GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Dynamic QTL analysis of protein content and glutamine synthetase activity in recombinant inbred wheat lines

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Genet. Mol. Res. 14 (3): 8706-8715 (2015)
Received October 30, 2015
Accepted April 28, 2015
Published July 31, 2015
DOI http://dx.doi.org/10.4238/2015.July.31.19

ABSTRACT. Protein content (PC) is a crucial factor that determines the end-use and nutritional quality of wheat (*Triticum aestivum*). Glutamine synthetase (GS), which is a major participant in nitrogen metabolism, can convert inorganic nitrogen into organic nitrogen. Although many studies have been conducted on PC and GS, a dynamic analysis of all of the filling stages has not been conducted. Therefore, 115 F_{9-10} recombinant inbred wheat lines of 'R131/R142' were used to analyze PC and GS activity during different developmental stages, using the conditional quantitative trait loci (QTL) mapping method. Twenty-two and six conditional QTL were detected for PC and GS actively, respectively. More QTL in leaf PC were detected during the early filling stages than in the later filling stages. Grain PC QTL displayed different dynamic variations to leaf PC QTL during the entire grain-filling stages. All of the QTL were expressed differently over time, and nine conditional QTL were detected across two filling stages. QTL with similar functions may have tended to group in specific locales. This study provides dynamic

genetic information on protein accumulation during grain-filling stages.

Key words: Wheat; Protein content; Conditional QTL; Glutamine synthetase; Dynamic QTL mapping

INTRODUCTION

Protein content (PC) is one of the main focuses of research into wheat breeding, because it is crucial in determining the quality of baked and boiled foods (bread, cookies, and noodles, etc.). It is very difficult to increase the PC in wheat because of its complex genetic system, and its sensitivity to the environment (Simmonds, 1995). To understand PC genetics at the molecular level, several studies have been conducted on PC quantitative trait loci (QTL), which have provided many valuable data for marker-assisted breeding (MAB) (Blanco et al., 1996; Prasad et al., 1999; Börner et al., 2002; Olmos et al., 2003).

During the grain-filling stage, a large number of amino acids from wheat leaves are transported into the grain and assembled into storage proteins. These storage proteins greatly affect wheat-processing quality, through protein synthesis and enrichment in the grain (Wang et al., 2008). Therefore, as the major organ in wheat nitrogen assimilation, the leaf (particularly the flag leaf) influences the final quality of the wheat product.

Enzyme content and activity are controlled by more than one gene during plant metabolism (Obara et al., 2001, 2004). For example, in addition to introducing a glutenin subunit gene, another gene is also needed to ensure its successful expression (Ren, 2002). In higher plants, ammoniacal nitrogen can be directly absorbed and assimilated into glutamic acid by glutamine synthetase (GS) and glutamate synthase, and finally converted into protein for biological activity (Ireland et al., 1999). Although GS plays a crucial role in converting inorganic nitrogen into organic nitrogen during nitrogen metabolism (Miflin and Habash, 2002), few studies have conducted a QTL analysis of GS (Sasaki et al., 2002; Limami et al., 2003; Obara et al., 2001, 2004).

Several QTL studies on wheat quality have been conducted; however, most have focused on a given period during the grain-filling stage (most at maturity), using traditional statistical analyses, and information on gene expression at different developmental stages is lacking (Han et al., 2011). Consequently, several studies have been conducted on developmental behavioral traits (Yan et al., 1998a; Li et al., 2010; Han et al., 2011; Zheng et al., 2011; Cui et al., 2012). Regarding dynamic QTL studies, Zhu (1995) suggested that conditional analyses are more powerful than unconditional analyses for ascertaining the gene expressions of quantitative developmental behavioral traits. Therefore, to elucidate the dynamic genetic mechanism involved, conditional analyses at different developmental stages should be conducted (Wang et al., 2008; Zheng et al., 2011).

Considering that 1) wheat protein accumulation during filling stages is a complex physicochemical process and cannot be completed in a single event, and 2) dynamic QTL analyses (particularly conditional analyses) are effective in evaluating the gene expressions of quantitative traits, in the present study the PC and GS activity (GSA) were studied during the filling stages, and the data were analyzed by conditional dynamic QTL analyses. Our aim was to understand the dynamic behavior of quantitative trait expression, and provide more desirable QTL for MAB (Han et al., 2011).

MATERIAL AND METHODS

Genetic material

The population consisted of 115 F_{9-10} recombinant inbred wheat lines (RILs), which were derived from filial generations of the wheat (*Triticum aestivum L.*) lines R131 and R142. The flowering stage of the two parents was different by 3 days in 2010 [R131 (3/28), R142 (3/25), where m/n mean month and day]. The flowering stage of the RILs was between 3/22 and 4/1, and 75.2% of them were between 3/25 and 4/1.

The RILs and their parents were planted with three replications in Qionglai District (30°25'N, 103°28'E and 493.3 m above sea level), Chengdu, Sichuan, China, between 2009 and 2010. Each plot was 3 m long and 25 cm apart, and contained two rows with 90 seeds in each row. The field management followed standard agricultural practice. At the flowering stage, 25 plants in each plot were marked with brands, and these marked plants had the same vigorous growth tendency and flowering stage. Flag leaves were harvested every 4 days from 4/5 to 5/10, and wheat ears were also harvested every 4 days from 4/15 to 5/5. All of the samples were stored at -75°C for 2 h.

Frozen wheat ears were fast-threshed to manually grain. Frozen leaves were fast-crushed by hand, and some were kept in a refrigerator for GSA analysis. The remaining leaves and grain were dried in a drying oven at 100°C for 5 min, and 45°C for 10 h, respectively. The fresh and dry weights of leaves and grain were measured using a moisture content (MC) assay. Dry grain and leaves were milled to powder by hand. Grain PC (GPC) was determined using a distillation unit (B-324, Buchi, Sweden). The GSA measurement was conducted as described previously (Rhodes et al., 1975; Kamachi et al., 1991; Miflin and Habash, 2002; Martin et al., 2006).

Map construction and QTL detection

DNA was isolated from fresh leaves at the seeding stage using the cetyltrimethylammonium bromide (CTAB) method (Maroof et al., 1984). Single-sequence repeat (SSR) primer pairs of Xwmc were catalogued in the GrainGenes database (http://wheat.pw.usda.gov). SSR analysis was conducted as described previously (Senior and Heun, 1993). QTL were analyzed by composite interval mapping (CIM), using Windows QTL Cartographer version 2.5 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). CIM was run using the forward regression method at a 10-cM window size. Permutation tests were conducted with 1000 repetitions at a 1-cM walk speed, and a significance level set at 0.05.

A consensus map was constructed using JoinMap® 4 (Van Ooijen, 2006), with microsatellite markers (Xwmc, Xgwm, and Xbarc) from three sets of mapping data (Somers et al., 2004; Quarrie et al., 2005; Xue et al., 2008). The results of the QTL analysis were projected onto the consensus map using BioMercator 2.1 (Arcade et al., 2004). The major procedures of consensus map construction and QTL projection were executed based on the descriptions by Li et al. (2013a).

Statistical analyses

The conditional phenotypic values (the net genetic effects of genes) were obtained by QGA Station 2.0 (Chen et al., 2012), following a mixed-model approach (Zhu, 1995). Basic

statistical analyses were conducted using the PASW statistical package, version 18.0 (http://www.spss.com.hk/statistics/). The phenotypic correlations were calculated using Pearson's correlation, and significant differences were investigated using two-tailed tests. The coefficient of variation was calculated using the means and standard deviations of the results of three replications.

RESULTS

PC and GSA analysis

In three consecutive years, the GPC of parent R142 was 2.39% higher than that of R131 (Table 1). The PC (leaves and grain) and GSA (leaves) of the RILs exhibited different trends at the filling stages (Table 2 and Figure 1). In addition, all the traits of the RILs at the different filling stages exhibited markedly variable means, high coefficient of variations, and various degrees of transgressive segregation (Table 2). The abundant heritable variance indicated that the RILs were suitable for QTL analysis and could breed a specific cultivar.

Table 1. Grain protein content of R131 and R142 over 3 years.

Parent	Protein content (%)			
	2008	2009	2010	Mean
R131	11.12	12.05	10.65	11.25*
	11.11	11.96	10.63	
R142	13.56	13.79	13.60	13.64*
	13.19	14.24	13.46	

*Significant difference ($P < 0.01$) between parents.

Table 2. Phenotypic values of protein content and glutamine synthetase activity for recombinant inbred lines (RIL) and their parents at grain-filling stages.

Trait	Filling stage (month/day)	Parent		RIL population			
		R131	R142	Mean	Min	Max	CV
Leaf protein content (%)	4/5	19.73	21.43	21.54	14.05	30.72	11.80
	4/10	21.35	24.58	22.55	5.34	28.04	13.31
	4/15	15.70	14.97	17.37	12.33	23.19	12.99
	4/20	14.74	14.56	16.60	11.68	24.81	11.82
	4/25	19.61	18.71	18.07	9.97	25.50	14.82
	4/30	11.61	10.96	13.08	6.31	28.36	26.15
	5/5	9.37	8.21	8.29	3.24	14.57	28.77
	5/10	3.98	3.12	4.46	0.71	8.17	22.42
Grain protein content (%)	4/15	8.82	11.08	11.37	8.82	14.43	9.72
	4/20	10.28	12.41	11.84	18.56	15.41	10.41
	4/25	11.02	11.48	11.77	8.21	23.15	16.95
	4/30	9.13	10.01	11.11	8.12	16.69	14.49
	5/5	10.40	11.02	11.07	8.14	14.19	11.03
Glutamine synthetase activity	4/5	0.78	0.55	0.74	0.47	2.34	27.13
	4/10	0.67	0.41	0.61	0.31	1.00	23.09
	4/15	0.48	0.66	0.74	0.26	3.04	39.27
	4/20	0.72	0.86	0.70	0.37	1.28	25.96
	4/25	0.25	0.43	0.46	0.19	0.85	30.94
	4/30			1.18	0.60	2.52	34.21
	5/5	1.28	1.87	1.37	0.50	2.79	31.20

CV = coefficient of variation.

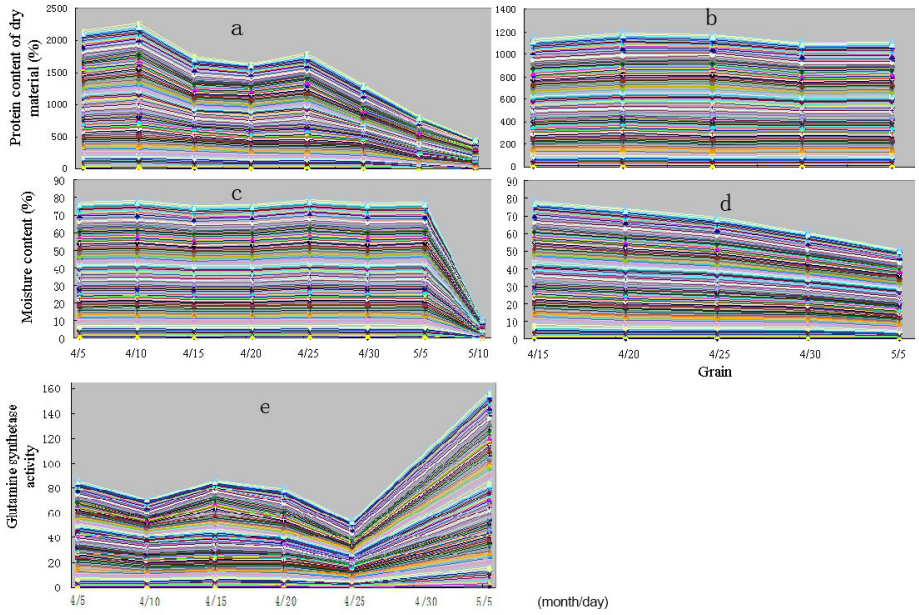

Figure 1. Accumulation line charts of protein content and glutamine synthetase activity in the R131 x R142 recombinant inbred wheat lines. **a.** Mean protein content of dry leaf; **b.** mean protein content of dry grain; **c.** mean moisture content of leaf; **d.** mean moisture content of grain; **e.** mean glutamine synthetase activity of leaf.

The leaf PC (LPC) of R142 was higher than that of R131 during the period 4/5 to 4/10, but the opposite pattern was found at the following filling stage. In the RILs and their parents, LPC generally declined, and only increased at 4/15-4/10 and 4/20-4/25, and decreased to a minimum on 5/10. R142 had higher GPC than R131. The GPC of the RILs was maintained at a stable level during the filling stages. During all of the filling stages, leaf MC kept at a steady level before decreasing sharply from 5/5, while grain MC exhibited a steady decrease. This suggests that grain dry matter accumulated at a steady rate during the filling stages.

In the RILs and their parents, the GSA exhibited a fluctuating trend during the period 4/5-4/25, and reached minima on 4/10 and 4/25. It increased rapidly from 4/25 to 5/5, and peaked on 5/5. This result indicates that QTL/gene expression has different time-phase - the expression level changes with time. The patterns of the changes in GSA were exactly opposite to those in LPC. This difference suggests that GS and LPC may be in the same enzymatic reaction system; the excess product (protein) would inhibit GSA.

QTL analysis of PC and GSA

Nine conditional QTL were detected in the GPC QTL analysis (Table 3 and Figure 2). There were three QTL on 1B chromosome and two on 4B chromosome. Three of the five GPC QTL (*Q.GPC-1B.1*, *Q.GPC-4B.1*, and *Q.GPC-1B.3*) were detected twice during the filling stages. *Q.GPC-1B.1* was detected firstly at time T_4 (LOD = 5.2, R^2 = 16.6), and its phenotypic variance was higher than that of the QTL detected the second time, T_7. *Q.GPC-4B.1* and *Q.GPC-1B.3* were detected twice in a series of time scales; they had opposite hereditary effects.

There were more GPC QTL detected at the end of the filling stage; this meant that QTL expression related to grain filling had a temporal specificity, and became active at later filling stages.

Table 3. Conditional quantitative trait loci (QTL) for the grain protein content at different filling stages.

QTL	Marker interval	T_3(4/15)			T_4(4/20|4/15)			T_5(4/25|4/20)			T_6(4/30|4/25)			T_7(5/5|4/30)		
		LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2
Q.GPC-3B	Xwmc777-Xgwm566	3.2	0.294	6.2												
Q.GPC-1B.1	XWMC216-wmc216.3eb				5.2	0.753	16.6							9.8	0.176	1.7
Q.GPC-2D	Xgwm296-Xwmc25ys							7.0	0.599	6.9						
Q.GPC-4B.1	Xwmc125-Xwmc349							6.9	-0.681	9.2	3.4	0.202	1.4			
Q.GPC-7B	Xwmc273							6.5	0.561	5.7						
Q.GPC-2A	Xwmc658-Xwmc179.2										3.5	-0.325	3.2			
Q.GPC-1B.2	Xwmc419-Xwmc419x													7.2	0.330	5.6
Q.GPC-1B.3	Xwmc419x-Xwmc44										3.5	-0.183	1.0	8.2	0.271	3.8
Q.GPC-4B.2	Xgwm251-Xwmc710													10.0	0.300	4.2

T_n(t|t-1) represents the conditional genetic effects from time (t-1) to t in the n^{th} filling stage. m/n = mean month/day.

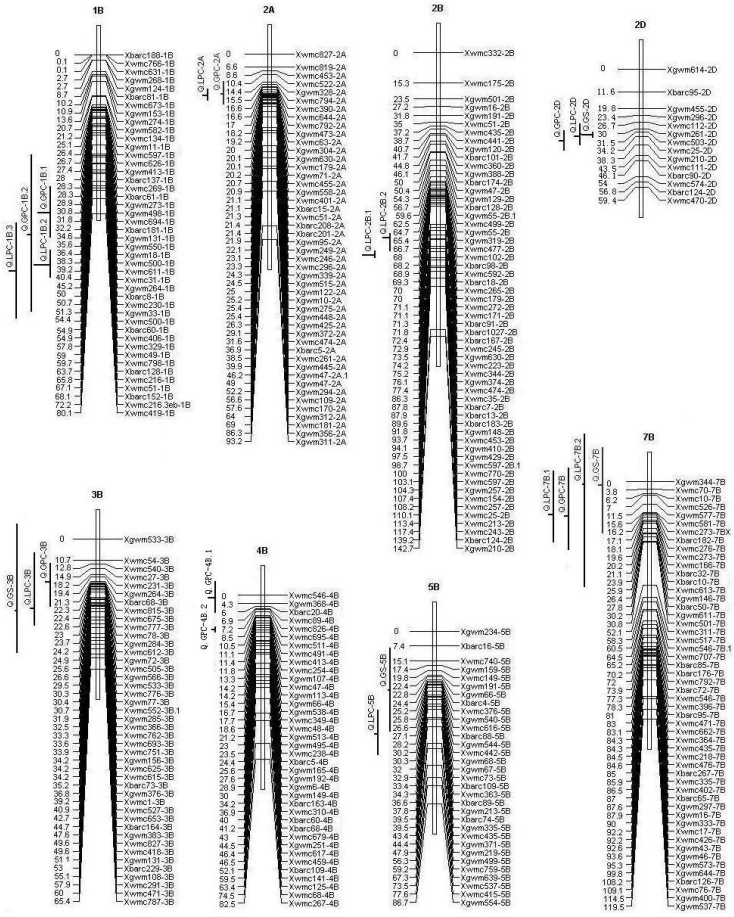

Figure 2. Positions of conditional quantitative trait loci associated with protein content and glutamine synthetase activity in the R131 x R142 recombinant inbred wheat lines.

Thirteen QTL were detected in the conditional QTL analysis of wheat flag LPC, including four QTL on 1B, one on 2B, and one on 7B (Table 4). *Q.LPC-2B.1* and *Q.LPC-1B.2* had high phenotypic variance (R^2 > 28%), and were detected twice at the grain-filling stages. *Q.LPC-7B.1* and *Q.LPC-1B.1* were also detected twice. Interestingly, *Q.LPC-7B.1* was detected at an early (T_2) and a later period (T_7). Most of the LPC QTL were detected at an early stage of the grain-filling stages, but no LPC QTL were detected at T_1 or T_4. It is possible that T_2 and T_3 (10 days) is an important period, during which flag leaves produce and store protein.

Table 4. Conditional quantitative trait loci (QTL) for the protein content of wheat leaves at different filling stages.

QTL	Marker interval	T_2(4/10|4/5)			T_3(4/15|4/10)			T_5(4/25|4/20)			T_6(4/30|4/25)			T_7(5/5|4/30)		
		LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2
Q.LPC-2B.1	Xwmc500-Xwmc453b	4.3	2.487	28.2							4.6	0.423	1.0			
Q.LPC-2B.2	Xwmc453b-Xgwm148	7.9	0.972	9.0												
Q.LPC-7D	Xwmc150.2-Xwmc438	8.4	0.845	5.2												
Q.LPC-7B.1	Xwmc273-Xwmc273x	4.6	-0.245	6.1										3.9	0.502	3.4
Q.LPC-7B.2	Xgwm344-Xwmc273	5.8	0.511	2.1												
Q.LPC-1B.1	Xwmc216-Xwmc216.3eb	5.7	0.552	2.1	4.0	0.575	3.3									
Q.LPC-1B.2	Xwmc419-Xwmc419x	5.2	0.532	2.2	8.1	-1.798	32.2									
Q.LPC-2D	Xwmc503S-Xgwm261				4.0	0.674	7.2									
Q.LPC-5B	Xwmc73-Xwmc149x							3.5	-1.388	4.2						
Q.LPC-1B.3	Xwmc419x-Xwmc44										5.0	-1.169	9.0			
Q.LPC-2A	Xgwm294-Xgwm71										5.9	0.957	6.5			
Q.LPC-3B	Xgwm376-Xwmc808										5.4	-0.843	5.5			
Q.LPC-1B.4	Xgwm18-Xwmc406													3.6	-0.458	3.4

T_n(t|t-1) represents the conditional genetic effects from time (t-1) to t in the n^{th} filling stage. m/n = month/day.

As GS is a key enzyme in nitrogen assimilation, it is important for wheat to accumulate enough protein. We detected six GSA QTL in wheat flag leaves using conditional QTL analysis (Table 5). *Q.GSA-5B* was detected twice at T_1 and T_3. Both *Q.GSA-5B* and *Q.GSA-3B*, detected at T_1, had high LOD values (>13). In addition, *Q.GSA-2D*, which was detected at T_2, had a high phenotypic variance (R^2 > 29%). It is strange that GSA remained high at T_4, but we did not detect QTL at this time (Table 5 and Figure 1a).

Table 5. Conditional quantitative trait loci (QTL) analysis for glutamine synthetase activity of wheat leaves at different filling stages.

QTL	Marker interval	T_1(4/5)			T_2(4/10|4/5)			T_3(4/15|4/10)			T_5(4/25|4/20)			T_7(5/5|4/25)		
		LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2
*Q.GSA-3*B	Xgwm376-Xwmc808	13.1	-0.036	2.6												
Q.GSA-5B	Xgwm544-Xwmc73	15.0	-0.032	2.3				4.6	0.020	0.9						
Q.GSA-2D	Xgwm296-Xwmc25ys				3.8	-0.249	29.4									
Q.GSA-1B	Xwmc216-Xwmc216.3eb							6.9	-0.047	3.4						
Q.GSA-1A	Xwmc24-Xwmc278										3.5	0.026	2.7			
Q.GSA-7B	Xgwm344-Xwmc273							4.7	-0.025	1.3				3.8	-0.112	5.5

T_n(t|t-1) represents the conditional genetic effects from time (t-1) to t in the n^{th} filling stage. m/n = mean month/day.

During the conditional QTL analysis of GPC, LPC, and GSA, the QTL of different traits were detected in the same molecular marker interval, which indicates that genes for these traits may co-localize in the same genomic location (Tables 3, 4, and 5). For example, 1) *Q.GPC-1B.3* and *Q.LPC-1B.3* were detected between the two molecular markers Xwmc419x

and Xwmc44; and 2), *Q.LPC-7B.2* and *Q.GSA-7B* were detected between the two molecular markers Xwmc344 and Xwmc273. This indicates that QTL with similar functions may gather in specific locales.

DISCUSSION

Genetic expression is closely related to trait development, which displays specific expression during ontogenetic development. Traditional QTL analysis is usually used for studying quantitative traits at a particular time, mainly during maturity (Wang et al., 2011; Li et al., 2013b; Park et al., 2013; Sandhu et al., 2013). As a static analysis method, it cannot accurately detect expressive QTL that correlate with GPC during all of the grain-filling stages, but can explain the accumulation effect, from expression to survey. Zheng et al. (2011) believe that dynamic QTL may be an effective method of revealing genetic information on protein accumulation during the grain-filling stages. By analyzing conditional genetic effects and variance components, Zhu (1995) perfected the precision of early dynamic QTL analysis, and obtained a net genetic variation value (phenotypic condition value) within the period (t-1) to t. Therefore, conditional QTL analysis can detect effective QTL, enhance our understanding of the time-dependent expression of QTL/genes, and provide information for MAB.

Some QTL of the three traits (GPC, LPC, and GSA) were detected twice, and exhibited the opposite genetic effect during the grain-filling stages. Other researchers have obtained similar results, which suggests that opposite genetic effects reduce differences in the accumulation effect and detection sensibility in unconditional QTL analysis (Yan et al., 1998b; Zheng et al., 2011). Yan et al. (1998a) reported that more tiller number QTL were detected by conditional QTL mapping than that by unconditional mapping. Therefore, conditional QTL analysis is a more efficient method for revealing the gene expression mechanisms of wheat at the filling stages. GSA was higher at T_4 than at the other filling stages, but conditional QTL were not detected at T_4, probably because the sampling interval was too long to separate the QTL by their opposite genetic effects (Yan et al., 1998a).

The conditional QTL analysis of PC and GSA revealed that the QTL of quantitative traits are expressed differently at different developmental stages. In this study, most QTL were detected at only one stage, and a few QTL were expressed in two continuous or separate stages. Sun et al. (2006) and Zheng et al. (2011) believe that this is the result of differential gene expression at different times. These results indicate that QTL/gene expression is time-dependent during the entire developmental process.

In this study, some QTL of different traits were detected in the same molecular marker interval. *Q.LPC-3B* and *Q.GSA-3B* were in the molecular marker interval Xgwm376-Xwmc808, and *Q.LPC-7B.2* and *Q.GSA-7B* were in Xgwm344-Xwmc273. *Q.GPC-1B.3* and *Q.LPC-1B.3* were detected in Xwmc419x-Xwmc44, and *Q.GPC-1B.3* was detected twice. These results demonstrate that QTL expression was time-specific, and QTL for similar traits tended to group in specific locales. Salih and Adelson (2009) stated that genes with similar functions may group in specific locales and contribute to QTL. Therefore, these locations must contain pleiotropic genes that control wheat-protein filling.

The LPC and GSA exhibited an oscillating and opposite trend at T_2 to T_5 (Figure 1). The probable reason for this is that GSA can promote protein accumulation in leaves during the filling stage, but is inhibited when the LPC reaches a certain level; when the LPC decreases by the nitrogen-transfer effect, the GSA increases again. From T_6 to T_7, the LPC decreased

while the GSA rapidly increased. This may have been because the wheat was in a fast-filling stage, and the rate of nitrogen transfer from the leaves to the grain was increased. Similar, unusual enzyme activity was reported by Hänsch et al. (2006). Therefore, wheat has a complicated enzymatic reaction system. Enzymes, due to protein accumulation, can be inhibited by their own substrates, leading to velocity curve changes that gradually increase to a maximum as the substrate concentration decreases, and vice versa (Reed et al., 2010).

The QTL for the LPC were mainly detected during the early filling stages, T_2 and T_3 (Table 4), while the QTL for the GPC were found in the later stages, T_5, T_6, and T_7 (Table 3). This suggests that wheat leaves store protein during the early filling stages. During these stages, PC genes are very active, and inorganic nitrogen is transformed into organic nitrogen in the leaves by assimilation. In the later filling stages, wheat leaves act as a protein source, and protein is transported from the leaves to the grain due to the high levels of gene expression in the grain. As can be seen in Figure 1, the last 10 to 15 days is an important period for filling, because QTL/genes for the GPC are very active, and grain protein accumulates quickly during this period.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#30730065, #31271722), the National ‘‘973’’ Wheat Breeding Program, the Wheat Breeding Project of Sichuan Province, and the Initiatives of Sichuan Education Department for Wheat Breeding.

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