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Full Length Research Paper

Identification of quantitative trait locus (QTLs) for γ-aminobutyric acid content in grain of barley

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r-Aminobutyric acid (GABA) has not only some physiological functions in plants and animals, but also has been used extensively in pharmaceuticals and functional foods for human health. To realize its genetic basis, in this study, a 192 plants F_2 population derived from the cross between Ziguang manluoerling (ZGMLEL) and Schooner was used for quantitative trait locus (QTL) mapping for GABA content in grain for barley (*Hordeum vulgare* L.). As a result, four QTLs (*qGABA3-1*, *qGABA4-1, qGABA4-2,* and *qGABA4-3*) were identified on chromosomes 3 and 4, of which these QTLs could explain phenotypic variance from 8.02 to 24.50%, due to additive effects for *qGABA3-1* and *qGABA4-1* from ZGMLEL on chromosome 3 and 4. Meanwhile, part dominance effects for *qGABA4-2* and *qGABA4-3* from ZGMLEL on chromosome 4, especially the marker interval (GBM1299-GBM1482) of *qGABA4-2* accounting for 24.50% of the phenotypic variance. To our knowledge, these are the first QTLs to be identified controlling GABA content in grains of barley. These results are beneficial for understanding the genetic basis of GABA and developing the markers linked with GABA for marker-assisted selection breeding in barley.

Key words: γ-Aminobutyric acid (GABA), quantitative trait locus (QTL), grain, barley.

INTRODUCTION

Global climate change is resulting in increased abiotic (example, drought and cold) and biotic (example, disease and insect) stresses on all crops. γ-Aminobutyric acid (GABA) is a four-carbon non-protein amino acid, produced in the cytosol (Breitkreuz and Shelp, 1995) via the decarboxylation of glutamate in a reaction catalysed by glutamate decarboxylase, a calcium/calmodulin-dependent enzyme, and acts as a major inhibitory neurotransmitter in the central nervous system (Krnjevic, 1974; Mazzucotelli et al., 2006). Cold acclimation causes a significant increase in amino acid pools and induces the expression of the GABA-shunt genes (Mazzucotelli et al., 2006). GABA may have a role in pH regulation, nitrogen (N) storage, plant development and the metabolite to both stress and signaling in plants (Oh, 2003; Fait et al., 2008; Clark et al., 2009). GABA also has several physiological functions in animals, such as neurotransmission, regulative blood pressure, treatment of epilepsy, preventing diabetes, diuretic and tranquilizer effects (Cohen et al., 2002; Huang et al., 2007; Jakobs et al., 1993; Mody et al., 1994). It has been used extensively in pharmaceuticals and functional foods (Huang et al., 2007). Many chronic diseases cause serious damages to human health, meanwhile, medicine therapies are effective and widely used in controlling, as well as curing chronic diseases worldwide. However, these medical treatments hold many associated problems, such as high costs and side effects.

Barley is not only the most stress tolerance of grain crop essential for the malting and brewing industries, as well as major food of Zang nationality, but also a key ingredient in strategies to reduce chronic diseases such as cardiovascular (example, GABA), obesity, diabetes

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and intestinal diseases (Newman and Newman, 2008; Nie et al., 2010). To accelerate the breeding of cultivars with improved malting quality, molecular markers and genetic linkage maps were used to localize these loci (Schmalenbach and Pillen, 2009). Amino aldehyde dehydrogenase probably plays a role in physiological processes connected to polyamine degradation, converting 4-aminobutanal to GABA (Petřivalský et al., 2007; Arikit et al., 2011). A γ-aminobutyrate transaminase with dual functions suggests the potential for interaction between GABA metabolism and photorespiratory glyoxylate production (Clark et al., 2009). However, the quantitative trait loci (QTLs) of many functional ingredient-related genes in crop grains still remain unclear.

Quantitative trait locus (QTL) mapping is an efficient approach to realize genetic basis of trait (Liu et al., 2010); QTL analysis has proven its usefulness to identify and localize favorable alles from exotic germplasm (Wang et al., 2010), such as yield-related traits (Saal et al., 2010), agronomic traits (Wang et al., 2010), malting quality (Schmalenbach and Pillen, 2009) and so on. The percentage of the total variation explained each marker ranged from 4.59 (*HVM2* associated with plant height) to 17.48% (Bmac 90 associated with density of main spike) (Sun et al., 2011). However, the QTLs of GABA-related traits still remain unclear. The objectives of this work were to identify QTLs, their effects and locations on chromosome for GABA in grains of F_2 populations of ZGMLEL × Schooner for barley.

MATERIALS AND METHODS

Genetic population

192 plants of F_2 population used to analyze the GABA locus in this study were derived from a cross between ZGMLEL × Schooner varieties. F_2 population and F_3 lines, as well as parents, were evaluated at Kunming in China in 2008 to 2010. ZGMLEL is a hullness two-rowed landrace with high GABA and the highest protein content, while Schooner is a hull two-rowed cultivar malting barley with low GABA and protein content.

Colorimetric determination of GABA and statistical analysis of traits

γ-Aminobutyric acid (GABA) was assayed using the procedures of Inatomi and Slaughter, (1971) with modifications as described by Zeng et al. (2010). The principle of this method is based on cyan compound under GABA and sodium hypochlorite action. The optical density of the mixture was measured at an absorbance of 645 nm and GABA data of conversion was drawn as the beeline based on GABA standard sample of production for Sigma Company. We weighed up to 500 mg of the barley grain powder for milling, surging and soaking for extraction as well as centrifugation, after constant volume 5 ml using distilled water. We took 1 ml of filtrate, added 0.6 ml borate buffer with 0.2 mol/L and 2.0 ml double distilled phenol solution 5%, and then added 1ml of 7.0% sodium hypochlorite solution at 20°C within 5 min of sufficient surging. After being kept at 20°C for 4~8 min, the mixture was heated in a boiling water bath for 10 min and promptly cooled in ice bath for 20 min, then 2 ml of 60% ethanol was added and kept at 20°C for 20~40 min. The optical density of the mixture was measured by the absorbance at 645 nm. To compute GABA content in grain by the standard curve ($y = 1230.18x - 6.71$), y served as the concentration of GABA, while x is absorbance at visible light and $R^2 = 0.9942$; this equation wasbased on 5 standard samples concentration; 0, 10, 20, 30, 40 and 50 mg/g).

All analyses were performed in duplicate. The associations among individual traits were determined with simple linear correlation. The descriptive statistics and correlation analysis was performed using the SPSS statistical software (version 16.0 for Windows, SPSS Inc. Chicago).

DNA extraction and polymerase chain reaction (PCR) amplification

Leaf tissue was harvested from young parent and 192 F_2 plants grown in the experimental farm of Yunnan Academy of Agricultural Sciences (YAAS), Kunming (altitude 1916 m). DNA was extracted from ground tissues by the CTAB method (Rogers and Bendich, 1988) with minor modifications (Zeng et al., 2009). A 15 µL reaction mixture containing 1.5 µL of PCR buffer (20 mM Tris, pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 50% glycerol), 50 ng of DNA, 330 nM of both forward and reverse primers, 250 mM of each dNTPs, and 0.8 U of Taq polymerase. The samples were prepared in a 96-well amplification plate for amplification using the GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR conditions were: 95°C for 5 min; 35 cycles of 95°C for 30 s; 55°C for 30 s; 72°C for 45 s; 72°C for 5 min. The amplified products were denatured at 95°C for 5 min, then loaded on 8% denatured polyacrylamide gels in 0.5 × TBE buffer. The gels were stained with silver nitrate (Varshney et al., 2007) to detect polymorphisms after electrophoresis. 137 SSR markers were screened against ZGMLEL and Schooner to identify polymorphisms. When the PCR products had the same band size for ZGMLEL, the high GABA bulks, but were different from those of low GABA bulks and Schooner. It was therefore assumed that the SSR marker was probably linked to a high GABA gene. We identified potential chromosome arm locations for many of GABA based on high or low GABA bulks and parents. 14 (EBmac0715, GBM1299, GBM1482, GBM1350, GBM1525, Bmag606, Hv5s, WMS6, Bmag0023, GBM1118, GBM1110, scssr25538, scssr07759 and GBM1434) of 137 SSR markers are probably linked to a high GABA gene. These markers were used for one-way ANOVA. All SSR markers showing polymorphisms were used to genotype the entire F_2 population.

Mapping and QTL analysis

This F_2 population is suitable for the Mapmaker program as follows. The statistical software SPSS for Windows, version 16.0 (SPSS Inc. Chicago, IL, USA) was used to analyze the phenotypic, molecular data and one-way ANOVA. Mapmaker/EXP3.0 (Lincoln et al., 1992) was used for linkage analysis and the Kosambi function was used to convert recombination values to genetic distances. Linkage groups were created with a LOD score of 3.0 and a recombination fraction of 0.4 using the GROUP command. QTLmapper V2.0, modified from QTLmapper V1.0 (Zhu et al., 1998), makes unbiased estimates of QTL additive and dominance effects in F_2 populations. GABA and nine phenotypic traits of F_3 were as phenotypic data for QTL analysis, respectively. A threshold probability of $P = 0.001$ was used for additive QTLs, while a LOD score of 3.0 was used as

Table 1. Mean and distribution of 192 lines and parent traits.

Agronomic trait	Mean of parent		192 plants of F_2 population for ZGMLEL × Schooner			
	ZGMLEL	Schooner	Mean ± SD	CV%	Skewness ± SE	Kurtosis ± SE
GABA (mg/100 g)	16.64 ± 0.39	6.04 ± 0.54	8.10 ± 1.91	23.58	0.38 ± 0.18	-0.38 ± 0.35
Plant height (cm)	121	109	102.37 ± 16.06	15.69	–0.09 ± 0.18	–0.44 ± 0.35
Panicle number per plant	12	18	14.88 ± 9.04	60.80	4.15 ± 0.18	33.14 ± 0.35
Tillers of per plant	14	19	16.25 ± 9.27	57.02	3.81 ± 0.18	29.30 ± 0.34
Panicle length (cm)	9.93	7.13	8.08 ± 2.10	25.92	0.87 ± 0.18	1.57 ± 0.35
Filled grains per panicle	20	24	22.86 ± 4.38	19.17	–0.22 ± 0.18	1.47 ± 0.25
Unfilled grains per panicle	3	1	2.01 ± 0.31	15.00	2.17 ± 0.18	5.36 ± 0.35
Total grains per panicle	23	25	24.86 ± 5.31	21.35	0.32 ± 0.18	1.17 ± 0.35
Spike density	10	16	13.93 ± 1.09	7.81	–0.06 ± 0.18	1.58 ± 0.35

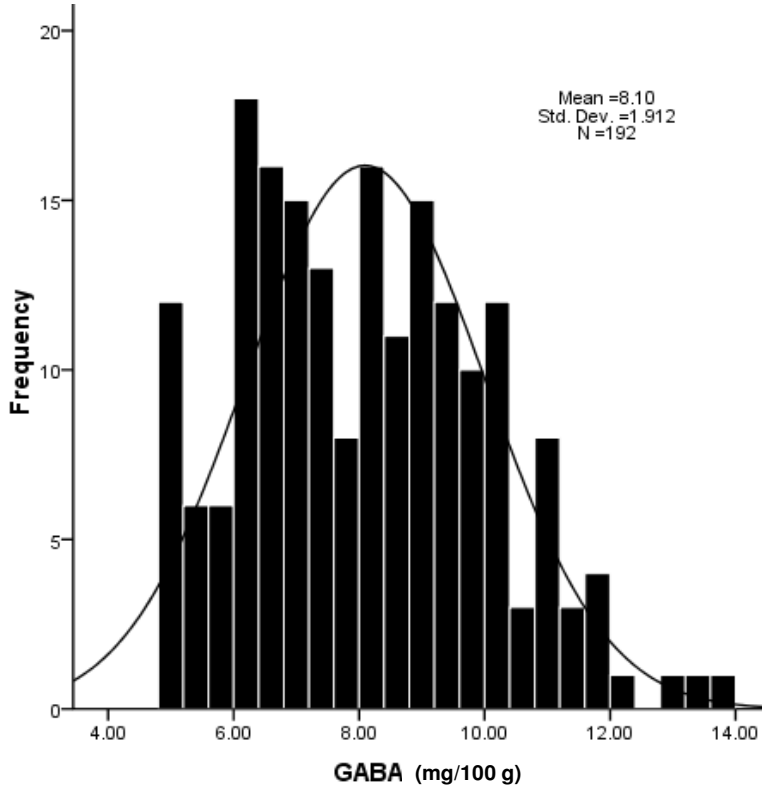

Figure 1. Histogram of GABA content in grains of 192 plants for F_2 population of ZGMLEL × Schooner.

another threshold to declare the presence of a putative QTL. The percentage variation explained (general contribution) by each QTL, and additive and dominance effects were estimated. Nomenclature of QTL name followed that of McCouch et al. (1997).

RESULTS

Agronomic traits of parent and 192 plants for F_2 population

The results of the descriptive statistics for F_2 population are displayed in Table 1. The six values of 192 plants for F_2 population, including mean, standard deviation (*SD*), coefficient of variation (*CV*%), skewness, kurtosis and standard error (*SE*) are shown. GABA content (16.64 mg/100 g) in grain of ZGMLEL is 2.75 times than that of Schooner (6.04 mg/100 g) and these values showed that these traits in parents and F_2 progeny have enough variation (8.10 ± 1.91 mg/100 g). GABA content in grain of 192 F_2 plants range from 4.81 to 13.73 mg/100 g, therefore, genetic analysis of GABA in grains of F_2 population for ZGMLEL × Schooner is very effective.

Table 2. F-value of ANOVA analysis between 14 markers and nine traits of 192 plants for F_2 population from ZGMLEL × Schooner.

Chromosome	Maker	GABA	Plant height	Panicle number per plant	Tillers per plant	Panicle length	Filled grains per panicle	Unfilled grains per panicle	Total grains per panicle	Spike density
4	EBmac0715	33.88**	0.63	0.37	0.31	0.46	2.24	2.18	3.43*	2.39
4	GBM1299	28.18**	2.07	0.27	0.29	1.05	0.30	0.42	0.02	0.43
4	GBM1482	32.35**	2.34	0.82	0.67	3.93*	6.35**	0.41	2.21	0.17
4	GBM1350	23.20**	1.86	1.08	1.47	2.54	2.37	1.72	3.56*	1.12
4	GBM1525	24.16**	2.32	0.91	0.66	0.40	0.76	0.50	0.05	1.43
4	WMS6	1.08	1.11	0.58	0.56	2.19	3.25*	0.32	2.59	1.46
3	Bmag606	42.92**	0.68	0.32	0.75	0.86	2.07	5.22**	4.86**	0.28
3	Bmag0023	76.36**	0.30	0.06	0.02	0.41	0.35	0.56	0.01	2.18
3	GBM1118	21.30**	4.06*	1.30	1.66	0.21	0.79	0.97	0.97	0.00
3	GBM1110	19.52**	0.32	0.99	1.27	1.20	0.39	0.36	0.48	0.33
3	scssr25538	19.76**	1.37	1.14	0.89	0.03	0.49	0.12	0.31	1.03
2	Hv5s	58.12**	4.07*	0.17	0.20	0.35	0.21	2.29	1.53	3.88*
2	scssr07759	16.09**	0.69	0.28	0.15	0.24	2.32	1.14	3.07*	0.03
1	GBM1434	22.44**	0.95	2.57	2.38	3.14*	4.47*	2.71	1.61	1.53

Furthermore, from the frequency distribution graphs (Figure 1) and skewness (0.38), as well as kurtosis (-0.38), GABA contents in grains of F_2 population could well agree with normal distribution, which meant that GABA is a quantitative trait controlled by multiple genes, hence, similar to some agronomic traits including plant height, panicle length, filled grains per panicle, total grains per panicle, spike density and so on (Table 1).

ANOVA analysis between single marker and GABA or nine agronomic traits

F-value of ANOVA analysis between 14 markers and nine traits of 192 plants for F_2 population from ZGMLEL × Schooner is shown in Table 2. One-way ANOVA showed that 13 markers were the most significantly associated with GABA content in grain of barley (P<0.01), GBM1118 and Hv5s were significantly associated with plant height (P<0.05), GBM1482 and GBM1434 were significantly associated with panicle length (P<0.05), 3 markers were significantly associated with filled grains per panicle (P<0.05), Bmag606 was significantly associated with unfilled grains per panicle (P<0.05), 4 markers were significantly associated with total grains per panicle (P<0.05) and Hv5s was significantly associated with spike density (P<0.05).

Linkage mapping and QTL analysis

The 14 markers were distributed on chromosomes 1, 2, 3 and 4. We constructed two linkage groups with eleven positive SSR markers (Figure 2). Four QTLs with effects ranging from 8.02 to 24.50% of the phenotypic variation were identified (Table 3 and Figure 2) and tentatively designated as *qGABA3-1*, *qGABA4-1, qGABA4-2, andqGABA4-3.* QTL analysis of GABA content in grain revealed a significant peak between markers EBmac0715 and GBM129 with a LOD = 5.93, and explaining 11.86% of the phenotypic variance, and we designated the locus *qGABA4-1*. Meanwhile, QTL analysis of GABA content in grain confirmed the most significant peak between markers GBM1299 and GBM1482 with a LOD = 12.27, the QTL accounting for 24.50% of the phenotypic variance, that is, ZGMLEL-derived allele contributed an increasing effect on GABA content in grain, and we designated the locus

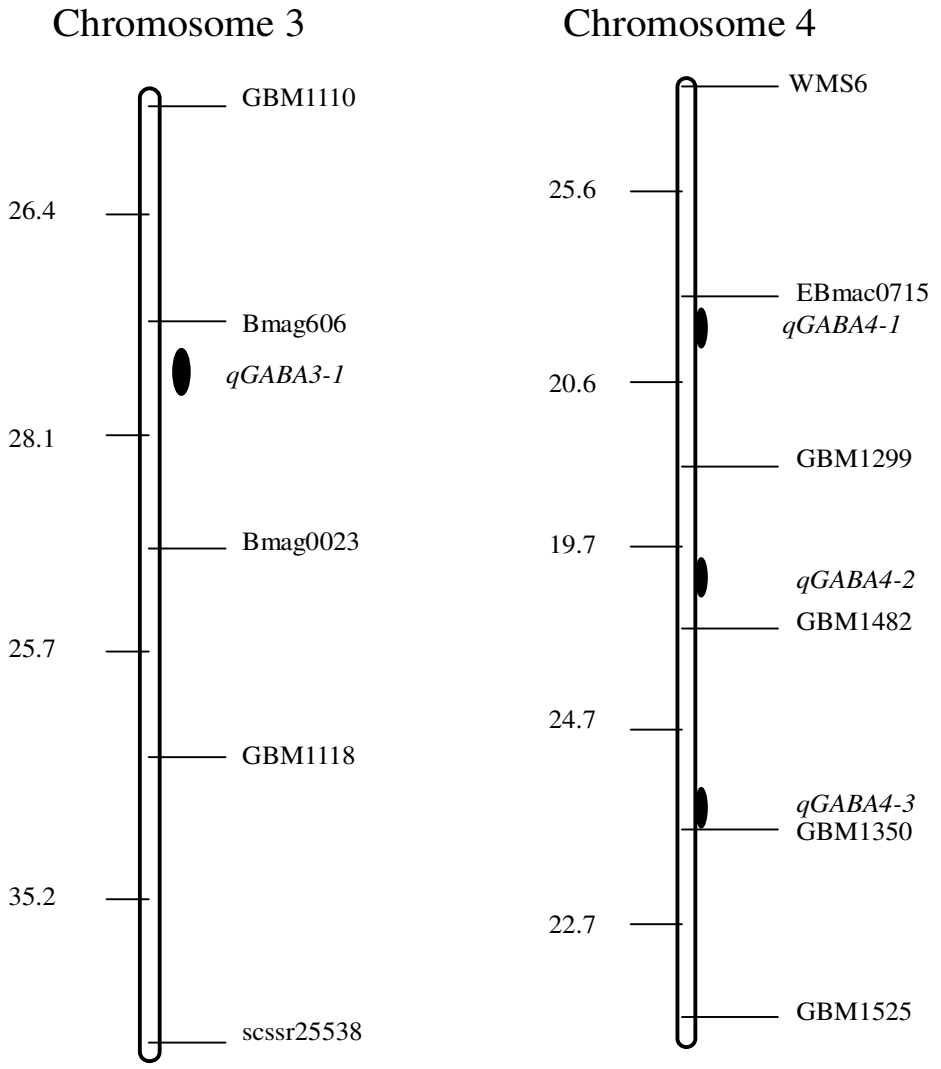

Figure 2. QTL affecting GABA content in grain of barley.

qGABA4-2 (Table 3). The above results demonstrated that *qGABA4-1* and *qGABA4-2* were responsible for GABA content in grain from ZGMLEL. *qGABA3-1* and *qGABA4-1* of ZGMLEL parent alleles were additive, leading to increasing GABA content in grain of barley. *qGABA4-2* and *qGABA4-3* of ZGMLE parent alleles were part dominance, leading to increased GABA content in grain of barley.

DISCUSSION

GABA is a quantitative trait controlled by multiple genes

GABA content in grain for barley is a quantitative trait controlled by multiple genes and this is quite similar to the earlier studies of some agronomic traits in barley including yield-related traits (Saal et al., 2010), flowering time and agronomic traits (Wang et al., 2010), malting quality (Schmalenbach and Pillen, 2009), drought tolerance (Szira et al., 2011), as well as cold tolerance at booting stage in rice (Zhou et al., 2010). GABA content (16.64 mg/100 g) in ZGMLEL grains is 2.75 times than that of Schooner (6.04 mg/100 g). Meanwhile, protein content in ZGMLEL grains is 2.3 times than Schooner. ZGMLEL is a landrace with the highest protein content (22.59%) in grain all over the world from Yunnan Province in China, although, Hiproly (19.15%) from Ethiopia and Risp1508 (14.92%) from Sweden are famous high protein barley in the world (Sun, 2001). But Schooner is a low protein content (9.80%) cultivar from University of Adelaide, which continues to be popular with Australia's brewing industry and has a long-standing

Table 3. QTL analysis of GABA content in grain and agronomic trait in barley.

QTLs	Marker interval	Interval Distance (cm)	LOD	Phenotypic variance (%)	Additive (%)	Dominance (%)	Resource of allele	Gene interaction
qGABA3-1	Bmag606-Bmag0023	28.1	3.04	8.02	-10.33	1.61	ZGMLEL	Additive
qGABA4-1	Bmaco0715-GBM1299	20.6	5.93	11.86	-10.09	1.43	ZGMLEL	Additive
qGABA4-2	GBM1299-GBM1482	19.7	12.27	24.50	-7.99	1.98	ZGMLEL	Part dominance
qGABA4-3	GBM1482-GBM1350	24.7	4.62	8.98	-8.17	3.41	ZGMLEL	Part dominance

market into China. Total amino acid including GABA had a high positive correlation (0.8316) with soluble protein (Nie et al., 2010). Therefore, barley of the highest protein content in grain may be likely to high GABA cultivar. In addition, barley is the most stress tolerant of crops, such that the rainfall of the growth stage for barley in Yunnan Province in 2009 to 2010 is less than 10 mm, which is the worst drought in a century, yield of 230,000 ha barley is 2.69 t/ha, but wheat is 1.20 t/ha for 600,000 ha in 2010 in Yunnan Province of China. Intracellular levels of GABA are typically low, but they rapidly increase in response to several abiotic stresses, such as hypoxia, drought, cold, heat shock, and mechanical stimulation (Bown and Shelp, 1997). Further research is needed for the relationship of GABA and protein content as well as drought for barley.

QTLs effecting GABA content in grains

To our knowledge, these are the first QTLs to be identified controlling GABA content in grains of barley, although, 2-acetyl-1-pyrroline (*Os2AP*) in aromatic rice chromosome 8 has accumulated several natural mutations in an amino aldehyde dehydrogenase that oxidizes γ-amino butylaldehyde to GABA (Vanavichit and Yoshihashi, 2010). Four QTLs with minor and major effects of GABA content in grains for barley ranging from 8.02 to 24.50% of the phenotypic variation were identified. This indicated that it inherited only some of high GABA genes from ZGMLEL and that GABA content in ZGMLEL was controlled by a large number (more than four) of QTLs with minor and major effects. The most interesting observation is that 3 QTLs in chromosome 4H (*qGABA4-1, qGABA4-2,* and *qGABA4-3*), especially the marker interval (GBM1299-GBM1482) of *qGABA4-2* accounting for 24.50% of the phenotypic variance.

GMB606 was not only near marker with a QTL that control GABA (*qGABA3-1*) at ZGMLEL, but also be affected to days to hedding (*QHD.umn-3H*) and *Fusarium graminearum* (*QFhs.umn*-3H.2) (Mesfin et al., 2003). EBmac0715 was not only near marker with a QTL that control GABA (*qGABA4-1*) at ZGMLEL, but also be affected to spot blotch (*QSb.Vabo*-3H) (Bilgic et al., 2006). GBM1482 and GBM1350 were not only near marker with a QTL that control GABA (*qGABA4-2* and*qGABA4-3*) at ZGMLEL, but also be affected to powdery mildew (*Rbgq1*)(Shtaya et al.,2006; Aghnoum et al., 2009). There are a lot of QTLs on chromosome 4H, including 5 malting quality QTLs (sol-total protein, grain protein, alpha amylase, diastatic power and malt extract), 6 agronomic QTLs and 5 disease resistance QTLs; but there is a lot of QTLs on chromosome 3H, including 3 malting quality QTLs (sol-total protein, grain protein, diastatic power), 6 agronomic QTLs, and 5 disease resistance QTLs(http://barleyworld.org/northamericanbarley/qtlsummary.php). There are also a lot of protein-related traits (sol-total protein, grain protein, and diastatic power) on chromosome 3H and 4H. Further research is therefore needed for the relationship of QTLs for GABA and protein content as well as malting quality especially to disease resistance for barley.

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