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Genetic and physiological bases for variation in water use efficiency in canola

Abstract

2020 The Authors. Food and Energy Security published by John Wiley & Sons Ltd on behalf of Association of Applied Biologists Drought stress due to water deficiency threatens production of canola worldwide. Carbon isotope discrimination ($\Delta^{13}\text{C}$), a trait that can be used to assess efficient water use, provides an opportunity to exploit natural variation in canola for stable production. Here, we show that substantial genetically controlled phenotypic variation in water use efficiency (WUE) component trait, $\Delta^{13}\text{C}$ (20.4 to 23.6‰) exists among accessions of canola. Quantitative trait loci (QTL) analysis revealed ten loci for $\Delta^{13}\text{C}$, each accounting for 2.5% to 16.5% of the genotypic variation. One of the significant QTL for $\Delta^{13}\text{C}$ was co-localized with a QTL for flowering time, a trait implicated in drought escape and was mapped in the vicinity of the FLOWERING LOCUS T (FT) on chromosome A07. Gene expression analyses revealed that among FT paralogs, BnC6.FTb expression was significantly correlated ($r = 0.33$, $p < .01$) with variation in $\Delta^{13}\text{C}$ across at least two environments in a canola DH population. Integration of data based on instantaneous single leaf gas exchange, dry matter $\Delta^{13}\text{C}$, and whole plant measurements suggests a possible trade-off between early flowering and WUE. Our findings provide insights into the complexity of $\Delta^{13}\text{C}$ and WUE which could enable the development of canola varieties resilient to drought and increasing canola productivity under water-limited conditions.

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Genetic and physiological bases for variation in water use efficiency in canola

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Abstract

Drought stress due to water deficiency threatens production of canola worldwide. Carbon isotope discrimination ($\Delta^{13}\text{C}$), a trait that can be used to assess efficient water use, provides an opportunity to exploit natural variation in canola for stable production. Here, we show that substantial genetically controlled phenotypic variation in water use efficiency (WUE) component trait, $\Delta^{13}\text{C}$ (20.4 to 23.6‰) exists among accessions of canola. Quantitative trait loci (QTL) analysis revealed ten loci for $\Delta^{13}\text{C}$, each accounting for 2.5% to 16.5% of the genotypic variation. One of the significant QTL for $\Delta^{13}\text{C}$ was co-localized with a QTL for flowering time, a trait implicated in drought escape and was mapped in the vicinity of the *FLOWERING LOCUS T (FT)* on chromosome A07. Gene expression analyses revealed that among *FT* paralogs, *BnC6.FTb* expression was significantly correlated ($r = 0.33$, $p < .01$) with variation in $\Delta^{13}\text{C}$ across at least two environments in a canola DH population. Integration of data based on instantaneous single leaf gas exchange, dry matter $\Delta^{13}\text{C}$, and whole plant measurements suggests a possible trade-off between early flowering and WUE. Our findings provide insights into the complexity of $\Delta^{13}\text{C}$ and WUE which could enable the development of canola varieties resilient to drought and increasing canola productivity under water-limited conditions.

KEYWORDS

Brassica napus, carbon isotope discrimination, flowering time, genetic analysis, genetic variation, seed yield

1 | INTRODUCTION

Drought (water deficit) is a major environmental stress that continues to threaten the sustainable production and profitability of various agricultural crops, including canola—the second most important oilseed crop used worldwide for healthy vegetable oil for human consumption, feed stocks,

and biodiesel production. In many countries, canola production largely relies on natural rainfall as water tables are often receding leaving an acute shortage of water for irrigation. Extreme weather events are also predicted to impact crop production (Korres, Norsworthy, Burgos, & Oosterhuis, 2017). Drought conditions affect all stages of plant development, from the rate of seed germination to seed and oil quality

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characteristics (Mailer & Cornish, 1987; Norouzi et al., 2008; Raman, Uppal, & Raman, 2019b). In order to cope with water deficit conditions, plants have evolved distinct strategies: drought escape, drought avoidance, and drought tolerance by altering phenological, developmental, and physiological traits (Blum, Shipiler, Golan, & Mayer, 1989; Hausmann et al., 2005; Ludlow, 1989). The drought escape strategy has been successfully exploited by using early flowering and early maturing varieties to achieve higher seed yield in canola under water-limited conditions, generally prevalent in semi-arid and arid environments. However, in recent years, canola varieties with a range of flowering windows (early to late flowering) that possess higher seed yield are being released for commercial cultivation in Australia and are likely to have other drought tolerance mechanism(s).

Breeding for water use efficiency (WUE defined as total biomass per unit transpiration) has been suggested as another strategy to produce higher yield per drop of water (Kijne et al. 2003; Passioura, 1977). However, breeding for this multidimensional trait is complicated by large seasonal variability that leads to large genotype by season effects, otherwise known as genotype (G) \times environment (E), and potentially low reliability (approximately heritability, H^2), making a direct selection for yield—the central component of WUE—difficult. In cereals and other crops, various component traits related to WUE such as CO_2 assimilation rate, stomatal conductance, biomass accumulation, harvest index, canopy temperature and leaf water content have been proposed as selection targets in plant improvement programs to enhance grain yields under water-limited conditions (Adiredjo et al., 2014; Blum et al., 1989; Christman et al., 2008; Condon, Farquhar, & Richards, 1990; Fischer et al., 1998; Rebetzke, Botwright, Moore, Richards, & Condon, 2004; Rebetzke, Rattey, Farquhar, Richards, & Condon, 2012; Richards, Rebetzke, Condon, & van Herwaarden, 2002). Most methods for measuring physiological attributes involved in WUE at whole plant and leaf levels are tedious and inefficient, rendering them unsuitable for rapid and large screening of breeding populations. Carbon isotope discrimination ($\Delta^{13}\text{C}$, the $^{12}\text{C}/^{13}\text{C}$ ratio of carbon in the plant divided by that in the atmospheric CO_2) or stable isotopic composition ($\delta^{13}\text{C}$, the $^{13}\text{C}/^{12}\text{C}$ ratio in plant carbon divided by that of a standard) has been proposed as a proxy for transpiration efficiency in plants during long-term carbon gain (Des Marais et al., 2014; Ehleringer, 1993; Farquhar, O'Leary, & Berry, 1982; Farquhar & Richards, 1984; Hubick, Farquhar, & Shorter, 1986; Krishnamurthy et al., 2013; Matus, Slinkard, & van Kessel, 1995, 1997).

Genetic analysis studies revealed quantitative trait loci (QTL) associated with $\Delta^{13}\text{C}$ in different crops including wheat, barley, rice, cotton, soybean, sunflower, tomato, and in a model plant, *Arabidopsis* (Dhanapal et al., 2015; Hall

et al., 2005; Hausmann et al., 2005; Juenger et al., 2005; Lazaa, Kondo, Idetaa, Barlaanb, & Imbea, 2006; Martin, Nienhuis, King, & Schaefer, 1989; Masle, Gilmore, & Farquhar, 2005; Rebetzke, Condon, Farquhar, Appels, & Richards, 2008; Saranga et al., 2001; TalamÉ et al., 2004; Thumma et al., 2001; Wu, Chang, & Jing, 2011). Studies in model plants have identified candidate genes such as *MPK12*, *FRIGIDA*, *ERECTA* as well as receptors for the abscisic acid (ABA) response to be involved in WUE (Des Marais et al., 2014; Karaba et al., 2007; Kuromori et al., 2016; Kuromori et al., 2010; Kuromori, Sugimoto, & Shinozaki, 2011; Lovell et al., 2013; Masle et al., 2005; Papacek, Christmann, & Grill, 2019; Zhang et al., 2016; Zhao, Hu, Li, Yao, & Liu, 2016; Zhao, Chan, et al., 2016). The knowledge of such pathways and their manipulation even in closely related major agricultural crops such as canola and Indian mustard is rather limited. A better understanding of the extent of natural variation and of the genetic and physiological bases for WUE (e.g., CO_2 assimilation, stomatal conductance) or effective water use (Sinclair, 2018) traits will facilitate the development of improved cultivars with other adaptation and productivity traits such as resistance to biotic and abiotic stresses.

Natural variation for $\Delta^{13}\text{C}$ and WUE has been reported in diploid *Brassica oleracea* ($2n = 2x = 18$), and *Brassica rapa* ($2n = 2x = 20$), and in limited accessions of amphidiploid *Brassica napus* (Knight, Livingston, & Van Kessel, 1994; Luckett, Cowley, Moroni, & Raman, 2011; Matus et al., 1995; McVetty, Austin, & Morgan, 1989; Pater, Mullen, McKay, & Schroeder, 2017). However, the genetic architecture underlying the $\Delta^{13}\text{C}$ variation and its associations with other phenological and productivity traits, such as flowering time, biomass accumulation, and grain yield under diverse environments, have not been reported. Moreover, canola is an indeterminate crop and loses leaves at maturity; therefore, relationships between physiological intrinsic WUE (*i*WUE), $\Delta^{13}\text{C}$, and yield have not been well understood.

This study describes (a) the extent of genetic variation for $\Delta^{13}\text{C}$ and seed yield among a diverse set of Australian canola accessions evaluated across Australian environments, (b) identification of genetic loci associated with $\Delta^{13}\text{C}$ and seed yield related traits, (c) interplay between *FLOWERING LOCUS T (FT)* paralogs and $\Delta^{13}\text{C}$ variation, and (d) the physiological basis of *i*WUE in selected lines of a DH canola population. We also provide evidence that the *FT* paralogs modulate $\Delta^{13}\text{C}$, flowering time and other plasticity traits such as early vigor, shoot biomass, plant height, and grain yield in a biparental doubled haploid (DH) population derived from a BC_1F_1 cross, Skipton/Ag-Spectrum//Skipton (Raman, Diffey, et al., 2016). Whole plant and plot-based data showed that flowering

time is negatively associated with harvest index (measured at maturity), plant height and seed yield, and $\Delta^{13}\text{C}$. We confirm that the early flowering allele is associated with large $\Delta^{13}\text{C}$, high CO_2 assimilation rate, and high stomatal conductance, especially under water stress conditions in a mapping population.

2 | MATERIALS AND METHODS

2.1 | Plant materials

To investigate the genetic and physiological bases of $\Delta^{13}\text{C}$ variation in canola, we carried-out four experiments under field and glasshouse conditions (Table 1). A total of 144 accessions of canola representing conventional open-pollinated varieties, non-conventional varieties such as Triazine (TT) and Clearfield (CL) tolerant, and hybrids were used in this study. Details of accessions are given in a Table S1. In order to understand genetic and physiological bases for $\Delta^{13}\text{C}$ variation, we utilized a DH population, comprising 145 lines derived from a BC_1F_1 cross, Skipton/Ag-Spectrum//Skipton, designated as SAgS DH population (Raman, Raman, Coombes, Song, Prangnell, et al., 2016). The parental lines and progenies of the SAgS DH population show segregation for a range of traits, including for flowering time, response to vernalization, resistance to blackleg, plant emergence, normalized difference in vegetative index, shoot biomass, plant height, and seed yield (Raman, Raman, Coombes, Song, Diffey, et al., 2016; Raman, Raman, Coombes, Song, Prangnell, et al., 2016; Raman, Raman, et al., 2019; Raman, Diffey, et al., 2016; Raman et al., 2012).

2.2 | Phenotypic evaluation and experiment designs

2.2.1 | Evaluation for $\Delta^{13}\text{C}$ variation among diverse lines

For experiment I, 144 to 150 canola diverse accessions were assessed for genetic variation in $\Delta^{13}\text{C}$ and their seed yield across diverse growing environments in 2013, 2014, and 2015 (Table S1). Accessions were sown in plots (2 m wide \times 10 m long) in two to three replicates over eight agricultural environments. We also used Australian canola varieties as “checks” in some experiments. Each plot consisted of 8 rows, spaced 25 cm apart, and 1,400 seeds of each accession (diverse accessions and SAgS DH population), counted with a Contador seed counter (Pfeuffer GmbH, Germany) were sown across sites; details of experimental designs are given in Table 2. At maturity, plots were harvested with a combine harvester (Kingaroy, Australia) and seed yield was measured from all experimental sites.

For $\Delta^{13}\text{C}$ measurement, ten leaves were taken from 10 randomly selected healthy plants (at first flowering stage, BBCH 60) from the middle row of each plot. Leaves were pooled in a paper bag (Southland Supply Group, Wagga Wagga, Australia) and dried at 70°C for 48 hr in a fan forced dehydrator (G.T.D, Australia). Dried leaf samples were then ground into a fine powder using a Cyclotec 1093X mill/blender (Foss, Australia) and sieved through 0.5 mm mesh. The $\delta^{13}\text{C}$ composition was determined by a Micromass Isochrom mass spectrometer (Middlewich, UK) at the Research School of Biology, Australian National University, Canberra using Vienna Pee Dee Belemnite (VPDB) as the

TABLE 1 Experiments conducted to determine genetic and physiological bases of variation in water use efficiency in canola. Details of accession and field sites are given in Table S1–S3

Experiment	Objective	Material	Environment	Traits investigated
I	Assessment of genetic variation in $\Delta^{13}\text{C}$	Diverse canola accessions (144 to 150 plus checks)	8 field trials	$\Delta^{13}\text{C}$ (this study) Seed yield ((this study)
II	Identification of loci for $\Delta^{13}\text{C}$ variation	145 DH lines of the Skipton/Ag-Spectrum//Skipton	4 field trials	$\Delta^{13}\text{C}$ (this study) Plant emergence, NDVI, shoot biomass, flowering time, plant height, seed yield (Raman, Raman, et al., 2019)
III	Relationship between $\Delta^{13}\text{C}$ variation and <i>FT</i> gene expression	145 DH lines of the Skipton/Ag-Spectrum//Skipton	1 field trial (2016)	$\Delta^{13}\text{C}$ (this study)
IV	Understanding physiological basis of WUE	22 DH lines with contrasting $\Delta^{13}\text{C}$ variation plus two parents of DH population (a subset of DH lines used in experiment 2)	1 glasshouse trial (2 irrigation treatments)	Gas exchange measurements, $\Delta^{13}\text{C}$, chlorophyll content, leaf thickness, stomata density, shoot biomass, flowering time, harvest index, seed yield (this study)

TABLE 2 Details of multiphase field experiments carried-out to determine the extent of natural variation in $\Delta^{13}\text{C}$ among canola accessions

Experiment	Phenotyping year	Experimental site	Field phase design			Lines used across replicates		
			Range	Rows	Plots	1	2	3
I (Diverse lines)	2013	Wagga	18	24	432	144	144	144
	2014	Condobolin	6	50	300	150	150	Nil
	2014	Finley	15	20	300	150	150	Nil
	2014	Rankin Springs	9	50	450	150	150	150
	2014	Kellerberrin	12	25	300	150	150	Nil
	2014	Yanco	9	57	513	171	171	171
	2015	Kellerberrin	6	50	300	150	150	Nil
	2015	Condobolin	6	50	300	150	150	Nil
II (SAGS DH population)	2013	Euberta	15	20	300	150	150	Nil
	2014	Wagga	18	24	432	144	144	144
	2015	Wagga	18	24	432	144	144	144
	2016	Wagga	9	48	432	144	144	144

^aFigures given in parenthesis are number of “Runs” used for analysis of samples.

^bIn 2013, the number of “Runs” was not recorded for observations.

ultimate reference. Since $\Delta^{13}\text{C}$ was measured on leaves taken from field plots and then analyzed, using a mass spectrometer in a laboratory, we implemented multiphase experimental designs to account for variation attributed to field and laboratory conditions (Table 2). The $\Delta^{13}\text{C}$ was measured separately for each experiment (Table 2, and Table S2) and in different “runs,” with each run consisting of 49 samples (including three to five standards at position 1st/2nd, 25th and 48th/49th of the carousel). At least twenty percent of field plot samples were duplicated for the measurement of $\Delta^{13}\text{C}$ in order to account for variation due to the laboratory process (such as between days of testing) and field variation. Carbon isotope discrimination ($\Delta^{13}\text{C}$) was calculated from the $\delta^{13}\text{C}$ values assuming the isotopic composition of CO_2 in the air to be -7.8% on the VPDB scale, as described previously (Condon, Richards, Rebetzke, & Farquhar, 2002; Rebetzke et al., 2008).

2.2.2 | Evaluation for $\Delta^{13}\text{C}$ variation in DH population

For experiment II (Table 1), the leaves for $\Delta^{13}\text{C}$ variation were collected from the SAGS DH lines, grown under field conditions across four years (2013–2016). Plots were essentially sown as described above in section 2.2.1. Experimental layout and statistical designs are described in our earlier studies (Raman, Raman, et al., 2019; Raman, Diffey, et al., 2016). The $\Delta^{13}\text{C}$ variation in the DH population was determined as described above and then used to identify QTL. The data collected from previous (2013–2016) experiments were

integrated with $\Delta^{13}\text{C}$ data for all SAGS DH lines (this study) to see the relationships of $\Delta^{13}\text{C}$ with productivity traits (seed yield, days to flowering) across environments.

2.3 | Physical mapping and *FT* expression analysis

Experiment III involved the assessment of whether *FT* expression could explain the genetic variation in $\Delta^{13}\text{C}$. We used Empirical Best Linear Unbiased Predictors (EBLUPS) of $\Delta^{13}\text{C}$ and considered their correlation with previously published *FT* expression data of SAGS DH population grown under field conditions (Raman, Raman, et al., 2019). Potential candidate genes underlying variation in the $\Delta^{13}\text{C}$ and WUE traits were identified following the procedure described previously (Raman, Raman, Coombes, Song, Diffey, et al., 2016). Plausible candidate genes were selected by their association with water use efficiency and flowering time, as identified in *A. thaliana*.

2.3.1 | Phenotyping for physiological traits

Determination of WUE at whole plant and single leaf levels

We first verified genetic variation for $\Delta^{13}\text{C}$, WUE, and a range of traits segregating among SAGS DH lines under contrasting water regimes (treatments; WET: 100% saturation field capacity, and DRY: 50% saturation field capacity) in controlled glasshouse conditions (Experiment IV). To

Laboratory phase design				
Range sampled for $\Delta^{13}\text{C}$ analysis	Row sampled for $\Delta^{13}\text{C}$ analysis	Duplicated samples for $\Delta^{13}\text{C}$ analysis	Total samples analyzed for $\Delta^{13}\text{C}$	^a Number of samples used in each carouse (Run)
18	8	48	192	32 (6)
6	28	17	185	37 (5)
15	11	20	185	37 (5)
4	50	18	218	43 (2) + 44 (3)
7	25	18	193	38 (2) + 39 (3)
9	57	51	564	38 (14) + 32 (1)
6	28	52	220	44 (5)
6	28	52	220	44 (5)
15	20	48	348	40 to 45 (^b)
7	24	22	190	38 (5)
12	24	64	352	44 (8)
6	48	64	352	44 (8)

demonstrate the link between $\Delta^{13}\text{C}$ and other WUE traits, a total of 22 DH lines of the SAgS DH population representing 11 low and 11 high $\Delta^{13}\text{C}$ lines (identified from field experiments), including parental lines Skipton and Ag-Spectrum, were evaluated in a glasshouse under both treatment regimes.

Prior to selected lines being allocated to pots, seeds of the selected 24 lines were sown in plastic trays (7 × 8 cells) under controlled environment conditions (21°C, 16 hr light). After 2–3 weeks of germination, plants were transplanted in plastic pots (22 × 22 × 260 mm), containing a mixture of commercial potting mix (Searle, Australia) and covered with a thick layer of silica beads (Qenos, Australia) to avoid evaporation from the soil surface. The 24 selected lines were allocated to pots in the glasshouse according to statistical design. Pots were arranged on 3 benches with each bench containing 96 pots in 4 column by 24 row rectangular array. Each half bench, that is, rows 1 to 12 and rows 13 to 24, was replicate blocks containing the full factorial of the 24 selected lines and the two treatment regimes.

We measured field capacity of the soil mix, following a 24 hr gravimetric technique in 12 pots across three benches. All plants were initially watered to 100% field capacity, and then, water was withheld for 2 weeks (dry-down cycle). We carried-out whole plant measurements by weighing each pot after every 48 hr over the entire growing period. For the WET treatment, plants were watered with 200 ml (90% of field capacity) whereas for the DRY treatment, 100 ml water (45% field capacity) was applied on the same day. Drought stress treatment commenced at the stem elongation stage and continued until the physiological maturity (BBCH stage 80) of all DH lines. Plants were fertilized with Thrive (Bunnings,

Australia) as necessary, without varying the water stress treatments.

All 24 lines were evaluated for variation at the whole plant level [seed yield, WUE (seed yield/unit of water applied), chlorophyll content, flowering time, plant height and shoot dry weight biomass at maturity] and at the leaf level ($\Delta^{13}\text{C}$, intrinsic *i*WUE, leaf water content, specific leaf weight, and specific leaf area) under both WET and DRY environments. Total water loss due to transpiration was measured as a sum of the entire life cycle of each DH line. Evaporative water loss was measured from the pots containing no canola plants and subtracted from transpiration loss. At the plant maturity stage (BBCH scale 92), WUE was estimated as the ratio of total above ground biomass to total water applied during the experiment. Glasshouse grown plants were hand-harvested, and yield components such as seed yield and harvest index were expressed on a per plant basis. Harvest index was determined as a ratio of seed and shoot biomass.

Gas exchange measurements

We determined *i*WUE at the single leaf level by measuring light saturated assimilation rate (*A*), internal CO₂ concentration (*C*_i), stomatal conductance to the diffusion of water vapor (*g*_{sw}), and transpiration rate (*E*). The 5th fully expanded leaf of each of the eight SAgS DH genotypes (from Experiment IV) which differed in $\Delta^{13}\text{C}$ composition was tagged, and *C*_i, *g*_{sw}, *E*, and *A* were measured four times at weekly intervals. In total, we measured gas exchange levels from 96 plants from both WET and DRY treatments across six replicates. The centre of the leaf was placed in a gas exchange cuvette (LI-6400XT, LICOR Inc.) between 10h and

16h (AEST). The light intensity in the cuvette was maintained at $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (photosynthetic photon flux density), the incoming CO_2 concentration at $400 \mu\text{mol/mol}$, the chamber temperature at 20°C , and relative humidity around 70%. $i\text{WUE}$ was calculated as the ratio of light saturated CO_2 assimilation rate to stomatal conductance (A/g_{sw}). Average means of all four measurements were used to establish relationships with WUE and other phenotypic traits on a whole plant basis.

Estimation of chlorophyll content

At 100% flowering stage, chlorophyll content of 5th leaf of each genotype was estimated from five measurements taken with SPAD-502 meter (Konica-Minolta) at weekly intervals and the average readings were recorded from each accession.

Estimation of leaf water content and thickness

Leaf thickness parameters have been used as alternative predictors of WUE (Vile et al., 2005; Wilson, Thompson, & Hodgson, 1999). Leaf water content (% dry weight) was determined as the difference between fresh and dry weights, expressed as a percentage of dry weight. Measurements were made from the same samples that were used for leaf thickness and cell density parameters. Leaves from DH accessions were dried at 80°C for 48 hr, until they reached constant mass. Specific leaf weight (SLW) was measured as leaf dry mass/leaf area. Its inverse, specific leaf area (SLA) was calculated as the ratio of leaf area to leaf dry weight. Leaf area was determined from digital scans of flattened excised leaves using the ImageJ program using thresholding and the magic wand tool (<http://www.imagej.nih.gov/ij/>).

Measurement of stomatal density and size

For density, three randomly selected fields of 0.14 mm^2 (Nikon) were counted on each epidermal peel. Guard cell area was calculated as that of an ellipse from the axes of measured guard cell length and width as described previously (Caine et al., 2019).

2.4 | Statistical and trait-marker association analyses

The diverse 144–150 lines multi-environment trials $\Delta^{13}\text{C}$ and seed yield data (Experiment I) were analyzed using a factor analytic mixed model (FA-LMM), where the model contains a fixed main effect of environment and factor analysis is used to model the genotype effects in each environment (Smith, Cullis, & Thompson, 2001). Non-genetic sources of variation accommodated in the model included field range and row for each environment and variance heterogeneity between laboratory runs of the mass spectrometer. The FA-LMM estimates the genetic variances and covariances

between environments using a small number of unknown factors. In the case of $\Delta^{13}\text{C}$, a factor analytic model of order 2 (FA2) was used and accounted for an average of 97.5% (with a range of 90.2% to 100%) of the genetic variation in each environment. Estimated genetic correlations between environments were all greater than 0.9. The across environment summary measures of overall performance (OP) and root mean square deviation (RMSD) proposed by Smith and Cullis (2018) were used to identify lines which differed in their $\Delta^{13}\text{C}$ response. For seed yield, non-genetic sources of variation included field replicate blocks and field spatial variability for each environment. The process described by Gilmour, Cullis, & Verbyla (1997) and the diagnostics of Stefanova, Smith, and Cullis (2009) were used to identify and account for sources of field spatial variability. In the case of seed yield, a factor analytic model of order 3 (FA3) was used and accounted for an average of 84.5% (with a range of 51% to 100%) of the genetic variation in each environment. Estimated genetic correlations between environments were all positive (range of 0.36 to 0.85). The regression form of line entry predictions (Smith, Ganesalingam, Kuchel, & Cullis, 2015) from the FA models for seed yield and $\Delta^{13}\text{C}$ is plotted against each other in Figure 2.

For QTL identification (experiment II), we utilized a genetic linkage map of SAgS DH population based on 7,716 DArTseq markers, representing 499 unique loci (Raman, Diffey, et al., 2016). QTL analysis for $\Delta^{13}\text{C}$ was proceeded, as detailed in our previous study (Raman, Raman, et al., 2019); this was based on genome scan approaches (Nelson et al., 2014; Raman, Diffey, et al., 2016). A “baseline” linear mixed model (LMM) was developed to accommodate both genetic and non-genetic sources of variation. The baseline model for $\Delta^{13}\text{C}$ can be written using the symbolic notation of Wilkinson and Rogers (1973) as

$$\Delta^{13}\text{C} \sim 1 + \text{nonDHL} + \text{DHL} + \text{Rep} + \text{Plot} + \text{Run}$$

where “1” represents the overall mean, and the term “nonDHL” represents the fixed effects for controls and commercial checks. Terms in bold font are fitted as random effects. The term “DHL” represents the random effects of DH lines with marker data, “Rep” represents field replicate block effects, and “Plot” is the effects of field plots (indexed by field range and row). The term “Run” is the random effects associated with laboratory runs, “Carousel” the effects of carousels and “Run. Carousel” the effects of carousels within runs. Associated with each random term is a variance parameter (often referred to as a variance component). The preferred method for estimating these parameters is residual (or restricted) maximum likelihood (REML) (Patterson & Thompson, 1971). In the second stage, the baseline LMM was extended to include marker information in order to determine genomic regions associated with genetic

variation in $\Delta^{13}\text{C}$. For the set of markers selected as potential QTL, p -values were obtained using the conditional Wald statistic testing the significance of the fixed effect of each marker and these were used for the calculation of LOD scores. The extent of genetic control of traits was investigated by calculating line mean H^2 (reliabilities/broad sense heritability) as the mean of the squared accuracy of the predicted DH lines effects as described previously (Cullis, Smith, & Coombes, 2006) and found to be dependent on environment.

The glasshouse experiment data (Experiment IV) was analyzed using linear mixed model technology. Terms fitted as fixed effects included the main effects of and interaction between the 24 selected lines and 2 treatment regimes. For $\Delta^{13}\text{C}$, additional sources of variation include those associated with runs of the mass spectrometer in the laboratory. Unlike ANOVA, the linear mixed model has the ability to accommodate a wide range of variance models, in particular spatial models. For some of the traits considered, there was evidence of spatial variability between pots within benches and variance heterogeneity between benches. For $\Delta^{13}\text{C}$, there was evidence of variance heterogeneity between runs of the mass spectrometer.

In all the models presented above, variance parameters associated with model terms fitted as random effects were estimated using residual (or restricted) maximum likelihood (REML) (Patterson & Thompson, 1971). All models were fitted using the statistical software package ASReml (Butler, Cullis, Gilmour, & Gogel, 2009) within the R (R Core Team 2019) computing environment.

3 | RESULTS

3.1 | Genetic variation for $\Delta^{13}\text{C}$

In experiment 1, we investigated the extent of genetic variation in $\Delta^{13}\text{C}$ among canola accessions across seven diverse agricultural environments in Australia. There was substantial variation for mean $\Delta^{13}\text{C}$ across environments, ranging from

21.4 to 22.5‰ (Table 3). Approximate H^2 for each environment ranged from 60.1% to 89.2%, except in Rankin Springs and Condobolin, where moderate H^2 values (34.6 to 54.2%) were observed in 2014 (Table 3). High H^2 values across different environments suggested that genetic gains for $\Delta^{13}\text{C}$ could be made in the canola breeding programs.

To identify accessions of interest, we used the summary measure overall performance (Smith & Cullis, 2018). Consistent with a previous study (Luckett et al., 2011), Triazine tolerant (TT) genotypes, such as SturtTT, SARDI524TT, ATR-Cobbler, TP001, and ATR-Signal (NMT052), exhibited 0.7 to 1.3‰ higher values for $\Delta^{13}\text{C}$ compared to non-TT accessions (Figure 1, Table S1). Of different accessions, AGC214 had the overall least discrimination ($\Delta^{13}\text{C}$: 20.85‰) and SturtTT the greatest discrimination ($\Delta^{13}\text{C}$: 23.28‰). Even within TT accessions the range in overall performance was approximately 1‰. A similar range of genetic variation in $\Delta^{13}\text{C}$ was also observed in *B. napus* (Pater et al., 2017). Multiple environment trial (MET) analysis suggests that $\Delta^{13}\text{C}$ is highly correlated across environments ($r > .9$) and that this trait is genetically determined and stable and that genetic gains for $\Delta^{13}\text{C}$ could be made in canola breeding programs and within chemistry groups such as TT.

3.2 | Relationship between $\Delta^{13}\text{C}$ and seed yield

To determine whether variation in $\Delta^{13}\text{C}$ has any relationship with seed yield, we analyzed pair-wise correlations based on data from experiment I. A range of correlations ($r = 0.08$ to 0.42) were observed between $\Delta^{13}\text{C}$ and yield across environments (Figure 2). There were moderate positive correlations between $\Delta^{13}\text{C}$ and seed yield in three trials conducted in low rainfall environments at Condobolin and Kellerberrin, Western Australia (14DHT-CON, 15DHT-WA), whereas weaker or neutral correlations were observed in moderate to WET environments at Wagga, Rankin Springs, Finley, Kellerberrin (14DHT-WA) and Yanco (Figure 2). Different

TABLE 3 Trait summaries showing descriptive statistics for variation in leaf $\Delta^{13}\text{C}$ signatures of diverse lines evaluated across seven environments in Australia

Phenotyping year	Location of trial Site	Number of lines evaluated	Mean $\Delta^{13}\text{C}$ (‰)	Range (‰)		Reliability (H^2 , %)
				Min	Max	
2013	Wagga Wagga	144	22.5 ± 0.2	21.4	23.6	64.1
2014	Condobolin	150	22.0 ± 0.2	20.6	23.6	54.2
2014	Finley	150	21.8 ± 0.2	20.4	23.1	60.1
2014	Rankin Springs	150	21.4 ± 0.2	20.5	22.3	34.6
2014	Kellerberrin	150	22.3 ± 0.2	21.5	23.3	61.1
2014	Yanco	150	22.1 ± 0.2	20.7	23.2	62.9
2015	Kellerberrin	150	21.8 ± 0.8	20.7	23.2	89.2

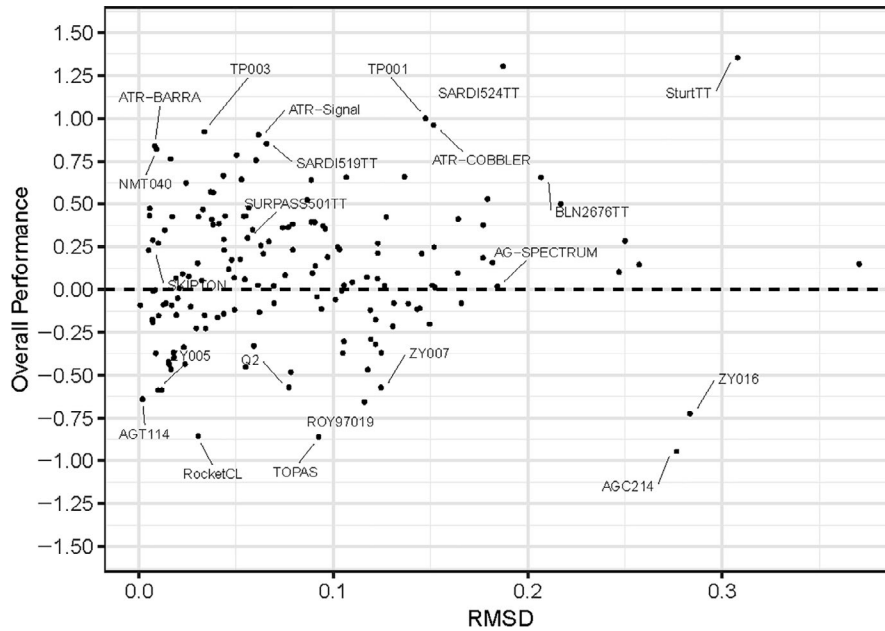


FIGURE 1 Natural variation for $\Delta^{13}\text{C}$ in *Brassica napus* accessions, evaluated under seven field environments. Overall performance (OP) plotted against a stability measure (RMSD) for all accessions (Experiment I). The highest and lowest overall $\Delta^{13}\text{C}$ accessions are labeled. Details of accessions are given in Table S1

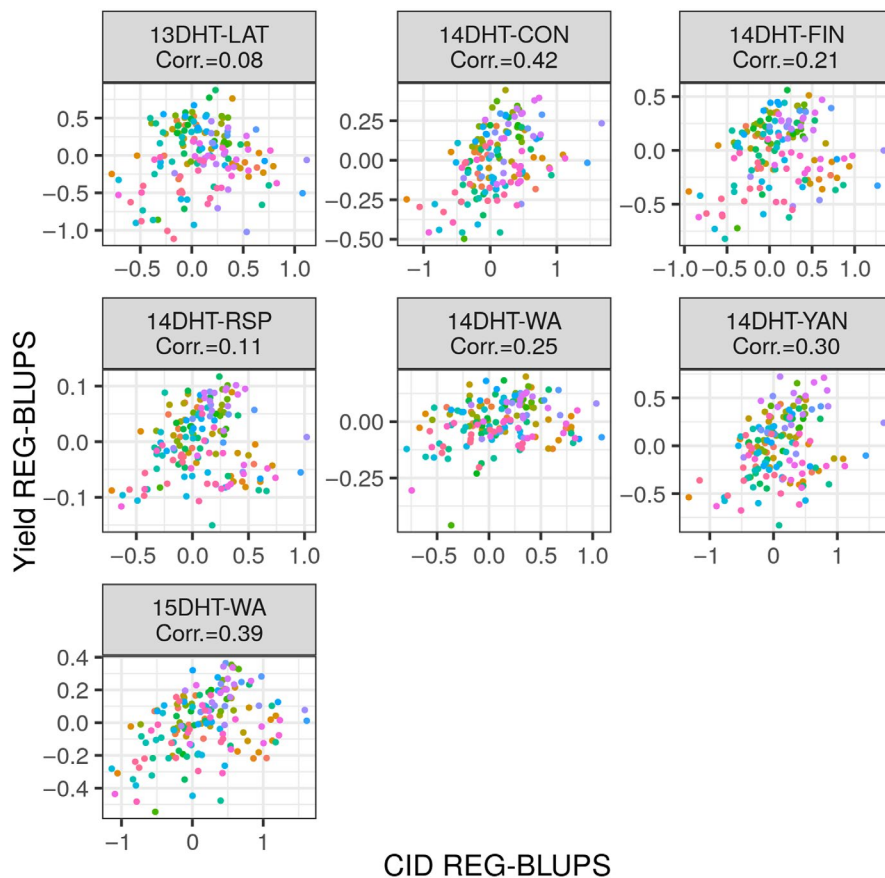


FIGURE 2 Relationship between $\Delta^{13}\text{C}$ and seed yield among diverse lines of *Brassica napus* (in different colors) evaluated across seven environments. 13DHT-LAT: Wagga Wagga, New South Wales (NSW, 2013); 14DHT-CON: Condobolin, NSW (2014); 14DHT-FIN: Finley, NSW (2014); 14DHT-RSP: Rankin Springs, NSW (2014); 14DHT-WA: Kellerberrin, Western Australia; 14DHT-YAN: Yanco, NSW (2014); 15DHT-WA: Kellerberrin (2015). Corr: Correlation coefficient; CID REG-BLUPS and Yield REG-BLUPS refer to regression form of line entry predictions from the FA models for $\Delta^{13}\text{C}$ and seed yield, respectively

sites experienced a range of drought stress (Figure S1a, Table S2). Under favorable environments (WET), yield up to 3.14 ton/h was achieved in AV-Jade (at Finley site, New South Wales), while under drought conditions, yield as low as 0.15

t/ha was achieved in CA001, followed by Monola NMT370 (at Kellerberrin, Western Australia, original data not shown). Generally, yield less than 0.5 t/ha is recognized as “uneconomical” in canola under Australian environments.

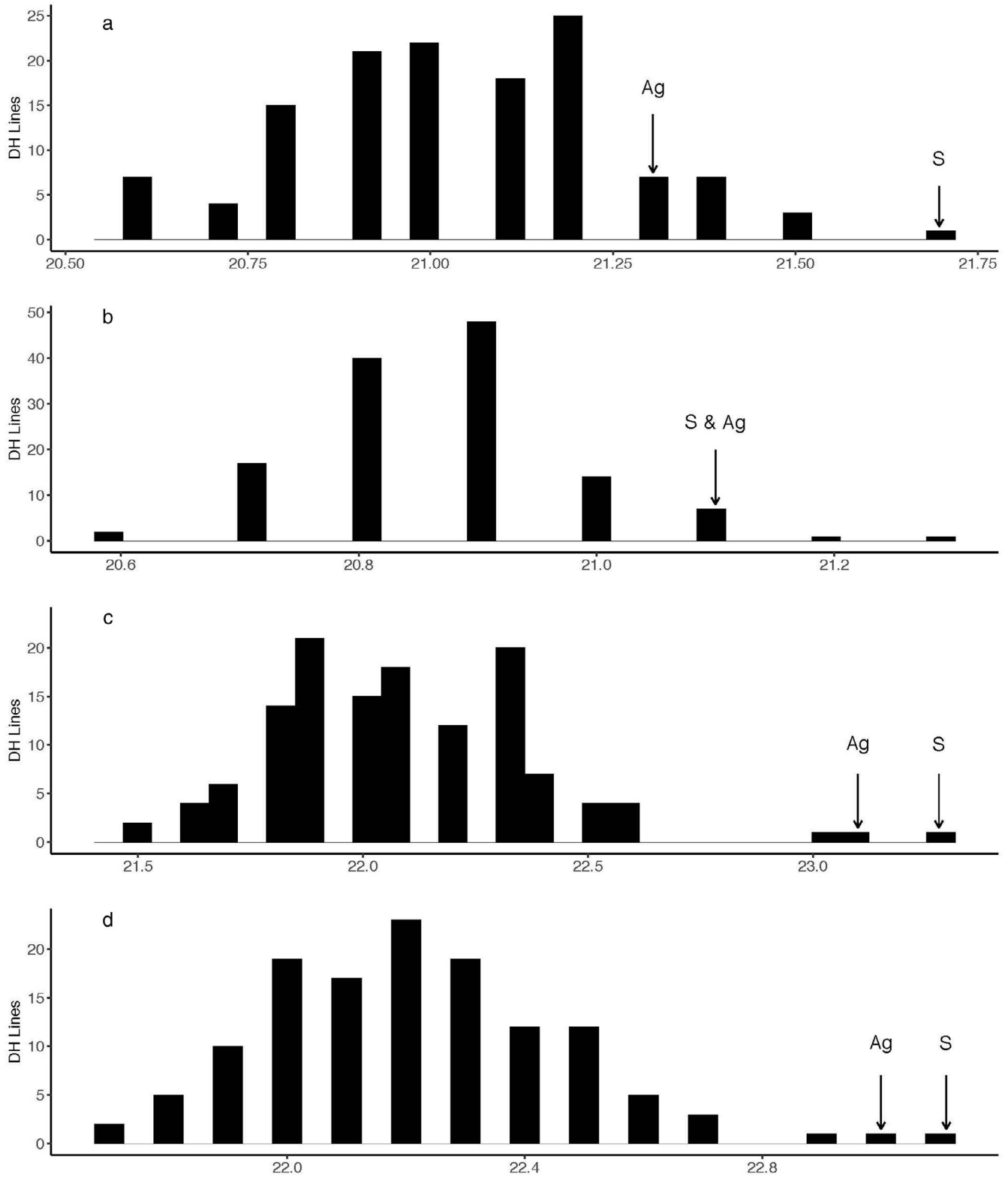


FIGURE 3 Frequency distribution of $\Delta^{13}\text{C}$ signatures (‰) in a doubled haploid population derived from a single BC_1F_1 plant of Skipton/Ag-Spectrum/Skipton, evaluated in plots under field conditions across four growing environments at Euberta (a: 2013) and Wagga Wagga (b: 2014, c: 2015 and d: 2016), NSW, Australia. S: Skipton; Ag: Ag-Spectrum. $\Delta^{13}\text{C}$ values are shown on X-axis

3.3 | Genetic control of $\Delta^{13}\text{C}$ variation in the SAgS DH population

To understand the genetic basis of $\Delta^{13}\text{C}$ variation, we analyzed a biparental DH population derived from non-TT parental lines, Skipton, and Ag-Spectrum (Raman, Diffey, et al., 2016) under field conditions (experiment II). Phenotypic analyses of the DH individuals revealed that the predicted means for $\Delta^{13}\text{C}$ are normally distributed (Figure 3) with moderate H^2 values (53.6% to 56.1%) except in the 2014 environment, suggesting that this trait is genetically determined, consistent with the results on diverse lines (Table 3, 4). Comparison of the parental means with the frequencies of DH means revealed a transgressive segregation, suggesting that phenotypic variation is under the control of multiple genetic loci. Large genotypic variation for $\Delta^{13}\text{C}$ was observed across four environments (Table 4). Mean $\Delta^{13}\text{C}$ values of DH population varied from 20.9 to 22.2‰. Consistent ranking of parental lines across most of the environments suggests that Skipton has higher leaf $\Delta^{13}\text{C}$ than Ag-Spectrum across environments. Stability of variation for $\Delta^{13}\text{C}$ in DH lines evaluated across environments was examined using Pearson pair-wise correlation; $\Delta^{13}\text{C}$ showed neutral to moderate correlations between different environments (Figure 4, Table S4).

3.4 | Possible pleiotropic links between $\Delta^{13}\text{C}$ and other agronomic traits in the SAgS DH population

In order to investigate whether variation in $\Delta^{13}\text{C}$ is associated with other traits, we first determined pair-wise genetic correlations between different phenotypes, including seed yield that we measured across environments in our previous study in the SAgS DH population (Raman, Raman, et al., 2019) (refer to Table S13). $\Delta^{13}\text{C}$ exhibited a range of correlation across different traits (Figure 4, Table S4). For example, it showed a positive correlation with seed yield, ranging from 0.1 to 0.3, in 2013 and 2014 environments, respectively while no correlation was found with seed yield in DH lines evaluated in 2016. Negative correlation (-0.2) was found in 2015. The magnitude of relationships was more pronounced

in 2013 and 2014, where the DH population was subjected to water stress at the reproductive stage (flowering and pod filling, Figure S1). The $\Delta^{13}\text{C}$ exhibited a range of negative correlation ($r = -0.1$ to -0.7) with flowering time depending on environment (Figure 4). Moderate to high correlations (at least in two dry environments) hint that some correlations between traits such as flowering time, $\Delta^{13}\text{C}$, and seed yield may result from pleiotropy and or tight linkage of underlying genes.

3.5 | QTL analysis reveals multiple genomic regions associated with leaf $\Delta^{13}\text{C}$ variation

To reveal loci underlying $\Delta^{13}\text{C}$ variation in canola, we carried-out QTL analysis of the SAgS DH population using a linear mixed model approach. Ten loci (2 significant QTL with $\text{LOD} \geq 3$, and 8 suggestive QTL with $\text{LOD} \leq 3$) were identified for variation in $\Delta^{13}\text{C}$ on different chromosomes: A01, A03, A07, C02, C03, C04, and C09 (Table 5). Both parental lines contain favorable alleles for $\Delta^{13}\text{C}$ variation, which suggests that both Skipton and Ag-Spectrum could be exploited to obtain alleles that can be used to increase WUE in canola. Of the QTL regions, marker 4121265 on chromosome C03 accounted for 33.1% of the total $\Delta^{13}\text{C}$ variation that was accounted for by all markers ($R^2 = 49.8\%$), suggestive of a major-effect QTL. We identified at least one genomic region delimited with 4121265 and 4119165 markers on C03 (mapped within 3 cM interval) which was detected for $\Delta^{13}\text{C}$ variation across two environments (Table 5, Figure 5).

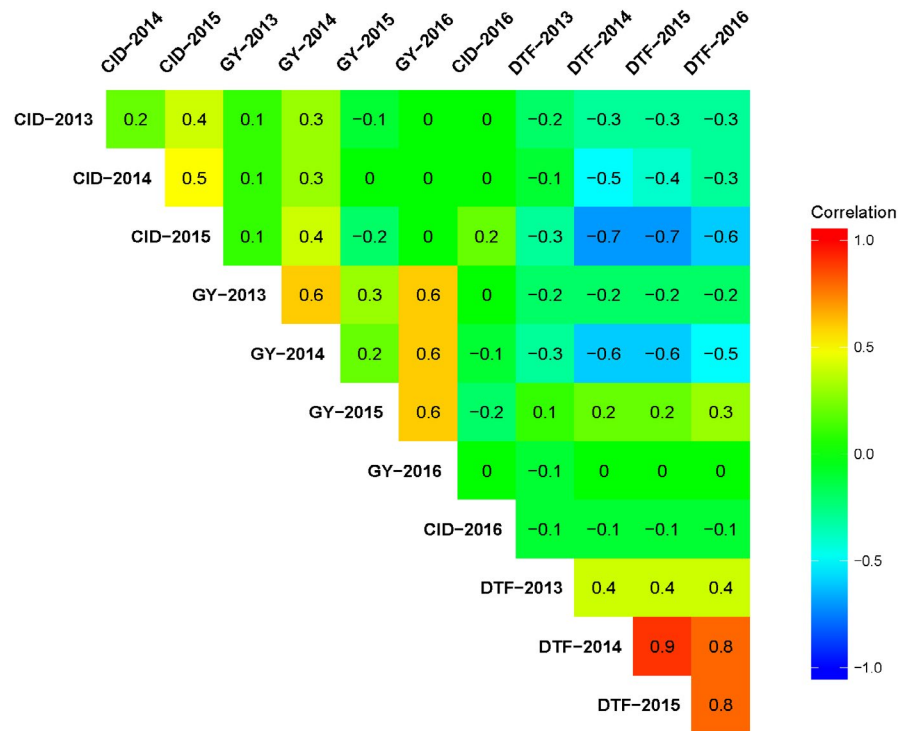
3.6 | QTL for $\Delta^{13}\text{C}$, flowering time, and seed yield are co-located near flowering genes

Recently, we reported at least three QTL for multiple traits that are located on chromosomes A07, and C03 in the SAgS DH population (Raman, Raman, et al., 2019). To investigate the localization of QTL regions for $\Delta^{13}\text{C}$ in relation to those multiple trait QTL, we mapped significant markers associated with $\Delta^{13}\text{C}$ onto the reference *B. napus* cv. Darmor-*bzh*. Two significant markers underlying QTL for both $\Delta^{13}\text{C}$ and multiple traits could be localized; the first for seed yield,

Experiment	Location	Mean $\Delta^{13}\text{C}$ (‰)	$\Delta^{13}\text{C}$ (range, ‰)	Reliability (H^2 , %)
2013	Euberta, NSW	21.0	19.7–22.4	53.7
2014	Wagga Wagga, NSW	20.9	19.4–23.0	22.9
2015	Wagga Wagga, NSW	22.1	20.4–24.7	56.1
2016	Wagga Wagga, NSW	22.2	21.0–24.4	53.6

TABLE 4 Summary of mean, range, and reliability values for $\Delta^{13}\text{C}$ measured among doubled haploid lines grown in field plots across four environments

FIGURE 4 Pearson correlation between $\Delta^{13}\text{C}$ (carbon isotope discrimination, ‰), flowering time, and yield related traits in a doubled haploid population derived from Skipton/Ag-Spectrum/Skipton. $\Delta^{13}\text{C}$.2013: $\Delta^{13}\text{C}$ (2013), DTF 2013: days to flower in 2013; $\Delta^{13}\text{C}$.2015: $\Delta^{13}\text{C}$ (2015); DTF.2014: days to flower in 2014; DTF.2015: days to flower in 2015; Delta13C.2016: $\Delta^{13}\text{C}$ (2016), SY.2013: grain yield in 2013; SY.2014: grain yield in 2014; SY.2015: grain yield in 2015; and SY.2016: grain yield in 2016. Empirical best linear unbiased predictions (E-BLUPs) of doubled haploid lines, estimated from separate analysis of each trait and each experiment are plotted



flowering time, and plant height, delimited with marker 3077306 on chromosome C03 (physical position 30.77 Mb to 30.90 Mb) was mapped near *ATCESA7*, *GRF6*, *FY*, *CAL1*, and *API* paralogs (Figure 5, Table S5). The second QTL associated with $\Delta^{13}\text{C}$ having LOD score of 4.86 was mapped to the multi-trait QTL region, flanked with 3110489 and 3075574 markers for shoot biomass, flowering time, plant height, and seed yield, on chromosome A07 (Figure 5). This potential pleiotropic QTL region accounted for 11.6% of total genotypic variance for $\Delta^{13}\text{C}$ and was located within 0.12 Mb of the *FLOWERING LOCUS T* (*BnA07FTb*), *ELF4-2*, *OST1B*, and *SMZ* paralogs in *B. napus*.

3.7 | Localization of a priori candidate genes for WUE in canola

Previous studies have shown that several candidate genes including *AtABCG22*, *ERECTA*, *MPK12*, and *HARDY* are implicated in WUE/transpiration efficiency in Arabidopsis and rice (Des Marais et al., 2014; Karaba et al., 2007; Kuromori et al., 2016; Kuromori et al., 2010; Kuromori et al., 2011; Lovell et al., 2013; Masle et al., 2005; Papacek et al., 2019; Zhang et al., 2016; Zhao, Chan, et al., 2016; Zhao, Chan, et al., 2016). To identify potential candidates involved in the $\Delta^{13}\text{C}$ variation, we compared the physical positions of 10 SNP associations for $\Delta^{13}\text{C}$ (Table 5) with the physical positions of genes involved in drought escape (flowering time) and drought tolerance (WUE) in Arabidopsis and other crops (Table S6). We are able to associate some potential candidate genes for the putative QTL for drought tolerance/WUE

in the SAS DH population. SNP markers were mapped in the vicinity (0.5 Mb) of *FT/FLC* (A01), *FLD*, and *H⁺ATPase 3* (A03); *H⁺ATPase* (A07); *FT* and *ELF4-L2* (A07), *CCT* (C02); *FY*, *CAL1*, *CESA7* (C03); *H⁺ATPase* (C04) and Vacuolar *H⁺ATPase* and *TOE1* (C09), genes underlying flowering time and WUE in the SAgS DH population grown in field experiments (Table S5).

3.8 | FT expression correlates with $\Delta^{13}\text{C}$ variation

Given *FT* plays a pleiotropic role across multiple traits and regulates stomatal opening (Kinoshita et al., 2011; Raman, Raman, et al., 2019), and given that WUE QTL ($\Delta^{13}\text{C}$, early vigor, shoot biomass, and seed yield) co-localize with *FT* (which display sequence variation between parental lines of the mapping population used herein (Raman, Raman, et al., 2019), we reasoned that *FT* expression may be associated with $\Delta^{13}\text{C}$ variation. To assess whether *FT* expression could explain the phenotypic variation in $\Delta^{13}\text{C}$, we calculated the correlations between $\Delta^{13}\text{C}$ levels and the expression levels of *FT* paralogs that we have recently reported (Raman, Raman, et al., 2019), with variation in $\Delta^{13}\text{C}$. A model that combined the expression levels of all paralogs displayed significant association with $\Delta^{13}\text{C}$ ($p < .001$), with different copies accounting for genetic variation for $\Delta^{13}\text{C}$ variably across environments. Expression levels of *FT* homeologues *BnA7.FTb* and *BnC6.FTb* localized near the multiple trait QTL on A07 were significantly correlated with phenotypic variation in $\Delta^{13}\text{C}$ at least in two environments. High *FT*

TABLE 5 Quantitative Trait Loci (QTL) for leaf $\Delta^{13}\text{C}$ variation measured in 144 doubled haploid lines from the BC₁F₁ cross: Skipton/Ag-Spectrum//Skipton, evaluated across four environments (2013–2016). LOD scores, additive effects, and proportion of variance (R^2) explained were estimated as described in Raman, Diffey, et al. (2016)

Phenotyping year	Marker locus	Chromosome	Distance (cM)	Allelic Effect	LOD	R^2 (%)	Physical map position on Darmor genome
Significant QTL							
2014	3090744	A07	51.63	0.34	3.5	11.57	A07
2016	3110489	A07	64.85	0.18	4.9	6.11	A07
Suggestive QTL							
2016	3146558	A01	0	0.11	2.4	2.76	A01_random
2014	4121491	A03	9.3	0.27	2.6	8.53	A03
2013	3117766_49:G > A	A03	20.19	0.1	1.9	8.5	A03
2013	3138613_65:T > G	C02	12.4	0.1	1.6	7.8	C02_random
2015	4119165	C03	6.2	0.13	2.2	2.5	A03
2013	4121265 ^a	C03	9.31	−0.14	2.2	16.5	C03
2015	3075169	C04	14.75	−0.16	2.3	2.5	C04
2015	3150149	C09	50.02	−0.12	2.6	3.1	C09

^aPhysical map position based on consensus map of *B. napus* (Raman et al unpublished). Positive and negative effects refer to Skipton and Ag-Spectrum, respectively.

expression was correlated with high $\Delta^{13}\text{C}$ (Figure 6). Our results reinforce the idea that $\Delta^{13}\text{C}$ is driven (at least partly) by a floral integrator, *FT* gene in the SAgS DH population.

3.9 | Physiological basis of WUE

3.9.1 | Interaction between $\Delta^{13}\text{C}$ and WUE at whole plant level

We first verified the phenotypic variation of $\Delta^{13}\text{C}$ and a range of traits segregating among SAgS DH lines under

contrasting water regimes (treatments = Wet: 100% saturation field capacity, and DRY: 50% saturation field capacity) in controlled glasshouse conditions. WUE was calculated from measurements of plant shoot biomass (dry weight) and seed yield, and water loss as a result of plant transpiration from both WET and DRY treatments. Water stress significantly affected all traits that we measured across genotypes; however, treatment \times genotype interactions were only significant ($p < .001$) for plant height, seed yield and *i*WUE (Table S6, Figure S2). Water stressed plants lost 62 to 90% of the seed yield in comparison with the control (WET) treatment. An almost linear relationship was observed between seed

Physical map position (bp)	Closest candidate	Distance from closest candidate gene (kb)	Function	Closest gene implicated in drought tolerance
20,791,615	BnaA07g28850D	0.75	Zein-binding domain	<i>PRR9</i> (0.02 Mb) <i>CAR9</i> (0.02 Mb)
22,661,368	BnaA07g32890D	0.57	Ankyrin repeat-containing domain	<i>FT</i> (0.12 Mb) <i>OSTIB</i> (0.59 Mb)
370,393	BnaA01g35060D	6.82	DREPP plasma membrane polypeptide	<i>FLC</i> (0.047 Mb) <i>BHP1</i> (0.48 Mb)
15,070,102	BnaA03g31180D	7.97	FLD	<i>PRR9</i> (0.9 Mb) <i>NCED5</i> (1.04 Mb) <i>AREB2</i> (0.81 Mb) <i>MPK12</i> (1.76 Mb)
17,022,812	BnaA03g34890D	2.71	Armadillo-type fold	<i>VRN1</i> (77.9 kb) <i>FMA</i> (0.31 Mb) <i>AREB2</i> (0.81 Mb) <i>H(+)-ATPASE2/3</i> (0.93 Mb)
1,220,892	BnaC02g45430D	5,909	Zinc finger, RING-type, conserved site	<i>CCT</i> (0.55 Mb) <i>EDF4</i> (1.07 Mb) <i>ATCESA7</i> (1.15 Mb)
3,269,876	BnaA03g07370D	2,934	Leucine-rich repeat 2	<i>GRF6</i> (0.53 Mb) <i>FY</i> (0.26 Mb)
3,315,711	BnaC03g09230D	197	Glycosyltransferase, family 8	<i>CAL1</i> (0.17 Mb) <i>API</i> (0.40 Mb) <i>ATCESA7</i> (0.10 Mb)
21,513,493	BnaC04g20400D	3,357	Pentatricopeptide repeat	<i>H + ATPase</i> (0.49 Mb) <i>PRR9</i> (0.53 Mb) <i>ATCYSC1</i> (1.33 Mb)
7,912,422	BnaC09g11310D	986	Gnk2-homologous domain	<i>JMJ17</i> (29.9 kb) <i>AAT2</i> (0.59 Mb) <i>AP2</i> (0.10 Mb) <i>TOE1</i> (0.10 Mb) <i>VHA-F1</i> (1.43 Mb)

yield and WUE ($R^2 = .99$), measured as seed yield per unit of water applied. This is because all plants within a particular treatment were given the same amount of water. However, there were significant differences in seed yield/WUE between WET and DRY treatments (Figure S3a,b). There was a negative correlation between flowering time and seed yield across treatment ($R^2 = .42$ to $.52$) as observed under field conditions (Raman, Diffey, et al., 2016); early flowering genotypes yielded higher than those flowering later (Figure S3c). Flowering time also showed a negative correlation ($R^2 = .35$) with shoot biomass (mainly stem biomass, as all canola leaves senesce at full maturity, Figure S3d). Plant height was

also negatively correlated ($R^2 = .37$) with seed yield under WET conditions, whereas it showed a poor relationship ($R^2 = .06$) under DRY treatment. Shoot biomass was positively correlated with seed yield ($R^2 = .22$ to $.57$) across both treatments. We have shown that genetic variation in $\Delta^{13}\text{C}$ indeed exists in canola and related with plant productivity traits in the SAgS DH lines of canola. However, while there was no correlation between $\Delta^{13}\text{C}$ and shoot biomass under both DRY and WET treatments, biomass is lost as leaves are shed. We found that both parental lines of DH population display phenotypic plasticity to water stress. For example, Sipton accumulated more biomass than Ag-Spectrum under

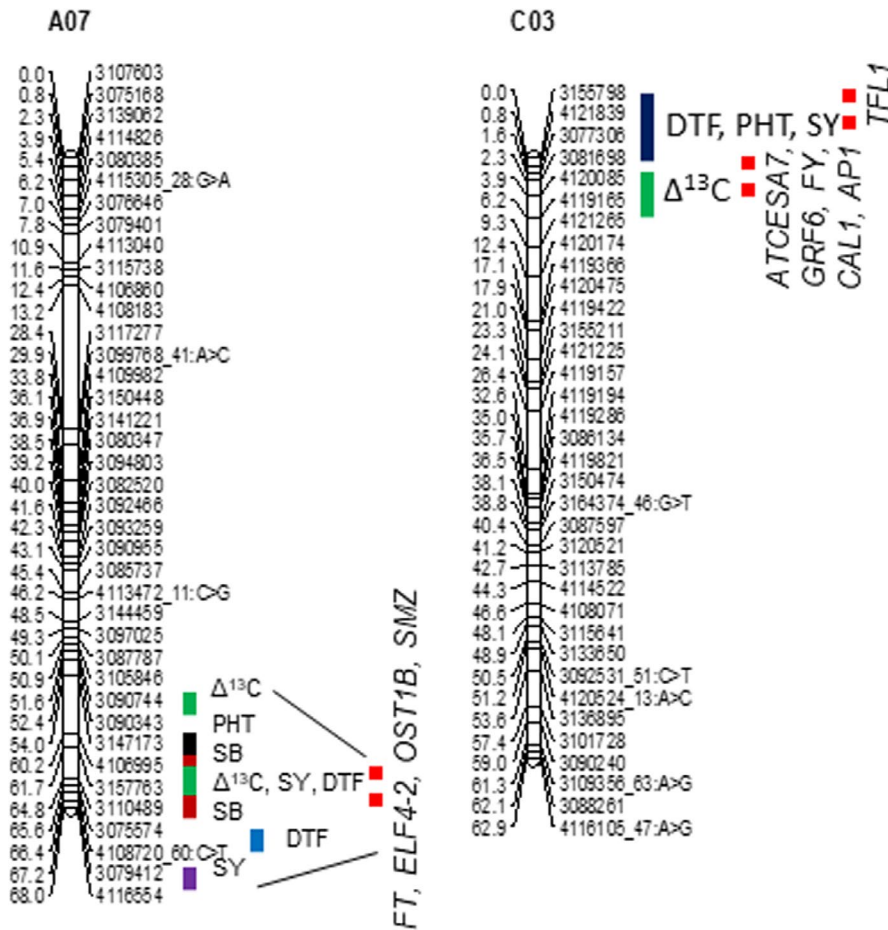


FIGURE 5 Graphical representation showing localization of multi-trait QTL associated with $\Delta^{13}\text{C}$ (‰), above ground shoot biomass (SB); flowering time (days to flower, DTF); plant height (PHT) and seed yield (SY) in a doubled haploid population from Skipton/Ag-Spectrum//Skipton. DArTseq markers and their genetic map positions are shown on right- and left-hand side, respectively. Solid lines (in different colors) represent markers that showed significant associations with traits of interest. Map distances are given in cM and displayed using the MapChart. The closest genes implicated in drought tolerance near significant marker associations are illustrated.

DRY treatments while Ag-Spectrum had more biomass than Skipton under WET environments. Both genotypes therefore led to fitness trade-off and were subject to $G \times E$ interaction. Similar results have been found in a RIL population of *B. rapa* when evaluated under well-watered and drought environments (El-Soda et al., 2014) as well as in *Arabidopsis* (Des Marais et al., 2014).

3.9.2 | Interaction between $\Delta^{13}\text{C}$ and WUE at single leaf level

Since *i*WUE measurements are laborious and time-consuming, and are dependent upon environmental conditions such as light intensity and vapor pressure deficit, we selected a set of six DH lines from the SAgS DH population (2 low $\Delta^{13}\text{C}$, low *FT* expression and 2 high $\Delta^{13}\text{C}$ with high *FT* expression levels, plus 2 parental lines: Skipton (high *FT* expression) and Ag-Spectrum (low *FT* expression) for single leaf based *i*WUE measurements.

Gas exchange measurements showed a significant variation in CO_2 assimilation rate, stomatal conductance, and *i*WUE (Table S6, Figure S2e). Water stress also had a significant effect on all gaseous exchange and $\Delta^{13}\text{C}$ measurements;

the CO_2 assimilation rate and stomatal conductance decreased with water stress (Figure 7). As expected, CO_2 assimilation rates were decreased under DRY treatment compared to WET treatment (Figure 7d), as stomata would have been partially closed, resulting in increased *i*WUE (Specht et al., 2001). CO_2 assimilation is an intrinsic part of *i*WUE, and so we investigated the variation in CO_2 assimilation between accessions under both treatments. Predicted means of CO_2 assimilation ranged from 5.2 to 13.5 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ and were positively correlated with $\Delta^{13}\text{C}$ ($R^2 = .26$ to $.67$) across treatments (Figure 7a). A similar positive relationship was observed between $\Delta^{13}\text{C}$ and stomatal conductance (Figure 7b). Different genotypes varied in the magnitude of stomatal conductance, which ranged from 0.05 to 0.34 $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$. The variation in $\Delta^{13}\text{C}$ and *i*WUE was driven more by variation in g_{sw} (~7-fold) than in *A* (~2-fold). The stomatal conductance showed a positive correlation ($R^2 = .11$) with shoot biomass under WET environments, whereas in the DRY environment, no correlation ($R^2 = .01$) was found between stomatal conductance and biomass, which may have been due to loss of leaves. There was a positive correlation ($R^2 = .59$) between stomatal conductance and seed yield under WET environment; however, poor relationship was found under DRY conditions ($R^2 = .08$). This was probably due to the timing of

FIGURE 6 FLOWERING LOCUS *T* (*FT*) is associated with variation in $\Delta^{13}\text{C}$ in canola varieties. Expression analysis of different *FT* paralogs *BnA7.FTb* and *BnC6.FTb* on leaves taken from field grown plants of around 80 doubled haploid lines of Skipton/Ag-Spectrum/Skipton, and its correlation with $\Delta^{13}\text{C}$ in canola. The relative expression levels of *FT* after normalization with the reference *UBC9* are plotted against EBLUPS of $\Delta^{13}\text{C}$

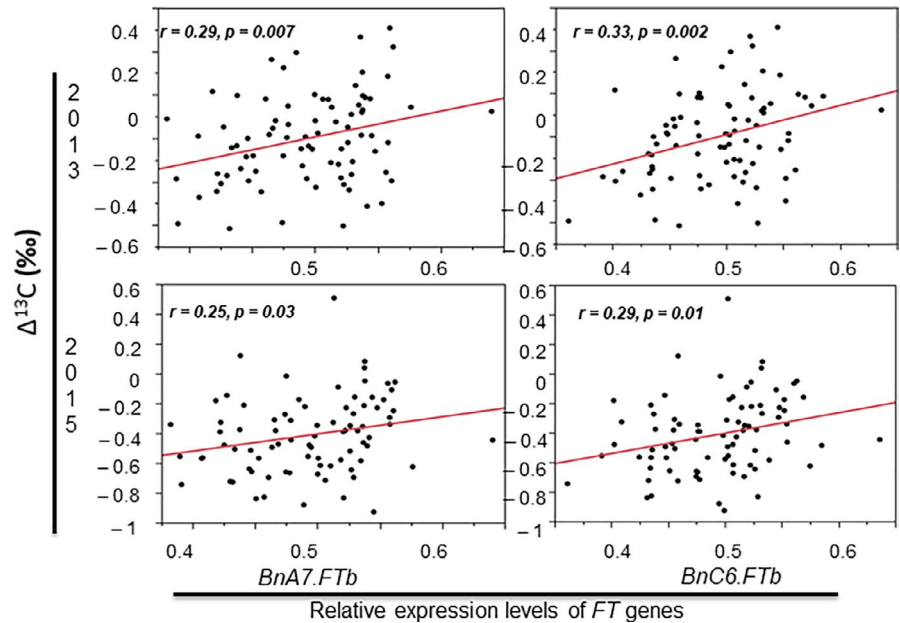
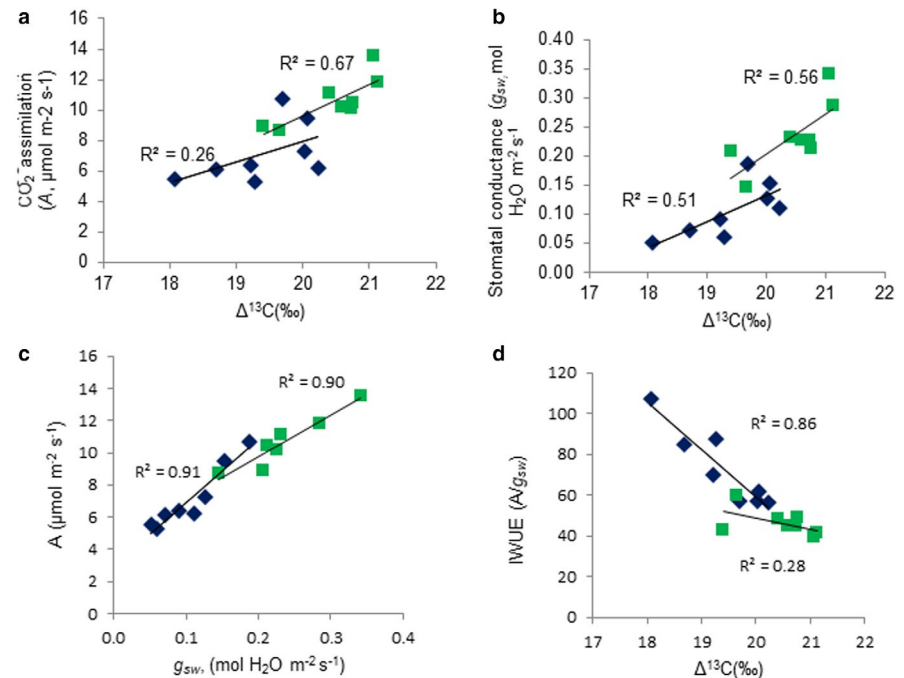


FIGURE 7 Relationships between $\Delta^{13}\text{C}$ and gas exchange measurements, CO_2 (assimilation *A*, *a*), stomatal conductance (g_{sw} , *b*), A/g_{sw} (*c*), and intrinsic transpiration efficiency (*iWUE*: A/g_{sw} , *d*) of selected doubled haploid lines of the Skipton/Ag-Spectrum/Skipton population represent to extremes (High and low values) in $\Delta^{13}\text{C}$. Response to drought stress on gas exchange at flowering stage is shown: Green square and Blue diamond represent predicted means for DH lines under WET and DRY treatments, respectively



the water stress treatment, as we imposed stress when plants were at the flowering stage and by then plants may have accumulated biomass for seed yield. There was a strong positive relationship between CO_2 assimilation and stomatal conductance ($R^2 = .90$ to $.91$), suggesting that rate of CO_2 assimilation is dependent on the stomatal conductance among tested genotypes (Figure 7c).

iWUE is essentially the ratio of CO_2 assimilation rate and stomatal conductance. The values of *iWUE* ranged from 39.6 to 59.7 $\mu\text{mol CO}_2$ per mol H_2O under WET, and 57.1 to 107.2 $\mu\text{mol CO}_2$ per mol H_2O under DRY treatment, suggesting that canola genotypes differ in their *WUE* especially under

water stress (Figure 7d). The maternal parent Skipton had the higher *iWUE* (85 $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{H}_2\text{O}$) and higher yield (3.4 g/plant) as compared to the paternal parent Ag-Spectrum under DRY treatment (57.1 $\mu\text{mol CO}_2$ per mol H_2O and seed yield of 2.3 g/plant). However, under WET conditions, Ag-Spectrum had almost similar *iWUE* (43.03 $\mu\text{mol CO}_2$ per mol H_2O) as compared to Skipton (45.1 $\mu\text{mol CO}_2$ per mol H_2O), suggesting genotypic plasticity in *iWUE*. Negative correlations were observed between $\Delta^{13}\text{C}$ and *iWUE* (transpiration efficiency) at the leaf level, as reported previously (Condon et al., 1990); there were stronger correlations among accessions in DRY treatment ($R^2 = .86$) compared to WET

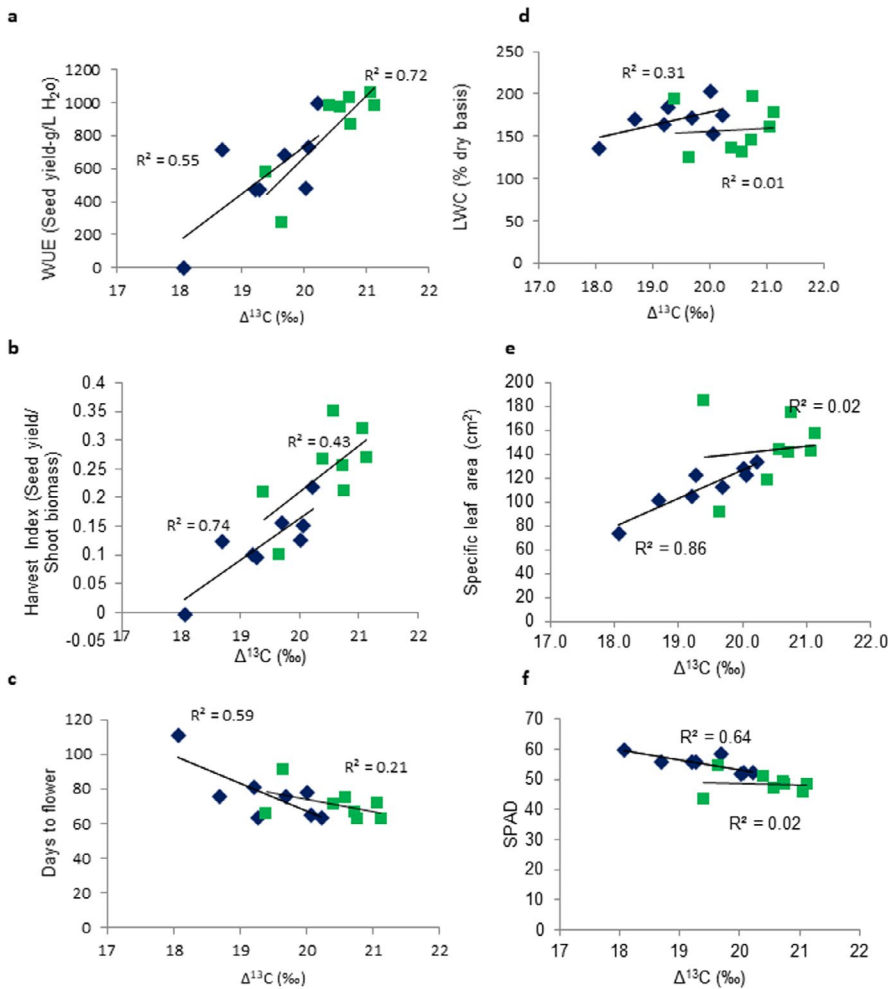


FIGURE 8 Relationship between $\Delta^{13}\text{C}$ (‰) and WUE-related traits. (a) WUE (seed yield/L water applied); (b) harvest index (ratio between seed yield (g)/shoot biomass measured at maturity); (c) Days to first flower; (d) leaf water content (LWC % dry weight); (e) chlorophyll content (measured with SPAD-502 meter) and (f) specific leaf area of selected doubled haploid lines of the Skipton/Ag-Spectrum/Skipton population represent to extremes in $\Delta^{13}\text{C}$. Green square and blue diamond represent predicted means for DH lines under WET and DRY treatments, respectively

treatment ($R^2 = .28$). The strong relationship suggests that difference in $i\text{WUE}$ corresponds negatively, as expected, to the genotypic variation in $\Delta^{13}\text{C}$. Among DH lines evaluated, DH107 had the highest $i\text{WUE}$ and lowest stomatal conductance under drought stress conditions. Gravimetric measurements also revealed a negative relationship between $\Delta^{13}\text{C}$ and WUE (DH lines with high $\Delta^{13}\text{C}$ had high water loss), suggesting that $\Delta^{13}\text{C}$ can be used as surrogate trait for selecting lines having high WUE under both treatments (Figure 7, Figure S4).

At the whole plant level, we observed moderate to high correlations between $\Delta^{13}\text{C}$ and seed yield, WUE, and days to flower under both WET and DRY treatments (Figure S4, Figure 8). Negative correlations were observed between $\Delta^{13}\text{C}$ and flowering time, while positive correlations were observed between $\Delta^{13}\text{C}$ and seed yield, WUE (seed yield/unit of water), harvest index, and leaf water content (Figure 8a-d, Figure S3). Strong positive correlation ($R^2 = .55$ to 0.72) was observed between whole plant WUE and $\Delta^{13}\text{C}$ across control (WET) and DRY (water stress) treatments (Figure 8a). While $\Delta^{13}\text{C}$ showed high correlation with harvest index (ratio between seed yield/shoot biomass, $R^2 = .43$ to $.74$, Figure 8b), there was no correlation between $\Delta^{13}\text{C}$ and shoot biomass

across both WET and DRY treatments (Figure S4e). This may reflect the loss of leaves between flowering and harvest.

3.9.3 | Relationship between $i\text{WUE}$ and stomata density and size

To determine whether variation in $i\text{WUE}$ is due to differences in $\Delta^{13}\text{C}$ and expression of different FT alleles on chromosome A07, we measured stomatal density and size among parental lines and their six DH lines grown under well-watered conditions across six replicates. No difference was observed between the maternal parent Skipton (having alleles for early flowering, high FT expression, high $\Delta^{13}\text{C}$, low WUE, and high yield under water stress conditions) and Ag-Spectrum (having allele for late flowering, low FT expression, low $\Delta^{13}\text{C}$, high $i\text{WUE}$ and low yield) in stomata size—a trait associated with drought avoidance mechanism. However, stomatal density showed a positive correlation with $i\text{WUE}$ ($R^2 = .15$) while no correlation was found with stomatal conductance (Figure S4d,f); this suggests that there is a role for the mesophyll cells in CO_2 assimilation for improved $i\text{WUE}$ among DH lines. It is yet to establish whether

and how reduced stomatal density and stomatal size affect WUE in canola.

3.9.4 | Relationship between $\Delta^{13}\text{C}$ and leaf thickness and chlorophyll content

At a single leaf level, there was a positive relationship between $\Delta^{13}\text{C}$ and leaf water content among accessions ($R^2 = .31$) under DRY treatment, while poor relationships between $\Delta^{13}\text{C}$ and LWC were found in the WET treatment (Figure 8d). SLA showed a positive relationship with $\Delta^{13}\text{C}$ under DRY conditions ($R^2 = .86$) while no correlation ($R^2 = .02$) was observed in WET conditions (Figure 8e). SLW showed a negative correlation with $\Delta^{13}\text{C}$ ($R^2 = .81$) under DRY conditions, whereas poor relationship ($R^2 = .09$) was observed under WET conditions (Figure S4c). There were high correlations between SLA and LWC under both DRY ($R^2 = .54$) and WET ($R^2 = .78$), suggesting that both these measures are suitable for characterizing leaf thickness in canola. Accessions that had greater $\Delta^{13}\text{C}$, and higher LWC and smaller SLA may be beneficial for sustainable cultivation of canola under drought stress conditions. $\Delta^{13}\text{C}$ showed a negative correlation with chlorophyll content under DRY treatments, while poor correlation was found under WET treatment (Figure 8f). It was interesting to note that plants with high $\Delta^{13}\text{C}$ had low chlorophyll and high leaf water content compared to those with low $\Delta^{13}\text{C}$ when exposed to water stress. The latter were dark green in color. Moderate to high correlations were observed between chlorophyll content and seed yield (Figure S4b).

3.10 | *FT*, a potential driver in WUE

We were interested whether differences in expression levels of *FT* paralogs and $\Delta^{13}\text{C}$ composition contribute to differences in WUE under contrasting water regime. We selected the low and high $\Delta^{13}\text{C}$ lines which differ in *FT* expression levels (high $\Delta^{13}\text{C}$ lines had earlier flowering and high *FT* expression) and compared seed yield reduction due to drought stress (WET-DRY). One DH line, SAgS55 (high *FT* expression, high $\Delta^{13}\text{C}$) showed the least reduction (62%) in seed yield. Our results suggest that WUE (seed yield) can be increased by 28% by incorporating alleles for high $\Delta^{13}\text{C}$. We found that the relationship between *FT* expression and $\Delta^{13}\text{C}$ across treatments was inconsistent, being a negative correlation under DRY treatment ($r = -.28$) while a positive correlation was found under WET treatment ($r = .23$). By noting that there is an advantage in being fast to flower in dry conditions, drought escape (Franks, 2011), but not in wet conditions, and so the observation suggests that there may be a very sophisticated association between $\Delta^{13}\text{C}$, flowering time, and *FT* expression.

4 | DISCUSSION

4.1 | Genetic basis of $\Delta^{13}\text{C}$ variation and relationship with seed yield

Deciphering the extent of genetically controlled phenotypic variation and the genetic architecture underlying effective water use is important for making gains in canola. In this study, we investigated the extent of $\Delta^{13}\text{C}$ variation and its genetic control in canola germplasm across seven environments in Australia. Our results showed that considerable genotypic variation exists for $\Delta^{13}\text{C}$ among canola accessions. While our results showed somewhat inconsistent relationships between $\Delta^{13}\text{C}$ and seed yield, correlations were observed under water stress conditions (Figures 2, 4; Table S4, Figure S4), suggesting that $\Delta^{13}\text{C}$ may be a useful trait that can improve seed yield under an unfavorable (drought) environment. Our results are partially inconsistent with those of Matus et al. (1995) who reported no significant phenotypic correlations between seed yield and $\Delta^{13}\text{C}$ among 10 diverse *B. napus* genotypes grown at five locations in Saskatchewan. The variable relationship between $\Delta^{13}\text{C}$ and seed yield suggests this trait is subject to some $G \times E$ interaction. In addition, we also included TT varieties in our multi-site field experiments as these are highly effective in controlling cereal weeds and therefore are quite popular among canola growers. These varieties generally yield less due to the mutation in chloroplast DNA and have high $\Delta^{13}\text{C}$ values, consistent with reduced rates of photosynthesis (A).

Of the various vegetative and reproductive traits evaluated such as plant emergence, fractional ground cover, shoot biomass, days to flower, plant height, and seed yield, $\Delta^{13}\text{C}$ only showed moderate to high, negative correlation ($r =$ up to $-.7$) with flowering time. Thus, early flowering DH lines (with short time to flower) had large $\Delta^{13}\text{C}$. They also had greater seed yield, and so there was negative correlation between time to flower and yield, and a positive correlation between $\Delta^{13}\text{C}$ and seed yield. Our results suggested that early flowering DH with high $\Delta^{13}\text{C}$ values can be selected for the development of canola varieties having high WUE of seed production and improved yield. Therefore, high $\Delta^{13}\text{C}$ does not mean low water use efficiency, as reported in other crops (Condon, Richards, Rebetzke, & Farquhar, 2004; Rebetzke et al., 2008). The high WUE lines with high $\Delta^{13}\text{C}$ may seem counter-intuitive, but it appears that such lines maintain a much greater proportion of their dry matter (less leaf loss) and also have a greater harvest index. In previous studies, it was shown that flowering time is negatively correlated with seed yield in canola (Raman, Raman, et al., 2019; Raman, Diffey, et al., 2016). Positive correlation shared between $\Delta^{13}\text{C}$ and seed yield in drought environments also suggested that accessions (diverse lines as well as DH lines of Skipton/Ag-Spectrum/Skipton) that used more water (low $\Delta^{13}\text{C}$) yielded less compared to

those that used less water (high $\Delta^{13}\text{C}$) yield higher. In this study, the water stress we imposed reduced seed yield by up to 89% (Figure S2). Drought stress reduced the CO_2 assimilation by up to 50%. Clearly, water is fundamental to survival and reproductive success of plants. Water availability affects plant development and productivity well as its physiology, as observed in this study. However, high $\Delta^{13}\text{C}$ lines had a yield advantage of up to 20% compared to the low $\Delta^{13}\text{C}$ lines. In contrast, the value of low $\Delta^{13}\text{C}$ as a surrogate trait has been demonstrated in wheat, where two varieties were released for commercial cultivation in rainfed Mediterranean-type environments in Australia (Rebetzke et al., 2008).

4.2 | Physiological basis of $\Delta^{13}\text{C}$ variation and relationship with seed yield

Our glasshouse study describes the long-term water use assessment and plant performance (Figures 6-7); results hinted toward increase in $i\text{WUE}$ were via reducing stomatal conductance and improved CO_2 assimilation rates. Our results reconfirmed that $\Delta^{13}\text{C}$ could be used as proxy for $i\text{WUE}$ in canola as proposed previously in other plant species (Farquhar & Richards, 1984). $\Delta^{13}\text{C}$ measurements are less prone to $G \times E$ interactions as well as have higher heritability (Table 3, 4). However, $\Delta^{13}\text{C}$ estimations are subject to experimental variation including laboratory/instrument as found in this study (Table 4, year 2014 environment). Deployment of multiphase experiments enabled to account for such variation and improved reliability values in this study.

4.3 | Role of other proxy traits in WUE

In addition to gas exchange measurements, specific leaf weight (SLW) and specific leaf area (SLA) were measured for genetic variation in leaf thickness. Positive correlation between $\Delta^{13}\text{C}$ and LWC (% dry weight) was observed under DRY treatment, whereas weak to nil correlation was observed under WET treatment (Figure 8d). Early flowering lines had high leaf water content ($R^2 = .31$) and high $\Delta^{13}\text{C}$ compared with late flowering ones under DRY conditions. High WUE was associated with lower leaf water content and specific leaf area (Des Marais et al., 2014). Accessions with thicker leaves had high SLW and lower SLA which may be responsible for greater rate of assimilation of CO_2 due to higher photosynthetic capacity and resulting in higher $i\text{WUE}$. Previous studies have shown positive correlation between TE and SLW in peanut and sunflower (Hubick et al., 1986; Virgona, Hubick, Rawson, Farquhar, & Downes, 1990).

Our genetic analysis provides two key results that improve our understanding of the genetic basis of $\Delta^{13}\text{C}$ variation. First, we identified ten QTL (significant and suggestive)

for $\Delta^{13}\text{C}$, suggesting that it is controlled by multiple genes. Second, detection of QTL was highly dependent upon the growing environment. The number of QTL detected, their magnitudes, and allelic effects were variable across phenotyping environments, suggesting that impacts of individual QTL for $\Delta^{13}\text{C}$ depended on $G \times E$ interactions. Breeding programs need to select favorable alleles across different environments to improve overall WUE in canola varieties. Of the ten genomic regions associated with $\Delta^{13}\text{C}$, only two QTL were mapped in the vicinity of flowering genes, *FT* (A07) and *TFL1* (C03) in a SAgS DH population of canola. In Arabidopsis, McKay, Richards, and Mitchell-Olds (2003) have also mapped QTL for $\Delta^{13}\text{C}$ near the flowering genes, *FRIGIDA* (*FRI*) and *FLC*. This study showed that near isogenic lines with functional *FRI* and *FLC* alleles had lower $\Delta^{13}\text{C}$ and greater WUE. The negative correlation between $\Delta^{13}\text{C}$ and flowering time has also been demonstrated in other studies in Arabidopsis (Easlon et al., 2014; Hausmann et al., 2005; Juenger et al., 2005; Kenney, McKay, Richards, & Juenger, 2014), wheat (Rebetzke et al., 2008), bean, and cowpea (Hall, Richards, Condon, Wright, & Farquhar, 2010).

4.4 | Physiological basis of $\Delta^{13}\text{C}$ variation and relationship with seed yield

Our glasshouse study describes the long-term water use assessment and plant performance (Figures 7-8); results hinted that the increase in WUE was via reduced g_{sw} and improved A . Our results reconfirmed that $\Delta^{13}\text{C}$ could be used as proxy for $i\text{WUE}$ in canola (Figure 7d) as proposed previously in other plant species (Farquhar & Richards, 1984). High $\Delta^{13}\text{C}$ showed strong correlation with higher WUE and seed yield (Figure 8a-b) under both WET and DRY environments.

4.5 | *FT* as a driver for improved WUE

We present two findings linking *FT* with improved WUE (on the basis of productivity as measured as seed yield) and intrinsic WUE (A/g_{sw}). First, through genetic analysis of the SAgS DH population, two QTL for multiple traits were localized in the vicinity of photoperiod genes: *FT* (A07) and *TERMINAL FLOWER1* (*TFL1* on C03). *FT* underlies the QTL accounting for significant variation in $\Delta^{13}\text{C}$ and traits involved in drought escape (flowering time, plant emergence, early vigor, shoot biomass) and grain yield, leading to improved WUE, at least in some environments (Figure 4). The second expression analysis revealed a significant positive correlation (up to 33%) between *FT* expression and $\Delta^{13}\text{C}$ (Figure 5) and positive correlation between *FT* expression and flowering time (Raman, Raman, et al., 2019). In addition, flowering time also strong correlation with $i\text{WUE}$ under both WET ($R^2 = .55$) and DRY ($R^2 = .41$)

conditions; early flowering lines had lower $iWUE$ as compared to late flowering ones, as observed in *B. rapa* (Franks, 2011). These observations provide evidence that *FT* may be driving variation in WUE in canola. Our findings are consistent with previous studies that found associations between $\Delta^{13}C$ and flowering time and productivity traits (Ferguson et al., 2019; Fletcher, Mullen, Heiliger, & McKay, 2015; McKay et al., 2003), suggesting that a cross-talk between drought tolerance and drought avoidance mechanisms contributes to effective water use in canola productivity. *FT* and *ELF* genes (candidates identified herein) have been shown to regulate stomatal opening in Arabidopsis via the regulation of $H^+ATPase$ by blue light in guard cells (Kinoshita et al., 2011). Recently, *FT* expression was also shown to alter stomatal patterning in Indian mustard (Tyagi et al., 2018). In the present study, we also found a positive correlation between stomatal density and $iWUE$ in parental lines as well as in selected eight DH lines differing in $\Delta^{13}C$ and *FT* expression. Coincidentally, we identified markers underlying QTL for $\Delta^{13}C$ near the candidate genes for WUE such as $H^+ATPase$ (A03); $H^+ATPase$ (A07); *FT* and *ELF4-L2* (A07), *CESA7* (C03); $H^+ATPase$ (C04); and Vacuolar $H^+ATPase$ (C09). Further research is required to establish the clear cause of stomatal patterning in diverse *B. napus* accessions. It was interesting that none of the *ERECTA* and *MPK12* paralogs that regulate WUE in Arabidopsis (Des Marais et al., 2014; Masle et al., 2005)—a relative of canola, was located in the vicinity of QTL for $\Delta^{13}C$ in the SAgS DH population. Overall, our results suggest that early flowering is associated with high *FT* expression, large $\Delta^{13}C$ (integrated WUE), improved WUE (above ground shoot biomass, height, and seed yield) under water stress conditions, whereas no such relationships were observed under non-stress field conditions. Nevertheless, canola lines with large $\Delta^{13}C$ could be selected for higher yield under water stress environment (low rainfall areas) in Australia and elsewhere where canola cultivation largely relies on stored ground water. Our results showed that there is no trade-off between high $\Delta^{13}C$ and seed yield under WET environment.

5 | CONCLUSIONS

In several countries including Australia, canola productivity relies heavily on seasonal pattern of water availability in dry-land farming. Dissection of WUE traits and interaction with plant adaptation and productivity traits is complex. In the present study, we characterized genetic variation for $\Delta^{13}C$ in Australian canola accessions and related it to seed yield across different environments. In addition, ten QTL having small to moderate allelic effects for $\Delta^{13}C$ variation were identified in an Australian DH population of canola, two stable QTL were detected in at least two environments on chromosomes A07 and C03. The relationship of $\Delta^{13}C$ in improved productivity

via $iWUE$ (A/g_{sw}) and WUE (seed yield/unit of H_2O) experiments was verified under two contrasting water environments. Our findings suggest that the combination of alleles involved in alternative strategies for reproductive success (drought escape and drought tolerance mechanisms) relying on early flowering varieties with high WUE (high $\Delta^{13}C$ or low $\delta^{13}C$, high seed yield) are likely to be beneficial for sustainable production of canola. Our results showed that improvement in $\Delta^{13}C$ could lead to an effective water use throughout the growing season for increasing yield in a DH population from Skipton/Ag-Spectrum. Canola germplasm in conjunction with molecular markers flanking the QTL for $\Delta^{13}C$ would provide valuable tools for improving WUE in canola varieties. The QTL regions would also become the basis for understanding the molecular basis of WUE in canola.

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CONFLICT OF INTEREST


There is no conflict of interest to declare.

AUTHOR'S CONTRIBUTION

HR designed the study. HR and RR performed field experiments and collected leaf samples for carbon isotope discrimination analysis. BM and HR conducted GH experiment; BM and HR measured $iWUE$. LB and SD analyzed data, HR, RR, AS, and SB carried-out molecular analyses. GF and HR provided inputs in interpreting gas exchange measurements. HR prepared manuscript with inputs from others. All authors read and approved manuscript for publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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