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Full Length Article

Mapping Freezing Tolerance Quantitative Trait Loci in Bread Wheat (*Triticum aestivum*) using a Doubled Haploid Population

Meng Zhou[1†], Yong Zhao[1†], Xuefang Yang[1], Guishun Chen[1], Ke Xu[1], Baohua Liu[2], Shuhua Zhang[1], Jichun Tian[3] and Xueju Yang[1*]

[1]*Huabei Key Laboratory of Crop Germplasm, Hebei Agricultural University, Baoding 071000, Hebei, China*
[2]*Handan Academy of Agricultural Sciences, 056001, China*
[3]*Shandong Agricultural University, Tai'an 271018, China*
[*]For correspondence: shmyxj@163.com
†These authors contributed equally to this work

Abstract

Exposure to cold conditions is a major abiotic stress affecting crop growth and productivity. Here, quantitative trait loci (QTLs) for antioxidant enzyme activity, ratio of free water to bound water (RFB), and malondialdehyde (MDA) content in six environments were identified using a doubled haploid wheat population derived from a cross between Huapei-3 and Yumai-57 wheat cultivars. These QTLs were analyed using an inclusive composite interval mapping approach. Consequently, 35 additive QTLs for specific traits were identified, including *QRFB-2A-1*, which was detected in three environments. Moreover, *QPOD-2D-2* and *QPOD-1B*, which were associated with peroxidase activity, were detected in two environments, with *QPOD-2D-2* responsible for more than 10% of the phenotypic variation. Additionally, *QCAT-6D-2* on chromosome 6D explained 16.80% of the phenotypic variation, with the positive effect contributed by Huapei-3. Furthermore, eight additive QTLs for MDA content were detected on chromosomes 1A, 2B, 3A, 4B, 5B.2, 6B and 7B, including *QMDA-6B* between markers *Xcfa2257* and *Xcfd48*, which was responsible for 20.21% of the phenotypic variation. By marker-assisted selection, the major QTLs detected in this study may be valuable for improving freezing tolerance in wheat.

Keywords: QTL; Ratio of free water to bound water; Antioxidant enzyme activity; Malondialdehyde

Introduction

Common wheat (*Triticum aestivum* L.) makes an important contribution to worldwide food security as it represents a staple cereal crop that feeds almost 35–40% of the global population (Li *et al*., 2010). Exposure to low temperatures at various growth stages can limit wheat grain yields. However, complex biochemical and physiological processes induce freezing tolerance in wheat plants (Ruelland and Zachowski, 2010; Jin *et al*., 2017). Thus, a thorough characterization of the physiological and molecular mechanisms underlying freezing tolerance is necessary for breeding new high-yielding wheat cultivars capable of tolerating cold stress conditions.

Previous studies revealed that the phenotypic variability of many complex traits is controlled by multiple quantitative trait loci (QTLs). Because they are useful for elucidating the genetic basis of diverse plant processes, QTLs have been used to investigate freezing tolerance in various plant species, including rice (Tai and Andaya, 2006; Shimono *et al*., 2012; Hur *et al*., 2016; Huang *et al*., 2017), tomato (Foolad *et al*., 2003, 2010; Wang *et al*., 2016), barley (Stockinger *et al*., 2006), maize (Engelen *et al*., 2003) and wheat (Båga *et al*., 2007; Iehisa *et al*., 2013; Gorji *et al*., 2014). Over the past several years, studies have confirmed that freezing tolerance is mediated by multiple genes, with strong interactions between the genes and environmental conditions. Moreover, there are reports describing freezing tolerance QTLs in wheat. For example, Fridovich (1978) identified relevant QTLs on chromosomes 4D, 5D, and 7A, while Liu (2005) identified several freezing tolerance QTLs on chromosomes 1A, 2A, 4B and 5A. Båga *et al.* (2007) uncovered a major QTL on chromosome 5A and a weaker QTL on chromosome 1D in a doubled haploid (DH) population. Ju (2012) detected 51 additive QTLs, with most chromosomes carrying at least one QTL, and Gorji *et al.* (2014) identified 27 QTLs on chromosomes 4A, 5B, and 5D. However, there are no reports regarding the analysis of freezing tolerance QTLs based on antioxidant enzyme activity (AEA), malondialdehyde (MDA) content, or the ratio of free water to bound water (RFB) in different environments.

In this study, we used a DH population derived from a cross between Huapei-3 (HP3) and Yumai-57 (YM57)

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wheat cultivars to analyze wheat traits associated with freezing tolerance. Our data may be useful for elucidating the genetic basis of freezing tolerance and may be relevant for wheat breeding programs.

Materials and Methods

Plant Materials

For the QTL analyses, we constructed a population of 168 DH lines derived from across between Chinese wheat cultivars HP3 and YM57, which were released in Henan province in 2006 (Hai and Kang, 2007) and nationally in 2003 (Guo *et al*., 2004), respectively. The freezing tolerance of HP3 plants (*i.e.*, weak spring wheat cultivar) differed from that of YM57 plants (*i.e*., semi-winter wheat cultivar).

Experimental Design

This study was conducted according to a randomized complete block design in Baoding (38.85°N, 115.5°E), Xingtai (37.13°N, 114.68°E), and Cangzhou (38.58°N, 116.82°E), China. Plants were grown in 3 row plots, with 2 m rows separated by 25 cm in the early October. We collected leaves that appeared to be growing normally during the over-wintering stage afer 7 d the average diurnal temperature <0°C in 2012 and 2013, and measured the free water (FW) and bound water (BW) contents to calculate the RFB. Additionally, we measured the AEA and MDA content of the normally growing leaves collected during the over-wintering stage after 7 d the average diurnal temperature <0°C in 2014 and 2015. The exact average temperature is shown in Fig. S1.

Trait Measurements

The traits were measured in 2012 and 2013 included total water (TW), free water (FW), and bound water (BW) contents. We used a hole-punch to obtain uniformly sized leaf discs. Three replicates of 50 leaves per DH line and their parents were weighed (W_1), dried to a constant weight in a drying oven set at 85°C, and then reweighed (W_2). The TW content was calculated as follows: $TW = (W_1 - W_2)/W_1$.

The FW content was determined according to a published method involving an Abbe refractometer (Pan, 2004). Briefly, we weighed an empty weighing bottle (W_3), after which 50 leaf discs obtained using a hole-punch were added and the bottle was weighed again (W_4). Finally, 5.0 mL sucrose (C_1) was added and the bottle was weighed again (W_5). The weighing bottles were incubated in darkness for 6 h, with gentle shaking, after which we determined the sucrose concentration (C_2) using the Abbe refractometer. The FW content was calculated as follows:

$$FW\% = \frac{(W_5 - W_4) \times (C_1 - C_2)}{(W_4 - W_3) \times C_2} \times 100$$

The TW and FW contents (%) were used to calculate the BW content according to the following equation: BW = TW − FW. The RFB was then calculated.

We measured the activity of specific antioxidant enzymes, namely superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as the MDA content in 2014 and 2015. Leaf samples (approximately 0.5 g) from the DH lines and their parents were ground to a fine powder in a mortar containing 5.0 mL phosphate buffer (pH 7.8). All processes were completed on ice. Samples were centrifuged at 2500 × g for 10 min at 4°C. The supernatants were collected and used to measure SOD, POD, and CAT activity as well as MDA content. Superoxide dismutase activity was assayed using a slightly modified published method (Zhang and Chen, 2008). Briefly, 50 μL 320 μM lactochrome and 50 μL supernatant were added to 4 mL reaction solution (*i.e*., phosphate buffer: methionine: nitro-blue tetrazolium: EDTA=4:1:2:1). One unit of SOD activity was defined as the amount of enzyme that decreased the reduction of NBT by 50% at 560 nm. Meanwhile, POD activity was assayed using a slightly modified published method (Chen and Tan, 2007). Briefly, 3 mL reaction solution (phosphate buffer: 20 m*M* guaiacol: 40 m*M* H_2O_2 = 291:5:2) was placed in a 50 mL tube containing 20 µL supernatant. After adding 20 μL 20% chloroacetic acid, we recorded the absorbance at 470 nm. Catalase activity was assayed using a slightly modified published method (Gao, 2006). We added 100 μL supernatant to 2.5 mL reaction solution (0.1 *M* H_2O_2: 0.1 *M* phosphate buffer (pH 7.0) = 1:4) and then recorded the absorbance at 240 nm at 1 min intervals. Additionally, the MDA content was measured using a slightly modified published method (Hao, 2004). Briefly, 1 mL supernatant was mixed with 2 mL 0.6% TBA and then incubated at 100°C for 15 min. Samples were cooled and centrifuged at 2500 × g for 5 min. We then recorded the absorbance at 600, 532, and 450 nm.

Analyses of Data and Quantitative Trait Loci

Data were analyzed using SPSS Statistics version 18.0 (SPSS Inc., Chicago, IL, USA). All data were initially assessed to ensure they were normally distributed. The linkage map for the DH population, which was established by Zhang *et al*. (2008), was used for the QTL analyses. The map comprised 357 markers (*i.e*., simple sequence repeats, expressed sequence tags, inter-simple sequence repeat loci, and HMW-GS loci) that were distributed on all wheat chromosomes, covering 2780.9 cM, with markers separated by an average distance of 7.79 cM. These markers formed 24 linkage groups over 21 chromosomes.

The IciMapping 3.3 program was used to map the QTLs. We also applied an inclusive composite interval mapping method with a forward–backward stepwise approach and a scanning interval of 10 cM. To detect a significant QTL for each trait–treatment combination, the

Table 1: Details regarding the ratio of free water to bound water (RFB), antioxidant enzyme activity, and MDA content in different environments

Trait	Environment[a]	Parent		DH population			
		HP3	YM57	Mean	CV%	Skewness	Kurtosis
RFB	E1	2.37	1.62	1.73	23.77	0.69	0.80
	E2	2.07	1.50	1.87	27.66	0.62	0.70
	E3	1.89	1.35	1.84	31.14	0.48	-0.66
	E4	3.93	3.27	3.77	12.16	0.49	0.21
	E5	3.65	3.26	3.58	15.44	0.38	0.49
	E6	3.85	2.28	3.23	33.39	0.85	0.37
SOD	E7	30.60	41.73	33.62	28.71	0.04	-0.21
(OD/g·fw)	E8	19.00	29.40	21.20	29.41	0.70	0.89
	E9	24.13	40.27	17.35	66.68	0.73	0.01
	E10	25.93	36.07	35.29	14.28	-0.74	0.91
	E11	19.27	29.93	26.75	22.63	0.78	-0.28
	E12	45.73	56.07	47.96	12.27	0.04	-0.18
POD	E7	94.33	119.00	105.59	22.90	0.22	0.61
(OD/g·fw)	E8	160.83	175.83	166.04	18.41	0.09	0.18
	E9	208.67	220.50	159.11	25.97	0.33	0.61
	E10	64.83	98.17	73.83	25.43	0.50	-0.25
	E11	57.33	85.17	78.73	25.72	0.78	0.46
	E12	140.50	154.83	144.10	42.54	0.79	-0.25
CAT	E7	16.33	20.37	12.66	15.41	0.09	0.54
($\triangle$A240·g^{-1}fw·min^{-1})	E8	10.57	16.53	10.25	28.55	0.47	-0.54
	E9	10.77	18.40	12.58	18.84	-0.05	0.01
	E10	7.07	9.67	8.52	17.06	0.34	0.92
	E11	8.17	10.00	8.57	12.45	0.63	0.87
	E12	4.70	8.50	6.22	25.64	0.48	-0.43
MDA	E7	23.60	18.30	18.80	18.62	0.36	-0.06
($\times10^{-3}$μmol·g^{-1}fw)	E8	23.00	17.70	20.40	28.92	0.79	0.76
	E9	33.80	17.60	20.10	37.81	0.64	0.21
	E10	12.20	8.10	11.30	19.82	0.63	0.96
	E11	16.00	12.40	11.20	15.73	0.31	0.12
	E12	16.70	12.50	17.50	37.14	0.97	0.33

[a] This study was completed in the following environments: E1, Cangzhou in 2012; E2, Xingtai in 2012; E3, Baoding in 2012; E4, Cangzhou in 2013; E5, Xingtai in 2013; E6, Baoding in 2013; E7, Cangzhou in 2014; E8, Xingtai in 2014; E9, Baoding in 2014; E10, Cangzhou in 2015; E11, Xingtai in 2015; E12, Baoding in 2015

Table 2: Correlations among the ratio of free water to bound water (RFB), antioxidant enzyme activity, and malondialdehyde (MDA) content

Variables	RFB[a]	SOD	POD	CAT
SOD	0.505			
POD	-0.703	0.281*		
CAT	-0.789**	-0.685	0.217*	
MDA	0.913**	-0.273	-0.895**	-0.521

* Significant at $p < 0.05$; ** Significant at $p < 0.01$

[a] Average value in six environments

thresholds were defined by 1000 permutations at $p \leq 0.05$ and a logarithm of odds (LOD) ≥ 2.5 (Churchill and Doerge, 1994).

Results

Phenotypic Variation

The results of the analysis of AEA, MDA content, and the RFB in the DH lines in six environments are provided in Table 1. The values for the parents were obviously different from the values for the DH lines for all examined traits. Additionally, the AEA values were higher for YM57 than for HP3 in all environments and MDA levels and the RFB were higher for HP3than YM57, which suggested that YM57 is better able to adjust to cold conditions than HP3. Moreover, the mean AEA, MDA content, and RFB values for the DH lines were between those of the parents. Our data were consistent with a significant transgressive segregation and polygenic inheritance, suggesting both parents have favorable alleles at different loci and the populations were suitable for QTL analyses.

Correlations among the Investigated Traits

Correlations among all traits were analyzed (Table 2). The

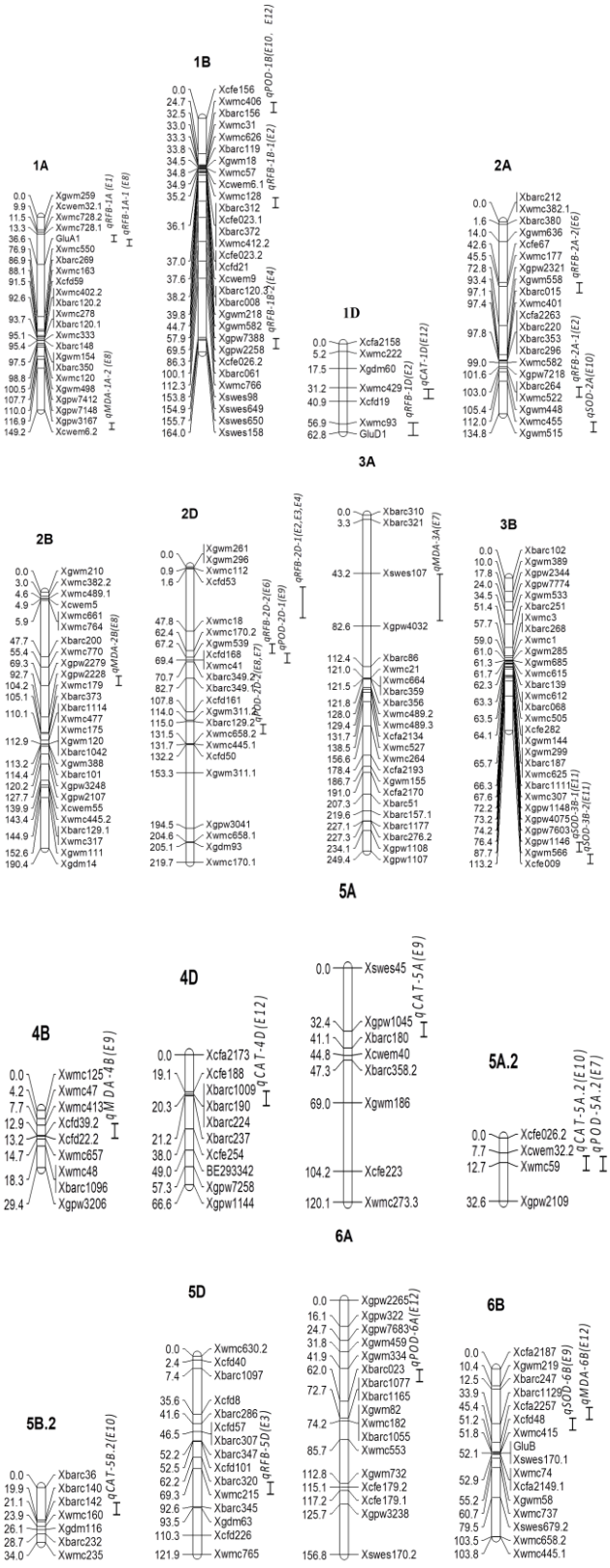

Fig. 1: Continued

RFB was positively correlated with MDA content ($p < 0.01$),

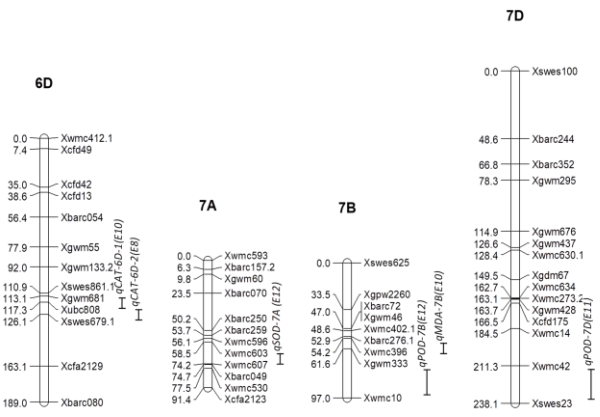

Fig. 1: Chromosomal positions of quantitative trait loci for antioxidant enzyme activity, malondialdehyde content, and the ratio of free water to bound water in wheat

negatively correlated with CAT activity ($p < 0.01$). Additionally, POD activity was correlated with SOD and CAT activities as well as with MDA content ($p < 0.05$ or $p < 0.01$).

Analysis of Quantitative Trait Loci

35 additive QTLs for five traits (RFB, SOD, CAT, POD, MDA) were detected across all chromosomes except for chromosomes 3D, 4A, 5B and 7B.2 (Table 3 and Fig. 1). Of these QTLs, nine were for the RFB, five were for SOD activity, seven were for POD activity, six were for CAT activity, and eight were for MDA content. The phenotypic variance explained by a single QTL ranged from 2.75–20.21%. Moreover, 16 QTLs explained more than 10% of the phenotypic variation, including 20.21% of the variation in MDA levels.

The nine additive QTLs for the RFB were mapped to chromosomes 1A, 1B, 1D, 2A, 2D and 5D. These QTLs were responsible for 5.06–18.98% of the phenotypic variation. Our analysis revealed that *QRFB-1D*, which was located between markers *Xwmc93* and *GluD1*, explained 18.98% of the phenotypic variation. Additionally, the alleles at the major QTLs (*QRFB-1D*, *QRFB-2D-1*, and *QRFB-5D*) associated with increased RFB values were inherited from YM57. We also detected *QRFB-2D-1* within the *Xcfd53–Xwmc18* marker interval in three environments.

Five additive QTLs for SOD activity were localized to chromosomes 2A, 3B, 6B and 7A. These QTLs explained 8.35–19.13% of the phenotypic variation observed in this study. *QSOD-3B-2* and *QSOD-7A* within the marker intervals *Xgwm566–Xcfe009* and *Xwmc603–Xwmc607* explained 8.49 and 9.31% of the phenotypic variation, respectively, and were derived from HP3 alleles. In contrast, the other three QTLs *QSOD-6B*, *QSOD-3B-1*, *QSOD-2A*, which explained 8.35–19.13% of the phenotypic variance was derived from YM57 allele.

Table 3: Summary of the additive quantitative trait loci for the ratio of free water to bound water (RFB), antioxidant enzyme activity, and malondialdehyde (MDA) content

Traits	Environment [a]	QTLs	Marker interval	LOD score	Additive (%) [b]	Var. (%)
RFB	E2	*qRFB-1B-1*	*Xwmc128 - Xbarc312*	3.31	-0.29	7.21
	E2	*qRFB-1D*	*Xwmc93 -GluD1*	8.28	-0.46	18.98
	E2	*qRFB-2A-1*	*Xbarc264 -Xwmc522*	4.05	-0.31	8.67
	E5	*qRFB-2D-1*	*Xcfd53 - Xwmc18*	3.27	-0.13	10.61
	E6	*qRFB-2A-2*	*Xgwm558- Xbarc015*	2.90	0.12	5.06
	E6	*qRFB-2D-2*	*Xgwm539 -Xcfd168*	2.77	-0.15	9.16
	E3	*qRFB-2D-1*	*Xcfd53-Xwmc18*	5.28	-0.18	11.59
	E3	*qRFB-5D*	*Xbarc320 - Xwmc215*	3.77	-0.29	12.38
	E1	*qRFB-1A*	*Xwmc728.1 –GluA1*	3.32	0.22	12.31
	E4	*qRFB-1B-2*	*Xgpw7388 -Xgpw2258*	2.53	0.17	8.27
	E4	*qRFB-2D-1*	*Xcfd53 - Xwmc18*	2.60	-0.17	9.10
SOD	E11	*QSOD-3B-1*	*Xgpw1146-Xgwm566*	3.13	-1.80	8.35
	E11	*QSOD-3B-2*	*Xgwm566-Xcfe009*	2.87	1.76	8.49
	E10	*QSOD-2A*	*Xwmc455-Xgwm515*	4.06	-1.71	11.80
	E9	*QSOD-6B*	*Xcfd48-Xwmc415*	4.23	-10.15	19.13
	E12	*QSOD-7A*	*Xwmc603-Xwmc607*	2.67	1.80	9.31
POD	E12	*QPOD-1B*	*Xwmc406-Xbarc156*	3.66	18.46	9.10
	E9	*QPOD-2D-1*	*Xcfd168-Xwmc41*	3.02	-11.90	8.46
	E12	*QPOD-6A*	*Xbarc023-Xbarc1077*	3.74	19.53	10.19
	E12	*QPOD-7B*	*Xgwm333-Xwmc10*	2.54	-17.01	6.87
	E10	*QPOD-1B*	*Xwmc406-Xbarc156*	2.62	5.15	7.55
	E7	*QPOD-2D-2*	*Xbarc129.2-Xwmc658.2*	4.29	-7.93	10.58
	E7	*QPOD-5A.2*	*Xcwem32.2-Xwmc59*	4.21	8.13	7.97
	E8	*QPOD-2D-2*	*Xbarc129.2-Xwmc658.2*	2.87	-8.70	10.96
	E11	*QPOD-7D*	*Xwmc42-Xswes23*	3.61	-9.28	12.18
CAT	E12	*QCAT-1D*	*Xwmc429-Xcfd19*	2.83	-0.50	9.30
	E12	*QCAT-4D*	*Xbarc1009-Xbarc190*	2.65	-0.65	7.40
	E9	*QCAT-5A*	*Xgpw1045-Xbarc180*	2.62	0.72	7.50
	E10	*QCAT-5A.2*	*Xcwem32.2-Xwmc59*	3.99	0.49	11.33
	E10	*QCAT-6D-1*	*Xgwm681-Xubc808*	2.59	-0.86	7.56
	E8	*QCAT-6D-2*	*Xubc808-Xswes679.1*	4.14	3.83	16.80
MDA	E9	*QMDA-4B*	*Xcfd39.2-Xcfd22.2*	2.75	0.2	2.75
	E12	*QMDA-6B*	*Xcfa2257-Xcfd48*	3.00	-0.6	20.21
	E7	*QMDA-3A*	*Xswes107-Xgpw4032*	2.51	0.1	8.96
	E10	*QMDA-5B.2*	*Xbarc142-Xwmc160*	3.68	0.1	9.61
	E10	*QMDA-7B*	*Xbarc276.1-Xwmc396*	2.79	-0.6	7.49
	E8	*QMDA-1A-1*	*GluA1-Xwmc550*	6.34	0.13	13.06
	E8	*QMDA-1A-2*	*Xgpw3167-Xcwem6.2*	2.56	-0.2	6.35
	E8	*QMDA-2B*	*Xgpw2228-Xwmc179*	4.34	0.2	11.66

[a] Environments are the same as those listed in the Table 1 footnote; [b] Positive additive effects (%) are associated with increased effects from Huapei-3 alleles, while negative additive effects (%) are associated with increased effects from Yumai-57 alleles

Seven additive QTLs associated with POD activity were detected on chromosomes 1B, 2D, 5A, 6A, and 7D. These QTLs were responsible for 6.87–12.18% of the phenotypic variation. *QPOD-1B*, *QPOD-5A.2*, and *QPOD-6A* were mapped in the marker intervals *Xwmc406–Xbarc156*, *Xcwem32.2–Xwmc59*, and *Xbarc023–Xbarc1077* on chromosomes 1, 5, and 6, respectively, with the positive effects provided by HP3 alleles. Additionally, the highest LOD score (4.29) was calculated for *QPOD-2D-2*, which accounted for 10.58% of the phenotypic variation. And *QPOD-2D-2* and *QPOD-1B* were detected in two environments. Six additive QTLs for CAT activity were identified on chromosomes 1D, 4D, 5A, and 6D, with *QCAT-6D-2* accounting for 16.80% of the phenotypic variation as the maximum value. These loci were characterized by LOD values of 2.59–4.14, and explained 7.4–16.80% of the phenotypic variation. The positive effects on CAT activity associated with *QCAT-5A*, *QCAT-5A.2*, and *QCAT-6D-2* within marker intervals *Xgpw1045–Xbarc180*, *Xcwem32.2–Xwmc59* and *Xubc808–Xswes679.1* respectively, were contributed by HP3 alleles, and explained 7.50, 11.33, and 16.80% of the phenotypic variation, respectively.

Eight additive QTLs for MDA content were mapped to chromosomes 1A, 2B, 3A, 4B, 5B, 6B and 7B, and controlled 2.75–20.21% of the phenotypic variation. *QMDA-1A-1*, *QMDA-6B*, and *QMDA-7B* were mapped in the marker intervals *Xgpw3167–Xcwem6.2*, *Xcfa2257–Xcfd48*, and *Xbarc276.1–Xwmc396* on chromosomes 1, 6, and 7, respectively, with positive effects shown by YM57. *QMDA-4B*, *QMDA-3A*, *QMDA-5B.2*, *QMDA-1A-1* and *QMDA-2B* were responsible for 2.75–13.06% of the phenotypic variation, with the positive effect derived from HP3.

Discussion

Plants can acquire freezing tolerance by exposuring to low, nonfreezing temperature, which is called cold acclimation, and then can survive in cold (<0°C). In this process, morphological, biochemical and physiological traits occur a big change. Researchers have used diverse traits such as LT50, cell membrane permeability and rate of survival to explore the physiological mechanisms underlying freezing tolerance, and have made important discoveries (Båga *et al*., 2007; Ju, 2012; Yoichi *et al*., 2013; Gorji *et al*., 2014). The AEA as well as the MDA, FW, and BW contents are reportedly significantly correlated with freezing tolerance (Zhou *et al*., 2005; Taşgin *et al*., 2006; Chen and Tan, 2007). However, as far as we know, no study has investigated freezing tolerance in wheat by focusing on the five traits and DH population examined in this study. We analyzed the wheat freezing tolerance QTLs using a DH population in different environments over 2 years. Additionally, our findings confirm these traits are useful for evaluating the extent of freezing tolerance. Furthermore, the mean values of some DH lines exceeded those of their parents, implying the DH population was affected by transgressive segregation.

An earlier study concluded that compared with the A genome, the D genome exerts a greater influence on the traits associated with freezing tolerance (Limin *et al*., 1997). In this study, 14 of 35 QTLs for five traits (*i.e*., the RFB, MDA content, and SOD, POD, and CAT activities) were located on D genome chromosomes.

In particular, chromosome 5A, 2A and 5D plays a key role in cold acclimation and frost tolerance (Fridovich, 1978; Sutka, 2001; Liu, 2005; Ju, 2012; Gorji *et al*., 2014). In this study, *QRFB-2A-1*, *QRFB-2A-2*, *QSOD-2A*, *QCAT-5A*, *QCAT-5A.2*, *QPOD-5A.2* and *QRFB-5D* were identified on these chromosomes and responsible for 5.06–12.38% of the phenotypic variation. Moreover, most of the QTLs uncovered in this study were located in new marker regions and were mapped to different chromosomes (*e.g*., 1D, 2B and 5B.2) from those mentioned in previous studies.

Because of the complexity of gene expression, the same QTLs were seldom detected for the same trait in different environments. However, three QTLs (*i.e*., *QRFB-2D-1* between markers *Xcfd53* and *Xwmc18*, which explained 9.10–11.59% of the phenotypic variation; *QPOD-2D-2* between markers *Xbarc129.2* and *Xwmc658.2*, which explained 10.58–10.96% of the phenotypic variation; and *QPOD-1B* between markers *Xwmc406* and *Xbarc156*, which explained 7.55–8.46% of the phenotypic variation) were identified in different environments. Additionally, *QRFB-2D-1* between markers *Xcfd53* and *Xwmc18* was identified in three environments and was responsible for 9.10–11.59% of the observed variation in the RFB. The *Xcfd53–Xwmc18* region is reportedly associated with freezing tolerance in wheat (Ju, 2012) as well as chlorophyll *b* content (Liang, 2009), accounting for more than 10% of the phenotypic variation. The results of another study suggest that chlorophyll content can be a useful indicator of freezing tolerance (Smillie and Hetherington, 1983). These results imply that *Xcfd53–Xwmc18* may be associated with freezing tolerance and play an important role. Therefore, the biological functions of the *QRFB-2D-1* should be elucidated further. Moreover, we detected two QTLs in the *Xwmc93–GluD1* marker interval, namely *QCAT-5A.2* (accounting for 11.33% of the phenotypic variation in environment 10) and *QPOD-5A.2* (accounting for 7.97% of the phenotypic variation in environment 7). Furthermore, Ju (2012) reported that a QTL (*QCMP-5A.2*-2) within the same marker interval considerably affects cell membrane permeability, with implications for cold resistance. There are also some QTLs located in adjacent marker regions, including *QRFB-2D-2* between *Xgwm539* and *Xcfd168*, *QPOD-2D-1* between *Xcfd168* and *Xwmc41*, *QSOD-3B-1* between *Xgpw1146* and *Xgwm566*, *QSOD-3B-2* between *Xgwm566* and *Xcfe009* (detected by Ju (2012) in two environments), *QSOD-6B* between *Xcfd48* and *Xwmc415*, *QMDA-6B* between *Xcfa2257* and *Xcfd48*, *QPOD-7B* between *Xgwm333* and *Xwmc10*, *QMDA-7B* between *Xbarc276.1* and *Xwmc396*, *QCAT-6D-1* between *Xgwm681* and *Xubc808*, and *QCAT-6D-2* between *Xubc808* and *Xswes679.11*. We observed that *QMDA-1A-1* within the marker interval *GluAl–Xwmc550* was responsible for 13.06% of the phenotypic variation. This genomic region is also reportedly associated with salt tolerance in wheat (Zhao *et al*., 2014). These results suggest that a locus may be associated with different types of stress tolerance.

It was revealed that 16 QTLs are responsible for more than 10% of the phenotypic variation, making these loci candidates for future marker-assisted selection experiments. To determine the roles of the identified QTLs, a larger segregating population and additional markers must be developed. Identifying QTLs associated with freezing tolerance is necessary for characterizing the genetic basis of freezing tolerance and for breeding new cold-tolerant wheat.

Conclusion

We analyzed the wheat freezing tolerance QTLs for five traits (ratio of free water to bound water, SOD, POD, CAT, MAD) using a DH population in different environments over 2 years. In summary, we revealed that 16 QTLs are responsible for more than 10% of the phenotypic variation, making these loci candidates for future marker-assisted selection experiments.

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