussel et al. (2009) concluded that since leaf $\Delta^{13}C$ is under tight genetic control, the difference in genotypic leaf $\Delta^{13}C$ might stem from differences in g_s. A review by Condon et al. (2002) suggests that when g_s is the main source of variation in WUE and when water is not a major limiting factor on crop growth, a high WUE may be disadvantageous. Considering that the genotypes used in the present study were a mixture of 2-row and 6-row types and different usage (feed

and malting), it is likely that their difference in biomass production and harvest index may have affected the relationship between leaf $\Delta^{13}C$ and grain yield. Selection for low leaf $\Delta^{13}C$ in wheat resulted in greater aerial biomass and grain yield in back-crossed populations evaluated under rain-fed conditions in Australia (Rebetzke et al. 2002). In field evaluations, low-$\Delta^{13}C$ hybrids of sunflower significantly out-yielded high-$\Delta^{13}C$ hybrids in three out of the four environments (Condon et al. 2004; Lambrides et al. 2004). Anyia et al. (2007) suggested that selection for improved biomass from a collection of barley genotypes was possible even in situations where the relationship between leaf $\Delta^{13}C$ and aerial biomass or grain yield was positive. But caution should also be exercised in the application of leaf $\Delta^{13}C$ in breeding programs aimed at yield improvement.

Physiological differences of genotypes with contrasting leaf $\Delta^{13}C$

Photosynthesis is one of the key determinants for plant survival, growth, and productivity, while g_s has been suggested as an indicator of the intensity of water stress (Flexas et al. 2004). When subjected to water deficit, all genotypes showed decline of A and g_s. A low value of leaf $\Delta^{13}C$ may arise from reduced g_s or increased A or both (Araus et al. 1997; Condon et al. 2004). Under well-watered conditions, low leaf $\Delta^{13}C$ genotype 'CDC Cowboy' had a significantly higher WUE_{ic} than the high leaf $\Delta^{13}C$ genotype 'Kasota', and they both had similar levels of A. Therefore the higher WUE_{ic} (or low leaf $\Delta^{13}C$) of 'CDC Cowboy' was achieved by both maintenance of a high A and low g_s. In contrast, low leaf $\Delta^{13}C$ of '170011' was achieved by the lowest g_s, which resulted in its lowest A among the four genotypes tested under well-watered conditions. Under water deficit, 'CDC Cowboy' showed the least percentage decline in A and g_s, which suggested that 'CDC Cowboy' still can maintain its productivity under progressive drought conditions.

Previous studies have shown that changes in the diffusion rate of CO_2 across stomata resulted in changes in C_i, and that a lower C_i can contribute to

increased WUE either by enhancing the driving force for CO_2 uptake or by increasing photosynthetic capacity through increased carboxylation, or decreased stomatal aperture or mesophyll conductance (Flexas and Medrano 2002). Genotype 'CDC Cowboy' had the significantly lowest C_i under both well-watered and water-deficit conditions. Under well-watered conditions, the high WUE_{ic} of 'CDC Cowboy' can be explained by the significantly lower C_i through increased *A*. During water deficit, 'CDC Cowboy' had the least percentage decline in g_s among four genotypes, and it also maintained greater stomatal conductance than the other three genotypes, therefore the observed decline in C_i of this genotype may be attributed to both increased carboxylation and decreased stomatal aperture, which resulted in increased WUE_{ic} of this genotype.

While low leaf $\Delta^{13}C$ arising from a low g_s resulted in relatively low biomass and grain yield of genotypes '170011' and 'W89001002003' under field condition, the biomass and grain yield of 'CDC Cowboy' was comparable to that of high leaf $\Delta^{13}C$ genotypes such as 'Niobe', 'H93174006' and 'I60049', suggesting that a low leaf $\Delta^{13}C$ achieved by a combination of high *A* and low g_s may not limit productivity even in years when moisture is not a limiting factor such as 2007 in Vegreville.

Specific leaf area has often been used to address how leaf morphology (density and thickness) responds to environmental changes (Marron et al. 2003). SLA also has been correlated with photosynthetic capacity and relative growth rate in many studies (Araus et al. 1997; Bort et al. 1998; Johan et al. 2006; Poorter and de Jong 1999). Generally, species with high SLA are characterized by a high rate of photosynthesis per unit leaf nitrogen (Lambers and Poorter 1992). In our study, SLA and *A* of all genotypes (except '170011') was similar under well-watered conditions. In contrast to other genotypes, '170011' displayed the highest SLA but the lowest assimilation rate, which may be attributed to the long vegetative growth of this genotype (under greenhouse conditions, data not shown). SLA of all genotypes decreased significantly in response to water deficit, however, 'CDC Cowboy' and 'Kasota' showed a lower percentage decline in SLA

compared with ‘Niobe’ and ‘170011’, which may be partly responsible for the relatively high assimilation rate of the genotypes under water deficit (Table 3.4).

It took up to four days for the four genotypes to reach an almost complete recovery in *A* and C_i after re-watering. ‘Niobe’ and ‘Kasota’ showed quicker recovery in *A*, g_s, C_i and *E* than ‘CDC Cowboy’ and ‘170011’, which suggested that ‘Niobe’ and ‘Kasota’ are more sensitive to re-watering. Overall, assimilation rates recovered faster than stomatal conductance. After recovery from water deficit imposed on stem elongation stage, plant biomass and grain yield increased, probably due to increased carbon assimilation as a compensatory strategy of plants under drought conditions.

Conclusions

The present study clearly demonstrated the genotypic rank consistency and stability of leaf $\Delta^{13}C$ in barley as a measure of WUE under the Canadian Prairies conditions. Sampling leaf $\Delta^{13}C$ during the vegetative stage has the advantage that breeding lines may be screened and crosses may be made within one growing season. Under well-watered conditions in a greenhouse, ‘CDC Cowboy’ – a low leaf $\Delta^{13}C$ genotype, maintained a high assimilation rate, which was similar to that of ‘Kasota’ (with a high leaf $\Delta^{13}C$) at a considerably lower stomatal conductance. The low g_s of ‘CDC Cowboy’ under field conditions did not restrict its biomass and grain yield, which were comparable to genotypes showing a high g_s. This result suggests that a low leaf $\Delta^{13}C$ or conversely a high WUE may not limit biomass or grain yield of this genotype when water is not a major limiting factor under target environments. Selection and breeding for low leaf $\Delta^{13}C$ genotypes such as ‘CDC Cowboy’ may be useful for maintaining productivity and yield stability under water limited conditions on the Canadian Prairies. In breeding programs aimed at yield improvement, co-selection for low leaf $\Delta^{13}C$ and other yield traits (such as HI and early seedling vigour) is recommended.

Table 3. 1 Genotypes used in field and subsequent greenhouse experiments

Genotype	Row type	2005 experiment[1]	2006 experiment	2007 experiment	Greenhouse experiment
I60049	6	√	√	√	
170011	6	√	√	√	√[2]
H97097006	6	√	√	√	
Kasota	6	√	√	√	√
M92081001	6	√	√	√	
W89001002003	6	√	√	√	
AC Metcalf	2	√	√	√	
CDC Cowboy	2		√	√	√
H93174006	2	√	√	√	
Merit	2	√	√	√	
Niobe	2	√	√	√	√
Xena	2	√	√	√	

[1] Data from Anyia et al. (2007)

[2] Genotype 170011 is a winter type, and failed in reproductive stage as it was not vernalized

Table 3. 2 Mean carbon isotope discrimination ($\Delta^{13}C$), biomass, grain yield, harvest index (HI), specific leaf area (SLA) and stomatal conductance (g_s) of twelve barley genotypes under field conditions

Genotype	Row type	2006 Veg.[1] $\Delta^{13}C$(‰)	2007 Veg. $\Delta^{13}C$(‰)	2007 Cas.[1] $\Delta^{13}C$(‰)	Biomass[2] (g m^{-2})	Grain yield[2] (g m^{-2})	HI[2]	SLA[2] (cm^2 g^{-1})	g_s[2] (mmol m^{-2} s^{-1})
I60049	6	19.60^{a}	21.34^{a}	21.05^{a}	534.8^{a}	303.8^{a}	0.55^{a}	258^{abc}	257.2^{a}
170011	6	16.86^{de}	19.61^{e}	20.38^{cd}	462.7^{a}	133.5^{b}	0.29^{f}	262^{ab}	143.4^{c}
H97097006	6	17.44^{cd}	20.79^{bc}	20.56^{abcd}	576.9^{a}	308.6^{a}	0.52^{abc}	261^{ab}	168.4^{c}
Kasota	6	18.41^{b}	21.34^{a}	20.81^{abc}	458.4^{a}	243.9^{ab}	0.53^{ab}	238^{bcd}	260.1^{a}
M92081001	6	18.11^{bc}	19.84^{de}	20.41^{cd}	620.7^{a}	297.1^{a}	0.48^{abc}	258^{abc}	175.5^{bc}
W89001002003	6	17.57^{cd}	20.13^{de}	20.55^{bcd}	463.7^{a}	189.1^{ab}	0.41^{cd}	233^{cd}	151.5^{c}
ACMetcalf	2	18.04^{bc}	21.06^{ab}	20.68^{abc}	536.8^{a}	250.4^{ab}	0.45^{abcd}	271^{a}	223.4^{ab}
CDCCowboy	2	16.52^{e}	20.02^{de}	20.07^{d}	589.0^{a}	269.9^{ab}	0.46^{abcd}	221^{de}	151.3^{c}
H93174006	2	18.56^{b}	21.18^{ab}	20.83^{abc}	576.1^{a}	221.7^{ab}	0.37^{de}	249^{abc}	191.9^{bc}
Merit	2	17.88^{bc}	20.31^{cd}	20.68^{abc}	634.6^{a}	282.0^{ab}	0.44^{bcd}	245^{abc}	178.7^{bc}
Niobe	2	17.93^{bc}	21.10^{ab}	21.00^{ab}	593.6^{a}	282.0^{ab}	0.47^{abcd}	253^{abc}	230.3^{ab}
Xena	2	18.17^{bc}	20.87^{ab}	20.86^{abc}	520.8^{a}	243.8^{ab}	0.47^{abcd}	204^{e}	256.9^{a}

[1] Veg., Vegreville; Cas., Castor; [2] Data from 2006 only; Means followed by a different letter are significantly different at $P < 0.05$

Table 3. 3 Mean leaf carbon isotope discrimination ($\Delta^{13}C$), water-use efficiency (WUE_{ie}), aerial biomass, grain yield, and specific leaf area (SLA) of four barley genotypes under well-watered (WW) and water-deficit (WD) conditions in the greenhouse

Treatment	Genotype	Leaf $\Delta^{13}C$ (‰)	WUE_{ie} (g kg^{-1})	Aerial biomass (g $plant^{-1}$)	Grain yield (g $plant^{-1}$)	SLA (cm^2 g^{-1})
ww	CDC Cowboy	23.11^{b}	3.71^{c}	23.8^{bc}	10.1^{bc}	472^{bc}
	Niobe	23.25^{ab}	3.96^{c}	25.3^{b}	11.4^{abc}	496^{b}
	170011	22.97^{bc}	4.01^{c}	n.a.[1]	n.a.	592^{a}
	Kasota	23.89^{a}	3.90^{c}	20.3^{d}	9.6^{c}	488^{b}
wd	CDC Cowboy	22.05^{d}	6.14^{a}	29.1^{a}	12.0^{ab}	396^{d}
	Niobe	22.54^{bcd}	5.53^{ab}	29.1^{a}	12.1^{a}	404^{cd}
	170011	22.32^{cd}	5.24^{b}	n.a.	n.a.	459^{bcd}
	Kasota	22.76^{bcd}	5.86^{ab}	20.9^{cd}	10.6^{abc}	419^{cd}

[1] n.a., not available, 170011 is a winter type, it failed in reproductive stage without vernalization

Means followed by a different letter are significantly different at $P < 0.05$

Table 3. 4 Leaf gas exchange parameters (assimilation rate (*A*), stomatal conductance (g_s), transpiration rate (*E*), internal CO_2 concentration (C_i)) of four barley genotypes on the 10th day of water-deficit (WD) and the 4th day of water recovery (WR)

Day after water treatment	Genotype	*A* (μmol CO_2 m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})	C_i (ppm)	*E* (mmol H_2O m^{-2} s^{-1})
10^{th} day of WD	*Well-watered*				
	CDCCowboy	22.5^{ab}	423.7^{b}	287^{c}	8.79^{b}
	Niobe	20.4^{b}	471.7^{b}	302^{a}	9.83^{ab}
	170011	16.5^{c}	379.2^{b}	315^{a}	9.01^{b}
	Kasota	23.2^{a}	625.2^{a}	316^{a}	10.68^{a}
	Water-deficit				
	CDCCowboy	6.2^{d}	40.3^{c}	124^{c}	1.50^{c}
	Niobe	1.9^{f}	14.7^{c}	176^{b}	0.60^{c}
	170011	2.5^{ef}	21.2^{c}	169^{b}	0.94^{c}
	Kasota	5.2^{de}	33.2^{c}	157^{b}	1.54^{c}

Table 3. 4 *continued*

4th day of WR	*Well-watered*				
	CDCCowboy	22[a]	518[abc]	284[bc]	9.26[ab]
	Niobe	21[a]	576.9[a]	313[ab]	10.38[ab]
	170011	17.6[b]	324.3[cd]	281[bc]	7.70[bc]
	Kasota	22.5[a]	718.3[a]	328[a]	10.24[a]
	Water-recovery				
	CDCCowboy	21[a]	355.8[bcd]	268[c]	7.06[c]
	Niobe	20.4[ab]	544.6[ab]	310[ab]	10.32[a]
	170011	14.3[c]	178.8[d]	256[c]	4.39[d]
	Kasota	21.8[a]	597.7[a]	318[a]	9.99[ab]

Means followed by a different letter are significantly different at $P < 0.05$

Table 3. 5 Correlations between assimilation rate (*A*), internal CO_2 concentration (C_i), stomatal conductance (g_s), transpiration rate (E), water-use efficiency (WUE_{ic}), leaf carbon isotope discrimination ($\Delta^{13}C$), specific leaf area (SLA), water-use efficiency (WUE_{ie}), biomass and grain yield of four barley genotypes on the 10th day of water-deficit under greenhouse conditions

	A	C_i	g_s	*E*	WUE_{ic}	$\Delta^{13}C$	SLA	WUE_{ie}	Biomass
Well-watered									
A	1.000								
C_i	-0.35	1.000							
g_s	0.71	0.39	1.000						
E	0.47	0.58	0.94	1.000					
WUE_{ic}	-0.30	-0.78	-0.85	-0.85	1.000				
$\Delta^{13}C$	0.69	0.43	0.99**	0.93	-0.88	1.000			
SLA	-0.94	0.57	-0.53	-0.32	0.02	-0.49	1.000		
WUE_{ie}	-0.69	0.79	-0.02	0.30	-0.31	-0.02	0.72	1.000	
Biomass	-0.88	-0.69	-0.86	-0.64	0.90	-0.89	0.11	0.005	1.000
Grain yield	-0.99*	-0.28	-0.54	-0.21	0.59	-0.59	0.55	0.46	0.89
Water-deficit									
A	1.000								
C_i	-0.90	1.000							
g_s	0.99*	-0.92	1.000						
E	0.95*	-0.77	0.96*	1.000					
WUE_{ic}	0.93	-0.99**	0.93	0.78	1.000				
$\Delta^{13}C$	-0.26	0.64	-0.35	-0.10	-0.57	1.000			
SLA	-0.46	0.50	-0.37	-0.22	-0.58	0.11	1.000		
WUE_{ie}	0.91	-0.86	0.86	0.76	0.91	-0.23	-0.79	1.000	
Biomass	-0.30	-0.17	-0.25	-0.53	0.14	-0.74	-0.94	-0.05	1.000
Grain yield	-0.35	-0.11	-0.31	-0.58	0.08	-0.70	-0.92	-0.11	0.99*

* and ** indicates 5% and 1% significant level respectively.

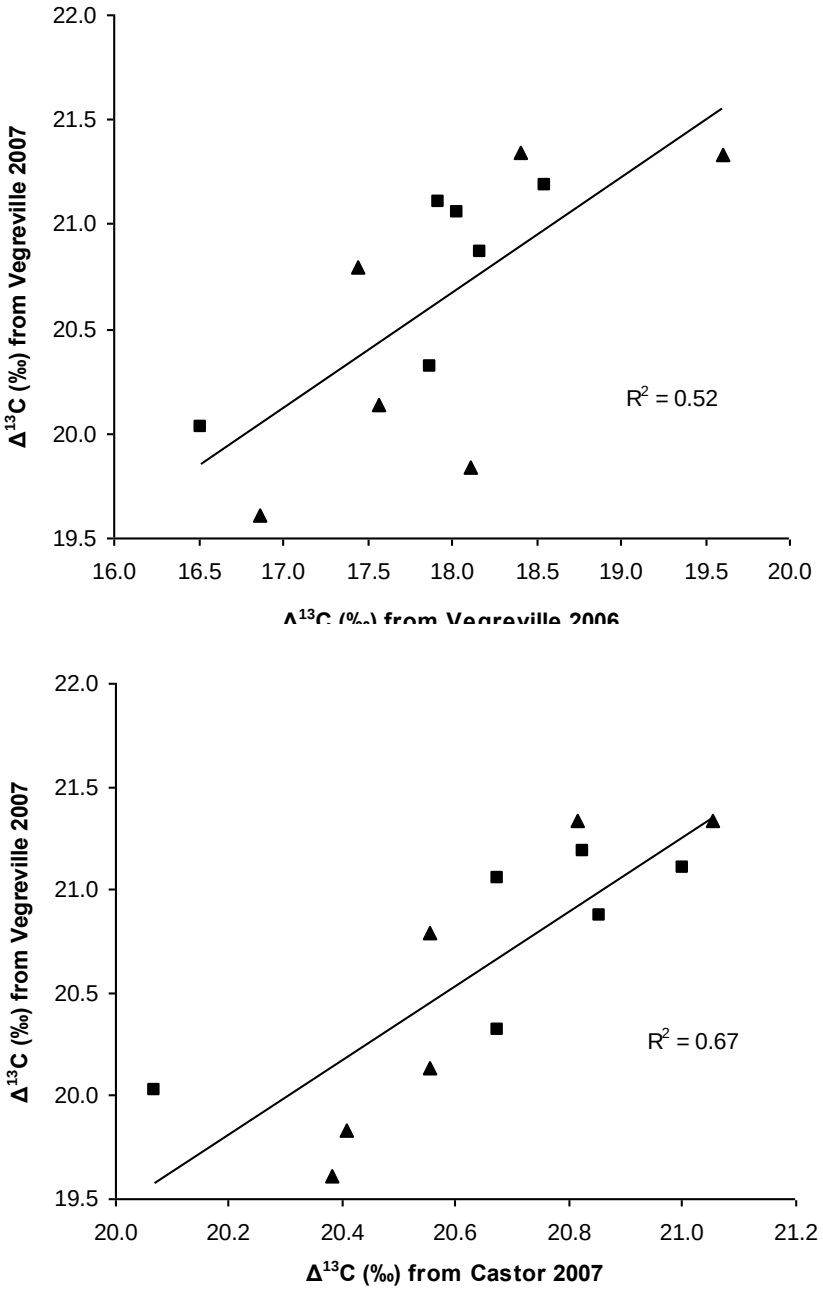

Figure 3. 1 Correlation between leaf carbon isotope discrimination ($\Delta^{13}C$) of 12 barley varieties measured in Vegreville during 2006 and 2007 growing seasons (top); Correlation between leaf $\Delta^{13}C$ of 12 barley varieties measured in Vegreville and Castor during 2007 growing season (bottom) (two-row barley, square; six-row barley, triangle).

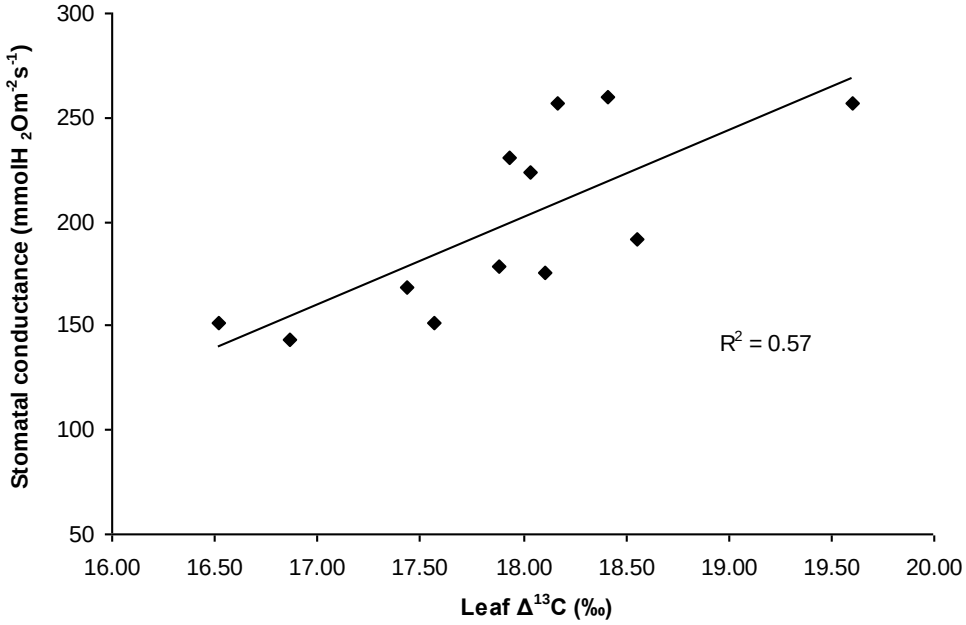

Figure 3. 2 Correlation between leaf $\Delta^{13}C$ and stomatal conductance of 12 barley varieties measured in Vegreville during the 2006 growing season.

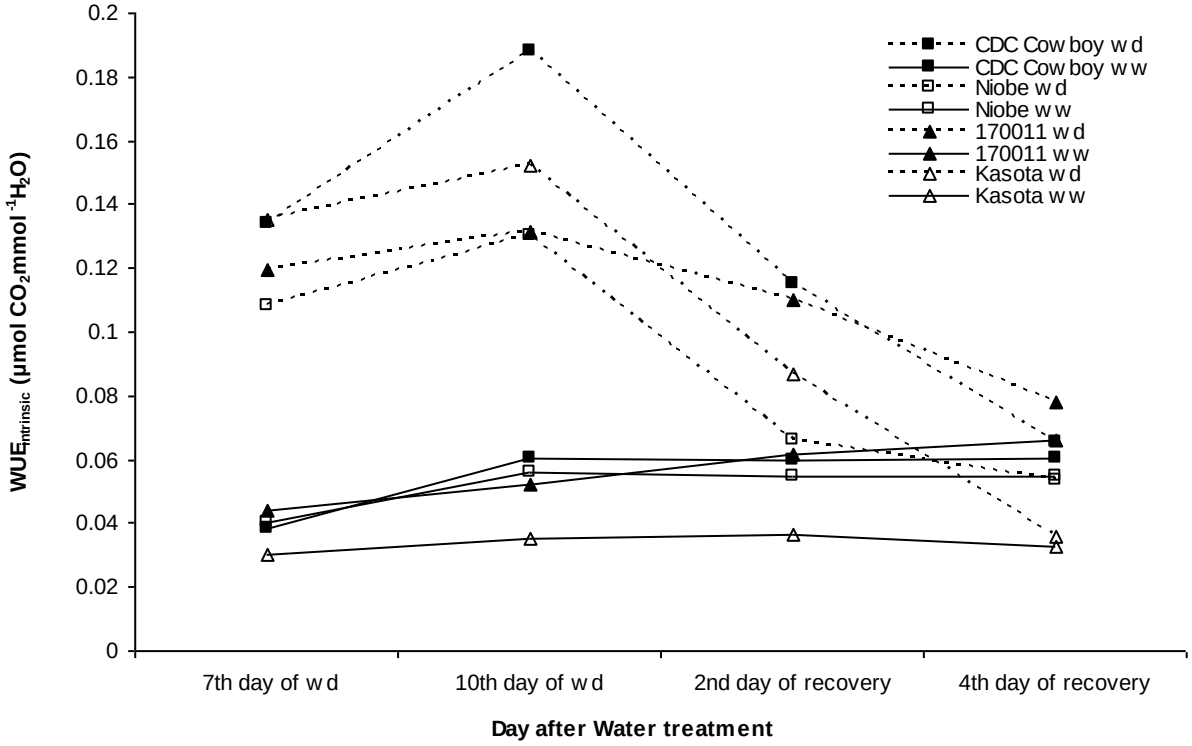

Figure 3. 3 Trends of WUE_{ic} over time under well-watered (ww), water-deficit (wd) and water recovery conditions

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Chapter 4 Quantitative trait loci for water-use efficiency in barley (*Hordeum vulgare* L.) under rain-fed conditions on the Canadian Prairies

Introduction

Drought continues to be a major constraint on the productivity of cereal crops and water deficit will increase in most arid and semi-arid regions under future climate-change scenarios (IPCC 2007; Wassmann et al. 2009). Agricultural production in Canada is mostly concentrated on the Prairies of western Canada (Canadian International Grains Institute (CIGI) 2004). The production of barley, one of the main crops grown in Canada, is centered in the three Prairie Provinces (Alberta, Saskatchewan and Manitoba), with an average of 12.3 million tonnes produced annually during 1986 – 2006 (FAOSTAT 2008). Both two- and six-row ear types of barley are commonly grown under rain-fed conditions for malting, livestock feed and food. During the short and dry growing seasons, sometimes with terminal heat stress (Anyia et al. 2008), barley relies on stored soil moisture and limited rainfall within the growing season. The unpredictable occurrence of drought can cause highly unstable barley yield across years. Therefore breeding for drought tolerant and water-use efficient varieties has been a critical area of agricultural research in Canada.

Water-use efficiency (WUE) has been proposed as a criterion for yield improvement under drought (Condon and Richards 1992; Condon et al. 2002; Rebetzke et al. 2002; Richards et al. 2002), and improved WUE can increase yield in certain environments. However, the application of WUE in breeding programs has been largely limited by the time-consuming and expensive screening process under field conditions for large populations. Carbon isotope discrimination ($\Delta^{13}C$), through its negative relationship with transpiration efficiency has been demonstrated to be a simple but reliable measure of WUE (Farquhar et al. 1982;

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Farquhar and Richards 1984), and their negative correlation has been used for indirect selection of WUE under selected environments (Cattivelli et al. 2008).

Selection efficiency of leaf $\Delta^{13}C$ could be enhanced with a better understanding of its genetic control. The advancement of DNA-based molecular markers and computational methods in the late 1980s and 1990s has greatly revolutionized the dissection of quantitative trait inheritance and genetic improvement of yield in dry environments (Baum et al. 2007). The first quantitative trait loci (QTL) identified for $\Delta^{13}C$ was reported in tomato (*Lycopersicon esculentum* and *L. pennellii*) by Martin and Nienhuis (1989) and subsequently QTL for $\Delta^{13}C$ have been reported in *Arabidopsis thaliana* (Hausmann et al. 2005; Juenger et al. 2005), barley (grain) (Teulat et al. 2002), cotton (*Gossypium hirsutum* and *G. barbadense*) (Saranga et al. 2001), rice (*Oryza sativa* L.) (Laza et al. 2006; Takai et al. 2006; This et al. 2010; Xu et al. 2009), soybean (*Glycine max* L.) (Specht et al. 2001) and bread wheat (*Triticum aestivum* L.) (Rebetzke et al. 2008). Ellis et al. (1997) discovered several AFLP (Amplified Fragment Length Polymorphisms) markers associated with whole-shoot $\Delta^{13}C$ of barley measured on 57 doubled haploids (DHs) from a cross between *H. vulgare* L. cv Lina and *H. vulgare* ssp. *spontaneum* HS92 in a hydroponic system under controlled and salt-stress conditions. The first QTL study for grain $\Delta^{13}C$ in barley was by Teulat et al. (2002) using 167 recombinant inbred lines (RILs) derived from two-row barley cultivars Tadmor and Er/Apm in three Mediterranean field environments, with a total of ten QTL identified in the study.

Different plant parts such as root, leaf, sheath, awn or grain, can be characterized with their own $\Delta^{13}C$ values and potential advantages for evaluating WUE. Leaf $\Delta^{13}C$ during the stem elongation stage, which is regarded as the formation of yield potential (Anyia et al. 2008), could reflect the integrated WUE and the vegetative establishment. Condon and Richards (1992) proposed that it would be most effective to assess $\Delta^{13}C$ at early stages in plant development under well-watered conditions. In addition, leaf $\Delta^{13}C$ measured before anthesis can

enable selection and crosses of varieties to be made within the same season, thereby speeding up the breeding process. According to Jiang et al. (2006), values of $\Delta^{13}C$ from different plant parts (flag leaf, awn and grain) were on average higher for six-row barley than those of two-row barley, suggesting a higher WUE of two-row barley compared with six-row barley under both irrigated and non-irrigated field conditions. So far, there has been no report on QTL analysis of $\Delta^{13}C$ on barley leaves or on two- and six-row barley examined under the same rain-fed environments.

In this study, two- and six-row barley RIL populations were grown under rain-fed conditions, and the penultimate leaves at stem elongation stage were sampled for $\Delta^{13}C$ analysis and QTL mapping. The objectives of the present study were to: 1) to determine the chromosomal regions and phenotypic effects of QTL associated with variations in leaf $\Delta^{13}C$ as well as agronomic traits; 2) to identify the common QTL regions across environments and populations; 3) to dissect the genetic control of leaf $\Delta^{13}C$ and potential alleles for further WUE improvement in barley.

Materials and methods

Field experiments and climatic conditions

The experiments were conducted at Lacombe (52 °28' N, 113 °45' W, 847.3 m altitude), having an Orthic Black Chernozemic soil (Canadian system of soil classification), Vegreville (53 °31' N, 112 °6' W, 639.3 m altitude), having the Malmo series of an Eluviated Black Chernozemic soil, and Castor (52 °8' N, 111 °54' W, 807.7 m altitude), with a Dark Brown Chernozemic soil, in Alberta, Canada, under rain-fed conditions. The three sites were considered three different environments characterized by distinct soil moisture conditions with Castor as the driest site and Lacombe as the wettest site. The average annual precipitation and within season rainfall (June to August) from 1977 to 2007 was 340 ±89 mm and

172±67 mm at Castor compared with Vegreville which had 382±62 mm and 193±52 mm, and Lacombe which had 440±84 mm and 230±63 mm, respectively (AgroClimatic Information Service (ACIS) 2009; Environment Canada 2009).

Mapping populations

One hundred and six diverse genotypes (advanced lines and commercial varieties) of barley were screened for variation in leaf $\Delta^{13}C$ at two field locations in Vegreville and Lacombe in 2005. Among these genotypes, two-row genotypes (Merit and H93174006) and six-row barley (W89001002003, hereafter referred to as W89 and I60049) showing contrasting levels (low versus high) of leaf $\Delta^{13}C$ were selected as parental materials for mapping population development after additional greenhouse and field experiments confirmed their leaf $\Delta^{13}C$ rank stability (Fig. 4.1). Two RIL mapping populations were developed, with one cross between W89 and I60049 (hereafter referred to as W × I) and the other cross between Merit and H93174006 (hereafter referred to as M × H) in the field during the summer of 2006. The parents with low leaf-$\Delta^{13}C$ (Merit and W89) were used as females since previous experience indicated that maternal inheritance may have an influence on $\Delta^{13}C$ and WUE traits (Dr. Richard Richards, CSIRO, personal communication). All F_1 seeds from each cross were planted in bulk in the field (F_2 populations) at Lacombe in 2007 and evaluated for segregation. The single seed descent (SSD) approach was used to advance the populations from F_3 to F_4 generation in the greenhouse at Lacombe during 2007 and 2008. The F_5 seeds from each F_4 plant were bulked and advanced to produce enough seeds for $F_{5:6}$ generation field trials.

Experiment design

The W × I mapping population

Two hundred F_5 RILs and two parental lines (W89 and I60049) were planted at Lacombe in 2008 (L08), Castor in 2009 (C09) and Vegreville in 2009 (V09).

For L08, the experiment used a completely randomized design with one replicate of RILs and four replicates of parental lines. Seeds of each line were planted in 2 rows in 0.3 x 2 m plots with a spacing of 15 cm within rows and 35 cm between plots. Winter triticale 'Pika' was seeded as guard row between plots. Synthetic fertilizer mix (6-25-30) was applied at 112.5 kg ha^{-1} prior to seeding. Plots were seeded on May 15 (Table 4.1), and harvested on September 5. Herbicides were applied at the rate of 29.7 g ha^{-1} for 'Refine' (33.35% thifensulfuron methyl, 16.65% tribenuron methyl) and 988.4 mL ha^{-1} for 'MCPA' (500 g L^{-1} amine, 500 g L^{-1} ester, 400 g L^{-1} K-salt and 300 g L^{-1} Na-salt) during the vegetative stage to control weed species.

For C09 and V09, the experiments used a randomized complete block design with three replicates of each $F_{5:6}$ RILs and parental lines. Seeds of each line were planted in 4 rows in 1 x 2 m plots with a spacing of 20 cm within rows and 20 cm between plots. Spring wheat 'AC Crystal' was seeded as a guard row between plots. Fertilizer was applied prior to seeding at a rate of 22.5 kg ha^{-1} P_2O_5 at Vegreville, and 31.4 kg ha^{-1} N and 18 kg ha^{-1} P_2O_5 at Castor based on results of soil test. Seeds were sown on May 13 at Castor and on May 22 at Vegreville, and both sites were harvested during the last week of September. Weeds were controlled manually at the 3-leaf stage (BBCH 13) (Zadoks et al. 1974) at Castor. For Vegreville, 'Round-up Weathermax' (540 g L^{-1} glyphosate) was sprayed at 1.66 L ha^{-1} before emergence on May 24.

The M × H mapping population

One hundred and twenty-seven F_5 RILs and two parental lines (Merit and H93174006) were planted at Vegreville in 2008 (V08), as well as C09, and V09.

For V08, the experiment was a completely randomized design with one replicate of RILs and four replicates of parental lines. 200 seeds of each line were sown in 2 rows of a 2 m long plot with a spacing of 20 cm between rows and 60

cm between plots. Fertilizer was applied at seeding by adding 38.25 kg P_2O_5 ha^{-1}, and 61.88 kg N ha^{-1}. Seeds were sown on May 16 and plants were harvested on September 18. Herbicide 'Round-up Weathermax' was sprayed at 1.66 L ha^{-1} before seeding, and 'Achieve 40DG' and 'Buctril M' were applied on June 4 at 494.2 g ha^{-1} and 988.4 mL ha^{-1}, respectively.

For C09 and V09, the experiment design was the same as for the W x I population described above. Seeds were sown on May 15 at Castor and on May 25 at Vegreville, and both sites were harvested during the last week of September.

Phenotypic data collection

Carbon isotope discrimination analysis: At the stem elongation stage (BBCH 36 to 39), five fully expanded penultimate leaves per plot were randomly collected, bulked, and dried in an oven at 70 ℃ for 48 h. Dried leaf samples were ground with a ball mill (Spex SamplePrep 8000D Mixer, Metuchen, NJ, USA) to fine power and analyzed for carbon isotope composition ($\delta^{13}C$) using a continuous-flow stable isotope ratio mass spectrometer (Thermo Finnigan Mat Gmbh, Bremen, Germany). The $\delta^{13}C$ was calculated as:

$$\delta^{13}C\ (‰) = (R_{sample}/R_{reference} - 1) \times 1000$$

Where R_{sample} and $R_{standard}$ are the ratios of $^{13}C/^{12}C$ measured in the plant material and the reference, respectively, and the reference material used was the belemnite carbonate standard (PDB) from the Pee Dee Formation. The $\Delta^{13}C$ was calculated according to Farquhar et al. (1989) as:

$$\Delta^{13}C\ (‰) = [(\delta^{13}C_a - \delta^{13}C_p) / (1 + \delta^{13}C_p /1000)] \times 1000$$

Where $\delta^{13}C_a$ and $\delta^{13}C_p$ refer to the C isotope ratios of atmospheric CO_2 (-8.0 ‰) and plant, respectively.

Leaf area index (LAI): LAI was measured in the 2009 field trials for the W × I population using a LAI-2000 Plant Canopy Analyzer (Li-Cor, Lincoln, NE, USA). A measurement cycle consisted of one reference measurement and four below-

canopy readings. Reference measurements were collected above canopy level of each experimental plot (between 1 and 1.5 m above ground) at the beginning of each cycle. The below-canopy measurements were carried out at a diagonal transect between rows in each plot to improve the spatial average. The fish-eye lens of the instrument was covered with a view cap with a 45 °opening, so that the reference measurements were not influenced by the operator and the angle of the sun (Li-Cor 1992). Measurements were taken at the stem elongation stage (BBCH 36 to 39) and grain filling stage (BBCH 71 to 75) under cloudy conditions.

Plant height, days to maturity, aerial biomass and grain yield: Plant height was measured from the ground level to the tip of the spike on 5 randomly selected plants per plot at the physiological maturity stage (BBCH 89) in the 2009 field trials for the W × I population. Days to maturity was recorded as the number of days from sowing till the stage that 90% of plants in the plot reached maturity (BBCH 91 to 92), this trait was only recorded at V09 for the W × I population. Biomass and grain yield were determined from 1 m^2 sub-plots. Grain was threshed, cleaned and air-dried for two weeks before weighing, with all grain yields expressed as oven-dried values, after the moisture content of the air-dried grains was determined. Harvest index (HI) was calculated as HI = Grain yield / total aboveground biomass.

Molecular marker assay

For the W × I population: Parents (W89 and I60049) were screened for polymorphism with a total of 516 simple sequence repeat (SSR) markers from the published literature (Chaabane et al. 2009; Karakousis et al. 2003; Li et al. 2003; Liu et al. 1996; Ramsay et al. 2000; Thiel et al. 2003; Varshney et al. 2007). Polymorphic SSR markers were chosen for scoring the entire W × I population.

Young leaf tissues from each plot in the field trials were collected for DNA extraction. Genomic DNA was isolated as described by Saghai-Maroof et

al. (1984), and quantified using a spectrophotometer (NanoDrop ND-1000 Spectrophotometer, Wilmington, Delaware USA), and qualified by electrophoresis. Regular PCR (polymerase chain reaction) was carried out in 20-μL reactions containing 5 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM forward prime, 0.2 μM reverse primer, 1 unit of *Go Taq* Flexi DNA polymerase (Promega Corporation, Wisconsin, USA), deionized water, and 40 ng genomic DNA in 96-well plates using a Eppendorf Mastercyler (Hamburg, Germany). The SSRs amplification was performed using three PCR conditions: 1) A "touchdown" PCR consisting of 18 cycles of 1 minute denaturing at 94 ℃, 30 s annealing at 62 ℃ or 69 ℃ and 1 minute extension at 72 ℃. Annealing temperatures were decreased by 0.5 ℃ per cycle, depending on the melting temperature (T_m) of primer pairs used, either from 62 ℃ to 53 ℃ or from 69 ℃ to 60 ℃. The reaction continued for 30 additional cycles with 1 minute at 94 ℃, 1 minute at 53 ℃ or 60 ℃, 1 minute at 72 ℃, and eventually 5 minutes at 72 ℃ as the final extension step; 2) A regular PCR was performed with 2 minutes at 94 ℃, followed by 35 cycles at 94 ℃ for 30s, 30s at optimal annealing temperature (ranging from 53 ℃ to 58 ℃), 1 minute at 72 ℃, and ended with 5 minutes at 72 ℃; 3) Condition was the same as those described by Röder et al. (1995), 35 cycles were performed with 1 minute at 96 ℃, 1 minute at 60 ℃ (varies with the T_m of primers) and 2 minutes at 72 ℃, and a final elongation step of 10 minutes at 72 ℃, in some cases the $MgC1_2$ concentration was increased to 2.5 mM.

Primers with similar annealing temperatures and non-overlapping ranges of amplified fragment sizes were combined in the same reaction. Amplified PCR products were separated on 2.5% agarose gels in 1X TAE buffer, stained with ethidium bromide and photographed. The entire population was genotyped for SSR markers using a multicapillary (12-channel) electrophoresis system (HAD-GT12, eGene, Irvine, CA, USA).

For the M × H population: A total of 373 SSR and 72 STS (Sequence-tagged-site) markers were screened for polymorphism between parental lines (Merit and H93174006). To get additional genomic coverage, extracted DNA of the RILs and parental lines were sent to Triticarte Pty Ltd (Canberra, Australia; http://www.triticarte.com.au/) for Diversity Arrays Technology (DArT®) genotyping, which is a novel genotyping method and hybridization-based technology as described by Wenzl et al. (2004).

QTL analysis and map construction

The segregating patterns of SSR amplifications at polymorphic loci in all lines were compared to parental lines, and scored as maternal (1), paternal (2) or heterozygous/missing (3) of an allele. Segregation markers in the RILs were examined by the chi-square (χ^2) test for goodness of fit. The expected ratio of individuals in each genotypic class (maternal or paternal) was 1:1 in the RILs. Mapmaker v3.0 (Lander et al. 1987) was used to construct linkage groups at a LOD (logarithm of odds ratio) threshold of 3.0 with a maximum Kosambi distance of 30 centiMorgans (cM), and markers were assembled to initial linkage groups, then the LOD score was reduced to bridge some intervals. The position and order of markers in linkage groups were compared with published barley consensus map (Varshney et al. 2007; Wenzl et al. 2006). Genetic linkage maps were imported into Windows QTL Cartographer V2.5 (Wang et al. 2010) to locate putative QTL for all traits using composite interval mapping method (CIM) (Zeng 1994). A threshold score for the QTL was determined to be 2.0 by 1,000 time permutations using the Zmapqtl program (Churchill and Doerge 1994). A confidence interval for QTL location was determined by a one-LOD drop from the peak position (Flint-Garcia et al. 2003), and overlapping confidence intervals were used to determine common QTL across environments. The final linkage maps were drawn using the MapChart software (Voorrips 2002).

Data analysis

Data were analyzed using version 9.1 of the SAS software (SAS Institute, Inc., Cary, NC). Each location-year combination of the field trials (L08, V08, C09, and V09) was treated as a single environment. Analysis of variance (ANOVA) was performed for each environment as well as combined environments. Variance components were estimated using the restricted maximum likelihood (REML) method of PROC MIXED.

According to Hanson et al. (1956), the broad-sense heritability (H^2) for leaf $\Delta^{13}C$ across environments was estimated as:

$$H^2 = \sigma^2_g / [\sigma^2_g + \sigma^2_{g\times e}/e + \sigma^2_r/(r\times e)]$$

Where variances were due to genotype (σ^2_g), genotype × environment ($\sigma^2_{g\times e}$), and residual (σ^2_r), e is the number of environments, and r is the number of replicates.

Homogeneity of variance and normality of distribution were tested before analysis of variance. Correlation analyses (PROC CORR) were performed to detect the relationships among traits, with means of each RIL combined over the environments. Pearson correlation coefficients were calculated between environments for leaf $\Delta^{13}C$, with means of each RIL based on each environment. An α value of 0.05 was chosen to indicate statistical significance.

Results

Environments and leaf $\Delta^{13}C$

Across eight different environments (location-year combinations) in this study, the leaf $\Delta^{13}C$ of W89 was consistently lower than that of I60049, except at Castor-2007 (Fig. 4.1). Although the leaf $\Delta^{13}C$ of Merit was only significantly lower than that of H93174006 at three out of seven environments, trends of difference were still observed across environments (Fig. 4.1). The correlation analysis

showed that leaf $\Delta^{13}C$ was significantly related to rainfall in June (data not shown, $r = 0.48$, $n = 122$, $p < 0.001$) and total within season precipitation (from June to August) (data not shown, $r = 0.42$, $n = 122$, $p < 0.001$). Significant differences were observed for leaf $\Delta^{13}C$ between environments for both populations (Table 4.2 and 4.3).

For the W × I population, leaf $\Delta^{13}C$ of RILs were significantly higher in L08, followed by V09 and then C09 (Table 4.2). For the M × H population, leaf $\Delta^{13}C$ for the experiments conducted in Vegreville was significantly higher in 2008 than in 2009. Positive and significant correlations of leaf $\Delta^{13}C$ were observed between C09 and V09 (Fig. 4.2, $r = 0.32$, $n = 200$, $p < 0.001$), C09 and L08 (Fig. 4.2, $r = 0.32$, $n = 200$, $p < 0.001$), L08 and V09 (Fig. 4.2, $r = 0.21$, $n = 200$, $p = 0.002$). The extreme genotypes differed by 3.38‰ at L08, 3.82‰ at C09 and 4.25‰ at V09. For the M × H population, a positive correlation of leaf $\Delta^{13}C$ was observed between V08 and V09 (Fig. 4.3, $r = 0.16$, $n = 127$, $p = 0.08$). The extreme genotypes differed by 3‰ at V08 and 3.14‰ at V09.

Leaf $\Delta^{13}C$ and agronomic traits

The two populations differed in leaf $\Delta^{13}C$, with the M × H population producing a significantly lower leaf $\Delta^{13}C$ than the W × I population at V09 (data not shown, $p < 0.001$). Leaf $\Delta^{13}C$ of progeny within each population were significantly different among the environments studied (Table 4.2 and 4.3). The Shapiro-Wilk test showed that leaf $\Delta^{13}C$ of both populations had typically normal distributions under all environments (Fig. 4.4). Most of the leaf $\Delta^{13}C$ values for the RILs were between those of the parents in each field trial, but transgressive segregation was also noted as some progeny extremes for leaf $\Delta^{13}C$ exceeded parental values. Some of the extreme RILs that showed transgressive segregation for leaf $\Delta^{13}C$ from the W × I population (such as '176' and '191') and the M × H population (such as '43', '110' and '162') were consistent across all locations. The H^2 for leaf $\Delta^{13}C$ was high (0.80) for the W × I population across all environments.

Under a single environment (C09 or V09), the H^2 for both populations reached 0.80 (data not shown).

Biomass, yield, HI, plant height, LAI, and days to maturity all showed continuous variation (Tables 4.2 and 4.3). For the W × I population, ANOVA revealed significant variability among the 200 RILs for a range of agronomic traits (Table 4.2) except LAI measured at grain filling stage (LAI-G). Interactions between genotype and environment (G × E) were not significant for leaf $\Delta^{13}C$, LAI-G, and HI. For the M × H population, genotypic variation was not significant for biomass, yield and HI (Table 4.3), and G × E was only significant for leaf $\Delta^{13}C$.

For the W × I population, leaf $\Delta^{13}C$ showed significant positive relationships with LAI at stem elongation stage (LAI-S), biomass, yield and HI at both C09 and V09 (Table 4.4). For the M × H population, there was also significant positive correlation between $\Delta^{13}C$ and biomass at V08 and V09 (Table 4.5), and significant positive relationship between $\Delta^{13}C$ and grain yield was only observed at V08.

Linkage map construction

Screening of the parental lines with SSR markers revealed a low level of polymorphism between parental lines. For the W × I population, only 148 (28.7%) out of 516 screened markers could be used for scoring the 200 RILs. For the M × H population, 55 (12.9%) out of 373 SSR markers and 196 (39.3%) out of 499 DArT markers were polymorphic between Merit and H93174006. In total, 104 SSR markers were mapped for the W × I population, and 209 loci were mapped for the M × H population. In general, there was good agreement for the marker order between maps from this study and maps published in the literature.

For the W × I population, the 104 SSR loci were grouped into ten linkage groups which were assigned to seven chromosomes (Fig. 4.5) based on alignments from previously published barley consensus maps (Karakousis et al.

2003; Varshney et al. 2007). Chromosome (Chr.) 1H, 2H, 3H, 4H, and 6H were each represented by a single linkage group, whereas Chr. 5H and 7H were split into two and three linkage groups respectively. The χ^2 test revealed highly biased segregation: 67 SSR markers (64.4%) showed segregation ratio which distorted from the expected Mendelian ratio for RILs. The 23 SSR markers (34.3% of the distorted markers) on Chr. 3H completely distorted towards the maternal line W89, while 23.9% of the distorted markers deviated significantly to paternal line I60049 on Chr. 5H ($p < 0.05$). Skewing in favor of maternal alleles (W89, 52.2%) at all the distorted loci was observed in this population.

For the M × H population, the 209 loci, including 21 SSR and 188 DArT loci, were grouped into seventeen linkage groups which were assigned to seven chromosomes (Fig. 4.6) according to previously published high-density consensus maps of barley (Varshney et al. 2007; Wenzl et al. 2006). Chr. 1H, 2H and 3H were split into three linkage groups, whereas Chr. 6H and 7H were split into four and two linkage groups, respectively, and Chr. 4H and 5H were each represented by a single linkage group. Chr. 4H showed a low level of polymorphism which resulted in only four loci being mapped. The χ^2 test of SSR and DArT markers showed distorted segregation ratios of 57.1 and 53.7%, respectively. Maternal alleles (Merit) were significantly overrepresented (76.1%) at all skewed loci.

QTL identified for leaf $\Delta^{13}C$

Table 4.6 describes the QTL identified for leaf $\Delta^{13}C$ in each environment separately. For the W × I population, a total of 12 QTLs clustering in nine chromosomal regions and explaining from 3.6 to 22% of the phenotypic variation were identified for leaf $\Delta^{13}C$, with 5 QTLs detected at L08, 3 QTLs at C09 and 4 QTLs at V09 by the CIM analysis. Eight of these 12 QTLs reduced leaf $\Delta^{13}C$ and the W89 allele was associated with low leaf $\Delta^{13}C$. One QTL located on Chr. 3H near SSR marker Bamg606 was detected consistently in all three environments. This QTL conferred main effect across all environments, with LOD values of

10.81, 5.86 and 5.27, explaining 22, 14.4 and 11% of the phenotypic variance in leaf $\Delta^{13}C$ at L08, C09 and V09, respectively. At this locus, leaf $\Delta^{13}C$ value was reduced by the W89 allele (Table 4.6).

For the M × H population, a total of 5 QTLs clustering in three chromosomal regions and explaining from 8.6 to 11.7% of the phenotypic variation were detected by the CIM analysis for leaf $\Delta^{13}C$, with 3 QTLs at V08 and 2 QTLs at V09. Two QTLs were detected consistently in two environments with one QTL located at Chr. 6H near DArT marker bPb1212, explaining 11.2 and 10.8% of the phenotypic variance in leaf $\Delta^{13}C$ at V08 and V09, respectively, and the other QTL on Chr. 7H close to DArT marker bPb9898, explaining 9.9 and 8.6% of leaf $\Delta^{13}C$ at V08 and V09, respectively. All of the five QTLs increased leaf $\Delta^{13}C$ value with allele from Merit (Table 4.6).

Identification of QTL for agronomic traits

As listed in Table 4.7, Fig. 4.5 and Fig. 4.6, a total of 38 QTLs were identified by CIM analysis for six agronomic traits under each environment.

Leaf area index: LAI was measured only in the W × I population at C09 and V09, and the QTLs for LAI were only found at V09. Two QTLs for LAI-S and four QTLs for LAI-G were detected. One QTL for LAI-S was co-located with QTL for LAI-G which was positioned on Chr. 3H near marker GBM1405. One QTL for LAI-G on Chr. 3H near Bmag606 co-located with QTL for leaf $\Delta^{13}C$, with W89 allele responsible for reduced LAI at this locus (Table 4.7).

Plant height: Plant height was measured only in the W × I population. Eleven QTLs were associated with plant height. Eight QTLs affecting plant height were detected at C09 and three were identified at V09. The QTL with the largest effect on plant height was located on Chr. 3H close to Bamg606, explaining 27.4% of the phenotypic variance in plant height at V09, and this QTL was detected at C09 as well. Increased plant height was associated with the W89 allele at this locus (Table 4.7). This QTL was also co-located with QTL for leaf $\Delta^{13}C$ on Chr. 3H.

Days to maturity: Days to maturity was recorded in the W × I population only at V09. Five QTLs were detected for days to maturity. One QTL located on Chr. 3H near Bamg606, explaining 16% of the phenotypic variance in days to maturity with a LOD score of 10.78, and this QTL was also co-located with the QTL for leaf $\Delta^{13}C$ on Chr. 3H. Another QTL on Chr. 5H near marker Bmac113 was co-located with QTL for leaf $\Delta^{13}C$, biomass and grain yield. The W89 allele of all QTLs detected at V09 contributed to early maturity (Table 4.7).

Total aboveground biomass: For the W × I population, four QTLs associated with total aboveground biomass were found. Only one QTL was detected at C09. The three QTLs detected at V09 explained from 3.7 to 6.3% of the phenotypic variance in total aboveground biomass, with LOD scores ranging from 2.21 to 3.76. For the M × H population, only two QTLs were detected, with one QTL at V08 and one at C09. Both loci increased total biomass with allele from Merit (Table 4.7).

Grain yield: For the W × I population, variation for grain yield was associated with six QTLs that clustered into five chromosomal regions. On Chr. 5H near marker GBM5008, overlapping QTLs from both locations were detected. This QTL interval included QTLs for biomass, HI, and LAI. The W89 allele was associated with increased grain yield, biomass, HI and LAI at this locus (Table 4.7). One QTL on Chr. 5H near marker Bmag323 was co-located with QTLs for leaf $\Delta^{13}C$ and biomass, with increased grain yield from the I60049 allele (Table 4.7). One QTL detected at C09 was co-located with QTLs for leaf $\Delta^{13}C$ on Chr. 3H, explaining 11.4% of the phenotypic variance in grain yield at a LOD value of 4.42, with the W89 allele responsible for reduced grain yield at this locus (Table 4.7).

For the M × H population, three QTLs associated with grain yield were identified. One QTL on Chr. 6H near marker bPb1212 was co-located with QTLs for leaf $\Delta^{13}C$. Another QTL on Chr. 7H near marker bPb6821 overlapped with QTLs for grain yield, biomass and leaf $\Delta^{13}C$. Both of these QTLs (near bPb1212 and bPb682) increased the grain yield by the Merit allele (Table 4.7).

Harvest index: For the W × I population, only two QTLs were detected for HI. One QTL on Chr. 5H was co-located with QTLs for LAI-G, biomass and grain yield with increased HI from the W89 allele. The other QTL for HI on Chr. 3H near marker Bmag606 was mapped around the same position with QTLs for LAI-G, leaf $\Delta^{13}C$, grain yield and plant height.

For the M × H population, four QTLs for HI were identified on Chr. 2H. One QTL near marker bPb4293 was co-located with the QTL for grain yield, which reduced the HI value by the Merit allele (Table 4.7).

Discussion

Leaf $\Delta^{13}C$ and rainfall across environments

Changes in the leaf $\Delta^{13}C$ of parental lines across years and locations were consistent with trends of the total growing season rainfall, suggesting environmental effects on leaf $\Delta^{13}C$ (Fig. 4.1). Teulat et al. (2002) reported that both total rainfall and the ratio of rainfall to evapotranspiration had a significant impact on $\Delta^{13}C$, explaining mainly the environment effects in their study. For water-limited environments such as the Canadian Prairies where crops rely on soil-stored moisture (Anyia et al. 2008), the temporal distribution of rainfall is critical for crop growth (Bonsal et al. 1999; Chakravartia 1972). In this study, leaf $\Delta^{13}C$ was significantly related to total within season precipitation ($r = 0.42$, $n = 122$, $p < 0.001$), especially the amount of rainfall during June ($r = 0.48$, $n = 122$, $p < 0.001$). Ivlev and Voronin (2007) reported that the dynamics of $\delta^{13}C$ in the annual rings of trees were found to be related positively with air temperature in June but negatively with precipitation in June from 1980 to 2005; even though trees are very different plant species from the barley I studied, their biological response to water availability should be similar.

Leaf $\Delta^{13}C$ was significantly reduced under drought conditions such as C09, which is in agreement with most reports on $\Delta^{13}C$ in C_3 crops (Craufurd et al. 1991;

Hall et al. 1990; Virgona et al. 1990). Leaf $\Delta^{13}C$ of extreme RILs differed more in the low-rainfall environment (such as C09) than the high rainfall environment (such as L08). This result is in agreement with previous reports, which suggest that $\Delta^{13}C$ can be used as a sensitive indicator for plant water status or water availability during the growing period (Bloch et al. 2006; Craufurd et al. 1991; Merah et al. 2001). Although the effect of environment was significant, significant correlations between leaf $\Delta^{13}C$ across environments were observed for both populations, demonstrating the stability of leaf $\Delta^{13}C$ across the tested environments.

Relationship between leaf $\Delta^{13}C$ and agronomic traits

Positive or neutral relationships are frequently reported between $\Delta^{13}C$ and grain yield and/or biomass in environments characterized with plentiful within-season rainfall or supplemental irrigation (Araus et al. 1998; Araus et al. 2003; Condon et al. 1987; Condon et al. 1993; Jiang et al. 2006; Merah et al. 1999; Merah et al. 2001; Monneveux et al. 2006; Morgan et al. 1993; Teulat et al. 2001c; Voltas et al. 1999). The correlations between leaf and/or grain $\Delta^{13}C$ and grain yield have been reported to be positive, negative or neutral, depending on the target environment and row type on the Canadian Prairies (Anyia et al. 2007). In this study, leaf $\Delta^{13}C$ was found to be positively correlated with aboveground biomass and grain yield for both populations under field conditions (Table 4.4 and 4.5, $p < 0.05$). One possible explanation of such relationship is the positive association between leaf $\Delta^{13}C$ and g_s (Condon et al. 1987), because more carbon could be fixed early in the season when stomatal opening was less limited by water deficit (therefore a high $\Delta^{13}C$). Positive relationship between leaf $\Delta^{13}C$ and g_s in barley under field and greenhouse (well-watered) conditions was observed in a previous study (Chen et al. 2011), and similar results have been reported in common bean (*Phaseolus vulgaris* L.) (Ehleringer 1990), rice (Kondo et al. 2004; Takai et al. 2009) and wheat (Monneveux et al. 2006). Takai et al. (2009) found that a QTL controlling

leaf $\Delta^{13}C$ on the long arm of chromosome 3 in rice was associated with g_s. Another hypothesis is that genotypes differ in their abilities to translocate stem carbohydrate reserves for grain filling, since high leaf $\Delta^{13}C$ genotypes tend to grow faster and convert more assimilates to grain than low $\Delta^{13}C$ genotypes (Monneveux et al. 2005). The positive relationship between leaf $\Delta^{13}C$ and HI (Tables 4.4 and 4.5) also suggested that genotypes with high leaf $\Delta^{13}C$ genotypes were more efficient in dry matter partitioning to grain (Teulat et al. 2001c).

The $\Delta^{13}C$ provides a long-term average estimate of cumulative WUE integrated over time and space (Condon et al. 2002). Leaves sampled for $\Delta^{13}C$ at the stem elongation stage, when there is usually little drought stress and low vapour pressure deficit, could reflect the integrated WUE during vegetative development and formation of yield potential, and evaluate vegetative establishment (Anyia et al. 2008; Chen et al. 2011; Condon and Richards 1992). There was also a positive relationship between leaf $\Delta^{13}C$ and LAI-S across locations in this study (Table 4.4). LAI is usually used to estimate early vigor (the early growth of leaf area and biomass, Richards (1996)), canopy photosynthesis, evapo-transpiration and final yield since it represents the percentage of incident solar radiation intercepted (Dale et al. 1980). The significant positive correlations among LAI-S, leaf $\Delta^{13}C$, biomass and grain yield in this study suggest that both leaf $\Delta^{13}C$ and LAI at the stem elongation stage could be used to estimate final yield.

Leaf $\Delta^{13}C$ and row type

Jiang et al. (2006) reported that values of $\Delta^{13}C$ from different plant parts (flag leaf, awn and grain) were on average higher for six-row than for two-row barley, suggesting a higher WUE of two-row than six-row barley under irrigated and non-irrigated field conditions. A proposed explanation for the difference in $\Delta^{13}C$ values between barley ear types is the difference in days to maturity, as low $\Delta^{13}C$ values appeared in late-maturing genetic lines (Craufurd et al. 1991; Sayrea et al.

1995). Another possible explanation suggested by Jiang et al. (2006) is that the intrinsic difference may exist in water/carbon metabolism at the whole tiller, flag leaf and ear levels, since flag leaves of two-row barley are generally much smaller than six-row barley. The six-row barley may fix more carbohydrates from flag leaf blades and stems, and therefore they were enriched in ^{13}C. We didn't find any significant difference in leaf $\Delta^{13}C$ between two-row and six-row barley in previous studies (Anyia et al. 2007; Chen et al. 2011). In the present study, the two populations differed in leaf $\Delta^{13}C$, with the M × H RILs producing a significantly lower leaf $\Delta^{13}C$ than the W × I RILs at V09 (data not shown, $p < 0.001$), which needs to be verified under multiple environments.

Six-row barley has more florets and therefore it is usually considered to be more fertile than two-row barley. In this study, the W × I RILs produced more biomass and grain yield than the M × H RILs at V09 ($p < 0.01$), but there was no significant difference in grain yield between these two barley populations when soil moisture was not sufficient at C09. According to Forster et al. (2004), six-row barley has an advantage over two-row barley since six-row type generally matures earlier and therefore it is valuable in breeding for drought escape for countries in North Africa such as Morocco. Different types of barley play different roles in market such as livestock feed, malt beverages and human diet, so it is necessary to rank the RILs with low leaf $\Delta^{13}C$ and high biomass or grain yield from different populations (two-row and six-row) against a wide range of varieties (such as local cultivars) in different field trials across the Canadian Prairies to select the best drought resistant genotype in order to meet the increasing market demand for barley supply.

Barley genetic map and segregation distortion

The markers used in this study were mostly SSRs due to their high specificity, co-dominance and fewer limitations than other markers (AFLP, RAPD, RFLP etc.), and SSRs have been proven to be the markers of choice in plant breeding and

genetic diversity studies (Li et al. 2008; Varshney et al. 2007). Lorieux et al. (1995a; 1995b) concluded that segregation distortion (SD) affected less on co-dominant markers than dominant markers when estimating recombination frequencies. The SSR marker polymorphism was low between parental lines in this study. Although DArT analysis generated a large number of polymorphic markers (39.3%), they tended to be clustered (Fig. 4.6). The distribution of markers on both maps was characterized by regions with tight cluster and regions with low density of markers, which resembled previously published barley maps (Hearnden et al. 2007; Li et al. 2003; Ramsay et al. 2000; Teulat et al. 2002; Teulat et al. 2003; Tondelli et al. 2006; Wenzl et al. 2004).

Marker distortion is commonly observed in crop mapping population, and the proportion of distorted markers varies among the species and population types (Lu et al. 2002; Xu et al. 1997). A high level marker distortion was observed in the present study that is comparable to previously reported values in other barley populations. For example, 41, 42, 44, and 47% marker distortions were found by Marcel et al. (2007), Li et al. (2008), Sayed et al. (2002) and Stein et al. (2007), respectively. In the present study, distorted markers were clustered in both populations, consistent with other reports on barley (Li et al. 2010; Marcel et al. 2007). The skewed deviation from the expected allele frequency could be influenced by genetic factors such as sterility or gametophytic genes carried by one of the parents. Konishi et al. (1990; 1992) studied the genetic control of SD in barley using isozyme markers and they identified the gametophyte gene (*Ga2*) as a factor. Li et al. (2010) identified segregation distorted regions (SDRs) on all seven barley chromosomes from four different double haploid (DH) populations, which may be due to the genetic processes related to position near centromeres. For the RIL population, distorted segregation was inferred to be related with environmental and artificial selection during the development of RILs (Song et al. 2006). It is difficult to distinguish genetic factors from environmental causes of distorted allele frequencies in RIL populations because the cumulative effect of both factors after multiple generations of selfing (Xu et al. 1997). However, the

influence of SD on QTL analysis could be negligible (Song et al. 2006) and even helpful to find informative QTL (Xu 2008; Xu and Hu 2009). Xu (2008) concluded that SD is not always a detriment to QTL mapping for additive effects and the SD loci is beneficial to QTL mapping around 44% of the time when it is a random event. Zhang et al. (2010) also concluded that SD generally will not produce more false QTL nor affect the estimation of QTL position and effect.

Heritability of leaf $\Delta^{13}C$

In the present study, the H^2 for leaf $\Delta^{13}C$ was high (0.80) for the six-row barley population across all environments. Under a single environment (C09 or V09), the H^2 for both populations reached 0.80. These results suggest that leaf $\Delta^{13}C$ is under strong genetic control and can be reliably used as a measure of WUE under field conditions. For many complex traits (such as biomass, HI and yield components), genetic gain is slow under water-limited environments due to a large interaction between genotype and environment and a low heritability (Rebetzke et al. 2008). The high H^2 of physiological or secondary traits that are correlated with yield, such as $\Delta^{13}C$, presents a good opportunity for plant breeding in drought-prone regions (Stiller et al. 2005). Although the expression of $\Delta^{13}C$ in leaf and other plant tissues varies with water-supply, and the spatial variability in soil water availability can reduce genetic variance of $\Delta^{13}C$ (Condon et al. 1992; Rebetzke et al. 2008), the heritability for $\Delta^{13}C$ is still high compared with other complex traits under drought conditions. Several studies have previously reported high H^2 values for $\Delta^{13}C$. Hubick et al. (1988) observed that the H^2 of $\Delta^{13}C$ (whole plant, excluding the pods and roots) in field-grown peanut cultivars was 0.81, and there was no significant interaction between genotype and environment for $\Delta^{13}C$. Condon and Richards (1992) found that the H^2 of $\Delta^{13}C$ in wheat was greatest for plant material sampled before or during early stem elongation (0.95 on genotype basis or 0.88 on a single-plot basis) as compared with plant parts formed near anthesis (flag leaf or plants tops at ear emergence). In another study on wheat, the

narrow-sense heritability of leaf $\Delta^{13}C$ ranged from 0.37 to 0.91 on a single environment basis and from 0.76 to 0.86 on genotype-mean basis in a QTL analysis using three wheat mapping populations across three years (Rebetzke et al. 2008).

QTL for $\Delta^{13}C$ across environments and populations

The CIM method revealed 12 putative QTLs associated with leaf $\Delta^{13}C$ for the W × I population, and 5 QTLs for leaf $\Delta^{13}C$ from the M × H population in the current study. Common QTLs across environments and populations can provide validation of the QTL and identify robust markers across different gene pools for marker-assisted selection. The significant low level of polymorphism between parental lines (especially in the M × H population) resulted in the low density and uneven distribution of markers in the QTL maps obtained in the present study. For example, Chr. 3H of the M x H map was split into three linkage groups and was only covered by 14 markers, which may have hindered the detection of putative QTL for this chromosome. The low marker density and the lack of common markers prevented us from identifying and comparing common QTLs across the two populations evaluated in the present study. However, it may be possible to identify a few common QTLs for leaf $\Delta^{13}C$ across the two mapping populations if more markers are placed to saturate the maps. Since the order and not the distance between markers is usually considered conserved between populations (This et al. 2010), 7 of the 12 QTLs regions detected in the W × I population in this study (region on Chr. 2H around marker EBmac558, Chr. 3H between marker Bmag606 and Bmag13, Chr. 5H near marker Bmac113 and on Chr. 6H near marker Bmag173) were identified to be similar to those previously reported for grain $\Delta^{13}C$ in the Tadmor × Er/Apm population under three Mediterranean field environments by Teulat et al. (2002). Specifically, the region of Chr. 5H near marker Bmac113 in our study overlapped with grain $\Delta^{13}C$ in the Tadmor × Er/Apm population under irrigated field conditions (Diab et al. 2004).

Ellis et al. (2002) reported similar chromosome regions for shoot $\Delta^{13}C$ (3H and 5H) in their Derkado × B83-12/21/5 DH population.

Although one QTL with a major effect located on Chr. 3H near marker Bamg606 on the W × I linkage map was detected consistently in all three field locations, its effect on leaf $\Delta^{13}C$ varied across environments, and this QTL explained 22% of the phenotypic value at L08, followed by C09 (14.4%) and then V09 (11%), with no significant G × E for leaf $\Delta^{13}C$ in the W × I population. In agreement with Teulat et al. (2002), the observed differential effects of this QTL across the different environments studied may be attributable to the total growing season rainfall. Condon et al. (1992) also pointed out that the environmental effects on $\Delta^{13}C$ can be attributed to stomatal closure in response to declining soil water and/or increasing vapour-pressure deficit. Overall, the QTLs identified in this study varied in size and accounted for small to modest amounts of the phenotypic variance, which is consistent with most of the previous studies in QTL mapping of $\Delta^{13}C$ across a range of plant species.

Effect of parental alleles

It is imperative to understand the inheritance of $\Delta^{13}C$ and the mode of gene action to develop cultivars with high WUE via selection for low $\Delta^{13}C$ lines. There was one report on cytoplasmic inheritance of $\Delta^{13}C$ in cultivated sunflower by Lambrides et al. (2004), but most studies provided strong evidence for nuclear genetic control of $\Delta^{13}C$, with QTL for $\Delta^{13}C$ assigned to specific chromosomes in several different species, such as *Arabidopsis thaliana* (Hausmann et al. 2005; Juenger et al. 2005), barley (Diab et al. 2004; Ellis et al. 2002; Ellis et al. 1997; Handley et al. 1994; Teulat et al. 2002), cotton (Saranga et al. 2001), rice (Laza et al. 2006; Price et al. 2002; Takai et al. 2006; This et al. 2010; Xu et al. 2009), soybean (Specht et al. 2001), tomato (Xu et al. 2008) and wheat (Rebetzke et al. 2008). Predominantly additive gene action of $\Delta^{13}C$ has been reported in alfalfa (*Medicago sativa* L.) (Johnson and Rumbaugh 1995), *Arabidopsis thaliana*

(Hausmann et al. 2005; Juenger et al. 2005), common bean (White 1993), wheat (Ehdaie and Waines 1994; Rebetzke et al. 2006) and rice (Takai et al. 2006).

In the present study, the low leaf $\Delta^{13}C$ parent W89 had favorable alleles for WUE at loci on Chr. 2H, 3H and 5H, and the high leaf $\Delta^{13}C$ parent I60049 contributed favorable alleles for WUE at loci on Chr. 1H, 6H and 7H. For the M × H population, the high leaf $\Delta^{13}C$ parent H93174006 had favorable alleles for WUE at loci on Chr. 6H and 7H. The complementary alleles at multiple loci contributed by the parents provided the most plausible explanation for transgression observed among the progeny (Tanksley 1993). The additive gene action underlying $\Delta^{13}C$ suggests that replacement and fixation of desirable alleles within a locus could be achieved by selecting lines with high or low $\Delta^{13}C$ (Rebetzke et al. 2008; Rebetzke et al. 2003). Independent alleles at multiple loci could also be pyramided to develop lines for further altered $\Delta^{13}C$.

Clusters of QTLs

Multiple regions controlling leaf $\Delta^{13}C$ co-located with the QTLs for agronomic traits (such as aboveground biomass and grain yield) were identified in both RILs populations in the present study, which is consistent with previous reports. Teulat et al. (2001a; 2001b; 2002) reported eight QTLs for grain $\Delta^{13}C$ co-located with QTLs for agronomic traits and traits related to plant water status and/or osmotic adjustment. Most QTLs for $\Delta^{13}C$ were previously reported to co-segregate with QTLs for agronomic traits such as heading time and/or plant height in barley (Forster et al. 2004).

Correlations (Table 4.4 and 4.5) between leaf $\Delta^{13}C$ and LAI-S, plant height, days to maturity, biomass, grain yield and HI were partially explained at the level of co-localized QTLs (Fig. 4.5 and 4.6). One of the genomic regions with the most overlapping traits was on Chr. 3H near marker Bmag606, with W89 alleles favoring lower leaf $\Delta^{13}C$, increased plant height, and reduced LAI, grain yield, HI and days to maturity. Around the same region, QTL for $\Delta^{13}C$ was

identified near the semi-dwarf gene *sdw1* in different barley mapping populations (Ellis et al. 2002; Teulat et al. 2002), which is homoeologous to rice Chr. 1 where $\Delta^{13}C$ is located. Another noteworthy region was on Chr. 5H near marker Bmac113, with W89 alleles favoring lower leaf $\Delta^{13}C$, reduced biomass and yield. In the same region, QTLs for shoot $\delta^{13}C$ and grain yield were associated with another semi-dwarf gene *ari-e.*GP (Ellis et al. 2002). The co-localization of QTLs may result from pleiotropic relationships between these traits or due to genetic linkage among the traits. Clustering regions are of interest in terms of plant breeding as they control both drought-adaptive traits such as $\Delta^{13}C$ and yield components.

Conclusions

The temporal variation of within-season rainfall distribution on the Canadian Prairies has a significant impact on barley leaf $\Delta^{13}C$. Sufficient genotypic variation, stability across environments, and a high H^2 indicate that leaf $\Delta^{13}C$ is a good surrogate for improved WUE in breeding for barley varieties under rain-fed conditions. The transgressive variation of leaf $\Delta^{13}C$ observed in our mapping population suggests that it may be possible to select progeny lines with reduced $\Delta^{13}C$ or increased WUE that contribute favorable alleles of value for improvement of WUE. This study has confirmed the polygenic inheritance of leaf $\Delta^{13}C$ in barley by detecting multiple QTLs controlling leaf $\Delta^{13}C$. Several QTLs for leaf $\Delta^{13}C$ were found to overlap with QTLs for important agronomic traits that would need further validation. A major QTL for leaf $\Delta^{13}C$ located on Chr. 3H near SSR marker Bmag606 was identified across all locations that overlapped with several agronomic traits, with W89 alleles favoring lower leaf $\Delta^{13}C$, increased plant height, and reduced LAI, grain yield, HI and days to maturity. Since overlapping or co-location of QTLs suggests pleiotropic relationship or genetic linkage, care must be taken to ensure that low $\Delta^{13}C$ does not impose penalty on grain yield under favorable conditions. Previous studies (Anyia et al. 2007; Chen et al. 2011)

suggest that it is possible to select high-yielding lines with low $\Delta^{13}C$. More research is needed to further dissect this major QTL region for low $\Delta^{13}C$ towards the identification of candidate genes as well as to understand the genetic mechanisms underlying these co-located traits.

Table 4. 1 Monthly precipitation (mm) over the growing season for the three field locations during 2008 and 2009

Location-year	Sowing date	Precipitation (mm)			
		June	July	August	Total
Lacombe-2008	May 15	45.8	48.8	55.5	150.1
Vegreville-2008	May 16	42.4	44.6	64.3	151.3
Vegreville-2009	May 22	32.2	44.6	25.2	102.0
Castor-2009	May 13	21.5	33.8	51.9	107.2

Table 4. 2 Means, standard deviations (SD), range (minimum and maximum values) of the 200 recombinant inbred lines (RILs) and their parental lines from the six-row (W89001002003 × I60049) barley population for traits measured in three field trials

Trait	Environ-ment	Parents				RILs						
		W89001002003		I60049								
		Mean	SD	Mean	SD	Mean	SD	Range	E^1	G	G × E	H^2
Leaf $\Delta^{13}C$ (‰)	L08[2]	20.71	0.23	22.40	0.48	21.35	0.61	19.84 – 23.22	***	***	ns	0.80
	C09	17.69	0.98	18.99	0.26	18.53	0.61	16.48 – 20.30				
	V09	18.72	0.56	19.97	0.95	19.71	0.65	17.94 – 22.19				
LAI-S[3] (m^2 m^{-2})	C09	1.39	0.30	1.37	0.55	1.37	0.46	0.40 – 2.84	***	*	*	0.61
	V09	4.52	0.79	4.60	1.26	3.96	0.96	1.95 – 6.96				
LAI-G[4] (m^2 m^{-2})	C09	1.34	0.19	1.71	0.14	1.63	0.33	0.82 – 2.74	***	ns	ns	0.63
	V09	4.54	0.59	4.89	1.02	4.34	0.89	2.30 – 7.04				
Plant height (cm)	C09	38	2.1	47	2.6	47	7.0	29 – 69	***	***	***	0.82
	V09	67	7.2	73	6.5	76	13.0	42 – 105				
Days to maturity (days)	V09	88	2.7	91	1.0	87	3.7	73 – 92	–	***	–	0.96

Table 4. 2 *Continued*

Biomass ($g\ m^{-2}$)	L08	1512	100.5	1229	60.4	1650	354.5	1072 – 3243				
	C09	236	22.9	265	43.3	261	56.1	143 – 520	***	***	***	0.75
	V09	1038	120.3	987	190.6	828	195.1	410 – 1518				
Grain yield ($g\ m^{-2}$)	L08	701	39.6	513	36.0	708	153.7	412 – 408				
	C09	113	21.6	128	6.1	129	35.2	60 – 294	***	***	***	0.74
	V09	529	69.5	521	90.7	424	114.0	180 – 834				
HI	L08	0.46	0.01	0.42	0.02	0.43	0.02	0.35 – 0.49				
	C09	0.48	0.04	0.49	0.06	0.49	0.06	0.30 – 0.66	***	**	ns	0.75
	V09	0.51	0.02	0.53	0.01	0.51	0.04	0.35 – 0.60				

[1] E: Environment effect; G: Genotype effect; G × E: interaction between environment and genotype.

[2] L08: Lacombe-2008, C09: Castor-2009, V09: Vegreville-2009; [3] LAI-S: leaf area index measured at stem elongation stage.

[4] LAI-G: leaf area index measured at grain filling stage.

*, **, *** indicate 5, 1, 0.1% significance levels, respectively; ns, not significant**.**

Table 4. 3 Means, standard deviations (SD), range (minimum and maximum values) of the 127 recombinant inbred lines (RILs) and their parental lines from the two-row (Merit × H93174006) barley population for traits measured in three field trials

Trait	Environment	Parents				RILs						
		Merit		H93174006								
		Mean	SD	Mean	SD	Mean	SD	Range	E[1]	G	G × E	H^2
Leaf $\Delta^{13}C$ (‰)	V08[2]	18.62	0.35	18.73	0.46	18.93	0.63	17.53 – 20.53	***	***	*	0.66
	V09	17.80	0.50	18.67	0.70	18.46	0.52	17.19 – 20.33				
Biomass ($g\ m^{-2}$)	V08	362	16.6	401	36.2	495	131.6	292 – 896	***	ns	ns	0.69
	C09	366	100.7	170	9.1	273	80.2	92 – 595				
	V09	572	117.5	662	38.2	665	132.4	305 – 1155				
Grain yield ($g\ m^{-2}$)	V08	99	11.6	104	7.4	137	43.9	65 – 305	***	ns	ns	0.63
	C09	194	59.0	68	6.5	127	47.6	51 – 307				
	V09	282	62.8	331	32.8	324	63.0	151 – 566				
HI	V08	0.28	0.02	0.26	0.01	0.27	0.03	0.19 – 0.35	***	ns	*	0.66
	C09	0.53	0.04	0.40	0.04	0.46	0.07	0.23 – 0.59				
	V09	0.49	0.01	0.50	0.03	0.49	0.03	0.34 – 0.66				

[1] E: Environment effect; G: Genotype effect; G × E: interaction between environment and genotype.

[2] V08: Vegreville-2008, C09: Castor-2009, V09: Vegreville-2009.

*, **, *** indicate 5 %, 1 %, 0.1% significance levels, respectively; ns, not significant.

Table 4. 4 Correlations among leaf carbon isotope discrimination ($\Delta^{13}C$), leaf area index at stem elongation stage (LAI-S), leaf area index at grain filling stage (LAI-G), plant height (PH), biomass, yield, harvest index (HI) and days to maturity at Castor and Vegreville in 2009 for the six-row (W89001002003 × I60049) barley population

	Leaf $\Delta^{13}C$	LAI-S	LAI-G	PH	Biomass	Yield	HI
Castor-2009							
LAI-S	0.20**						
LAI-G	0.13^{ns}	0.41***					
PH	-0.002^{ns}	0.26***	0.30***				
Biomass	0.31***	0.45***	0.49***	0.29***			
Yield	0.28***	0.45***	0.40***	0.21**	0.78***		
HI	0.20**	0.24***	0.07^{ns}	-0.12^{ns}	0.28***	0.59***	
Vegreville-2009							
LAI-S	0.28***						
LAI-G	0.35***	0.77***					
PH	0.11^{ns}	0.40***	0.50***				
Biomass	0.44***	0.62***	0.63***	0.52***			
Yield	0.46***	0.56****	0.58***	0.38***	0.96***		
HI	0.30***	0.11^{ns}	0.13^{ns}	-0.26***	0.36***	0.58***	
Days to maturity	0.11^{ns}	0.29***	0.35***	0.06^{ns}	0.28***	0.29***	0.13^{ns}

*, **, *** indicate 5 %, 1 %, 0.1% significance level, respectively;

ns, not significant.

- , no record available for days to maturity for Castor-2009.

Table 4. 5 Correlations among leaf carbon isotope discrimination ($\Delta^{13}C$), biomass, yield and harvest index (HI) at Vegreville in 2008 and 2009 for the two-row (Merit × H93174006) barley population

	Leaf $\Delta^{13}C$	Biomass	Yield
2008			
Biomass	0.65***		
Yield	0.61***	0.95***	
HI	0.20*	0.31***	0.58***
2009			
Biomass	0.28**		
Yield	0.02^{ns}	0.34***	
HI	-0.08^{ns}	0.001^{ns}	0.94***

*, **, *** indicate 5 %, 1 %, 0.1% significance levels, respectively; ns, not significant.

Table 4. 6 QTL identified for carbon isotope discrimination ($\Delta^{13}C$) with QTL Cartographer 2.5 by the composite interval mapping (CIM) method using data from three locations (Castor, Lacombe and Vegreville) for the six-row (W89001002003 × I60049) barley population and data from Vegreville for the two-row (Merit × H93174006) barley population in 2 years (2008-2009)

Population	E[1]	Position (marker + distance to the maximum LOD value) (cM)	Ch[2]	LOD	R^2 (%)[3]	Additive effect[4]
Six-row	L08[5]	Bmac32 + 2.04	1H	2.13	3.9	0.13
		Bmag606 + 2.03	3H	10.81	22	-0.36
		EBmac708 – 2.37	3H	3.78	7.3	-0.20
		Bmag751 + 0.06	5H	4.77	7.3	-0.18
		Bmag807 – 1.72	6H	2.18	3.6	0.12
	C09	EBmac558 – 0.36	2H	2.34	4.1	-0.08
		Bmag603 + 0.04	3H	2.44	4.1	-0.08
		Bmag606 + 3.03	3H	5.86	14.4	-0.17
	V09	Bmag606 – 0.87	3H	5.27	11	-0.18
		Bmac113 + 0	5H	2.93	5.3	-0.11
		GBM1022 + 0.04	6H	2.12	3.8	0.09
		EBmac755 + 0	7H	2.38	4.3	0.09
Two-row	V08	bPb1212 + 0	6H	3.48	11.2	0.29
		bPb7872 – 0.04	6H	3.65	11.7	0.32
		bPb9898 + 0.98	7H	3.11	9.9	0.20
	V09	bPb1212 + 0	6H	3.60	10.8	0.16
		bPb9898 + 1.98	7H	2.54	8.6	0.10

[1] E: environment; [2] Ch: chromosome.

[3] R^2: proportion of the phenotypic variance explained by each QTL.

[4] Additive effect of the allele from W89001002003 compared with I60049.

[5] L08: Lacombe-2008; C09: Castor-2009; V08: Vegreville-2008; V09: Vegreville-2009.

Table 4. 7 QTL identified for leaf area index measured during stem elongation stage (LAI-S), leaf area index measured during grain filling stage (LAI-G), plant height (PH), days to maturity, biomass, yield and harvest index (HI) with QTL Cartographer 2.5 by the composite interval mapping (CIM) method using data from two locations (Castor and Vegreville) for the six-row (W89001002003 × I60049) and the two-row (Merit × H93174006) barley populations in 2 years (2008-2009)

Population	Trait	E[1]	Position (marker + distance to the maximum LOD value) (cM)	Ch[2]	LOD	R^2 (%)[3]	Additive effect[4]
Six-row	LAI-S	V09[5]	GBM1405 + 2.04	3H	2.61	5.4	-0.15
			Bmac156 + 0.01	7H	3.02	5.6	-0.14
	LAI-G	V09	GBM1405 + 1.04	3H	2.41	4.4	-0.13
			Bmag606 – 0.87	3H	2.11	4.2	-0.13
			GBM5008 + 0	5H	3.83	6.9	0.17
			GBMS139 + 2.02	7H	6.82	13.6	0.20
	PH	C09	Bmac32 – 3.76	1H	7.53	15.0	-2.15
			Bmac32 + 4.04	1H	7.96	14.5	-2.10
			Bmag606 + 4.03	3H	5.73	13.6	2.48
			EBmac708 – 5.37	3H	2.94	6.6	1.58
			Scssr25538 – 10.05	3H	3.23	8.4	1.68
			GBM1506 – 5.82	5H	5.18	10.7	-1.80
			GBM1355 – 11.79	6H	2.09	5.8	-1.32
			GBMS139 – 6.28	7H	2.98	6.9	1.42
		V09	Bmag606 + 5.03	3H	11.94	27.4	6.62
			GBM1506 – 3.82	5H	6.24	11.0	-3.42
			GBMS139 – 2.28	7H	5.00	9.3	3.22

Table 4. 7 *Continued*

Six-row	Biomass	C09	GBMS139 + 0.02	7H	2.73	5.6	8.90
		V09	Bmag337 – 0.01	5H	3.76	6.3	-32.16
			GBM1231 + 0.03	5H	3.55	6.3	34.08
			Scssr5599 – 0.01	6H	2.21	3.7	24.23
	Yield	C09	Bmag606 + 2.03	3H	4.42	11.4	-9.79
			GBM5008 + 0	5H	2.06	4.1	5.04
		V09	Bmag518 + 0.03	2H	2.91	5.0	-16.81
			Bmag323 + 0.05	5H	2.93	5.0	-17.07
			GBM1231 + 0.03	5H	5.62	10.2	25.63
			Scssr5599 – 0.01	6H	2.76	4.7	16.19
	HI	C09	GBM5008 + 0	5H	5.47	10.5	0.01
		V09	Bmag606 + 0.03	3H	5.18	10.5	-0.01
	Days to maturity	V09	Bmac32 – 4.76	1H	3.29	5.0	-0.68
			Bmag606 + 0.03	3H	10.78	16.0	-1.50
			Bmag740 + 0	4H	3.33	4.7	-0.65
			Bmac113 + 0	5H	4.06	5.5	-0.71
			Bmag323 + 4.05	5H	4.42	7.2	-0.80
Two-row	Biomass	V08	bPb6821 – 2.65	7H	2.32	9.7	41.79
		C09	bPb8213 – 1.67	7H	2.74	9.0	14.01
	Yield	V08	bPb6821 – 2.65	7H	2.06	9.2	13.57
		C09	bPb2279 + 0.04	2H	2.38	7.7	-12.22
		V09	bPb1212 + 0	6H	2.88	9.1	17.65
	HI	V08	bPb2501 – 0.77	2H	3.43	10.7	0.01
		C09	bPb8721 + 1.98	2H	2.04	7.4	0.01
		V09	bPb4293 + 0.01	2H	2.30	7.3	-0.07

[1] E: environment.

[2] Ch: chromosome.

[3] R^2: proportion of the phenotypic variance explained by each QTL.

[4] Additive effect of the allele from W89001002003 compared with I60049.

[5] C09: Castor-2009; V08: Vegreville-2008; V09: Vegreville-2009.

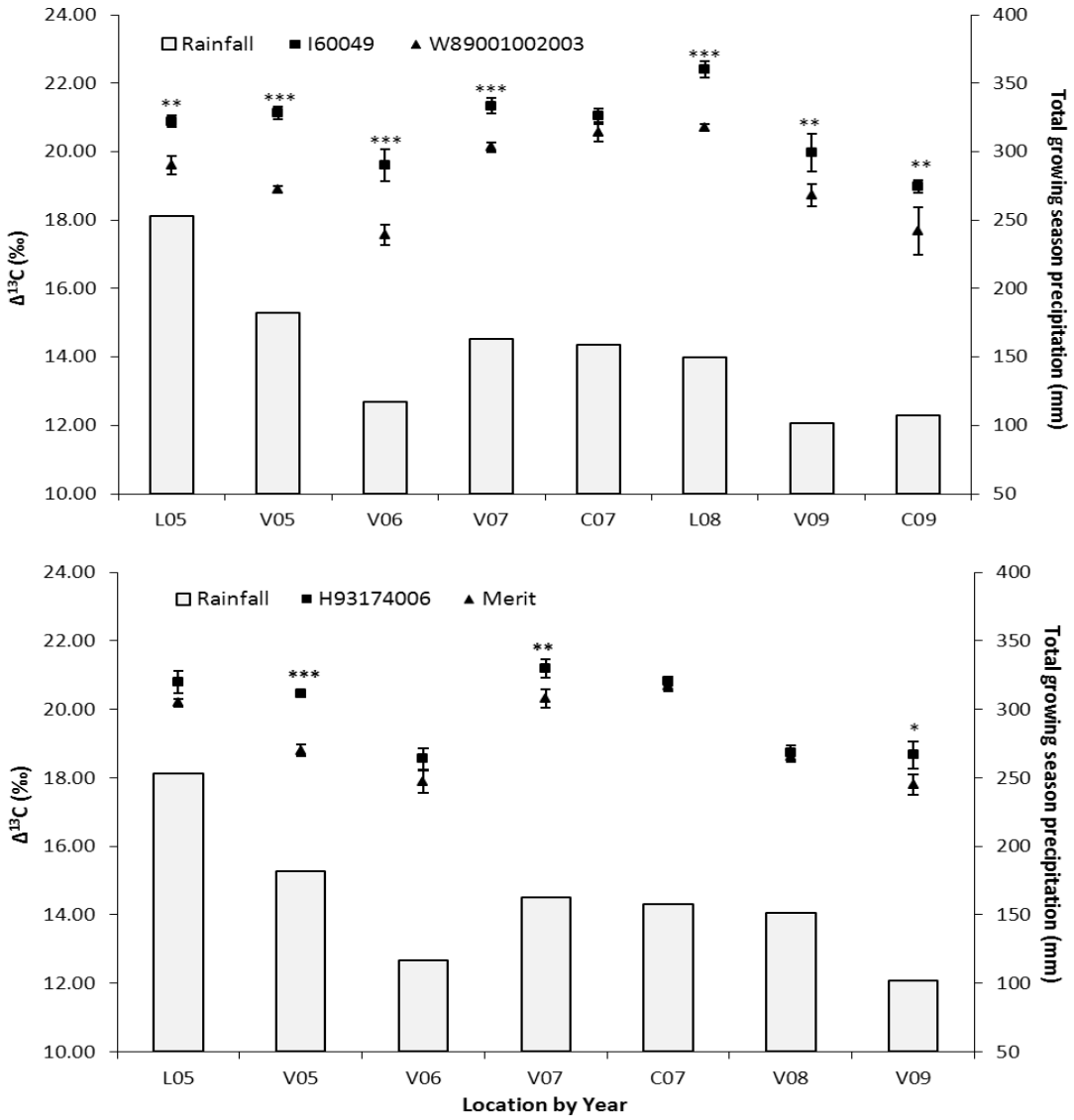


Figure 4. 1 Leaf carbon isotope discrimination ($\Delta^{13}C$) of parental lines W89001002003 and I60049 (top), Merit and H93174006 (bottom) across locations and years characterized by different total growing season rainfall. Significant differences of leaf $\Delta^{13}C$ between parental lines were indicated with stars, with *, ** and *** stand for $p < 0.05$, 0.01 and 0.001, respectively. L05, L08, C07, C09, V05, V06, V07, V08 and V09 stand for Lacombe-2005, Lacombe-2008, Castor-2007, Castor-2009, Vegreville-2005, Vegreville-2006, Vegreville-2007, Vegreville-2008 and Vegreville-2009, respectively.

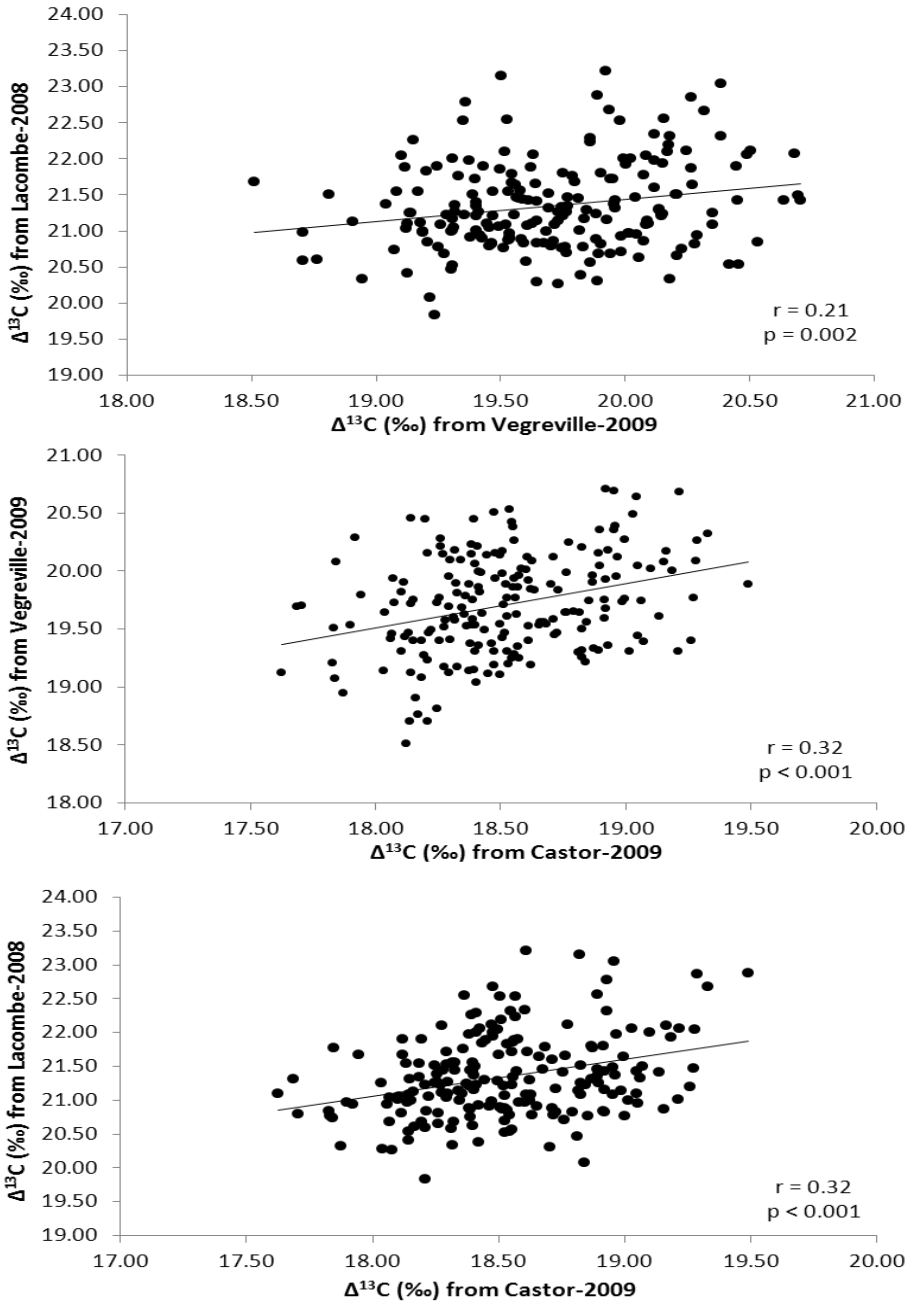


Figure 4. 2 Correlation between leaf carbon isotope discrimination ($\Delta^{13}C$) of 200 recombinant inbred lines (RILs) from the six-row (W89001002003 × I60049) population measured at Lacombe in 2008, Castor in 2009 and Vegreville in 2009.

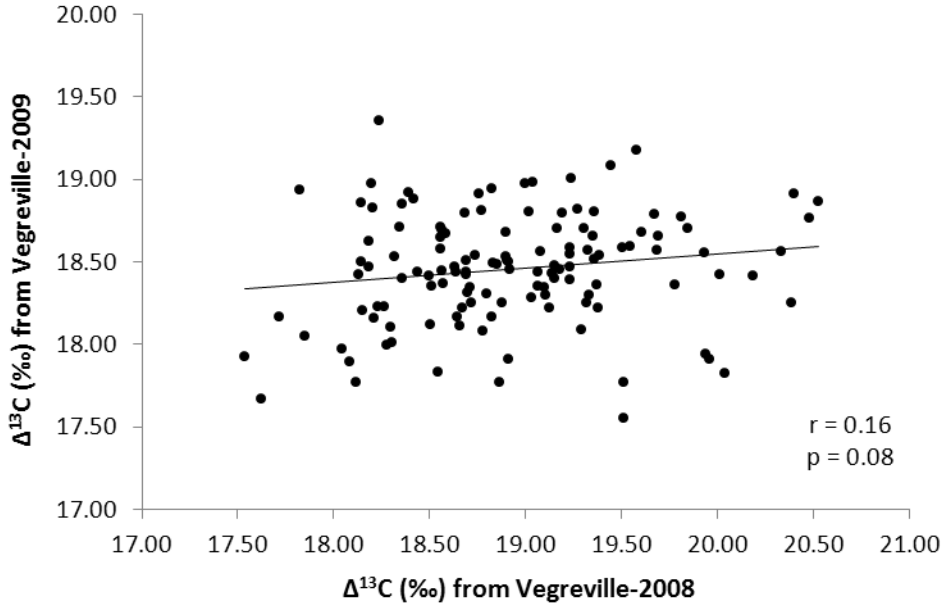


Figure 4. 3 Correlation between leaf carbon isotope discrimination ($\Delta^{13}C$) of 127 recombinant inbred lines (RILs) from the two-row (Merit ×H93174006) population measured at Vegreville in 2008 and 2009.

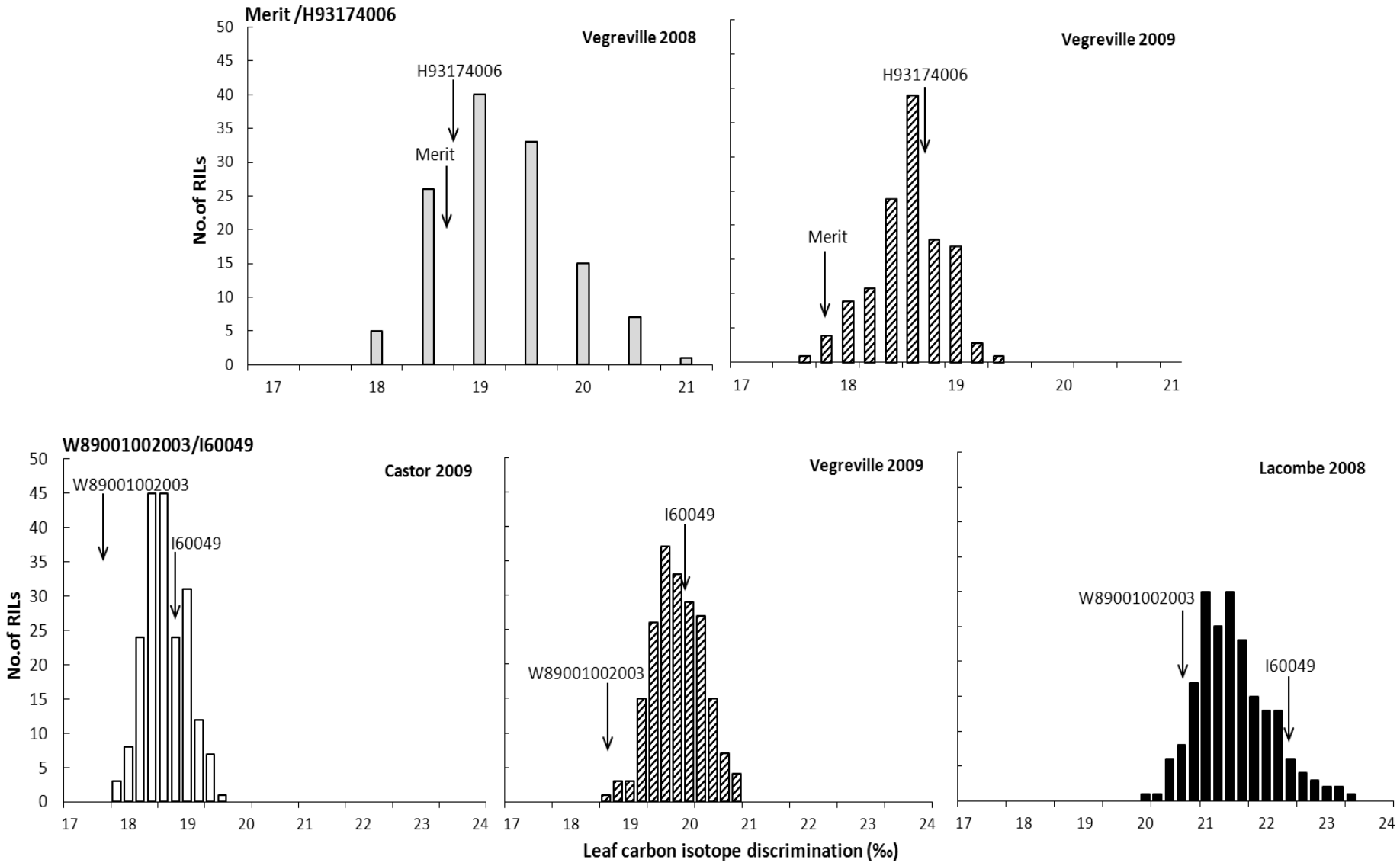
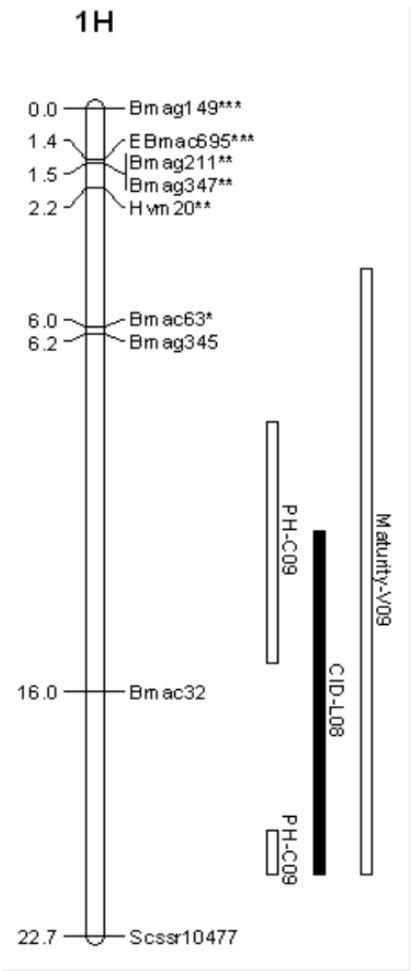
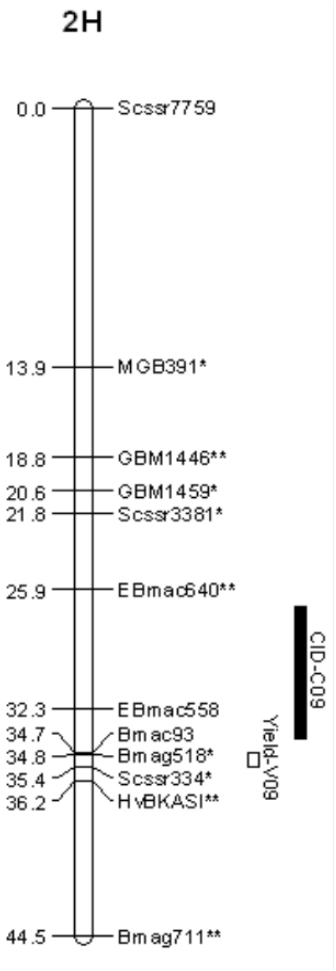
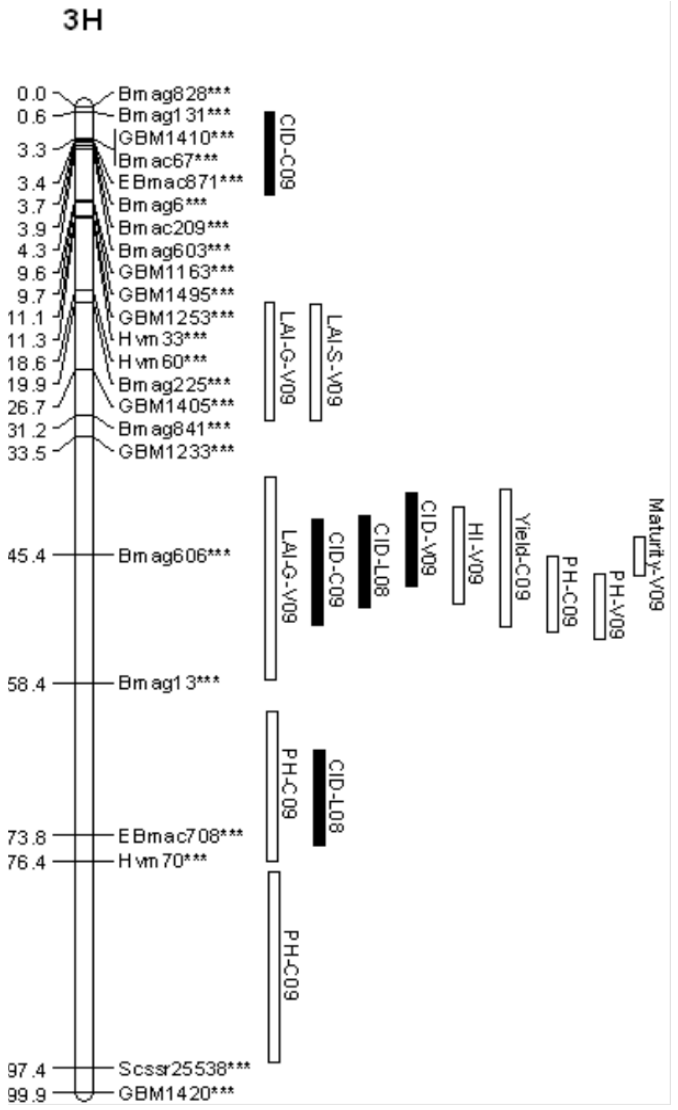
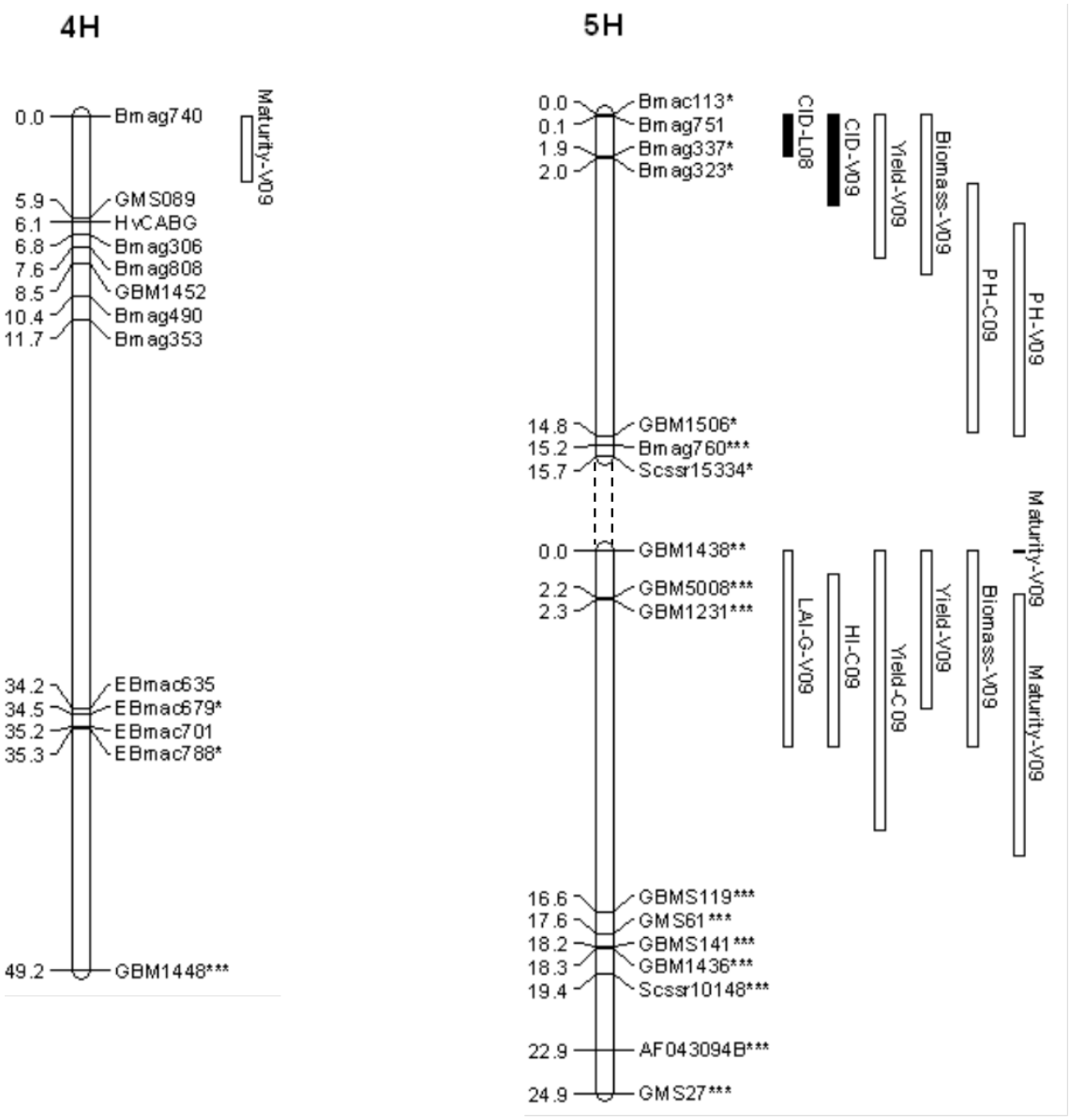

Merit /H93174006
Vegreville 2008
Vegreville 2009
H93174006
Merit
No.of RILs
W89001002003/I60049
Castor 2009
Vegreville 2009
Lacombe 2008
W89001002003
I60049
Leaf carbon isotope discrimination (‰)

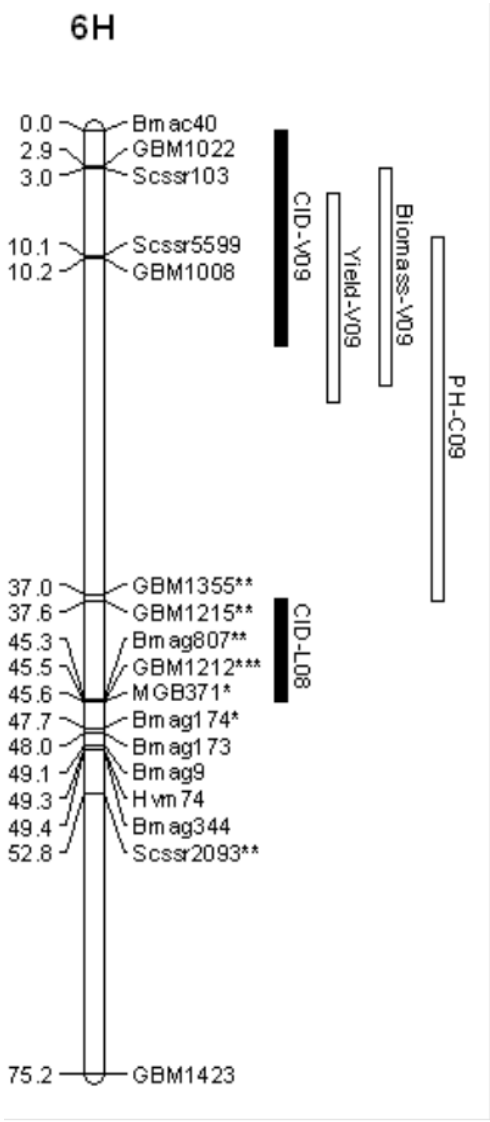
Figure 4. 4 Frequency distribution of mean leaf carbon isotope discrimination ($\Delta^{13}C$) measured on the recombinant inbred lines (RILs) from the two-row (Merit × H93174006) and the six-row (W89001002003 × I60049) mapping populations grown at Lacombe and Vegreville in 2008, Castor and Vegreville in 2009. Parental means are indicated for each environment.


1H
0.0 Bmag149***
1.4 EBmac695***
Bmag211**
1.5 Bmag347**
2.2 Hvm20**
6.0 Bmac63*
6.2 Bmag345
16.0 Bmac32
22.7 Scssr10477
PH-C09
CID-L08
PH-C09
Maturity-V09


2H
0.0 Scssr7759
13.9 MGB391*
18.8 GBM1446**
20.6 GBM1459*
21.8 Scssr3381*
25.9 EBmac640**
32.3 EBmac558
34.7 Bmac93
34.8 Bmag518*
35.4 Scssr334*
36.2 HvBKASI**
44.5 Bmag711**
CID-C09
Yield-V09


3H
0.0 Bmag828***
0.6 Bmag131***
3.3 GBM1410***
Bmac67***
3.4 EBmac871***
3.7 Bmag6***
3.9 Bmac209***
4.3 Bmag603***
9.6 GBM1163***
9.7 GBM1495***
11.1 GBM1253***
11.3 Hvm33***
18.6 Hvm60***
19.9 Bmag225***
26.7 GBM1405***
31.2 Bmag841***
33.5 GBM1233***
45.4 Bmag606***
58.4 Bmag13***
73.8 EBmac708***
76.4 Hvm70***
97.4 Scssr25538***
99.9 GBM1420***
CID-C09
LAI-G-V09
LAI-S-V09
LAI-G-V09
CID-C09
CID-L08
CID-V09
HI-V09
Yield-C09
PH-C09
Maturity-V09
PH-V09
PH-C09
CID-L08
PH-C09


4H
0.0 Bmag740
5.9 GMS089
6.1 HvCABG
6.8 Bmag306
7.6 Bmag808
8.5 GBM1452
10.4 Bmag490
11.7 Bmag353
34.2 EBmac635
34.5 EBmac679*
35.2 EBmac701
35.3 EBmac788*
49.2 GBM1448***
Maturity-V09
5H
0.0 Bmac113*
0.1 Bmag751
1.9 Bmag337*
2.0 Bmag323*
14.8 GBM1506*
15.2 Bmag760***
15.7 Scssr15334*
0.0 GBM1438**
2.2 GBM5008***
2.3 GBM1231***
16.6 GBMS119***
17.6 GMS61***
18.2 GBMS141***
18.3 GBM1436***
19.4 Scssr10148***
22.9 AF043094B***
24.9 GMS27***
CID-L08
CID-V09
Yield-V09
Biomass-V09
PH-C09
PH-V09
LAI-G-V09
HI-C09
Yield-C09
Yield-V09
Biomass-V09
Maturity-V09
Maturity-V09


6H
0.0 Bmac40
2.9 GBM1022
3.0 Scssr103
10.1 Scssr5599
10.2 GBM1008
37.0 GBM1355**
37.6 GBM1215**
45.3 Bmag807**
45.5 GBM1212***
45.6 MGB371*
47.7 Bmag174*
48.0 Bmag173
49.1 Bmag9
49.3 Hvm74
49.4 Bmag344
52.8 Scssr2093**
75.2 GBM1423
CID-V09
Yield-V09
Biomass-V09
PH-C09
CID-L08

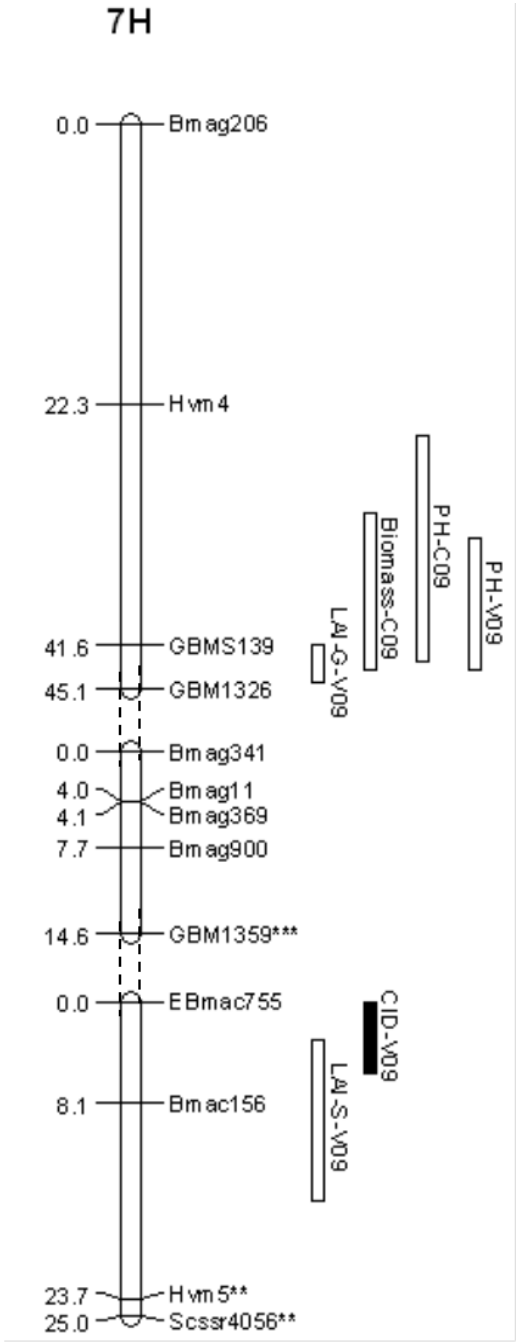
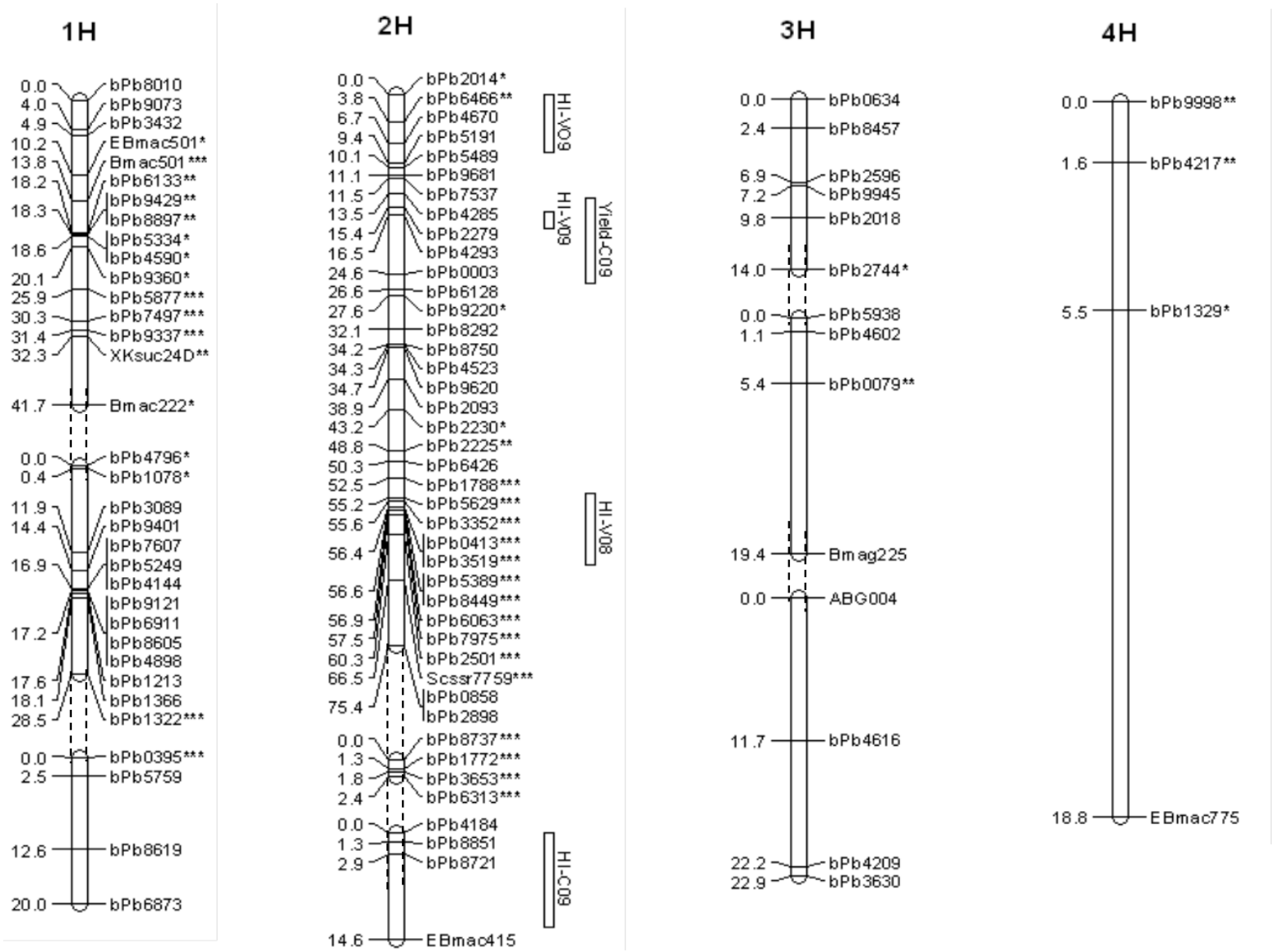
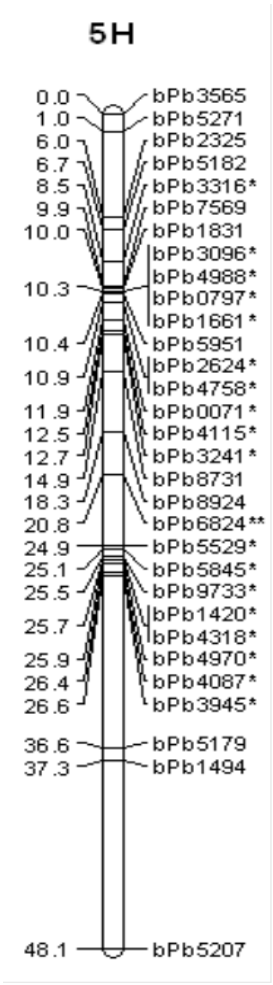

7H
0.0 Bmag206
22.3 Hvm4
41.6 GBMS139
45.1 GBM1326
0.0 Bmag341
4.0 Bmag11
4.1 Bmag369
7.7 Bmag900
14.6 GBM1359***
0.0 EBmac755
8.1 Bmac156
23.7 Hvm5**
25.0 Scssr4056**
LAI-G-V09
Biomass-C09
PH-C09
PH-V09
CID-V09
LAI-S-V09

Figure 4. 5 A linkage map of barley based on 200 $F_{5:6}$ recombinant inbred lines (RILs) from the cross between W89001002003 and I60049 constructed with 104 SSR markers. Numbers on the left of linkage groups indicate the cumulative map distances in cM (Kosambi). Marker loci are shown on the right of linkage groups. Co-segregating markers are listed next to each other in a vertical line on the right side of the linkage group. Markers with segregation distortion are indicated with stars, with *, ** and *** stand for $p < 0.05$, 0.01 and 0.001, respectively. QTL confidence intervals (peak LOD scores minus one) are shown to the right of the linkage group bar with QTL for carbon isotope discrimination ($\Delta^{13}C$) indicated by black rectangles and all others indicated by white rectangles as follows: leaf area index during stem elongation stage (LAI-S), leaf area index during grain filling stage (LAI-G), plant height (PH), days to maturity, biomass, yield and harvest index (HI). L08, C09 and V09 stand for Lacombe-2008, Castor-2009 and Vegreville-2009, respectively.


1H
0.0 bPb8010
4.0 bPb9073
4.9 bPb3432
10.2 EBmac501*
13.8 Bmac501***
18.2 bPb6133**
bPb9429**
18.3 bPb8897**
bPb5334*
18.6 bPb4590*
20.1 bPb9360*
25.9 bPb5877***
30.3 bPb7497***
31.4 bPb9337***
32.3 XKsuc24D**
41.7 Bmac222*
0.0 bPb4796*
0.4 bPb1078*
11.9 bPb3089
14.4 bPb9401
bPb7607
16.9 bPb5249
bPb4144
bPb9121
17.2 bPb6911
bPb8605
bPb4898
17.6 bPb1213
18.1 bPb1366
28.5 bPb1322***
0.0 bPb0395***
2.5 bPb5759
12.6 bPb8619
20.0 bPb6873
2H
0.0 bPb2014*
3.8 bPb6466**
6.7 bPb4670
9.4 bPb5191
10.1 bPb5489
11.1 bPb9681
11.5 bPb7537
13.5 bPb4285
15.4 bPb2279
16.5 bPb4293
24.6 bPb0003
26.6 bPb6128
27.6 bPb9220*
32.1 bPb8292
34.2 bPb8750
34.3 bPb4523
34.7 bPb9620
38.9 bPb2093
43.2 bPb2230*
48.8 bPb2225**
50.3 bPb6426
52.5 bPb1788***
55.2 bPb5629***
55.6 bPb3352***
bPb0413***
56.4 bPb3519***
bPb5389***
56.6 bPb8449***
56.9 bPb6063***
57.5 bPb7975***
60.3 bPb2501***
66.5 Scssr7759***
bPb0858
75.4 bPb2898
0.0 bPb8737***
1.3 bPb1772***
1.8 bPb3653***
2.4 bPb6313***
0.0 bPb4184
1.3 bPb8851
2.9 bPb8721
14.6 EBmac415
HI-V09
HI-V09
Yield-C09
HI-V08
HI-C09
3H
0.0 bPb0634
2.4 bPb8457
6.9 bPb2596
7.2 bPb9945
9.8 bPb2018
14.0 bPb2744*
0.0 bPb5938
1.1 bPb4602
5.4 bPb0079**
19.4 Bmag225
0.0 ABG004
11.7 bPb4616
22.2 bPb4209
22.9 bPb3630
4H
0.0 bPb9998**
1.6 bPb4217**
5.5 bPb1329*
18.8 EBmac775


5H
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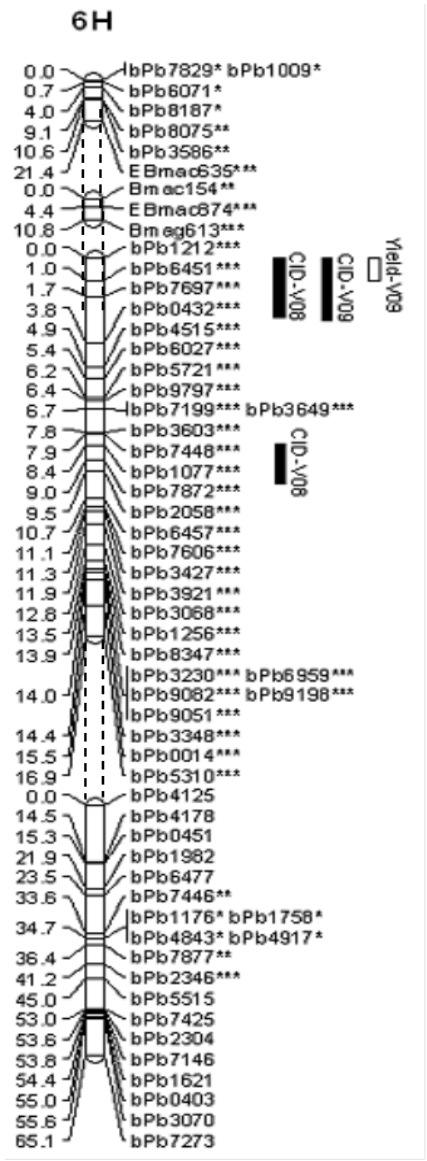
1.0 bPb5271
6.0 bPb2325
6.7 bPb5182
8.5 bPb3316*
9.9 bPb7569
10.0 bPb1831
bPb3096*
bPb4988*
10.3 bPb0797*
bPb1661*
10.4 bPb5951
10.9 bPb2624*
bPb4758*
11.9 bPb0071*
12.5 bPb4115*
12.7 bPb3241*
14.9 bPb8731
18.3 bPb8924
20.8 bPb6824**
24.9 bPb5529*
25.1 bPb5845*
25.5 bPb9733*
25.7 bPb1420*
bPb4318*
25.9 bPb4970*
26.4 bPb4087*
26.6 bPb3945*
36.6 bPb5179
37.3 bPb1494
48.1 bPb5207


6H
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0.7 bPb6071*
4.0 bPb8187*
9.1 bPb8075**
10.6 bPb3586**
21.4 EBmac635**
0.0 Bmac154**
4.4 EBmac874***
10.8 Bmag613***
0.0 bPb1212***
1.0 bPb6451***
1.7 bPb7697***
3.8 bPb0432***
4.9 bPb4515***
5.4 bPb6027***
6.2 bPb5721***
6.4 bPb9797***
6.7 bPb7199*** bPb3649***
7.8 bPb3603***
7.9 bPb7448***
8.4 bPb1077***
9.0 bPb7872***
9.5 bPb2058***
10.7 bPb6457***
11.1 bPb7606***
11.3 bPb3427***
11.9 bPb3921***
12.8 bPb3068***
13.5 bPb1256***
13.9 bPb8347***
14.0 bPb3230*** bPb6959***
bPb9082*** bPb9198***
bPb9051***
14.4 bPb3348***
15.5 bPb0014***
16.9 bPb5310***
0.0 bPb4125
14.5 bPb4178
15.3 bPb0451
21.9 bPb1982
23.5 bPb6477
33.6 bPb7446**
34.7 bPb1176* bPb1758*
bPb4843* bPb4917*
36.4 bPb7877**
41.2 bPb2346***
45.0 bPb5515
53.0 bPb7425
53.6 bPb2304
53.8 bPb7146
54.4 bPb1621
55.0 bPb0403
55.6 bPb3070
65.1 bPb7273
Yield-V09
CID-V08
CID-V09
CID-V08

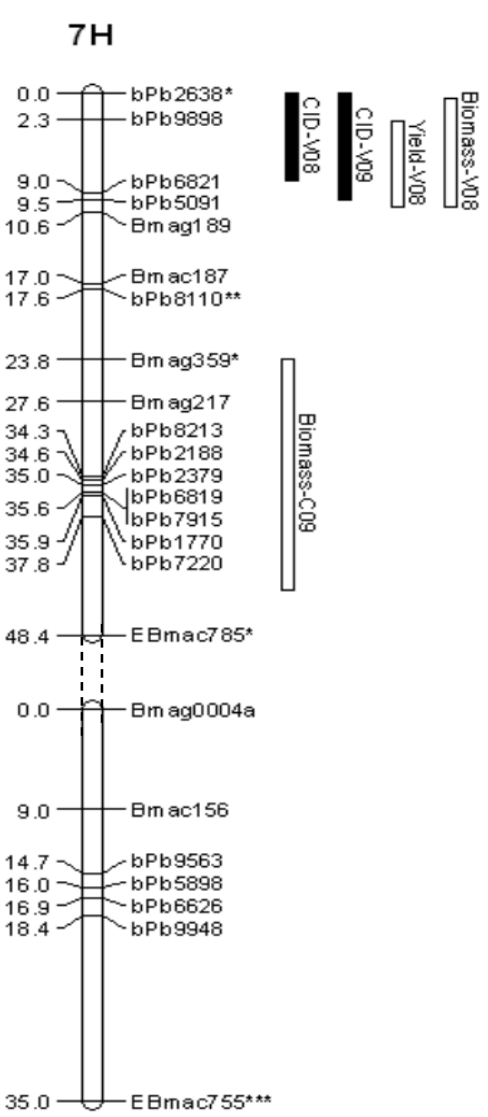
7H
0.0 bPb2638*
2.3 bPb9898
9.0 bPb6821
9.5 bPb5091
10.6 Bmag189
17.0 Bmac187
17.6 bPb8110**
23.8 Bmag359*
27.6 Bmag217
34.3 bPb8213
34.6 bPb2188
35.0 bPb2379
35.6 bPb6819
bPb7915
35.9 bPb1770
37.8 bPb7220
48.4 EBmac785*
0.0 Bmag0004a
9.0 Bmac156
14.7 bPb9563
16.0 bPb5898
16.9 bPb6626
18.4 bPb9948
35.0 EBmac755***
Biomass-V08
CID-V08
CID-V09
Yield-V08
Biomass-C09

Figure 4. 6 A linkage map of barley based on 127 $F_{5:6}$ recombinant inbred lines (RILs) from the cross between Merit and H93174006 was constructed with 21 SSR and 156 DArT markers. Numbers on the left of linkage groups indicate the cumulative map distances in cM (Kosambi). Marker loci are shown to the right of linkage groups. Co-segregating markers are listed next to each other in a vertical line on the right side of the linkage group. Markers with segregation distortion are indicated with stars, with *, ** and *** stand for $p < 0.05$, 0.01 and 0.001, respectively. QTL confidence intervals (peak LOD scores minus one) are shown to the right of the linkage group bar with QTL for carbon isotope discrimination ($\Delta^{13}C$) indicated by black rectangles and all others indicated by white rectangles as follows: biomass, yield and harvest index (HI). The C09, V08 and V09 stand for Castor-2009, Vegreville-2008 and Vegreville-2009, respectively.

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Chapter 5 Physiological characterization of recombinant inbred lines of barley with contrasting levels of carbon isotope discrimination

Introduction

Canada is one of the world's largest barley (*Hordeum vulgare* L.) producers and exporters, with an average of 12.3 million tonnes produced annually between 1986 and 2006 (FAOSTAT 2008). Barley production is mostly concentrated on the Prairies in western Canada, which is characterized by short and dry growing seasons with frequent drought and heat stress later in the growing season (Anyia et al. 2008). As a consequence of seasonal and temporal moisture deficits, barley yields and production are highly variable across locations and years. As drought continues to be one of the main abiotic constraints on agricultural productivity worldwide, and water deficit is likely to increase in most arid and semi-arid regions under future climate change scenarios (Barnabás et al. 2008), the development of barley varieties with high water-use efficiency (WUE) and drought tolerance that can maintain yield under water-limited conditions has been a key area of agricultural research around the world.

Plant physiological research has provided new insights to yield improvement in drought-prone environments. Considerable research and substantial breeding efforts have been devoted to identifying and selecting for morpho-physiological traits that increase WUE and yield under water-limited conditions (Blum 1996; Richards 1996; Richards et al. 2002). Physiological traits such as carbon isotope discrimination ($\Delta^{13}C$), a measure of the $^{13}C/^{12}C$ ratio in plant material relative to the ratio in atmospheric CO_2 (Hall et al. 1994), has been intensively exploited and demonstrated to be a simple but reliable indicator of WUE, and their negative correlation has been used for indirect selection of WUE under selected

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environments (Cattivelli et al. 2008). Genotypic variation of WUE and $\Delta^{13}C$ have long been reported in C_3 crops, but the complexity underlying drought resistant mechanisms and our poor knowledge of the genetic and physiological basis of yield have hindered the breeding process in drought environments (Passioura 2002; Tuberosa and Salvi 2006). Improved WUE and drought resistance without yield penalty offers a promising way to sustain agricultural production and land use in semi-arid regions (Karaba et al. 2007). Although the relationship between $\Delta^{13}C$ and WUE has been well described and verified by many authors, there are questions regarding the type of relationship between $\Delta^{13}C$ and biomass or grain yield (Anyia et al. 2007). Significant correlations between $\Delta^{13}C$ and biomass or grain yield have been reported, which were either positive or negative, depending on the plant tissue analyzed and the type of environment sampled (Condon et al. 2002; Anyia et al. 2007). In Australia, selection for low leaf $\Delta^{13}C$ resulted in improved wheat (*Triticum aestivum* L.) yield (Rebetzke et al. 2002). Under the terminal-drought in a Mediterranean-type environment, the correlation between $\Delta^{13}C$ and grain yield has been mostly positive and a high $\Delta^{13}C$ or low WUE was thought to be most appropriate in this region (Condon et al. 2004; Merah et al. 2001; Voltas et al. 1999). However, for stored-moisture environments such as eastern Australia and Canadian Prairies, yield improvements through a combination of low $\Delta^{13}C$ or high WUE and greater early vigor have been suggested (Condon et al. 2002; Anyia et al. 2008).

Multivariate procedures have been largely used in the assessment of genetic divergence in crop and grass species based on morphological and agronomic characteristics (Capo-chichi et al. 2005; Loos 1993; Matthewa et al. 1994; Riggs 1973; Vaylay and van Santen 2002). When each trait is considered separately in univariate analysis, considerable overlap of genetic variation may occur (Vaylay and van Santen 2002). During multivariate canonical discriminant analysis (CDA), all independent traits are viewed simultaneously to differentiate target groups (e.g., genotypes). The resulting differentiation extracts maximum genetic variability (between groups) compared with the environmental variability

(within groups) (Riggs 1973). There were few reports on discriminating genotypes with different level of WUE (measured as $\Delta^{13}C$) using the CDA method.

The objectives of this study were: 1) to examine the relationships among grain yield, leaf $\Delta^{13}C$ (WUE) and leaf gas exchange parameters of selected recombinant inbred lines (RILs) of barley with contrasting levels of leaf $\Delta^{13}C$; and 2) to classify the drought tolerance patterns of the RILs and their parents using CDA analysis based on agronomic and physiological traits.

Materials and methods

Plant materials

Ten six-row barley breeding lines (8 RILs and their parents) with contrasting levels of leaf $\Delta^{13}C$ (Table 5.1) were used in this study. The two parental lines (W89001002003 and I60049) were previously surveyed for leaf $\Delta^{13}C$ at three field locations from 2005 to 2009. Data from 2005 to 2006 were obtained from Anyia et al. (2007) and Chen et al. (2010). Based on consistent differences in leaf $\Delta^{13}C$ of W89001002003 (hereafter referred to as W89) and I60049, they were used to produce a RIL mapping population using the single seed descent (SSD) approach in 2006. Then eight progeny lines were selected from the 200 RILs developed at F_5 generation for this detailed greenhouse study. The selection of the eight progeny lines was based on their observed contrasting levels of leaf $\Delta^{13}C$ in previous field evaluations at 3 locations in 2008 and 2009.

Field experiments

Field experiments were conducted at Lacombe (52 °28' N, 113 °45' W, 847.3 m altitude, Orthic Black Chernozem, soil names based on the Canadian system of soil classification), Vegreville (53 °31' N, 112 °6' W, 639.3 m altitude, the Malmo

series of an Eluviated Black Chernozem) and Castor (52 °8' N, 111 °54' W, 807.7 m altitude, Dark Brown Chernozem) in Alberta, Canada, under rain-fed conditions. The three sites were characterized by distinct soil moisture conditions with Castor as the driest site and Lacombe as the wettest site among the three sites. The average annual precipitation and within season rainfall (June to August) from 1977 to 2007 was 340 ±89 mm and 172 ±67 mm at Castor compared with Vegreville which had 382 ±62 mm and 193 ±52 mm, and Lacombe which had 440 ±84 mm and 230 ±63 mm, respectively. The climate normals information was obtained from Environment Canada (2009) and AgroClimatic Information Service of Alberta (2009).

Field experiment in 2008: The experiment was conducted in Lacombe between May and September using a completely randomized design with one replicate of each RIL and four replicates of each parental line. Seeds of each genotype were planted in 2 rows in a 30 cm wide by 2 m long plot with 15 cm row spacing. Winter triticale 'Pika' was seeded between plots as a guard row (35 cm wide). Synthetic fertilizer (6-25-30) was applied at 112.5 kg ha^{-1} prior to seeding. Plots were seeded on May 15th (Table 5.2), and harvested on September 5th. Herbicides were applied at the rate of 29.7 g ha^{-1} for 'Refine' (33.35% thifensulfuron methyl, 16.65% tribenuron methyl) and 988.4 mL ha^{-1} for 'MCPA' (500 g L^{-1} amine, 500 g L^{-1} ester, 400 g L^{-1} K-salt and 300 g L^{-1} Na-salt) during the vegetative stage.

Field experiment in 2009: The 2009 field experiments conducted at Vegreville and Castor used a randomized complete block design with three replicates per genotype (RILs and parental lines). Each genotype was planted in 4 rows in 1 x 2 m plots with a 20 cm row spacing and a seeding rate of 215 seeds per square meter. Spring wheat 'AC Crystal' was seeded as a guard row between plots using the same row spacing of 20 cm. Fertilizer was applied prior to seeding at a rate of 22.5 kg ha^{-1} P_2O_5 at Vegreville, and 31.4 kg ha^{-1} N and 18 kg ha^{-1} P_2O_5 at Castor based on results of soil tests. Plots were seeded on May 13th (Castor) and May

22nd (Vegreville), and plants were harvested during the last week of September. Weed control was done by hand weeding at the 3-leaf stage (BBCH 13) at Castor. For Vegreville, herbicide 'Round-up Weathermax' (540 g L^{-1} glyphosate) was sprayed at 1.66 L ha^{-1} on May 24th before emergence.

Carbon isotope discrimination analysis: Fully expanded penultimate leaves were sampled for measurement of carbon isotope composition ($\delta^{13}C$) at the stem elongation stage (BBCH 36 to 39) according to the Zadoks et al. (1974) scale. Samples were randomly collected from 5 plants per plot and bulked. All samples were oven dried at 70 ℃ for 48 hours and then ground to fine powder with a ball mill (Spex SamplePrep 8000D Mixer, Metuchen, NJ, USA). Samples were analyzed for $\delta^{13}C$ using a continuous-flow stable isotope ratio mass spectrometer (Thermo Finnigan Mat Gmbh, Bremen, Germany). Carbon isotope composition was calculated as $\delta^{13}C$ (‰) = $(R_{sample}/R_{reference} - 1) \times 1000$, where R is the ratio of $^{13}C/^{12}C$, and the reference material is the belemnite carbonate standard (PDB) from the Pee Dee Formation. The $\Delta^{13}C$ was calculated according to Farquhar et al. (1989) as $\Delta^{13}C$ (‰) = $(\delta_a - \delta_p) / (1 + \delta_p) \times 1000$, where δ_a and δ_p refer to the C isotope ratios of atmospheric CO_2 (-8.0 ‰) and plant, respectively.

Plant height, aerial biomass and grain yield: Plant height was measured on 5 plants per plot at physiological maturity (BBCH 89). Biomass and grain yield were determined from a single 1 m^2 sub-plot within each plot. The harvested plant materials were air dried to a constant weight. Harvest index (HI) was calculated as HI = grain weight / total aboveground biomass.

Leaf area index (LAI): LAI was measure by a LAI-2000 Plant Canopy Analyzer (Li-Cor, Lincoln, NE, USA) over several occassions. A measurement cycle consisted of four below-canopy readings and one reference measurement. Reference measurements were collected above canopy level of each experimental plot (between 1 and 1.5 m above ground) at the beginning of each measurement

cycle. The below-canopy measurements were carried out at a diagonal transect between rows in each plot to improve spatial coverage. The fish-eye lens of the instrument was covered by a view cap with a 45 °opening, so that the reference measurements were not influenced by the operator and the sun (Li-Cor 1992). Measurements were taken during the stem elongation stage (BBCH 36 to 39) and grain filling stage (BBCH 71 to 75) under cloudy conditions.

Greenhouse experiment

The experiment was conducted in a greenhouse at Alberta Innovates - Technology Futures in Vegreville, Alberta, Canada, with a 16-hour photoperiod of natural illumination and supplemented lighting using sodium halide light bulbs, with 25 and 15 ℃ day and night temperatures, respectively. Seeds of each genotype were sown on December 9th 2009 and plants were harvested in mid March 2010.

Plants were grown in pots (21 cm diameter × 21 cm tall) filled with 3.5 kg of soil mix containing field topsoil and peat moss (Promix 'BX') in a 1:3 ratio (v:v). All pots were flushed with 2 L tap water and drained overnight to determine field capacity before seeding. Time Domain Reflectometry (TDR, Soil Moisture Equipment Corp., Santa Barbara, CA) probes (20-cm long stainless steel 3-rod configuration) was used to regularly monitor soil moisture. Eight seeds were sown at 3 cm depth in each pot and later thinned to four seedlings per pot two weeks after emergence. After thinning, the soil surface of each pot was covered with a 2 cm layer of perlite to minimize evaporation. All pots were then weighed and kept well-watered until the beginning of the drought treatment.

Each genotype was subjected to well-watered (ww) and water-deficit (wd) treatments with four replicates using a randomized complete block design. The well-watered pots were maintained at field capacity (ca. 28.8 ± 1.3 % volumetric soil moisture content) during the experiment. The water-deficit treatment was imposed for 7 days from the stem elongation stage (BBCH 31). Average soil moisture content at the end of the water-deficit treatment on day 7 was 10.9 ± 2.7

vol %. At the end of the water-deficit treatment, all pots were re-watered to pre-deficit levels and were then maintained under well-watered conditions until grain maturity. During the water-deficit treatment, pots were weighed every day to monitor water use. Four control pots containing similar amounts of soil and perlite but without plants were used to determine soil surface evaporation based on successive weight differences.

Plant measurements: At the beginning and end of the water-deficit treatment, one plant per pot was cut at ground level and oven-dried at 70 ℃ to a constant weight, to determine dry matter production. For WUE calculation, initial plant weights were subtracted from final plant weights during the water-deficit treatment period. The WUE was determined by dividing aerial biomass production by the cumulative water transpired during the seven days. On the last day of the water-deficit treatment, one penultimate leaf per pot was sampled for $\delta^{13}C$ analysis as described above for the field experiment. Leaf nitrogen and carbon contents were measured on separate subsamples of the same ground leaf material used for $\delta^{13}C$ analysis. A 5 – 7 mg sample was analyzed using an elemental analyser (NA 2500, CE Instruments, Milan, Italy) coupled by a Conflo II interface (Finnigan MAT, Bremen, Germany) to the stable isotope ratio mass spectrometer. Similar leaves to those sampled for $\delta^{13}C$ determination were used to measure leaf relative water content (RWC), and specific leaf area (SLA). The RWC was determined according to Barrs and Weatherley (1962) as: RWC (%) = (FW – DW) / (TW – DW) × 100%, where FW is fresh weight, DW is dry weight and TW is turgid weight. After measuring the FW, leaves were placed in distilled water for 24 hrs at room temperature in darkness to achieve complete rehydration. TW was measured by taking the leaves out of water, wiping any surface moisture quickly and lightly with a paper towel, and weighed immediately. The SLA was calculated as the ratio of leaf area to leaf dry weight. Leaf area was calculated as leaf length × leaf width × 0.8, where 0.8 is an empirical coefficient (Rebetzke and Richards 1999). At maturity, plant height was measured and the remaining two

plants per pot were harvested and oven dried at 70 ℃ for 2 days to determine aerial biomass and grain yield.

Gas exchange measurements: The topmost fully expanded and sun-exposed leaf (one leaf per pot) on the main stem was selected for gas exchange measurements on the last day of the water-deficit treatment, using a Li-Cor 6400 portable photosynthesis system (Li-Cor Inc, Lincoln, NE, USA). Gas exchange measurements were conducted between 9 am and noon under good light conditions. Air temperature inside the leaf chamber was maintained at ambient levels. The Li-Cor 6400's LED light source was used to set PPFD at 1000 μmol m^{-2} s^{-1} for all measurements while reference CO_2 was set at 400 ppm and flow rate was kept constant at 500 μmol s^{-1}. Measurements were made after the reading of parameters became relatively stable. Net CO_2 assimilation (*A*), transpiration rate (*E*), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), $WUE_{intrinsic}$ or WUE_{ic} (the ratio between *A* and g_s) was calculated from the gas exchange measurements.

Statistical analysis

Data were analyzed using SAS, version 9.1 (SAS Institute, Inc., Cary, NC). Each year-location combination of the field trials (Lacombe-2008, Castor-2009 and Vegreville-2009) was treated as an environment. Homogeneity of variance and normality of distribution were tested before analysis of variance (ANOVA). Differences among genotypes and between water treatments were examined using the General Linear Model (GLM) procedure. When ANOVA showed significant effects, means were separated by Bonferroni post hoc tests. Correlation analysis was performed to evaluate the relationship between traits using the CORR procedure in SAS. An α value of 0.05 was chosen to indicate statistical significance. In order to ascertain the most discriminant traits between groups, stepwise discriminant analysis was performed. At the first step all traits were

reviewed and evaluated to determine which one contributed significantly to the discrimination between groups, and the trait(s) that contributed most significantly to the discrimination were kept. The process was repeated until no more significant contribution from the remaining traits was found. In the end, all the statistically significant traits from stepwise discriminant analysis were used in multivariate CDA to determine which series of correlated traits best discriminate the genotypes. The squared Mahalanobis distance (D^2) was used to express the resemblance or separation between target groups (Loos 1993).

Results

Field performance of parental lines across locations and years

The difference of leaf $\Delta^{13}C$ between parental lines (W89 and I60049) was consistently significant across years and locations (Fig. 5.1). The average biomass and grain yield for W89 and I60049 at Lacombe-2008 (1370 and 607 g m^{-2}, respectively) and Vegreville-2009 (1012 and 525 g m^{-2}) were significantly higher than those at Vegreville-2006 (499 and 246 g m^{-2}, data from Chen et al. 2010) and Castor-2009 (251 and 121 g m^{-2}). The absolute biomass and grain yields of W89 were higher than those of I60049 at Lacombe-2008 (Table 5.3) and Vegreville-2009, but less than those of I60049 at Vegreville-2006 and Castor-2009. The HI of W89 ranged from 0.41 to 0.51, and that of I60049 ranged from 0.42 to 0.55 (Table 5.3 and data from Chen et al. 2010) across four location-year combinations. On average, I60049 had a higher HI than W89. During grain filling, I60049 tended to have a higher LAI than that of W89 ($p = 0.054$ at Vegreville-2009, $p = 0.64$ at Castor-2009). In 2009, plant height and LAI were significantly reduced at Castor as compared with Vegreville. Of the two parental lines, I60049 tended to be taller than W89 at Vegreville-2009 ($p = 0.33$), but significantly taller at Castor-2009 ($p = 0.011$).

Performance of RILs under field conditions

There were significant differences in leaf $\Delta^{13}C$ between the high-$\Delta^{13}C$ and low-$\Delta^{13}C$ groups (Table 5.1). All genotypes had higher value of leaf $\Delta^{13}C$ at Lacombe-2008, followed by Vegreville-2009 and Castor-2009. Considering location means, $\Delta^{13}C$ was 1.10 ‰ lower in Castor-2009 than in Vegreville-2009, 2.72 ‰ lower in Castor-2009 than in Lacombe-2008. The difference in leaf $\Delta^{13}C$ between the highest and lowest RILs was 2.83, 1.64 and 1.73‰ at Lacombe-2008, Castor-2009, and Vegreville-2009, respectively.

The ten barley genotypes (RILs and their parents) did not exhibit significant differences in any of the traits measured at Vegreville-2009, but they differed significantly in plant height, biomass and grain yield at Castor-2009 (Table 5.3). RIL '147' had higher biomass and grain yield than any other genotype at Castor-2009. In contrast, RIL '196' had the lowest biomass, grain yield and plant height at Castor-2009. Amongst all genotypes, RILs '144' and '116' had above average performance in biomass, grain yield and LAI at both locations in 2009. RIL '85' was consistently low in biomass, grain yield, HI and LAI-S in 2009.

Leaf $\Delta^{13}C$ showed significant positive correlations with biomass and grain yield under field conditions (Table 5.4). At Vegreville-2009, leaf $\Delta^{13}C$ was also positively correlated with HI, LAI-G and plant height (Table 5.4).

Performance of RILs under greenhouse conditions

The RILs from the high-$\Delta^{13}C$ group exhibited significantly higher leaf area, leaf N_m (nitrogen content per unit dry mass) and plant height than those of the low-$\Delta^{13}C$ group under both well-watered and water-deficit conditions (Table 5.5). Under water-deficit conditions, the high-$\Delta^{13}C$ group had significantly higher biomass and leaf C_m but significantly lower WUE compared with the low-$\Delta^{13}C$ group.

Substantial genotypic diversity was observed under both water availability conditions for biomass, grain yield, HI, WUE, LA, leaf N_m, plant height and leaf $\Delta^{13}C$ (Table 5.5). The drought treatment significantly reduced biomass, SLA, RWC, plant height, *A*, g_s and leaf $\Delta^{13}C$ (Table 5.5 and 5.6). Among all ten genotypes, RIL '147' showed the lowest decline in *A*, g_s, internal CO_2 concentration (C_i), and transpiration rate (*E*) on day 7 of withholding irrigation, while RIL '116' exhibited the largest decline in the four gas exchange parameters (Table 5.6). However, the genotypic ranking for biomass, grain yield, HI, LA, leaf N_m, plant height and leaf $\Delta^{13}C$ remained stable between well-watered and water-deficit conditions (Table 5.5).

Leaf $\Delta^{13}C$ was negatively correlated with WUE ($p < 0.01$), and positively correlated with SLA and leaf N_m (Table 5.7) under both well-watered and water-deficit conditions. Significant positive relationships among leaf $\Delta^{13}C$, biomass and grain yield under well-watered conditions were observed in the greenhouse experiment (Table 5.7). A strong positive relationship was found between *A* and g_s under water-deficit conditions ($r = 0.89$, $n = 32$, $p < 0.01$).

Performance of barley lines under both field and greenhouse conditions

Stepwise discriminant analysis showed that the most discriminant traits were plant height, leaf $\Delta^{13}C$, HI, grain yield and biomass (Table 5.8). The multivariate test for differences among the genotypes was significant ($p < 0.01$), whereas the univariate analyses for the combined field and greenhouse data failed to achieve the significant level ($p = 0.16$, 0.99, 0.99 and 0.18 for plant height, biomass, grain yield and leaf $\Delta^{13}C$, respectively) except for HI ($p = 0.01$). The first two canonical discriminant functions (CAN) accounted for 90% of the genotypic variance, with 59% from the first CAN (CAN1). The canonical correlations between the genotypes and CAN1 ($r_c = 0.64$), the genotypes and the second CAN (CAN2, $r_c = 0.52$) were both significant (Table 5.8). The HI and leaf $\Delta^{13}C$ had

large loadings on CAN1 (0.45 and 0.37, respectively). The CAN2 was dominated by large loading from plant height and HI (0.38 and 0.48, respectively).

Three clusters of genotypes were identified from the CAN1 × CAN2 plane (Fig. 5.2). Cluster I included genotypes '18', '116', '176', '196' and I60049, and cluster II contained RILs '85', and '144', and cluster III had RILs '127' and '147'. Cluster III was the overlapped part between cluster I and cluster II as suggested by the probability of Mahalanobis distance. The pair-wise distances between RIL '127' and all the genotypes in cluster I were not significant except with I60049 ($p < 0.01$), and RIL '127' clustered with the RIL '85' instead of '144' ($p = 0.02$) in cluster II. RIL '147' fitted into cluster II and also was not different from '18' and '196' in cluster I. The scatter plot showed that W89 was separated from the other genotypes, except with I60049 ($p = 0.054$).

Discussion

Stability of leaf $\Delta^{13}C$ across environments

Across eight different environments (location-year combinations) I studied, the leaf $\Delta^{13}C$ of W89 was consistently lower than that of I60049, suggesting that the trait could be intrinsic and under strong genetic control (Anyia et al. 2007; Chen et al. 2010). This finding is consistent with several other reports on different crops (Hubick et al. 1988; Condon and Richards 1992; Rebetzke et al. 2008; Stiller et al. 2005). For example, Hubick et al. (1988) observed that the broad sense heritability of $\Delta^{13}C$ (whole plant, excluding the pods and roots) in field-grown peanut cultivars was 0.81, and there was no significant interaction between genotype and environment for $\Delta^{13}C$. Condon and Richards (1992) found that the broad-sense heritability of $\Delta^{13}C$ in wheat was greatest for plant material sampled before or during early stem elongation (0.95 on genotype basis or 0.88 on a single-plot basis). In another study on wheat, the narrow-sense heritability of leaf $\Delta^{13}C$ ranged from 0.37 to 0.91 on a single environment basis and from 0.76 to

0.86 on genotype-mean basis in a quantitative trait loci analysis using three wheat mapping populations across three years (Rebetzke et al. 2008). In a study on cotton, the broad-sense heritability for leaf $\Delta^{13}C$ (0.68) was higher than that of net photosynthesis (0.65) and lint yield (0.56) (Stiller et al. 2005). These results suggest that leaf $\Delta^{13}C$ is under strong genetic control and can be reliably used as a measure of WUE under field conditions.

Leaf $\Delta^{13}C$ and temporal rainfall distribution across environments

On the Prairies in western Canada, barley relies on stored-moisture from snow melt to deal with the low rainfall situation within the growing season (Anyia et al. 2008). Overall, the difference of the leaf $\Delta^{13}C$ between the extreme genotypes was smallest at Castor-2009, largest at Lacombe-2008 and intermediate at Vegreville-2009, and such difference can be ascribed to the total precipitation (Table 5.2), especially the temporal rainfall distribution, which is critical for crop growth (Bonsal et al. 1999; Chakravartia 1972). As rainfall during June and July (Table 5.2) was higher at Lacombe-2008, followed by Vegreville-2009 and Castor-2009, so was the order of mean leaf $\Delta^{13}C$ values of the genotypes. The correlation analysis showed that leaf $\Delta^{13}C$ was significantly related to June rainfall ($r = 0.46$, $n = 120$, $p < 0.01$) and total precipitation ($r = 0.45$, $n = 120$, $p < 0.01$). The low rainfall during June and July may explain the low leaf $\Delta^{13}C$ value and narrow leaf $\Delta^{13}C$ differences for the extreme genotypes grown at Castor-2009.

Relationships between leaf $\Delta^{13}C$ and agronomic performance

Positive or neutral relationships between $\Delta^{13}C$ and grain yield or biomass are often reported in environments with large within-season rainfall or supplemental irrigation, such as wheat and barley grown in the Mediterranean (Araus et al. 2003; Condon et al. 1993; Jiang et al. 2006; Merah et al. 1999; Teulat et al. 2002;

Teulat et al. 2001; Voltas et al. 1999). For stored-moisture environments such as eastern Australia and the Canadian Prairies, negative association between $\Delta^{13}C$ and grain yield has been reported (Anyia et al. 2007; Condon et al. 1993; Rebetzke et al. 2002). In our study, leaf $\Delta^{13}C$ was found to be positively correlated with biomass and grain yield under field conditions (Table 5.4). A possible explanation of such relationship is suggested by the positive association between leaf $\Delta^{13}C$ and g_s, which has previously been reported in barley (Chen et al. 2011), common bean (*Phaseolus vulgaris* L.) (Ehleringer 1990) and rice (Kondo et al. 2004; Takai et al. 2009). Plants with a higher g_s are associated with a higher photosynthetic capacity (Wong et al. 1979), which could in turn increase biomass production and thereby final yield. Low g_s might constrain plant performance under dry environments due to reductions in carbon fixation per unit leaf area as stomata close. Low $\Delta^{13}C$ or high WUE under dry conditions can occur at the expense of absolute yield performance due to the fact that drought-stressed plants wilted far more than unstressed plants (Bloch et al. 2006; Clover et al. 2001). A significantly lower g_s in W89 than I60049 grown in field environments (Chen et al. 2011) and in the current greenhouse experiment (Table 5.6) may account for differences in performance between W89 and I60049.

The leaf $\Delta^{13}C$ reflects the time integrated measure of the transpiration efficiency (aerial biomass/water transpired) over the period when leaf tissue was formed (Rebetzke et al. 2002). The leaf $\Delta^{13}C$ in the present study reflected the integrated WUE from emergence to stem elongation, a critical stage for yield formation in barley (Anyia et al. 2008). Of the three field locations tested, leaf $\Delta^{13}C$ values of all genotypes were lowest at Castor-2009, highest at Lacombe-2008, and intermediate at Vegreville-2009 (Table 5.1), so was the order of the mean performance in biomass and grain yield of all genotypes (Table 5.3), which also reflected the average soil available moisture for these locations. Bloch et al. (2006) also suggested that $\Delta^{13}C$ can be used as a sensitive indicator for water availability during the growing period. The poor performance of all genotypes at

Castor-2009 can be ascribed to lower soil moisture resulting in the lower leaf $\Delta^{13}C$ values at this location, compared to leaf $\Delta^{13}C$ at other locations (Table 5.1).

In a previous study, Chen et al. (2011) reported that a low leaf $\Delta^{13}C$ genotype 'CDC Cowboy' maintained its biomass and grain yield at a low g_s, which were comparable to genotypes with high g_s under field conditions. In this study, the performance of RIL '147' with low-$\Delta^{13}C$ was similar to that of 'CDC Cowboy'. RIL '147' was the most productive one among all the ten genotypes tested under drier conditions at Castor (Table 5.2), and it also showed the least decline in biomass and grain yield between Vegreville and Castor during the 2009 growing season. Genotypes such as RIL '147' may be suited for the Canadian Prairies, where crops rely heavily on stored soil moisture (with limited rainfall) within the growing season. In low soil moisture environments such as Castor-2009, genotypes with low leaf $\Delta^{13}C$ such as '147' that can maintain relatively higher biomass and grain yield at a low g_s than other genotypes should be targeted in breeding programs to achieve a higher stability of yield across locations.

Physiological differences under drought conditions

In the greenhouse study, all genotypes showed reduced A and g_s when subjected to the water deficit treatment. The positive relationship between A and g_s under water-deficit conditions suggested that the decline in A was driven by stomatal limitations. Similar results have been reported for other crops (Monneveux et al. 2006; Scartazza et al. 1995; Xu et al. 2009). One of the most basic questions regarding $\Delta^{13}C$ is whether a low value of leaf $\Delta^{13}C$ may arise from reduced g_s or increased photosynthetic capacity or both (Araus et al. 1997; Condon et al. 1990; Condon et al. 2004; Morgan and LeCain 1991). A proportional change in both A and g_s might have no effect on WUE_{ic}, while a comparable change in A with g_s that remains constant would cause a substantial variation in WUE_{ic}, and vice versa. In this study, both A and g_s decreased under water-deficit conditions with a strong positive correlation, but the variation in g_s was proportionally greater than A,

which suggested that g_s drove the variation in $\Delta^{13}C$. A similar pattern has been found by Xu et al. (2009). Roussel et al. (2009) concluded that leaf $\Delta^{13}C$ is under tight genetic control, and genetic difference of leaf $\Delta^{13}C$ and WUE_{ic} can be ascribed to differences in g_s and stomatal density instead of *A*. RIL '147' maintained the highest g_s among the ten genotypes on the last day of the water-deficit treatment, which may be responsible for the low leaf $\Delta^{13}C$ value of this line.

Genotypic ranking for leaf area, leaf N_m, plant height and leaf $\Delta^{13}C$ was consistent between the well-watered and water-deficit treatments. The leaf $\Delta^{13}C$ was positively correlated with leaf N_m (Table 5.7) in this study, and similar results have also been reported in rice (This et al. 2010; Xu et al. 2009). As suggested by This et al. (2010), the positive relationship between leaf $\Delta^{13}C$ and N_m suggests a tradeoff between WUE and nitrogen use efficiency through regulation between *A* and g_s. The positive relationship between leaf $\Delta^{13}C$ and leaf N_m also implies that diversity in photosynthetic capacity may contribute to the variability of WUE among genotypes. Leaf $\Delta^{13}C$ and SLA were also positively correlated under both water conditions, but the genotypic ranking for SLA was not stable between well-watered and water-deficit conditions (Table 5.5), which may limit its application as a less expensive alternative to leaf $\Delta^{13}C$.

Similarities and differences between genotypes

Three distinct clusters of genotypes were identified from CDA primarily based on plant height, HI and leaf $\Delta^{13}C$, which were mostly consistent with our selection based on their contrasting level of leaf $\Delta^{13}C$ (Figure 5.2). Four out of five genotypes in the high-$\Delta^{13}C$ group (Table 5.1) were assigned to cluster I except RIL '127', while the five genotypes in the low-$\Delta^{13}C$ group were separated into three clusters by CDA (Figure 5.2), with W89 and RIL '116' in cluster I, RIL '85' and '144' in cluster II, and RIL '147' in cluster III. This diversity of the low-$\Delta^{13}C$ group suggests that genotypes with low leaf $\Delta^{13}C$ may differ in their level of

WUE and drought tolerance. The ability to increase WUE or decrease leaf $\Delta^{13}C$ is necessary to achieve drought tolerance, but leaf $\Delta^{13}C$ alone is not sufficient to explain the genotypic diversity in drought tolerance as indicated by the multiple patterns through the CDA analysis. The CDA discriminated genotypes largely based on leaf $\Delta^{13}C$, HI and plant height, suggesting HI and plant height also contributed to the overall performance of the genotypes.

When assessing the performance of crop, the trait of ultimate importance is grain yield under the target environment. In this study, different drought tolerance levels were observed as suggested by the clustering patterns from CDA. Cluster I produced more grain yield than the other two clusters at Vegreville-2009, but less grain yield than any other cluster at Castor-2009, suggesting cluster I was drought sensitive. Cluster III showed intermediate performance in grain yield at Vegreville-2009 as compared with clusters I and II. Under low moisture conditions during early to mid-season at Castor-2009, cluster III performed better than clusters I and II, suggesting that cluster III was drought tolerant.

Even RILs within the same cluster responded to the low moisture conditions differently. For example, RIL '176' showed less declines in biomass (57%) and grain yield (54%) between the two field locations than the other five genotypes in cluster I, which may be explained by its early maturity than the other genotypes (data not shown). In contrast, the three most productive genotypes (RIL '18', I60049 and W89) at Vegreville-2009 were very sensitive to drier conditions experienced at Castor-2009, with the biomass and grain yield declined by 73 and 72%, (RIL '18'), 72 and 75%, (I60049), and 77 and 78%, (W89), respectively, at Castor compared with Vegreville. RIL '127' was assigned to cluster III due to its average performance under field and greenhouse conditions. RIL '147' from cluster III was relatively more productive than other genotypes under drier conditions at Castor. It showed the least decline in biomass (56%) and grain yield (52%) among all ten genotypes between Vegreville and Castor, by maintaining greater stomatal opening, and a higher *A* than other genotypes evaluated under water-deficit conditions (Table 5.7).

Conclusions

This study demonstrated the stability of leaf $\Delta^{13}C$ of RILs derived from parental lines with contrasting levels of leaf $\Delta^{13}C$. The overall performance of RILs was consistent with their leaf $\Delta^{13}C$ grouping. The correlation between leaf $\Delta^{13}C$ and yield was positive. In this study, a low leaf $\Delta^{13}C$ genotype (RIL '147') was identified as a high yielding line that showed the least decline in biomass and grain yield between Vegreville and Castor during the 2009 growing season. Genotypes such as RIL '147' may be of interest for achieving yield stability on the Canadian Prairies, where crops rely on stored soil moisture due to limited rainfall within the growing season. Additional field testing is needed to further evaluate the yield stability of the parental lines and their RILs used in this study.

Table 5. 1 Genotypes used in the greenhouse experiment were based on previous field survey of leaf carbon isotope discrimination ($\Delta^{13}C$) in 2008 and 2009

Group	Genotype	Lacombe-2008[1]	Castor-2009	Vegreville-2009
		$\Delta^{13}C$ (‰, mean ± S.E.)		
High-$\Delta^{13}C$	18	21.43	18.39 ± 0.74	20.45 ± 0.49
	127	21.09	18.96 ± 0.15	20.35 ± 0.52
	176	22.67	19.33 ± 0.18	20.32 ± 0.96
	196	22.19	18.51 ± 0.21	20.17 ± 0.64
	I60049	22.40 ± 0.24	18.99 ± 0.15	19.97 ± 0.55
	Mean	21.96	18.84	20.25
Low-$\Delta^{13}C$	85	20.61	18.17 ± 0.40	18.77 ± 0.15
	116	21.25	18.37 ± 0.39	19.14 ± 0.12
	144	19.84	18.21 ± 0.05	19.24 ± 0.30
	147	20.47	18.81 ± 0.10	19.30 ± 0.44
	W89[2]	20.71 ± 0.09	17.69 ± 0.56	18.72 ± 0.33
	Mean	20.58	18.25	19.03
Significance				
Group			***	
Location-year			***	
Group × Location-year			ns	

[1] Data was only from one replicate (except parental lines).

ns, not significant; *** indicates 0.1 % significant level

[2] W89: W89001002003

Table 5. 2 Monthly precipitation (mm) over the growing season for the three field locations during 2008 and 2009. The mean, minimum, and maximum air temperature (Tm) for ten-day periods after sowing are also provided.

Location-year	Sowing date	Precipitation (mm)				Mean Tm (℃)	Mean min.Tm (℃)	Mean max.Tm (℃)
		June	July	August	Total			
Lacombe-2008	May 15th	45.8	48.8	55.5	150.1	14.2	6.7	20.7
Vegreville-2009	May 22nd	32.2	44.6	25.2	102.0	13.1	3.4	20.6
Castor-2009	May 13th	21.5	33.8	51.9	107.2	5.8	-1.3	13.1

Table 5. 3 Mean biomass, grain yield, harvest index (HI), leaf area index during stem elongation stage (LAI-S), leaf area index during grain filling stage (LAI-G) and plant height (PH) of ten barley lines under field conditions

Group	Genotype	Biomass ($g\ m^{-2}$)			Grain yield ($g\ m^{-2}$)			HI			LAI-S ($m^2\ m^{-2}$)		LAI-G ($m^2\ m^{-2}$)		PH (cm)	
		L-08[1,2]	V-09[1]	C-09[1]	L-08	V-09	C-09	L-08	V-09	C-09	V-09	C-09	V-09	C-09	V-09	C-09
High-$\Delta^{13}C$	18	1564	958	244[b]	668	495	124[bc]	0.43	0.51	0.50	3.98	1.34	5.62	1.64	86	45[abc]
	127	1274	807	244[b]	524	410	124[bc]	0.41	0.50	0.51	3.54	1.47	4.27	1.57	82	48[abc]
	176	1540	723	299[ab]	663	364	153[ab]	0.43	0.49	0.51	3.28	1.83	4.00	1.69	71	51[a]
	196	2403	800	174[c]	983	427	75[d]	0.41	0.53	0.43	3.43	1.02	4.23	1.29	73	36[c]
	I60049	1229	987	265[b]	513	521	128[bc]	0.42	0.53	0.49	4.60	1.37	4.89	1.71	73	47[abc]
	Mean	1602	855	245	670	444	121	0.42	0.51	0.49	3.77	1.41	4.60	1.58	77	45

Table 5. 3 *Continued*

	85	1811	596	238^{bc}	651	271	107^{cd}	0.36	0.45	0.45	3.33	1.09	3.83	1.62	72	51^{a}
	116	1790	919	289^{b}	850	487	131^{bc}	0.48	0.53	0.45	4.61	1.48	4.68	1.65	77	45^{abc}
Low-	144	2606	946	292^{ab}	978	487	139^{bc}	0.38	0.51	0.48	4.19	1.51	4.64	1.97	88	54^{a}
$\Delta^{13}C$	147	1549	837	356^{a}	681	421	194^{a}	0.44	0.50	0.54	4.53	1.63	5.11	1.58	74	49^{ab}
	W89[3]	1512	1038	236^{bc}	701	529	113^{bcd}	0.46	0.51	0.48	4.52	1.39	4.54	1.34	67	38^{bc}
	Mean	1854	867	282	772	439	137	0.42	0.50	0.48	4.23	1.42	4.56	1.63	76	47
Significance																
Group			ns			ns			ns			ns		ns		ns
Location-year			***			***			***			***		***		***
Group × Location-year			ns			ns			ns			ns		ns		ns

[1] L-08, V-09, and C-09 stand for location by year (environment), Lacombe-2008, Vegreville-2009, and Castor-2009;

[2] Data from Lacombe-2008 were only from one replicate (except parental lines), and therefore it was not subjected to ANOVA. Genotypic differences in biomass, grain yield and plant height were only found at Castor-2009, means followed by a different letter are significant at $P < 0.05$; [3] W89: W89001002003; ns, not significant; *** indicates 0.1% significance level.

Table 5. 4 Correlations between biomass, grain yield, harvest index (HI), leaf area index during stem elongation stage (LAI-S), leaf area index during grain filling stage (LAI-G), plant height (PH) and leaf carbon isotope discrimination ($\Delta^{13}C$) of 10 barley genotypes at Castor and Vegreville in 2009

	Biomass	Grain yield	HI	LAI-S	LAI-G	PH
Castor-2009						
Grain yield	0.94***					
HI	0.41*	0.69***				
LAI-S	0.54**	0.60***	0.45*			
LAI-G	0.46*	0.38*	ns	0.55**		
PH	0.61***	0.56**	ns	0.44*	0.51**	
$\Delta^{13}C$	0.38*	0.41*	ns	ns	ns	
Vegreville-2009						
Grain yield	0.99***					
HI	0.69***	0.80***	ns			
LAI-J	0.72***	0.68***	ns			
LAI-G	0.64***	0.59***	ns	0.78***		
PH	0.64***	0.63***	0.41*	0.54**	0.67***	
$\Delta^{13}C$	0.48**	0.51**	0.44*	ns	0.53**	0.66***

*, ** and *** indicate 5, 1 and 0.1% significance levels, respectively;

ns, not significant

Table 5. 5 Abbreviations, descriptions and mean values for plant productivity, leaf structure and leaf function traits measured under well-watered (WW) and water-deficit (WD) conditions in the greenhouse study

Variable	Description	Group difference						Genotypic diversity				Drought effect	
		ww			wd			ww		wd		ww vs wd	
		H	L	H vs L	H	L	H vs L	Mean	p	Mean	p	p	r
Biomass	g $plant^{-1}$	20.3	19.2	ns	19.2	17.5	*	19.8	**	18.4	**	**	0.41*
Grain yield	g $plant^{-1}$	9.2	8.3	ns	8.8	7.9	ns	8.7	***	8.3	***	ns	0.68***
HI	Harvest index	0.45	0.43	ns	0.46	0.45	ns	0.44	***	0.45	**	ns	0.72***
PH	Plant height (cm)	90	83.9	*	88	80	**	86.8	***	84	***	*	0.79***
WUE	Water-use efficiency (g kg^{-1})	3.9	4.0	ns	3.6	4.2	*	4.1	**	3.9	*	ns	ns
LA	Penultimate leaf area (cm^2)	67.1	57.2	***	63.4	56.3	**	62.1	**	59.9	***	ns	0.50**
N_m	Leaf nitrogen content per dry mass (mg g^{-1} DW)	53.8	48.2	*	53.6	47.6	**	50.8	**	50.6	**	ns	0.43*
C_m	Leaf carbon content per dry mass (mg g^{-1} DW)	420	416	ns	419	411	**	417.6	ns	415	ns	ns	ns
SLA	Specific leaf area (cm^2 g^{-1})	433	442	ns	416	404	ns	438	ns	410	ns	**	ns
RWC	Relative water content (%)	95.7	95.3	ns	88.6	89.9	ns	95.5	ns	89.3	ns	***	ns
A	Assimilation rate (μmol CO_2 m^{-2} s^{-1})	15.07	13.62	*	7.03	8.71	ns	14.4	ns	7.4	ns	***	ns

Table 5. 5 *Continued*

g_s	Stomata conductance (mmol H_2O m^{-2} s^{-1})	644.7	569.9	ns	75.7	118.0	ns	608.9	ns	83.2	ns	***	ns
WUE_{ic}	Intrinsic water-use efficiency (μmol mol^{-1})	0.02	0.03	ns	0.12	0.10	ns	0.02	ns	0.11	ns	***	ns
$\Delta^{13}C$	Leaf carbon isotope discrimination (‰)	22.1	21.4	**	21.6	21.0	**	21.7	***	21.3	***	***	0.48**

All the traits were recorded on the full set of 10 barley lines. For each trait, general means and significance level were indicated for group and treatment effects. H and L stand for high-$\Delta^{13}C$ group and low-$\Delta^{13}C$ group. The effects of drought on each trait and genotypic ranking were estimated by ANOVA and correlations (r). *, ** and *** indicate 5, 1 and 0.1% significant level, respectively. ns for non-significant values. DW, dry weight.

Table 5. 6 Leaf carbon isotope discrimination ($\Delta^{13}C$) and leaf gas exchange parameters (assimilation rate (*A*), stomatal conductance (g_s), internal CO_2 concentration (C_i), transpiration rate (*E*)), and intrinsic water-use efficiency (WUE_{ic}) of ten barley genotypes on the 7th day of water-deficit (WD) and well-watered (WW) conditions in a greenhouse study

Treatment	Group	Genotype	$\Delta^{13}C$ (‰, mean ± S.E.)	*A* (μmol CO_2 m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})	*Ci* (ppm)	*E* (mmol H_2O m^{-2} s^{-1})	WUE_{ic} (μmol CO_2 $mmol^{-1}$ H_2O)
WD	High-$\Delta^{13}C$	18	21.22	6.98	56.6	176	1.52	0.124
		127	20.90	6.15	48.0	165	1.35	0.132
		176	21.91	8.38	94.2	185	2.17	0.117
		196	21.49	7.85	84.9	194	2.04	0.112
		I60049	22.76	5.79	94.4	213	2.10	0.103
		Mean	21.66	7.03	75.7	187	1.84	0.118
	Low-$\Delta^{13}C$	85	21.32	10.22	149.9	252	3.41	0.074
		116	21.52	5.05	38.4	158	1.10	0.139
		144	20.70	9.87	135.3	238	3.02	0.083
		147	20.84	10.95	204.5	264	3.99	0.067
		W89[1]	20.26	7.49	62.1	173	1.66	0.126
		Mean	20.93	8.71	118.0	217	2.64	0.098

Table 5. 6 *Continued*

WW	High-$\Delta^{13}C$	18	22.61	15.33	669.4	330	8.68	0.021
		127	21.97	16.72	691.6	319	8.25	0.025
		176	21.91	13.69	535.2	323	7.45	0.026
		196	21.11	15.86	637.9	320	7.91	0.026
		I60049	22.64	13.73	689.3	336	8.78	0.018
		Mean	22.05	15.07	644.7	325	8.21	0.023
	Low-$\Delta^{13}C$	85	21.14	14.36	474.5	317	7.48	0.029
		116	22.26	14.09	629.1	326	7.87	0.024
		144	21.16	12.69	675.4	337	8.18	0.019
		147	21.08	13.03	543.5	324	7.32	0.027
		W89	21.31	13.95	526.9	321	7.50	0.028
		Mean	21.39	13.62	569.9	325	7.67	0.025

[1]W89: W89001002003

Table 5. 7 Linear correlations (Pearson's coefficients) among biomass, grain yield, harvest index (HI), water-use efficiency (WUE_{ie}), penultimate leaf area (LA), leaf nitrogen content (N_m), leaf carbon content (C_m), specific leaf area (SLA), relative water content (RWC), plant height (PH), assimilation rate (*A*), stomatal conductance (g_s), intrinsic water-use efficiency (WUE_{ic}) and leaf carbon isotope discrimination ($\Delta^{13}C$) of ten barley genotypes under well-watered and water-deficit conditions in the greenhouse study

	Biomass	Yield	HI	WUE_{ie}	LA	N_m	C_m	SLA	RWC	PH	*A*	g_s
Water-deficit												
Yield	0.81***											
HI		0.79***										
LA		0.36*	0.39*									
N_m				-0.45**	0.46**							
SLA				-0.41*		0.39*	-0.57***					
PH	0.39*						0.36*					
A									0.43*			
g_s									0.38*		0.89***	
WUE_{ic}											-0.83***	-0.90***
$\Delta^{13}C$				-0.58***	0.47**	0.62***		0.64***				

Table 5. 7 *Continued*

	Biomass	Yield	HI	WUE_{ie}	LA	N_m	C_m	SLA	RWC	PH	*A*	g_s
Well-watered												
Yield	0.88***											
HI	0.44**	0.81***										
WUE_{ie}	-0.62***	-0.59***	-0.32*									
N_m		0.32*										
SLA				-0.35*			-0.60***					
PH					0.45**							
A			0.32*		0.39*					0.34*		
WUE_{ic}											0.33*	-0.83***
$\Delta^{13}C$	0.39*	0.39*		-0.51***		0.56**		0.51**				

*, ** and *** indicate 5, 1 and 0.1 % significant level, respectively.

Table 5. 8 Canonical loadings of the independent variables on the first two canonical discriminate variates of the ten genotypes. The study was conducted under field (Vegreville and Castor) and greenhouse conditions in 2009

Trait	Canonical discriminant function	
	CAN1	CAN2
Plant height	-0.178	0.379
Biomass	0.018	-0.095
Yield	0.036	-0.099
HI	0.452	0.476
Leaf $\Delta^{13}C$	0.366	0.141
Canonical correlation	0.636	0.515
P level of significance	<0.001	0.020
Variance account for	0.590	0.310

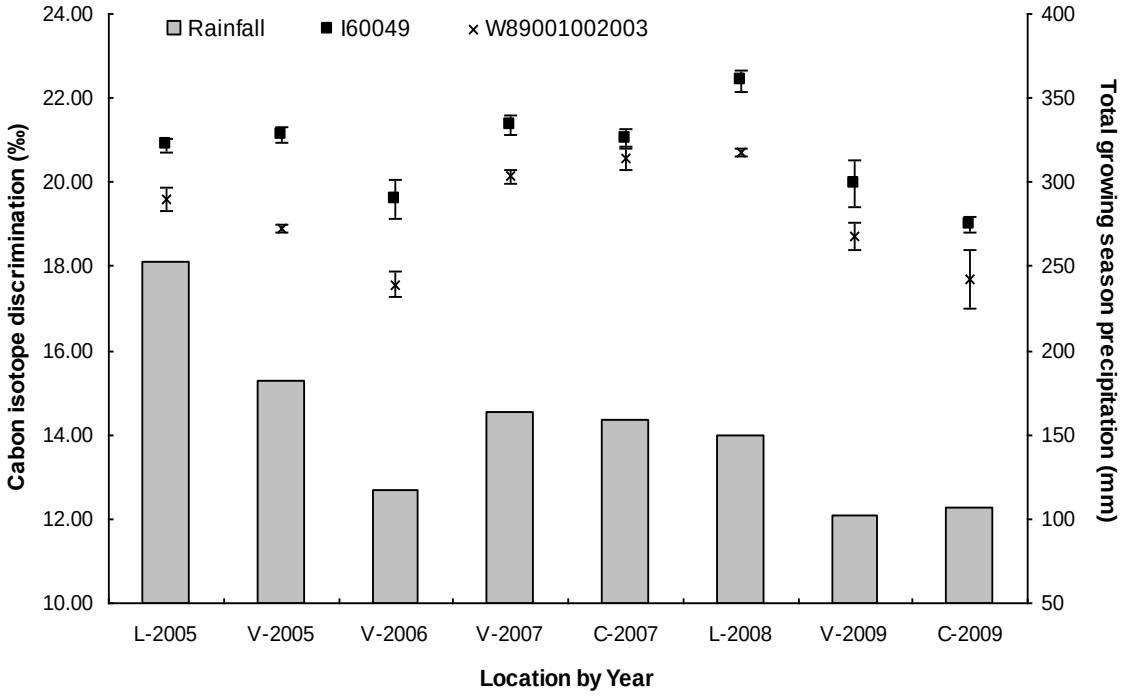

Figure 5. 1 Location and year variation of leaf $\Delta^{13}C$ of two six-row barley parental lines (W89001002003 and I60049) used for a RIL mapping population as a function of growing season rainfall. Vertical bars indicate standard errors of means. C, L, and V stand for the locations of Castor, Lacombe, and Vegreville.

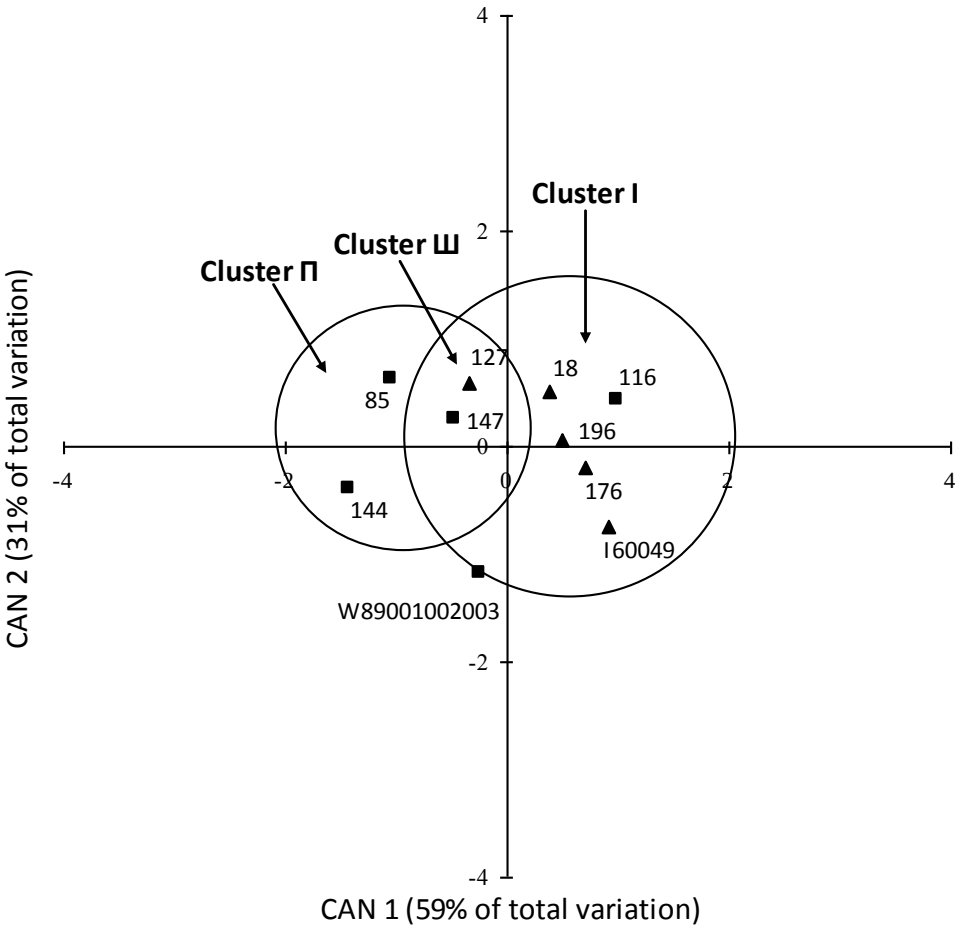

Figure 5. 2 Canonical discriminant analysis scatter plot separates the ten barley genotypes into three clusters based on plant height, harvest index and leaf carbon isotope discrimination under field and greenhouse conditions

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Chapter 6 Synthesis, Conclusions and Future Research

Overview of study objectives

Carbon isotope discrimination ($\Delta^{13}C$) is an important physiological trait that is based on the $^{13}C/^{12}C$ ratio in plant material relative to the ratio of the atmospheric CO_2 (Hall et al. 1994) and has been extensively exploited and demonstrated to be a simple but reliable indicator of water-use efficiency (WUE, the ratio of dry matter production to transpired water) in C_3 plants. The negative correlation between $\Delta^{13}C$ and WUE has been used for indirect selection of crops for improved WUE under selected environments (Cattivelli et al. 2008). However, there are some challenges associated with the application of $\Delta^{13}C$ in improving WUE in plant breeding programs, and information was scarce on the physiological processes involved in the $\Delta^{13}C$ variation in crops.

The overall goal of this research was to study the genetic and physiological determinants of $\Delta^{13}C$ variation in barley towards identifying the genetic loci and their linked molecular markers for use in a marker-assisted selection approach for the breeding of elite barley varieties with improved WUE.

Summary and synthesis of the research results

Leaf $\Delta^{13}C$ as a measure of WUE in barley

Plant parts sampled for $\Delta^{13}C$ analysis can be awn, grain, leaf, sheath, stem or root, each with its own characteristic $\Delta^{13}C$ value and potential advantages for evaluating WUE under different environments. Leaf $\Delta^{13}C$ during the stem elongation stage, which is regarded as the beginning of yield potential (Anyia et al. 2008), could reflect the integrated WUE and the vegetative establishment (May to June), and therefore leaf $\Delta^{13}C$ is informative for stored-moisture environments such as the Canadian Prairies. Moveover, it is less destructive to sample leaf than stem. In our study penultimate leaf was sampled for $\Delta^{13}C$ analysis without

harming flag leaf, because flag leaf is one of the primary leaves contributing to grain yield in cereal crops (Sicher 1993; Thorne 1965; Xue et al. 2008). In addition, leaf $\Delta^{13}C$ measured before maturity can enable selection and crosses to be made within the same season thereby speeding up the breeding process.

Stability of leaf $\Delta^{13}C$

In this study, the leaf $\Delta^{13}C$ performances of parental lines were examined both in the greenhouse and field since 2005, which showed consistent differences across environments (Chapters 3, 4 and 5). Leaf $\Delta^{13}C$ ranking among the genotypes evaluated in the field study was consistent across years and locations (Chapters 3 and 4). For the six-row barley RIL population, the broad-sense heritability (H^2) for $\Delta^{13}C$ was high (0.80), which is high compared with other complex traits such as grain yield under drought conditions in this and previous studies (Araus et al. 1998; Voltas et al. 1998). The high $H^2$of leaf $\Delta^{13}C$ has been previously reported in crops (Araus et al. 1998; Çağirgan et al. 2005; Condon and Richards 1992; Ehdaie et al. 1991; Hall et al. 1990; Hubick et al. 1988; Rebetzke et al. 2008; Specht et al. 2001; Stiller et al. 2005; Voltas et al. 1998). In agreement with our results, non-significant interactions between genotype and environment for $\Delta^{13}C$ have also been reported previously (Hubick et al. 1988; Peleg et al. 2009; Rajabi et al. 2009). These results suggest that leaf $\Delta^{13}C$ is under strong genetic control and can be reliably used as a measure of WUE under field conditions.

We found that the value of leaf $\Delta^{13}C$ was usually reduced under drought conditions (Chapters 3, 4 and 5), which is in agreement with most reports on $\Delta^{13}C$ in C_3 crops (Anyia et al. 2007; Craufurd et al. 1991; Hall et al. 1990; Virgona et al. 1990). Leaf $\Delta^{13}C$ of RILs across environments were related to total precipitation, especially the temporal rainfall distribution during the growing season (Chapters 4 and 5), which is not surprising because the leaf $\Delta^{13}C$ reflects the time-integrated measure of the transpiration efficiency (TE, aerial biomass/water transpired) over

the period when leaf tissue is formed (Rebetzke et al. 2002). The dynamics of $\delta^{13}C$ (carbon isotope composition) in the annual rings of trees were found to be related positively with air temperature in June but negatively with precipitation in June from 1980 to 2005 (Ivlev and Voronin 2007).

Physiological characterization of leaf $\Delta^{13}C$

To investigate the response and physiology of leaf $\Delta^{13}C$ to soil water availability, four barley genotypes with contrasting levels of leaf $\Delta^{13}C$ (Chapter 3) and eight F_5 RILs with contrasting levels of leaf $\Delta^{13}C$ (high vs low) together with their parental lines (Chapter 5) were chosen for greenhouse experiments with water deficit treatments. Water deficit significantly increased WUE, and a significant negative relationship between WUE and leaf $\Delta^{13}C$ was observed as predicted by the theory of Farquhar et al. (1982) (Chapter 5). Leaf $\Delta^{13}C$ between extreme genotypes differed more at the low-rainfall site than the site with relatively more rainfall (Chapter 3 and 4). This was in agreement with previous reports that $\Delta^{13}C$ can be used as a sensitive indicator for plant water status or water availability during the growing period (Bloch et al. 2006; Craufurd et al. 1991; Merah et al. 2001). Moreover, the increased difference in leaf $\Delta^{13}C$ between extreme genotypes at low-raifall site reflected their different responses to water deficit, because a low value of leaf $\Delta^{13}C$ may arise from reduced stomatal conductance (g_s) or increased photosynthesis rate (A) or both (Condon et al. 2004), and

When subjected to the water deficit treatment, all genotypes significantly reduced photosynthesis rate (A) and stomatal conductance (g_s) (Chapters 3 and 5). In this study, the strongly positive relationship between A and g_s under water-deficit conditions and the proportionally greater variation in g_s than A suggested that g_s mainly responsible for causing the variation in $\Delta^{13}C$. Similar patterns have been reported in cowpea (*Vigna unguiculata* L. Walp.) (Hall et al. 1992) and rice (*Oryza sativa* L.) (Xu et al. 2009).

Emphasis has been placed on the g_s when explaining the $\Delta^{13}C$ variability in the context of photosynthetic response to drought (Earl 2002; Monclus et al. 2006). Sekiya and Yano (2008) found a significantly negative correlation between $\Delta^{13}C$ and stomatal density in cowpea (*Vigna sinensis*) under various environments that included different amounts of soil phosphorus, water and atmospheric CO_2. Roussel et al. (2009) also found that genetic differences of $\Delta^{13}C$ and intrinsic water use efficiency ($WUE_i = A/g_s$) can be ascribed to differences in g_s and stomatal density instead of *A*. Gene expression studies have also indicated that plants tend to increase drought resistance and WUE through decreasing transpiration via stomatal closure, reducing stomatal opening size or number (density) (Bhatnagar-Mathur et al. 2007; Karaba et al. 2007; Masle et al. 2005; Nilson and Assmann 2010; Yoo et al. 2010).

Leaf $\Delta^{13}C$ and agronomic traits

The relationship between $\Delta^{13}C$ and aerial biomass or grain yield has been reported to be positive, negative or neutral, depending on the target environment and crop species (Anyia et al. 2007; Condon et al. 2004; Jiang et al. 2006; Tambussi et al. 2007). In this study, leaf $\Delta^{13}C$ was found to be positively correlated with biomass and grain yield of barley under field conditions (Chapters 4 and 5). One possible explanation of such a relationship is suggested by the positive association between leaf $\Delta^{13}C$ and g_s, since low g_s might constrain plant performance under dry environments due to reduction in carbon fixation per unit leaf area as stomata close. A positive relationship between leaf $\Delta^{13}C$ and g_s was observed under field and greenhouse (well-watered) conditions (Chapter 3). This relationship has previously been reported (Ehleringer 1990; Kondo et al. 2004; Monneveux et al. 2006; Takai et al. 2009). Monclus et al. (2006) also found that $\Delta^{13}C$ was correlated positively with g_s under moderate drought. Takai et al. (2009) found that a QTL that controlled leaf $\Delta^{13}C$ on the long arm of chromosome 3 in rice was associated with g_s. Another hypothesis is that genotypes differ in their abilities to

translocate stem carbohydrate reserves for grain filling, since high leaf $\Delta^{13}C$ genotypes tend to grow faster and convert more assimilates to the grain than low $\Delta^{13}C$ genotypes (Monneveux et al. 2005). Finally, Craufurd et al. (1991) suggested that early flowering contributed to the positive relationship between grain $\Delta^{13}C$ and grain yield, because varieties with early ear emergency may fix more carbon early in the season when stomatal opening was less limited by water deficit (and therefore a high $\Delta^{13}C$).

Genotype 'CDC Cowboy' (two-row) and RIL '147' (six-row) with low leaf $\Delta^{13}C$ achieved high WUE by having a lower percentage decline in A and g_s than the other genotypes in the greenhouse study, with comparable biomass and grain yield to genotypes showing a high g_s under field conditions (Chapters 3 and 5). These results suggest that genotypes with low leaf $\Delta^{13}C$ can achieve drought tolerance by maintaining a high A at a low g_s. Monclus et al. (2006) also discovered that a genotype with low $\Delta^{13}C$ had a high productivity and a high level of tolerance to a moderate drought constraint.

A positive correlation between leaf $\Delta^{13}C$ and leaf area index measured at the stem elongation stage (LAI-S) was found. LAI is usually used to estimate early vigor (the early growth of leaf area and biomass, Richards (1996)), canopy photosynthesis, evapo-transpiration and final yield since it represents the percentage of incident solar radiation intercepted (Dale et al. 1980). The significant positive correlations among LAI-S, leaf $\Delta^{13}C$, biomass and grain yield in this study suggest that both leaf $\Delta^{13}C$ and LAI-S could be used to estimate the integrated WUE during the vegetative establishment and the final yield (Chapter 4).

QTL identified for leaf $\Delta^{13}C$

The composite interval mapping method revealed 12 putative QTLs associated with leaf $\Delta^{13}C$ for the six-row barley population, and 5 QTLs for leaf $\Delta^{13}C$ for the two-row barley population. These QTLs varied in size and accounted for small to

modest amounts of the phenotypic variance, which is consistent with most of the pioneering work in QTL mapping of $\Delta^{13}C$ across a range of plant species (Diab et al. 2004; Ellis et al. 2002; Ellis et al. 1997; Handley et al. 1994; Hausmann et al. 2005; Juenger et al. 2005; Laza et al. 2006; Martin and Nienhuis 1989; Peleg et al. 2009; Rebetzke et al. 2008; Saranga et al. 2001; Specht et al. 2001; Takai et al. 2006; Teulat et al. 2002; This et al. 2010; Xu et al. 2008; Xu et al. 2009). However, due to the narrow genetic base of modern varieties and breeding lines, the level of polymorphism between parental lines of both RIL populations was very low. The low density and uneven distribution of markers hindered the detection of more QTLs in this study. The unsaturated map and the lack of common markers also prevented us from identifying and comparing common QTLs across the two types of mapping populations studied, however, there might be possibilities to find repeatable QTL for leaf $\Delta^{13}C$ across mapping populations and environments, since the order, instead of the distance between markers is usually considered conserved between populations (This et al. 2010).

Two regions controlling leaf $\Delta^{13}C$ detected in the two-row population were co-located with agronomic traits (biomass and grain yield) in the present study. Six regions controlling leaf $\Delta^{13}C$ were identified in the six-row population and were located in the same region or in a similar position with agronomic traits (LAI, maturity, plant height, biomass, grain yield and harvest index (HI)), in agreement with previous reports that most QTL for $\Delta^{13}C$ in barley co-localized with QTL for heading date and/or plant height (Forster et al. 2004). Correlations between leaf $\Delta^{13}C$ and LAI-S, plant height, maturity, biomass, grain yield and HI were partially explained at the level of co-localized QTL. Previous QTL analysis also reported potential pleiotropic relationships between $\Delta^{13}C$ and flowering time and/or heading date and/or plant height in different plants (Juenger et al. 2005; McKay et al. 2003; Peleg et al. 2009; Rebetzke et al. 2008; Specht et al. 2001; Teulat et al. 2002).

One QTL for leaf $\Delta^{13}C$ with a major effect located on chromosome 3H near marker Bamg606 on the six-row barley linkage map was detected in all three

field trials, its effects on leaf $\Delta^{13}C$ varied across environments (from 11 to 22%), suggesting an environmental impact on this locus or loci. This QTLwas co-localized with the QTL for plant height, LAI-G (measured during the grain filling stage), grain yield, HI and maturity, suggesting a pleiotropic relationship between these traits or genetic linkage among the traits. It is noteworthy that a QTL for $\Delta^{13}C$ was previously identified near the semi-dwarf gene *sdw1* around the same region in different barley mapping populations (Ellis et al. 2002; Teulat et al. 2002), which is homoeologous to rice chromosome 1 where $\Delta^{13}C$ is located.

Additive gene action of $\Delta^{13}C$ was found in this study, with parental lines contributing favorable alleles for WUE on different chromosomes. The complementary alleles at multiple loci contributed by the parents provided the most plausible explanation for transgression observed among the progeny (Tanksley 1993). However, $\Delta^{13}C$ is a genetically complex trait and its expression in different plant tissues and organs varies with water supply (Rebetzke et al. 2008), future screening of populations should be conducted under favorable and well-watered conditions to maximize genetic variance and heritability and improve QTL detection for $\Delta^{13}C$.

Conclusions

This study demonstrated the genotypic diversity and stability of leaf $\Delta^{13}C$ as a measure of WUE in barley, which was consistent with reports for other C_3 species. The high H^2 of leaf $\Delta^{13}C$ across environments suggested that it is an intrinsic trait under tight genetic control and can be reliably used as a measure of WUE under field conditions. The temporal rainfall distribution had a significant impact on barley leaf $\Delta^{13}C$ across the different locations. This result suggest that leaf $\Delta^{13}C$ during the stem elongation stage may be used as an indicator for water availability during the growing period.

The ability to increase WUE in response to water deficit is necessary for achieving a high level of drought tolerance (Monclus et al. 2006), and barley

genotypes responded differently to low soil moisture conditions in this study. Two genotypes 'CDC Cowboy' (two-row) and '147' (six-row) with low leaf $\Delta^{13}C$ achieved drought tolerance by maintaining high *A* at low g_s, and they both showed comparable biomass and grain yield to genotypes showing a high g_s under field conditions. However, further work should be undertaken to examine the similarity and difference between these two genotypes under the same experimental conditions.

The transgressive variation and high H^2 of leaf $\Delta^{13}C$ observed in the mapping populations increased confidence in directly selecting progeny lines with high productivity and enhanced WUE, and these superior lines might contribute valuable alleles to WUE improvement in barley. Since different types of barley have different uses such as for livestock feed, malt beverages, or human diet, it is necessary to rank the genotypes with low leaf $\Delta^{13}C$ and superior high biomass or grain yield such as 'CDC Cowboy' and '147' against a wide range of varieties (such as local cultivars and landraces) at different field trials to select the best drought tolerant genotype in order to meet the increasing market demand for barley supply.

A major QTL for leaf $\Delta^{13}C$ co-located with several agronomic traits on chromosome 3H near SSR marker Bmag606 was identified across environments. This is may be useful for plant breeding because this QTL controls both drought-adaptive traits such as $\Delta^{13}C$ and yield components. Furthermore, it is necessary to place more markers to saturate the map, increase population size and replicate field trials across more locations and seasons to achieve a more stable and reliable prediction of QTL position and effect. Further validation, fine mapping and dissection of the identified genomic region is needed to identify more closely linked markers that can be used for marker assisted selection in barley breeding programs on the Canadian Prairies.

Suggestions for future research

Based on the results of the present and other studies of leaf $\Delta^{13}C$, I propose the following for future studies:

1) Many efforts have been dedicated to understanding the physiological mechanism underlying $\Delta^{13}C$ by studying the morphological, phenological and ecophysiological traits related to WUE. Further physiological characterization of the relationship between leaf $\Delta^{13}C$ and gas exchange parameters by incorporating more factors, such as oxygen and hydrogen isotope composition (expressed as $\delta^{18}O$ and δ D), would be valuable and beneficial for breeding programs. Barbour et al. (2000) proposed that the $\delta^{18}O$ measured in plant matter can be used to indicate genetic differences in g_s and predict grain yield in wheat. Scheidegger et al. (2000) developed a conceptual isotope model to describe the long-term effects of environmental factors on carbon-water relations and to distinguish the response of A and g_s using $\delta^{18}O$ and $\delta^{13}C$ data. Cabrera-Bosquet et al. (2009) demonstrated the utility of combining $\Delta^{18}O$ and $\Delta^{13}C$ to assess plant growth and total transpiration. As suggested by Reichstein et al. (2002), leaf mesophyll conductance to CO_2 (g_m) should be incorporated into current gas-exchange models when interpreting WUE, because g_m may be more limiting for A than g_s (Galmés et al. 2007). As pointed out by Flexas et al. (2008), WUE could be more efficiently improved by modifying g_m than g_s, and errors would be induced by neglecting g_m when interpreting A and $\Delta^{13}C$. Barbour et al. (2010) suggested that g_m has unexplored potential to provide TE improvement within crop breeding programmes. The question whether the relationship between g_s and g_m is linear or curvilinear remains unresolved, and it is important to elucidate g_m effects on WUE under water or salinity stress (Warren and Adams 2006).
2) The inconsistencies in leaf $\Delta^{13}C$ between two-row and six-row barley lines need further research. No significant differences in leaf $\Delta^{13}C$ between two-row and six-row barley lines were found in previous studies (Anyia et

al. 2007; Chapter 3), but the two-row RILs had a significantly lower leaf $\Delta^{13}C$ than the six-row RILs at Vegreville in 2009 (Chapter 4). Jiang et al. (2006) suggested that two-row barley had a higher WUE than six-row barley under both irrigated and non-irrigated field conditions based on $\Delta^{13}C$ values from different plant parts (flag leaf, awn and grain). A proposed explanation for differences in $\Delta^{13}C$ values between barley ear types is the difference in date to maturity (Sayrea et al. 1995). Jiang et al. (2006) suggested that an intrinsic difference in $\Delta^{13}C$ values between two-row and six-row barley may exist in water/carbon metabolism at the whole tiller, flag leaf and ear levels, since flag leaves of two-row barley are generally much smaller than six-row barley.

3) The identified putative QTL and associated molecular markers for barley leaf $\Delta^{13}C$ in this study would need to be validated before they can be deployed in breeding programs. Association mapping (Salvi and Tuberosa 2005) can be used as a validation approach. Germplasm with diverse genetic backgrounds and different selection histories likely differ in their QTL alleles (Bernardo 2008), but the same QTL would be expected to be present in different mapping populations assuming that the particular QTL in this study is stable or consistent.
4) The interval between the major QTL and SSR marker identified in this study is around 10 centiMorgans (cM), which may contain many genes, since barley has such a large size of genome (5,300 Mbp). It is necessary to fine-scale mapping this interval to confine the QTL to a 2-3 cM (or even shorter) region, and eventually clone the candidate gene underlying the QTL for WUE. Fine-scale mapping also can help to distinguish the pleiotropy and physical linage for QTL co-localization (Hausmann et al. 2005). Comparative analysis of QTL results also provides valuable opportunities for positional cloning and to identify candidate genes for WUE in cereals through exploration of related species using sequence co-linearity from small (such as rice) to large genomes (such as wheat)

(Sorrells et al. 2000). Once barley genome sequencing is finished, it will also accelerate the identification of candidate genes responsible for WUE.

5) The candidate gene controlling leaf $\Delta^{13}C$ may function in a similar way as *ERECTA* gene, which regulates plant transpiration efficiency in *Arabidopsis* (Masle et al. 2005). Once the candidate gene is isolated in the future, its function and mechanism can be further characterized, and it may be introduced into cultivars by employing genetic transformation approaches to improve crop water-deficit tolerance. Constitutively over-expression of certain plant proteins in vegetative tissues has been demonstrated to play roles in drought tolerance. For example, a group 3 late embryogenic abundant (LEA) proteins encoded by barely *HVA1* gene demonstrated tolerance to water deficit and salt stress in transgenic rice plants (Xu et al. 1996). When *HVA1* gene was expressed in wheat, the biomass and WUE of transgenic lines were improved under water deficit conditions (Sivamani et al. 2000). Another approach to elucidate the underlying molecular mechanism for candidate gene is called eQTLs as reviewed by Kliebenstein (2009). Application of microarray technology to obtain genome-wide expression profiling using selected RILs from the mapping population can help to identify expression polymorphisms underlying $\Delta^{13}C$. The obtained information can also be used to develop new genetic markers to increase the linkage map density.
6) The phenomenon of segregation distortion (SD) identified in this study needs to be further dissected to enhance our knowledge of its mechanism, because SD is intimately linked to the potentially specific recombinants of interest in breeding populations (Xu et al. 1997). The understanding of the direction and rate of SD would increase the probability to obtain desired recombinants (Li et al. 2010). The genetic basis of SD may be the abortion of male or female gametes or zygotes, or preferential fertilization of gametic genotypes (Lyttle 1991). Molecular marker analysis in multiple populations would be useful to find common regions with SD and

identify genetic factors controlling SD in these regions (Lu et al. 2002). The development of high-density genetic maps will also speed up this progress, such as a 2383 locus linkage map has been available in barley (Szűcs et al. 2009).

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