Theoretical and Applied Genetics (2022) 135:1843–1854
https://doi.org/10.1007/s00122-022-04076-1

ORIGINAL ARTICLE

Leaf thickness of barley: genetic dissection, candidate genes prediction and its relationship with yield-related traits

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Received: 2 September 2021 / Accepted: 7 March 2022 / Published online: 29 March 2022

Abstract

Key message **In this first genetic study on assessing leaf thickness directly in cereals, major and environmentally stable QTL were detected in barley and candidate genes underlying a major locus were identified.**

Abstract Leaf thickness (LT) is an important characteristic affecting leaf functions which have been intensively studied. However, as LT has a small dimension in many plant species and technically difficult to measure, previous studies on this characteristic are often based on indirect estimations. In the first study of detecting QTL controlling LT by directly measuring the characteristic in barley, large and stable loci were detected from both field and glasshouse trials conducted in different cropping seasons by assessing a population of 201 recombinant inbred lines. Four loci (locating on chromosome arms 2H, 3H, 5H and 6H, respectively) were consistently detected for flag leaf thickness (FLT) in each of these trials. The one on 6H had the largest effect, with a maximum LOD 9.8 explaining up to 20.9% of phenotypic variance. FLT does not only show strong interactions with flag leaf width and flag leaf area but has also strong correlations with fertile tiller number, spike row types, kernel number per spike and heading date. Though with reduced efficiency, these loci were also detectable from assessing second last leaf of fully grown plants or even from assessing the third leaves of seedlings. Taking advantage of the high-quality genome assemblies for both parents of the mapping population used in this study, three candidate genes underlying the 6H QTL were predicted based on orthologous analysis. These results do not only broaden our understanding on genetic basis of LT and its relationship with other traits in cereal crops but also form the bases for cloning and functional analysis of genes regulating LT in barley.

Keywords Leaf thickness · QTL mapping · Gene prediction · Barley

Communicated by Maria von Korff.

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Introduction

Leaves are the most important organ in plant photosynthesis (Van Camp, 2005; White et al. 2016), and their characteristics also affect plant adaptations to different environments (Wright et al. 2004; Donovan et al. 2011). Plants with thicker leaves tend to contain higher chlorophyll, nitrogen and photosynthetic content per unit leaf area (Yin et al. 1999a; Murchie et al. 2002; Li et al. 2009). Strong relationships exist between leaf thickness (LT) and photosynthesis ability (Smith et al. 1998; Taiz and Zeiger 2006; Li et al. 2009; Tsukaya 2013), relative water content (Afzal et al. 2017) and yield potential of crop cultivars (Sexton et al. 1997; White and Montes-R 2005; Peng et al. 2008; Liu et al. 2014). Plants adapted to arid environments tend to have thicker leaves (Wright et al. 2004; Poorter et al. 2009). Not surprisingly, LT has been intensively studied in different species (e.g., Diaz et al. 2004; Vile et al. 2005; Li

et al. 2009; Tsukaya 2013; Coneva et al. 2017; Coneva and Chitwood 2018).

Due to its relatively small dimension, LT can be difficult to measure directly in some plant species. To overcome the difficulty, several surrogates have been used to estimate LT. These include specific leaf area (SLA, the ratio of leaf area to leaf dry mass), leaf dry matter content (LDMC, the ratio of leaf dry mass to saturated fresh mass = 1- leaf water content) and leaf mass per area (LMA, the ratio of leaf dry mass to leaf area) (Witkowski and Lamont 1991; Roderick et al. 1999; Poorter et al. 2009; Muir et al. 2014). With the use of these surrogates, QTL have been detected in various species including cereals. In barley, the numbers of QTL for SLA detected among different studies varied. In analysing QTL related to yield potential in spring barley, Yin et al. (1999a) detected loci for SLA on chromosomes 2H, 3H and 4H based on the evaluation of a population consisting of 94 recombinant lines (RILs). In a study on QTL affecting growth-related traits in wild barley (*Hordeum spontaneum*), Elberse et al. (2004) detected loci for SLA on chromosomes 3H and 4H based on assessments of an F3 population. In a recent publication studying traits related to seedling vigour in barley, Capo-chichi et al. (2021) detected as many as 26 loci for SLA based on an analysis of a RIL population, and these loci were distributed on each of the seven chromosomes.

Studies on LT based on direct measurements have been reported on several plant species in recent years. They included the studies on the natural variation of LT and its correlation with yield traits in rice (Liu et al. 2014), on the genetic architecture and molecular networks underlying LT in desert-adapted tomato (Coneva et al. 2017), on the influence of LT on canopy reflectance and physiological traits in cotton (Pauli et al. 2017) and the genetic and developmental basis for increased LT in *Arabidopsis* (Coneva and Chitwood 2018). As expected, available data showed that results from direct measurements do not always agree with those from indirect estimations (Coneva et al. 2017). However, genetic studies based on direct measurements of LT have not been reported in any cereal crop species yet. We, thus, made such an attempt and measured LT directly for QTL detection in a barley population consisting of 201 RILs. Following the successful detections of large-effect loci across different trials, we analysed candidate genes underlying a locus with the largest effect and assessed possible interactions between LT and other traits of agronomic importance. Results obtained from the study are reported in this publication.

Materials and methods

Plant materials

Results reported here were based on a population of recombinant inbred line (RILs)s. The population consisting of 201 F8 RILs was developed in an earlier study from a cross between Morex and AWCS276 (Zhou et al. 2021) using the single-seed descendent method based on the fast generation technique (Zheng et al. 2013). Morex is a six-row malting spring barley variety, and AWCS276 is a two-row wild barley with winter habit.

Phenotypic evaluation

Data on flag leaf (FL) and second last leaf (2LL) were collected from two field trials and two glasshouse trials. The two parents were assessed with the RIL population together in each of the field and glasshouse trials. In making sure that all lines could reach flowering stage, these trials were all conducted using vernalized seedlings. For vernalization, seeds were germinated in Petri dishes on two layers of filter paper saturated with water and placed in a 4 °C cold room with constant lighting for five weeks.

The field trials were conducted at CSIRO Gatton Research Station (27°33′*S*, 152°16′*E*), one in 2019 and the other in 2020 (designated as FD19 and FD20, respectively). Each of the field trials contained two replicates, each replicate with ten spaced planted (20-cm-apart) seedlings in a single row with 25 cm row spacing. The two glasshouse trials were conducted at Queensland Bioscience Precinct (QBP), one in 2019 and the other in 2020 (designated as GH19 and GH20, respectively). Settings for the glasshouse were: photoperiod 20 h, 25/18 (± 5) °C day/night temperature and 65/80 (± 5)% day/night relative humidity. Each of the glasshouse trials consisted of three replicates. Three plants, each in a separate 2.0 L pot with steam sterilized University of California mix C (UC mix) (50% sand and 50% peat v/v), were used in each of the replicates. A random block design was used for all the trials. Measurements of flag leaf thickness (FLT), flag leaf length (FLL), flag leaf width (FLW), flag leaf area (FLA), flag leaf length to width ratio (FLWR) and the second last leaf thickness (2LLT) were taken from the main tiller of each plant after anthesis.

As LT is sensitive to leaf water status, a standardised protocol described by Garnier et al. (2001) was applied on samples for rehydration. Briefly, leaf samples were collected at least 2–3 h after sunrise and 3–4 h before sunset and were immediately wrapped in moist paper bags and conserved in a cold box until return to the lab. Then, the

bags were placed into water and stored in a dark and cold room (4 °C) for at least 6 h before measurement. LT was measured by an electronic thickness gauge (SIDA, model SD-201) as the thickness in the middle section of the leaf on both sides as near the main midrib as possible. The leaf midrib was avoided, and average of two readings was used to represent the thickness of the leaf; leaf length (LL) was measured as the distance from the leaf ligule to tip; leaf width (LW) was measured as the width of the widest section of the leaf. Leaf area (LA) and leaf length to width ratio (LWR) are derivative characters, and their algorithms are LA = LW × LL × 0.75 (Spagnoletti Zeuli and Qualset 1990) and LWR = LL/LW (Zhang et al. 2015).

Heading date (HD) was recorded on the day on which approximately 50% of spikes emerged from main tillers in a trial. Spike row type (SRT) was determined by 2 or 6 rows. Data on kernel number per spike (KNPS), fertile tiller number (FTN) and thousand kernel weight (TKW) were collected from five plants in the middle section of each row from the field trials or each of the three plants from the glasshouse trials. TKW was based on the average of three replicated measurements of 300 randomly chosen kernels from the selected plants for each line used in the 2020 trails. Data on TKW from the 2019 trials were obtained from the previous study (Zhou et al. 2021).

To investigate if any similar loci can be detected from young seedlings, two trials were conducted in 2021 (designated as GH21a and GH21b, respectively) at QBP glasshouse with two replicates in each trial and each replicate contained seven seedlings. A random block design was used for both trials. Settings for the glasshouse were described above. Seeds with similar size were soaked in 70% ethanol for 30 s to sterilise and then washed two or three times with distilled water. Sterilized seeds were germinated in petri dishes on two layers of filter paper saturated with water under room temperature for 1–2 days. Seedlings with coleoptiles about 0.5 cm were planted into square punnets of a 56-well tray (Rite Grow Kwik Pots, Garden City Plastics, Australia) containing steam sterilized UC mix. Measurements of the 3rd leaf thickness (S3LT), length (S3LL), width (S3LW), area (S3LA) and length to width ratio (S3LWR) were taken from each of the seven seedlings when the collars of the 4 th leaf become visible on about 50% of the plants as described before. The average values from the seven seedlings in each replicate were used for further analysis.

Statistical analysis

The average values of five plants from the field trials and three plants from glasshouse trials for each line were employed in the subsequent analysis during 2019 to 2020, while the average value of seven plants from the seedling trials for each line was employed in 2021. The best linear unbiased prediction (BLUP) of target traits and the broad-sense heritability (H^2) were calculated using SAS V8.0 (SAS Institute, Cary, NC, USA; https://www.sas.com). To estimate random in statistics, the BLUP for the phenotypic values were calculated according to the model: $Y_i = X_i f + a_i + e_i$, where f = a vector of fixed effects, Xi = an incidence vector, e_i = the environmental deviation and a_i = the phenotypic value (Goddard 1992). H^2 for each trait was estimated as $H^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_{ge}/n + \sigma^2_e/nr)$, where σ^2_g is the genetic variance, σ^2_{ge} is the G × E variance, σ^2_e is the error, n is the number of environments, and r is the number of replicates. SPSS18.0 software (SPSS, Chicago, IL, USA) was used to perform normal distribution test, Student's t test ($P < 0.05$) and correlation analysis of phenotype values in different trials.

QTL analysis

A high-density genetic map of this population based on genotyping by sequencing (GBS) data was constructed according to the previous study (Zhou et al., 2021). The total length of the linkage map is about 1022.4 cM with an average distance of 0.7 cM. MapQTL 6.0 (Van Ooijen 2009) was used for QTL analysis. For each trial, a test of 1000 permutations was performed to identify the LOD threshold corresponding to a genome-wide false discovery rate of 5% ($P < 0.05$). Interval mapping was then used to identify QTL. A linkage map showing the QTL positions was drawn using MapChart (Voorrips 2002).

Identification of candidate genes underlying QTL for leaf thickness

Markers flanking QTL were used to delineate the physical intervals. Tag sequences in GBS dataset were used to blast on genome assemblies of barley pseudomolecules Morex (Mascher et al. 2017) to get physical positions. Coding sequence and protein sequences of predicted genes in the identified QTL regions were downloaded from ftp://ftp.ensemblgenomes.org/pub/release-44/plants/gff3/ hordeum_vulgare for Morex and NCGR wild barley database http://db.ncgr.ac.cn/wild_barley/ for AWCS276 (Liu et al. 2020). Gene sequences related to leaf size, leaf development, organ development and cell elongation were collected from rice and used to blast against the genome assembly of Morex. Variant calling of the candidate genes within the targeted interval and its functional annotation were carried out using Snippy v4.3.6 with default settings (https://github.com/tseemann/snippy), and the output from the Snippy analysis for each gene was integrated as a table using the tidyverse package in R v3.6 (Wickham 2019). For analysing the protein sequences, reciprocal best hits were identified with DIAMOND v2.0 (Buchfink et al. 2021). Only the genes

differing in sequences between the two parental genotypes were treated as candidates.

Results

Phenotypic data analysis and correlations

LT values of Morex measured from each of the three different leaves (flag and 2nd last leaves from fully grown plants and 3rd leaf from young seedlings) were significantly higher than those of AWCS276 in each of the trials conducted (Table 1). Transgressive segregation with normal distribution for each set of these values was detected based on the Shapiro–wilk test (Fig. 1, Fig. S1 and S2). The broad-sense heritability ranged from 0.68 to 0.86 for the three characteristics (Table 1). Significant and positive correlations were detected between FLT and 2LLT among different trials as well as S3LT. Correlations between trials were generally higher for FLT than for 2LLT (Table S1).

Similar correlations between LT and other traits were found between the measurements of FL and 2LL. LT obtained from these two leaves showed very strong correlation with FTN, SRT and KNPS as well as FLW. They also

Table 1 Phenotypic variation and heritability of leaf thickness for the parents and population assessed in different environments

#Trait	Trial	Parents		Population					
		Morex	AWCS276	Min	Max	Mean	SD	CV(%)	H^2
FLT(μm)	FD19	254.2	145.2	108.8	272.5	195.0	26.2	13.4	0.83
	FD20	236.8	136.9	120.6	266.8	199.0	26.5	13.3	
	GH19	270.9	130.5	108.5	295.0	191.3	28.2	14.8	0.87
	GH20	257.3	129.9	120.6	265.4	196.4	26.2	13.9	
2LLT(μm)	FD19	289.9	143.8	110.0	309.7	207.3	37.1	17.9	0.72
	FD20	284.9	124.4	118.2	328.0	190.4	30.9	16.2	
	GH19	279.2	125.3	118.9	306.5	206.7	36.1	17.5	0.75
	GH20	295.7	143.6	95.0	324.5	203.3	37.8	18.6	
S3LT(μm)	GH21a	233.3	196.1	150.2	288.6	221.2	23.8	10.7	0.68
	GH21b	239.4	185.6	150.7	266.7	215.8	22.2	10.3	

#*FLT* flag leaf thickness, *2LLT* the second last leaf thickness, *S3LT* 3rd leaf thickness from seedling, *SD* standard deviation, *CV* coefficient of variation, H^2 the broad-sense heritability

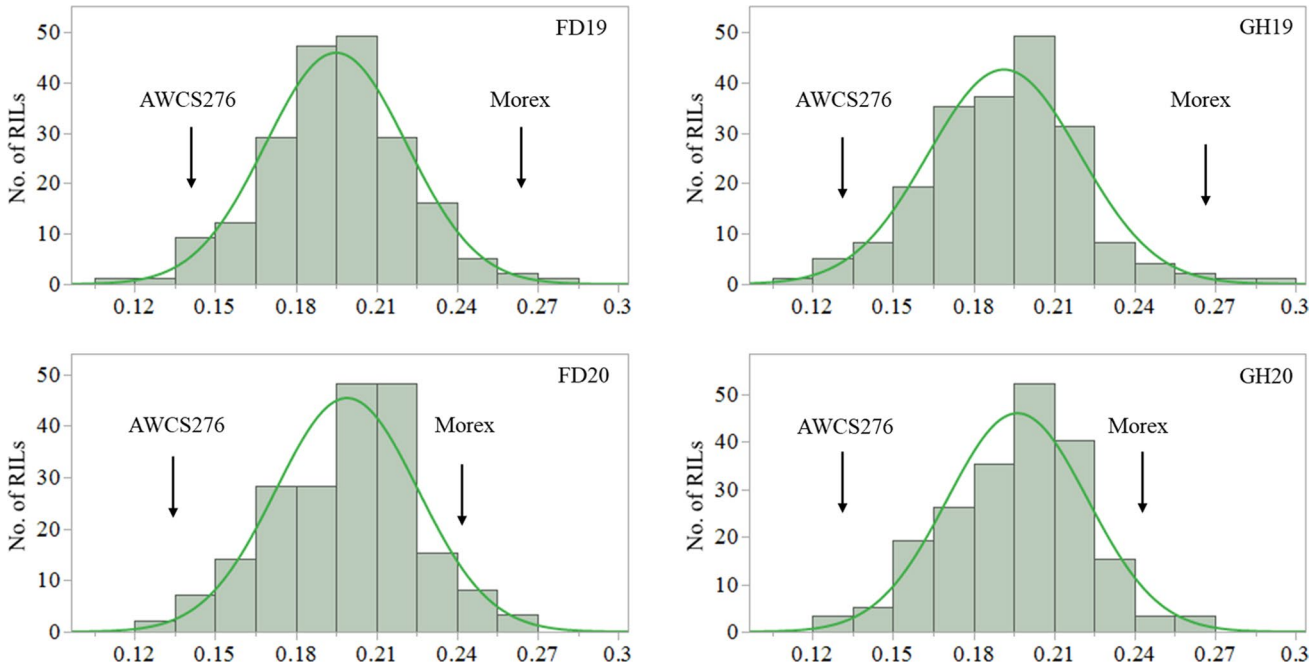

Fig. 1 Frequency distributions for flag leaf thickness (FLT) obtained from the populationof Morex/AWCS276 in different trials

Table 2 Coefficients of pairwise Pearson correlations between leaf thickness and other traits#

Group	Trait	FLT	2LLT	S3LT	FLL	FLW	FLA	FLWR	S3LL	S3LW	S3LA	S3LWR	TKW	KNPS	FTN	SRT	HD
LT traits	FLT	1.00															
	2LLT	0.94***	1.00														
	S3LT	0.25**	0.24**	1.00													
Other leaf traits	FLL	0.12	0.12	0.03	1.00												
	FLW	0.34***	0.30***	0.28***	0.46***	1.00											
	FLA	0.27**	0.24**	0.20**	0.82***	0.85***	1.00										
	FLWR	− 0.22**	− 0.23**	− 0.26**	0.22**	− 0.69***	− 0.25***	1.00									
	S3LL	0.16*	0.17*	0.56***	0.26***	0.06	0.19*	0.14	1.00								
	S3LW	0.22*	0.20**	0.68***	0.18*	0.44***	0.37***	− 0.33***	0.59***	1.00							
	S3LA	0.20**	0.21**	0.65***	0.26***	0.23**	0.29***	− 0.04	0.93***	0.83***	1.00						
	S3LWR	0.05	0.07	0.22**	0.15*	− 0.25***	− 0.06	0.42***	0.74***	− 0.07	0.46***	1.00					
Yield-related traits	TKW	− 0.05	− 0.04	0.08	0.20**	− 0.07	0.07	0.21**	0.28***	0.28***	0.31***	0.13	1.00				
	KNPS	0.29***	0.30***	0.01	0.02	0.33***	0.24***	− 0.29***	− 0.26***	− 0.12	− 0.22**	− 0.21**	− 0.63***	1.00			
	FTN	− 0.34***	− 0.36***	− 0.11	0.04	− 0.32***	− 0.18*	0.35***	0.17*	− 0.06	0.09	0.23**	0.25***	− 0.49***	1.00		
	SRT	0.29***	0.28***	0.03	− 0.05	0.34***	0.20	− 0.34***	− 0.29***	− 0.14	− 0.25**	− 0.23**	− 0.70***	0.95***	− 0.51***	1.00	
	HD	0.20**	0.23**	0.21**	0.10	0.09	0.13	0.03	0.18*	0.19*	0.19***	0.06	0.24***	− 0.02	− 0.06	− 0.02	1.00

#Correlations were calculated using BLUP datasets. *FLT* flag leaf thickness, *2LLT* the second last leaf thickness, *FLL* flag leaf length, *FLW* flag leaf width, *FLA* flag leaf area, *FLWR* flag leaf length and width ratio, S*3LT* 3rd leaf thickness from seedling, *S3LL* 3rd leaf length from seedling, *S3LW* 3rd leaf width from seedling, *S3LA* 3rd leaf area from seedling, *S3LWR* 3rd leaf length and width ratio from seedling, *TKW* thousand kernel weight, *KNPS* kernel number per spike, *FTN* fertile tiller number, *SRT* spike row type, *HD* heading date

Note: '*', '**' and '***' refer to significant correlations ($P < 0.05$, $P < 0.01$, $P < 0.001$)

showed strong correlation with HD, FLA and FLWR. However, LT measured from these leaves were not correlated with FLL and TKW (Table 2).

Correlations between LT and other traits are very different between the results obtained from S3LT and the other two leaves. The only similarity among measurements from the three different leaves is that LT was significantly correlated with HD. Apart from that, S3LT correlates strongly only with other leaf characteristics including those taken from either seedlings (S3LL, S3LW and S3LA) or fully grown plants (FLW, FLA and FLWR). Different from FLT and 2LLT, S3LT was not correlated with any of the yield-related traits including FTN, SRT and KNPS (Table 2).

QTL for leaf thickness

Permutation tests found that a LOD score of 2.9 was the threshold for the trials conducted in this study. Based on this threshold, a total of five QTL controlling FLT were detected across the first four trials. They were located on chromosomes 2H, 3H, 5H and 6H, respectively (Table 3). Four of these five QTL were consistently detected in each of the four trials as well as with the use of the BLUP values. Among them, the most significant QTL (designated as *Qflt.caf-6H*) was identified on chromosome 6H. This locus had a LOD value of 9.8 and explained up to 20.9% of phenotypic variance (Table 3; Fig. 2). Phenotypic

Table 3 QTL for FLT and 2LLT identified in the population of Morex/AWCS276#

Traits	Trials	QTL	Linkage map Interval (cM)	Physical map interval (Mb)	Left marker	Right marker	LOD	PVE (%)
FLT	FD19	2H	80.5–97.1	554–649	GBS_MST1178	GBS_MST1324	4.2	9.2
		3H	49.2–59.8	45–103	GBS_MST1659	GBS_MST2019	4.3	9.5
		6H	43.9–60.9	350–482	GBS_MST4486	GBS_MST4086	9.8	20.9
	GH19	2H	81.5–97.1	556–649	GBS_MST1202	GBS_MST1324	3.9	8.4
		3H	49.2–59.8	45–103	GBS_MST1659	GBS_MST2019	4.2	9.1
		5H.1	39.9–48.9	26–94	GBS_MST3835	GBS_MST3752	3.7	8.1
		6H	46.9–60.9	355–482	GBS_MST4482	GBS_MST4086	7.4	16.7
	FD20	2H	81.5–97.1	556–649	GBS_MST1202	GBS_MST1324	5.1	10.9
		3H	49.2–59.8	45–103	GBS_MST1659	GBS_MST2019	4.1	8.9
		5H.1	39.9–48.9	26–94	GBS_MST3835	GBS_MST3752	3.4	7.6
		5H.2	118.0–127.2	571–581	GBS_MST3270	GBS_MST3245	3.2	7.0
		6H	40.9–60.9	347–482	GBS_MST4489	GBS_MST3979	9.0	19.2
	GH20	2H	81.5–97.1	554–649	GBS_MST1202	GBS_MST1324	3.8	8.4
		3H	49.2–59.8	45–103	GBS_MST1659	GBS_MST2019	5.4	11.5
		5H.1	39.9–48.9	26–94	GBS_MST3835	GBS_MST3752	3.2	7.0
		5H.2	118.0–127.2	571–581	GBS_MST3270	GBS_MST3245	3.3	7.3
		6H	43.9–60.9	350–482	GBS_MST4486	GBS_MST4086	8.3	17.9
	BLUP	2H	81.5–97.1	556–649	GBS_MST1202	GBS_MST1324	4.6	10.1
		3H	49.2–59.8	45–103	GBS_MST1659	GBS_MST2019	4.2	9.1
		5H.1	39.9–48.9	26–94	GBS_MST3835	GBS_MST3752	3.3	7.3
		6H	43.9–60.9	350–482	GBS_MST4486	GBS_MST4086	9.5	20.6
2LLT	FD19	2H	81.5–97.1	554–649	GBS_MST1202	GBS_MST1324	3.6	8.0
		5H.1	39.9–48.9	26–94	GBS_MST3835	GBS_MST3752	3.7	8.2
		5H.2	118.0–127.2	571–581	GBS_MST3270	GBS_MST3245	4.2	9.2
		6H	43.9–60.9	350–482	GBS_MST4486	GBS_MST4086	6.2	11.4
	GH19	2H	81.5–97.1	554–649	GBS_MST1202	GBS_MST1324	3.8	8.5
		5H.2	118.0–127.2	571–581	GBS_MST3270	GBS_MST3245	4.4	9.6
		6H	31.6–80.9	310–482	GBS_MST4489	GBS_MST3979	5.3	11.7
	FD20	6H	31.6–80.9	310–482	GBS_MST4489	GBS_MST3979	6.7	15.0
	GH20	6H	42.4–53.6	356–413	GBS_MST4467	GBS_MST4142	7.0	15.5
	BLUP	2H	81.5–97.1	554–649	GBS_MST1202	GBS_MST1324	4.8	10.3
		3H	48.9–54.7	44–96	GBS_MST1635	GBS_MST1986	3.6	7.8
		6H	43.9–60.9	350–482	GBS_MST4486	GBS_MST4086	10.0	20.8

#*FLT* flag leaf thickness, *2LLT* the second last leaf thickness, *GH* glasshouse trial, *FD* field trial, *BLUP* best linear unbiased prediction, *cM* centimorgan

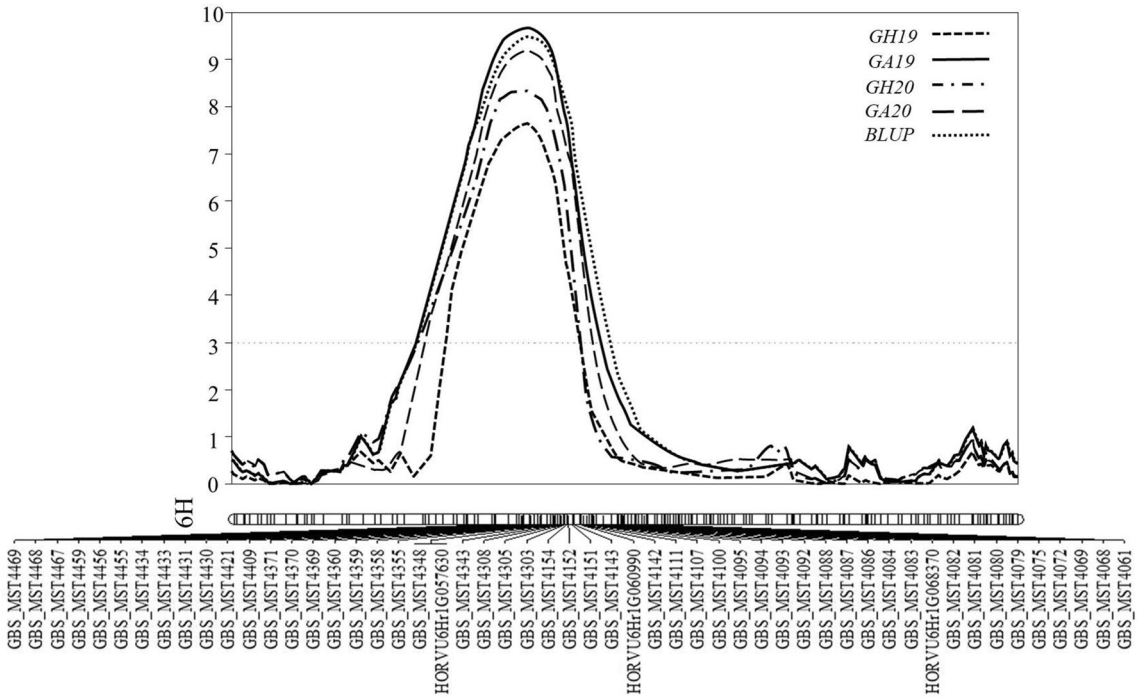

Fig. 2 QTL conferring flag leaf thickness detected on chromosome 6H with interval mapping from the population of Morex/AWCS276. The LOD values from each centimorgan of the chromosome were plotted against the chromosome, and the vertical dotted line indicates the average significant threshold (LOD = 2.9) derived from permutation test

variances explained by the other three QTL ranged from 7.3 to 11.5% with the LOD scores between 3.2 and 5.4 (Table 3).

For 2LLT, five QTL sharing similar positions with those for FLT were detected (Table 3). However, only the QTL on chromosome 6H was constantly detected in all the four trials. LOD values of the locus on 6H varied from 5.3 to 10.0, explaining between 11.4 and 20.8% of phenotypic variance. Of the remaining QTL, the two located on chromosomes 3H and 5H were only detected in one trial and the ones on chromosomes 2H and 5H in only two of the trials. Surprisingly, the locus on 3H was not detected from any of the trials but it was picked up with the use of BLUP values (Table 3).

To validate whether QTL detected from fully grown plants could also be found from seedlings, two additional trials were conducted. Three of the five loci detected by assessing FL and 2LL from fully grown plants were detected in these two seedling trials. However, none of them were detected from both seedling trials. Two of them, located on chromosomes 3H and 6H, were detected in one of the trials, while only the locus on chromosome 2H was detected in the other trial. When BLUP values were used, all three loci were detected (Fig. 3). Like the results obtained from measuring fully grown plants, the locus on chromosome 6H again gave the largest effect, explaining up to 9.7% phenotypic variance with a LOD value of 4.0 (Table S2).

QTL for other characteristics of flag leaf and yield-related traits and their relationship with those for leaf thickness

BLUP data were used to detect QTL for other characteristics of flag leaf and yield-related traits. QTL identified for these traits were detected on all but chromosome 4H (Fig. 3; Table S3). Three of the four QTL controlling LT co-located with some of these loci, and the exception was the one on chromosome 5H. Loci for FLW, FLWR, KNPS, SRT, FTN and TKW were detected in a similar region for the LT locus on chromosome 2H. Loci for FLW and FLWR were detected near the LT locus on chromosome 3. The LT locus on chromosome 5H located closely with a locus for HD but they do not overlap. The LT locus on chromosome 6H overlapped with those for FTN, TKW and FLL (Fig. 3).

Candidate genes underlying the major locus on chromosome 6H

As the QTL on chromosome 6H did not only show the largest effect but was also consistently detected, candidate genes underlying this locus were searched based on an orthologous analysis. Based on physical positions of the flanking markers, a total of 697 genes were detected in the QTL interval. Of these genes, 257 possessed sequence variants between the two parental genotypes. Sequence variants

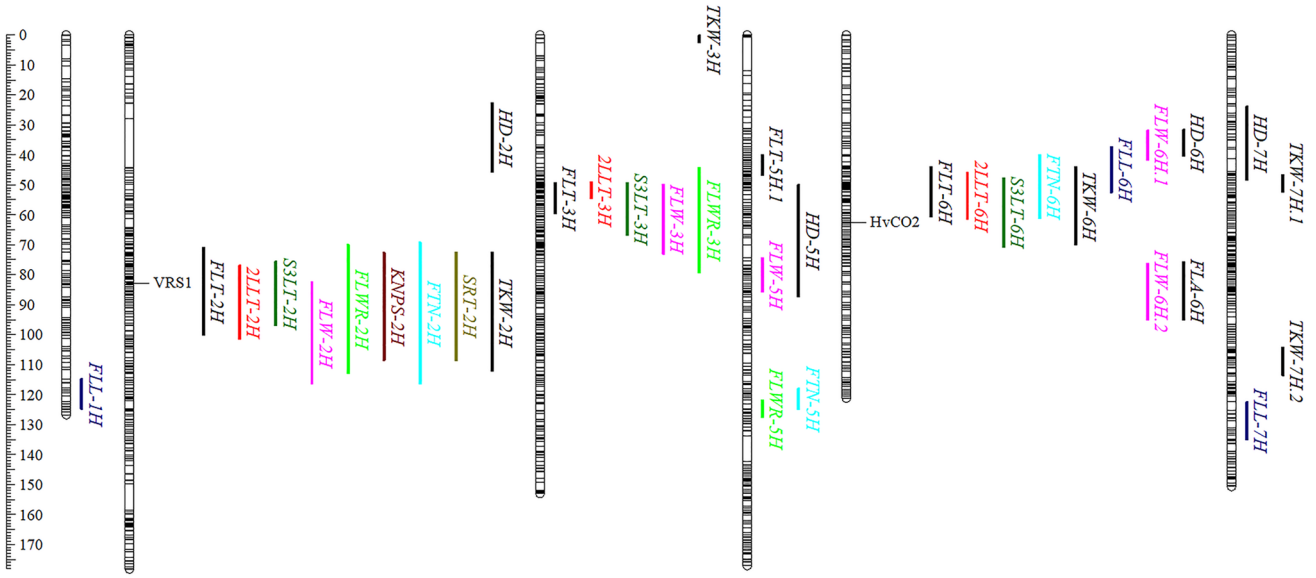

Fig. 3 QTL for leaf thickness, other flag leaf traits and yield-related traits identified in the population of Morex/AWCS276 using BLUP datasets. *FLT* flag leaf thickness, *2LLT* the second last leaf thickness, *S3LT* 3rd leaf thickness from seedling, *FLL* flag leaf length, *FLW* flag leaf width, *FLA* flag leaf area, *FLWR* flag leaf length and width ratio, *TKW* thousand kernel weight, *KNPS* kernel number per spike, *FTN* fertile tiller number, *SRT* spike row type, *HD* heading date

for 178 of these genes led to changes in protein functions (Table S4). Sequences for 161 genes related to leaf size, leaf development, organ development and cell elongation from rice were also identified (Table S5). Three candidate genes which may be related to the leaf thickness in the 6H interval were predicted, including *HORVU6Hr1G057630, HORVU6Hr1G060990 and HORVU6Hr1G068370*. They were orthologous to rice genes *OSPRR1*, *OsVPE3* and *OsGRF4*, respectively. Five SNPs in exons of *HORVU6Hr1G057630* were detected between Morex and AWCS276. Two of them were non-synonymous mutations, producing amino acid residue substitutions at positions 1219 (Threonine → Alanine) and 1994 (Serine → Proline), respectively. Four SNPs were identified in exons of *HORVU6Hr1G060990.* Two of them were non-synonymous mutations (T → G transversion at position 95 and A → G transition at position 99, respectively) producing amino acid residue substitutions at positions 32 (Isoleucine → Methionine) and 34 (Arginine → Glycine). The other two were synonymous mutations (G → A transition at position 122 and C → T transition at position 3933, respectively). Only one SNP (G → T) was detected between the two parental genotypes for *HORVU6Hr1G068370*, producing an amino acid substitution (Aspartic acid → Tyrosine) (Table 4). The estimated genetic distances between

Table 4 Candidate genes and their orthologs underlying the locus controlling leaf thickness on chromosome 6H

Barley Orthologs	Physical position (bp)	Rice gene	Identity	SNP[#]	Amino acids[#]
HORVU6Hr1G057630	chr6H: 374,866,561–374,869,556	OsPRR1	76.1	T/C(108)	T/A(215)
				A/G(1219)	S/P(434)
				C/T(1705)	
				G/T(1690)	
				T/C(1994)	
HORVU6Hr1G060990	chr6H: 407,203,000–407,209,104	*OsVPE3*	84.0	T/G(95)	I/M(32)
				A/G(99)	R/G(34)
	chr6H: 473,135,773–473,139,363			G/A(122)	
				C/T(3933)	
HORVU6Hr1G068370		*OsGRF4*	80.7	G/T(1111)	D/Y(371)

[#]The numbers in brackets represent the positions of differences in nucleotide or amino acid sequences between Morex and AWCS276 relative to initiation codons

these three genes to the peak of the QTL are 1.3 cM, 4.7 cM and 6.9 cM, respectively.

Discussion

The importance of LT in plant adaptation and crop production has been well documented. Due to the limited dimensions of LT, previous genetic studies on this characteristic in cereals have all been based on indirect estimations. In the study reported here, we demonstrated for the first time that targeting LT directly in the genetic studies is now feasible. By assessing the RIL population consisting of 201 lines, we did not only detect QTL for LT in barley but also showed that QTL detected for FLT are larger and more stable compared with those for other leaf characteristics including length, width and area (Table S3). Although with reduced magnitudes, QTL for LT with similar locations were also detected from measuring 2LL after anthesis as well as from measuring the 3rd leaves of developing plants. These results indicate that the thicknesses of different leaves in a plant are correlated, and it likely has a simpler inheritance than other leaf characteristics. The importance of LT is shown by its strong correlation with HD, FTN, SRT, KL and KNPS. Taken advantage of the high-quality genome assemblies for both parents of the mapping population used in this study, we also identified candidate genes underlying the most significant QTL on chromosome 6H based on the orthologous analysis.

In addition to the major locus on chromosome 6H, several other loci detected from fully grown plants were also detected from measuring S3L of seedlings especially with the use of the BLUP values. However, the magnitudes of the loci detected from seedlings were all significantly smaller. Importantly, the strong correlations between LT and yield-related traits obtained from measuring leaves of fully grown plants were not detected from measuring seedlings. One of the possible reasons for these differences could be caused by the likelihood that HD could have a larger effect on the third leaf in developing seedlings compared with that on leaves of fully grown plants. Another likely contributing factor is that leaves from developing plants are more difficult to measure accurately, reflected by the fact that higher inheritance for the various leaf characteristics was inevitably detected from FL measurements. Our results suggested that, where possible, data from FL should be collected when studying on LT.

In mapping loci for traits related to seedling vigour, Capochichi et al. (2021) detected multiple QTL for SLA on each of the seven chromosomes in barley. Of them, six were on chromosome 6H. It is likely that one of these six loci shares a similar location with the one on 6HL detected in this study. However, none of the six loci reported earlier comes close to the latter in regarding to either the magnitude or stability. Loci for SLA have been reported previously based on assessing either plants after anthesis (Yin et al. 1999a, b) or young seedlings (Elberse et al. 2004; Poorter et al. 2005). However, loci on chromosome 6H were not detected in any of these studies. The different results between the study reported here and those earlier ones could be due to direct vs indirect measurements as found in the study on desert-adapted tomato (Coneva et al. 2017). As only loci segregating in a population can be detected, another likely reason for the different results is due to the different materials used among these studies.

High-quality genome assemblies are available for both parental genotypes of the mapping population used in this study (Liu et al. 2020), which made it easier to identify candidate genes targeting a given region based on orthologous analysis (Zhou et al. 2021). In regarding to the gene underlying the LT locus on chromosome arm 6HL, anyone that locates in the targeted interval and differs in sequences between the two parents (Table S4) can be a candidate. Based on the functions of their orthologs, three of the genes can be treated as primary targets. One of them, *HORVU6Hr1G057630*, is orthologous to *OSPRR1* in rice which is involved in tiller bud outgrowth (Strable 2020). The orthologs of this gene are involved in photoperiodic flowering response in barley and *Arabidopsis* (Matsushika et al. 2000; Pruneda-Paz et al. 2009; He et al. 2019). The second gene *HORVU6Hr1G060990* is homologous with *OsVPE3* in rice. It has been reported that suppression of this gene could decrease the leaf width and guard cell length (Lu et al. 2016). The ortholog for the third gene *HORVU6Hr1G068370* is *OsGRF4* in rice, and it is a positive regulator of genes that promote cell proliferation (Hu et al. 2015; Sun et al. 2016) and activates transcription of expansin promoters in protoplasts leading to a potential function in cell expansion (Liebsch and Palatnik 2020). Orthologs of this gene have also been found to be involved in multiple development processes in various species (Liebsch and Palatnik 2020). Importantly, these three genes all contain non-synonymous variations in their exons between the two parental genotypes which lead to amino acid substitutions (Table 4).

Strong correlations between LT and several other traits were detected and three of the four QTL for LT detected in this study overlapped with loci for other traits including SRT, HD and FLW (Table 2; Fig. 3). Previous studies showed that LMA was significantly lower in six-rowed genotypes than in two-rowed genotypes in barley (Alqudah and Schnurbusch 2015). The row-type gene *VRS1* was known to affect leaf width (Thirulogachandar et al. 2017), and it was located within the region of the LT QTL on the chromosome 2H (Fig. 3). It has also been reported that genes influencing flowering time could affect leaf size (Digel et al. 2016). In the study reported here, a flowering promotor (*HvCO2*) located near the LT QTL on chromosome 6H (Fig. 3) (Wang

et al. 2010). There is no evidence showing that *HvCO2* affects leaf characteristics, but the gene interacts with *VRN-H2* and *Ppd-H1* (Campoli et al. 2014; Mulki et al. 2016), thus, may indirectly affect leaf characteristics. However, it is well known that QTL mapping based on assessing segregation populations only provides limited resolutions (Paterson et al. 1988), thus it cannot be effectively used to determine whether genes controlling different traits in a similar chromosomal region are controlled by closely linked genes or by the same gene(s) with pleiotropic effects. Near isogenic lines (NILs) have been effectively used to study the effect of a given locus for different traits in various plant species (Liu et al. 2010; Yan et al. 2011; Ma et al. 2012; Habib et al. 2016; Gao et al. 2019; Chen et al. 2021). With the adoption of techniques in rapidly generating materials with high-level of homozygosity (Zheng et al. 2013; Liu et al. 2016; Yan et al., 2017Wanga et al. 2021), generating NILs for a given locus in many plant species is not a time-consuming process anymore. The size and stability of the loci detected for LT in this study suggest that developing NILs for some of these loci can be straightforward. As only two isolines need to be compared, effects of a given LT locus in multiple genetic backgrounds can be conveniently and accurately assessed in different environments once a few sets of NILs become available.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00122-022-04076-1.

Acknowledgements The authors wish to thank Caritta Eliasson for her technical supports.

Author contribution statement JJ conceived the study. CL, ZZ and HH designed the experiments. ZZ, HH, SG, HZ, WL, and UK conducted the experiments, collected, and analysed data. ZZ, CL and HH prepared the first draft of the manuscript. All authors read and approved the final manuscript.

Funding The funding was provided by CSIRO (Grant No. R-10191-01), and the Key Scientific and Technological Research Project of Henan Province (Grant No. 192102110030).

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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