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Full Length Research Paper

Quantitative trait loci analysis for chlorophyll content of cucumber (*Cucumis sativus* L.) seedlings under low-light stress

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An increase in chlorophyll content is an adaptive response to low-light stress and can be used to evaluate low-light tolerance. The effects of low-light stress (100 μmol·m^{-2}.s^{-1}) on the chlorophyll content of cucumber (*Cucumis sativus* L.) were investigated in a set of 123 $F_{2:3}$ lines in the seedling stage in the autumn of 2008 and spring of 2009. Quantitative trait loci (QTL) analysis was undertaken on the basis of a genetic linkage map of the corresponding F_2 population that was constructed using composite interval mapping. $F_{2:3}$-based QTL analysis of the chlorophyll-a (chl.a), chlorophyll-b (chl.b) and chlorophyll-a+b (chl.a+b) content in the 2 environments revealed 21 QTLs located on the linkage groups 1, 2, 3, 4, 6 and 7, which accounted for 4.8 - 17.3% of the phenotypic variation. In the spring of 2009, the total phenotypic variation among the $F_{2:3}$ lines accounted for by the QTLs for chl.a, chl.b and chl.a+b were 44.5, 29.4 and 39.0%, respectively. In the autumn of 2008, 11 QTLs were identified, which accounted for 4.8 - 14.9% of the observed phenotypic variation and an additive effect of -8.10 to 20.85. Four major-effect QTLs (chla2.1, chlb2.2, chlb3.1 and chla+b2.2) were detected under both conditions. The QTL information presented in this research, together with the data from our previous study on heredity of low-light tolerant traits, will facilitate the breeding of low-light-stress-resistant cucumbers.

Key words: Cucumber (*Cucumis sativus* L.), seedling, quantitative trait loci analysis, low-light stress, chlorophyll content.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable plants grown worldwide; an increasing yield of cucumbers is needed to match the supply to their demand. Low-light intensity is a major stress factor that results in poor cucumber yields in northern China. Low-light stress causes slower growth, higher fruit abscission rate and decline in production (Liebig and Krug, 1990). In addition, the total chlorophyll content increases with a larger increase in chlorophyll b (chl.b) content, which is an adaptive response to the low-light stress. Thus, leaf chlorophyll content can be used as an early indicator of aging and physiological stress responses (Shen, 2001; Chen et al., 1995). Considerable effort has been directed toward the development of low-light-resistant cucumbers. Breeding large numbers of cucumber cultivars with high-yield and high-quality is always the goal of the breeders; however, conventional methods are often time-consuming. Marker-assisted selection (MAS) can accelerate the breeding process and is dependent on a saturated genetic linkage map with the mapped genes and quantitative trait loci (QTLs) for the targeted traits.

Recent technological advances have accelerated genetic mapping and QTL analysis development in several crop

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Abbreviations: QTL, Quantitative trait loci; **chl.a,** chlorophyll-a; **chl.b,** chlorophyll-b **chl.a+b,** chlorophyll-a+b; **MAS,** marker-assisted selection; **RAPD,** random amplified polymorphic DNA; RIL, recombinant inbred lines; **SSR,** simple sequence repeat; **SRAP,** sequence-related amplified polymorphism; **SSD,** single-seed descent; **CTAB,** cetyl trimethylammonium bromide; PCR, polymerase chain reaction; **LOD,** log-likelihood; **CIM,** composite interval mapping.

species to include QTL detection of important horticultural traits (Li et al., 2008), resistance to powdery mildew in cucumber (Liu et al., 2008), stalk tunneling in maize (Krakowsky et al., 2004) and bacterial leaf streak in rice (*Oryza sativa* L.) (Tang et al., 2000). For example, QTL analysis of specific horticultural traits (gender expression, trunk length, fruit weight, etc.) were detected using 1520 random amplified polymorphic DNA (RAPD) markers, resulting in the formation of more than 180 polymorphism bands and detection of 80 loci from 73 primers; however, the contribution rates for these traits were low (approximately 4 - 10%) (Serquen et al., 1997). Recombinant inbred lines (RIL)-based genetic mapping and QTL analysis of the number of important horticultural traits revealed a genetic map with 7 groups and 131 genetic loci covering 706 cM with an average interval of 5.6 cM. The location of F and de was identified by genetic linkage and QTL analysis and was associated with the simple sequence repeat (SSR) loci CSWCT28 and CSWCTT14 at 5.0 and 0.8 cM, respectively (Fazio et al., 2003). The 4 QTLs pm1.1, pm2.1, pm4.1 and pm6.1 for powdery mildew resistance were identified and located in the linkage groups (LGs) 1, 2, 4 and 6, respectively, accounting for 5.2 - 21.0% of the phenotypic variation (Liu et al., 2008). Genetic analysis revealed that in cucumbers, low-light resistance is controlled by 1 or 2 major genes plus a polygene and that low-light resistance is over dominant to susceptibility (Li et al., 2009). This genetic feature would likely map and detect QTLs of low-light tolerant traits in cucumbers. There are few reports on QTLs for low-light tolerance and only 5 QTLs for leaf area growth were detected, including la-1, la-2, la-3, la-4 and la-5 (Zhang et al., 2004). Therefore, it is necessary to carry out QTL analysis of other important traits related to low-light stress in cucumbers, such as hypocotyls, height and chlorophyll content.

In this study, SSR and sequence-related amplified polymorphism (SRAP) technology, which was developed by Ren et al. (2009) and Li and Quiros (2001), respectively, was used to detect QTLs for low-light-tolerant traits and their effects on chlorophyll content. One population of $F_{2:3}$ were produced using 2 cucumber species displaying low-light intensity characteristics: Low-light stress tolerance line M_{22} and a sensitivity line M_{14}. The identification of QTLs for low-light-tolerant traits may be useful in marker-assisted breeding in cucumbers.

MATERIALS AND METHODS

Plant materials

The cucumber low-light stress tolerance line M_{22} was crossed with the low-light sensitivity line M_{14}. M_{22} grew normally and exhibited acclimation when exposed to low-light stress. In contrast, M_{14} was a shorter plant with severe abnormalities. The parents were selected after screening a large number of cucumber germ plasms native to north China, south China and Europe. The F_1 generation between M_{22} and M_{14} was self-pollinated to produce 152 F_2 progeny, which were then self-pollinated by single-seed descent (SSD) to obtain 123 $F_{2:3}$ families.

Field evaluation and plant characteristics

The 2 parental lines (M_{22} and M_{14}), their F_1 generation and the $F_{2:3}$ families were evaluated in a greenhouse at the Horticulture School, Shenyang Agricultural University, Shenyang, China. The $F_{2:3}$ families were grown in 2 seasons: The autumn of 2008 (A) and the spring of 2009 (S). The soil media comprised 25% peat, 25% cinder and 50% per liter. The $F_{2:3}$ families were arranged in a randomized complete block design with 3 replications. Each replicate had 4 plants and individual plants were planted in a bowl (base area: 8 × 8 cm) and spaced 5 cm apart in the bowl. Evaluation of low-light tolerance characters (chl.a, chl.b and chl.a+b) in the seedling stage was carried out under low-light intensity. Low-light intensity was simulated by double layers of having lock in the greenhouse, with the day average light intensity of approximately 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and day/night average temperature of 25°C/15°C. The temperature was regulated using heating equipment and aeration. The low-light treatment of 30 days was carried out in two-leaf stage of seedlings.

Marker analysis

Leaves (weight: approximately 0.5 g) from 2-week-old F_2 (123), F_1, and parent seedlings were collected and preserved in an ultra-low temperature freezer. The tissue was then immediately lyophilized for DNA extraction using the cetyl trimethylammonium bromide (CTAB) method described by Zhao and Pan (2004). SRAP and more recently developed SSR markers were selected for genomic analysis of the parents. Polymerase chain reaction (PCR) analyses for SRAP (Zhao et al., 2009) and SSR markers were performed in 10 µL volumes of a uniform reaction mixture [SRAP: 1 µL of 10 × PCR commercial buffer, 20 mM $MgCl_2$, 2 mM dNTPs, 15 - 20 ng of DNA sample, 2.5 µM of each primer, and 1.75 U *Taq* DNA polymerase (Shanghai Promega); SSR: 1 µL of 10 × PCR commercial buffer, 20 mM $MgCl_2$, 2 mM dNTPs, 15 - 20 ng of DNA sample, 2.5 µM of each primer, and 1 U *Taq* DNA polymerase(Shanghai Promega)], incorporating 10 µL of a light-weight mineral oil overlay. The amplified products were resolved in 4.0% denatured polyacrylamide gels (6.0% for SSR) (Life Technologies, Gaithersburg, MD, USA) by silver staining. The gels were visualized using an image scanner III.

Mapping and quantitative trait loci analysis

Linkage mapping was performed using the Join Map 3.0 software (Ooijen and Voorrips, 2001) on the basis of F_2 data, with a log-likelihood (LOD) threshold ≥ 3.0 and Kosambi map function (Kosambi, 1944). To identify putative loci associated with chlorophyll content under low-light stress, QTL loci were carried out for the $F_{2:3}$ families and M_{22} and M_{14} populations using Win QTL Cart 2.5 (Zeng, 1993). A LOD threshold of 3.0 and $P < 0.05$ was applied to confirm the significance of a putative QTL. Least-square means for traits were calculated according to location and obtained using the mixed-models procedure (PROCMIXED) in the statistical analysis system (SAS) (Littell et al., 1996). The column diagram of frequency distribution for chlorophyll content in the $F_{2:3}$ families were examined for normal distribution using the statistical package for the social sciences (SPSS) 16.0 software (Liu and Li, 2008).

QTL analysis was performed using the Win QTL Cart 2.5 software (Basten et al.,2001), with the following parameters for LOD 2.5, composite interval mapping (CIM), model 6 with a walking speed of 1 cM, and background control: control marker number, 15; window size, 3 cM; and regression method, positive. The QTLs were named “chl.” for chlorophyll content, followed by 2 digits (separated by a dot), which represent the LG number and QTL number in the same group, respectively.

Table 1. Parent values and distribution in $F_{2:3}$ lines of traits in the autumn of 2008 and spring of 2009.

Chlorophyll	Autumn(2008)				Spring(2009)			
	Parents		$F_{2:3}$ lines		Parents		$F_{2:3}$ lines	
	$P_1(M_{22})$	$P_2(M_{14})$	Mean	SD	$P_1(M_{22})$	$P_2(M_{14})$	Mean	SD
Chl.a	9.840 ± 0.13	6.166 ± 0.01	7.742	2.719	8.973 ± 0.07	6.758 ± 0.10	6.984	1.940
Chl.b	5.181 ± 0.04	2.980 ± 0.04	3.303	1.150	4.997 ± 0.03	2.980 ± 0.08	3.543	0.984
Chl.(a+b)	15.021 ± 0.10	9.146 ± 0.003	11.034	3.472	13.960 ± 0.17	9.738 ± 0.05	10.513	2.512

Values are represented in mean ± standard deviation (SD) of chl.a, chl.b, and chl.a+b in parents.

RESULTS

Means and phenotypic variation in chlorophyll content

Findings of analysis of variance (ANOVA) proved that 3 trait values of the 2 parental lines were significantly different (P = 0.05 or P = 0.01) for each season (Table 1). M_{22} had greater chlorophyll content than M_{14} in both seasons. The mean chlorophyll-a (chl.a) content of the 123 $F_{2:3}$ families in autumn was also greater than that in spring; however, the chl.b and chlorophyll-a+b (chl.a+b) content in autumn were lesser than those in spring (Table 1), indicating that environmental conditions may influence the chlorophyll content. The ANOVA findings also indicated that the genotype significantly influenced the variation in the chlorophyll content traits. Furthermore, the chlorophyll content of the $F_{2:3}$ population in the 2 seasons segregated continuously and followed a normal distribution (Figure 1) and the absolute standard deviation values of the total chlorophyll content were 0.9-3.5 (Table 1), which indicated suitability of the data for QTL analysis. Although the distributions were skewed, original data were used for QTL analysis for chl.b and chl.a+b in autumn.

Linkage mapping and QTL analysis

The linkage map used in this study included 117 molecular markers (106 SRAPs and 12 SSRs) resulting in a linkage map of 7 linkage groups covering a genetic distance of 483.9 cM and a mean marker interval of 4.1 cM, which was suitable for QTL analysis. A total of 17 QTLs for chlorophyll content were identified in both seasons; 11 and 10 QTLs were detected in the autumn of 2008 and spring of 2009, respectively (Table 2 and Figure 2 and 3). These QTLs were located in LGs 1, 2, 3, 4 and 7, respectively (Figure 3). In the autumn of 2008, a total of 4, 3 and 4 QTLs were identified for chl.a (chla1.1, chla1.2, chla2.1 and chla4.1), chl.b, and chl.a+b, respectively (Figure 3). The 4 QTLs for chl.a were mapped on 1.9, 7.8, 5.5 and 18.3 cM, accounting for 4.8 - 14.9% of the observed phenotypic variation and -8.10 to 20.85 of the additive effect. The 3 QTLs for chl.b were identified at 2.5, 11.7, and 12.3 cM and had an R^2 value of 59.1%. Further, 6 QTLs for chl.a+b had R^2 values ranging from 9.2% to 17.3%. Among them, the QTL chla+b2.2 had the maxi- mum additive effect of 21.68, which was the maximum positive effect for the chlorophyll content.

In the spring of 2009, in the analysis of the $F_{2:3}$ families grown in the spring, 4 QTLs for chl.a were detected, and 3 QTLs were identified for chl.b and chl.a+b (Figure 3). The QTL chla3.1 exhibited a phenotypic variation of 16.1% and an additive heritability of 12.00, indicating that the M_{14} allele had a positive effect on chl.a content accumulation. In the autumn of 2008 and spring of 2009, there were 4 QTLs (on LG2 and LG3) for chlorophyll content positioned in the same intervals and with different R^2 values. Thus, the 4 QTLs were given the same names: chla2.1, chlb2.2, chlb3.1 and chla+b2.2. Among the $F_{2:3}$ lines, the total phenotypic variation for chl.a, chl.b and chl.a+b were 44.5, 29.4 and 39.0%, respectively, and the additive effect showed a greater number of small negative values, indicating a decrease in the chlorophyll content. As stated above, 21 QTLs were detected for chlorophyll content (Table 2); the QTLs chla2.1, chlb2.2, chlb3.1 and chla+b2.2 were more consistent with the same interval. Their effect over the environments tested contributed similarly to the phenotypic variation and additive effect direction between the seasons. Other QTLs also had similar effects on the phenotypic variation and additive effect, suggesting that they may be within the loci of the above mentioned traits.

DISCUSSION

Chlorophyll is the major pigment in plants for absorbing, transmitting and transforming luminous energy within a certain range. Leaf chlorophyll content is an important quantitative trait that positively correlates with the photosynthesis rate (Pan and Dong, 1995). The chloro- phyll content of leaves can be used as a measure of physiological responses resulting from stress conditions. Many studies have reported physiological mechanisms such as the effect of calcium on the photosynthesis of cucumbers under low-light intensity and sub-optimal temperature (Ai et al., 2006). Additionally, there are a few reports on heredity, especially at the molecular level, with the exception of a large number of studies on rice, wheat

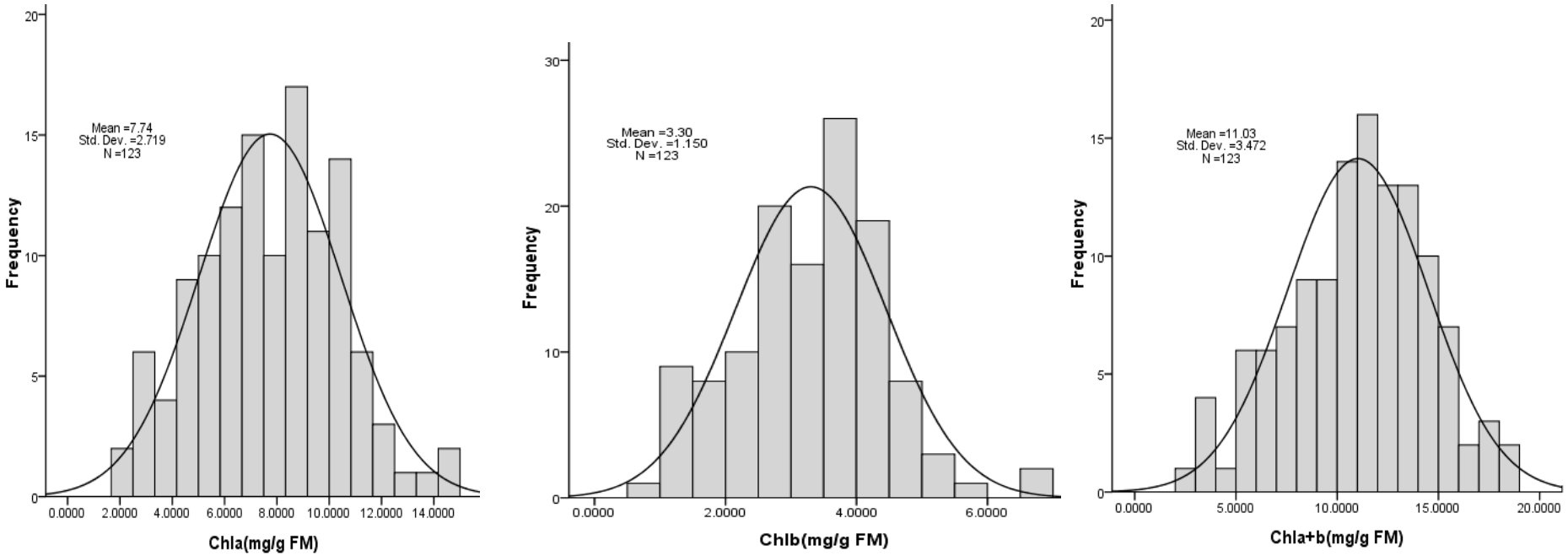

Figure 1. Frequency distribution in $F_{2:3}$ families for chlorophyll content in autumn (2008). Sta Dev= Standard deviation, N=number of $F_{2:3}$ population.

Table 2. Quantitative trait loci controlling chlorophyll content and their effect on cucumbers in 2 seasons.

Traits/QTLs[a]	LG[b]	Flanking loci	Position (cM)[c]	Autumn 2008			Spring 2009		
				LOD	R2 (%)[e]	Additive effect[d]	LOD	R2 (%)[e]	Additive effect[d]
Chl.a									
Chla1.1	1	E22SA18-ME23EM17 (ME3SA17)	1.9	1	5.4	-8.10	-	-	-
Chla1.2	1	ME10EM7-PM8EM5b	7.8	0.7	4.8	-9.78	-	-	-
Chla2.1	2	ME1EM8-ME2EM8	5.5	0.8	14.9	20.85	-	-	-
Chla2.1	2	ME1EM8-ME2EM8	5.5	-	-	-	2.4	15.3	-0.95
Chla2.2	2	ME3SA14 -ME8GA33	9.7	-	-	-	1.4	5.7	-1.15
Chla3.1	3	ME9EM4a-M42EM4	5.1	-	-	-	3.8	16.1	12.00
Chla4.1	4	ME9GA2-E23MSP15	18.3	1.4	7.1	11.10	-	-	-
Chla4.2	4	M42SA14	4.5	-	-	-	1.9	7.4	11.27

Table 2. Cont'd

Chl.b									
Chlb2.1	2	ME3EM8-ME7SA18	6.5	-	-	-	1.4	6.8	-0.29
Chlb2.2	2	ME1EM6-ME2EM3	11.7	0.9	14.6	10.48	-	-	-
Chlb2.2	2	ME1EM6-ME2EM3	11.8	-	-	-	2.5	5.7	-0.28
Chlb3.1	3	ME7SA7-SSR15698	2.5	1.4	7.4	8.70	-	-	-
Chlb3.1	3	ME3EM3-ME7SA7	1.7	-	-	-	2.9	6.9	-2.34
Chlb3.2	3	PM8GA2b	12.3	1.9	5.8	-0.51	-	-	-
Chl.a+b									
Chla+b2.1	2	ME4EM6	0.0	2.7	9.2	1.73	-	-	-
Chla+b2.2	2	ME1EM8-ME2EM8	6.3	1.4	17.3	21.68	-	-	-
Chla+b2.2	2	ME1EM8-ME2EM8	5.9	-	-	-	1.2	16.7	-0.48
Chla+b4.1	4	E23GA2-SSR15914	12.2	-	-	-	0.1	5.8	-0.77
Chla+b4.2	4	E23MSP15-ME6MSP15	19.4	1.1	14.9	11.16	-	-	-
Chla+b7.1	7	ME8EM5-ME22EM9c	4.0	1	14.7	-12.31	-	-	-
Chla+b7.2	7	ME9EM14b-ME3EM2	10.0	-	-	-	1.3	16.5	-14.90

[a]Abbreviation of the QTL trait name + linkage group number + QTL serial number; [b]linkage group; [c]position of the LOD peak value, "/": not detected; [d]"+": the add-effect derived from the parental line M_{22}, "-": the add-effect derived from the parental line M_{14}; [e]percentage of the phenotypic variation explained.

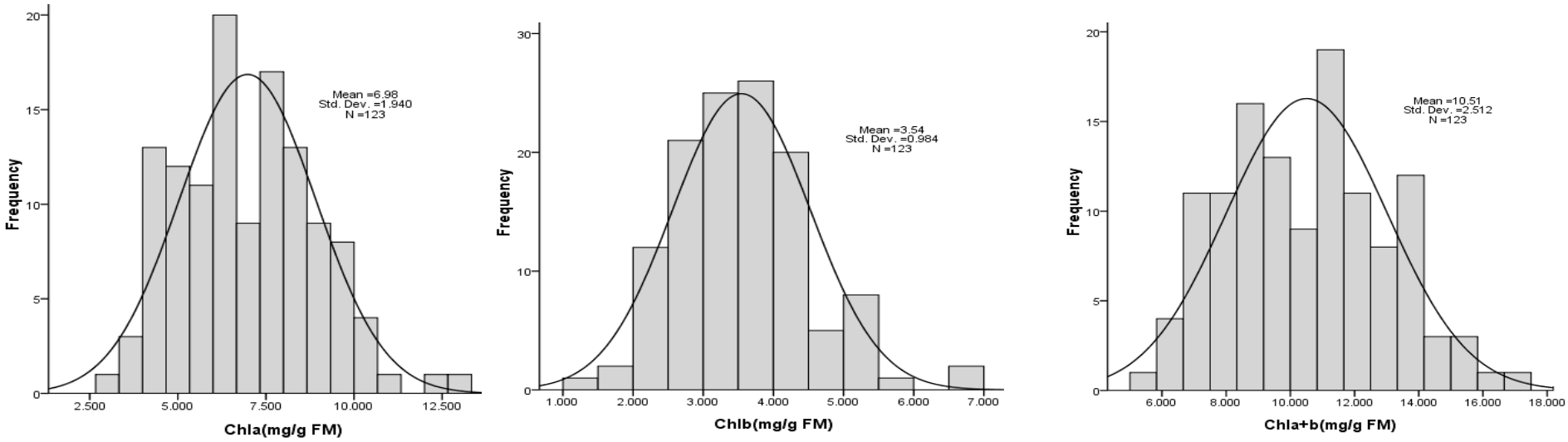

Figure 2. Frequency distribution in $F_{2:3}$ families for chlorophyll content in spring (2009). Sta Dev= Standard deviation; N=number of $F_{2:3}$ population.

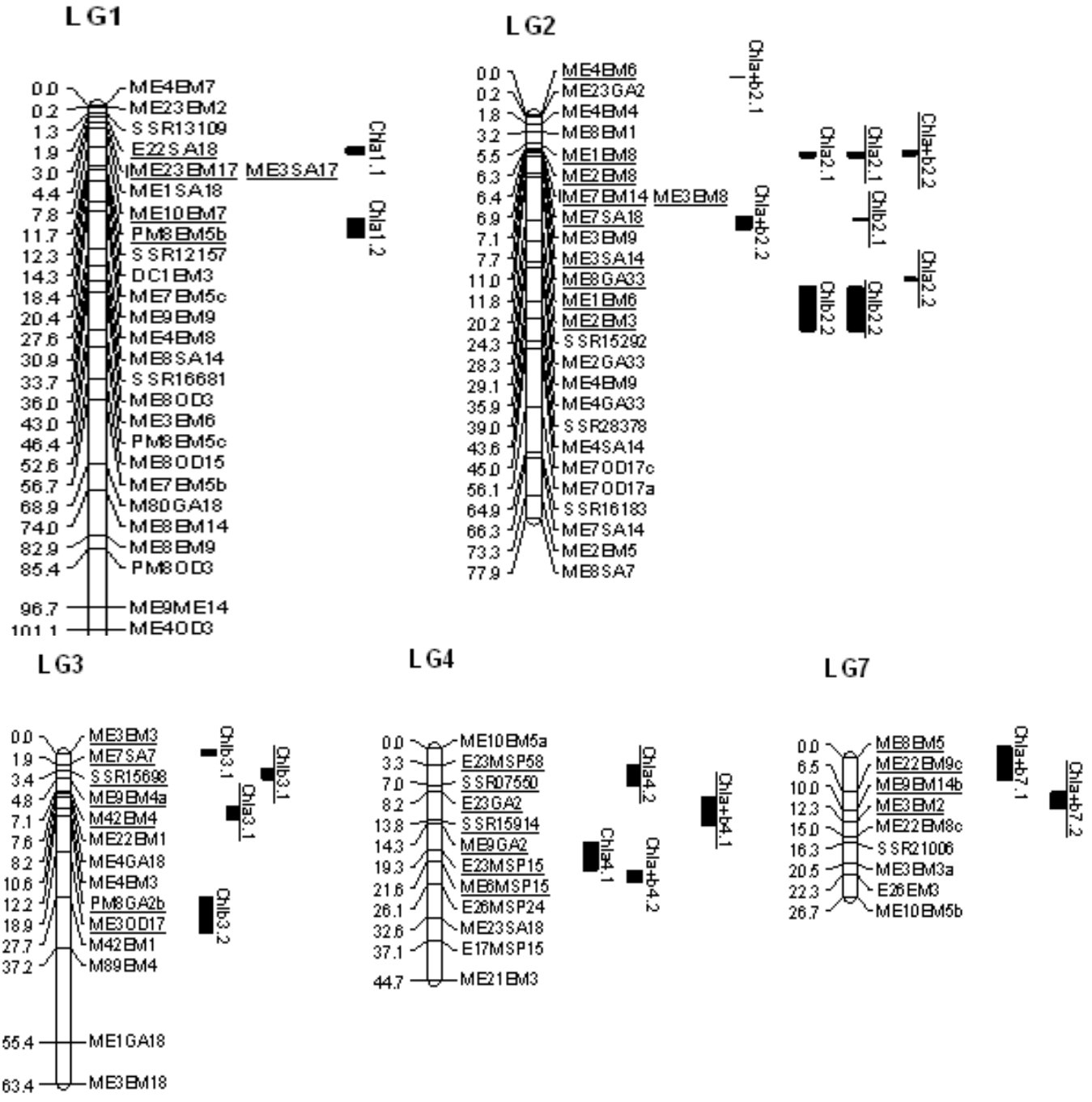

Figure 3. Linkage maps and location of QTLs for chlorophyll content in cucumber. Linkage groups (LGs) are designated by the linkage group number (e.g., LG1); starred markers are SRAP or SSR markers; bars and underlined bars indicate QTLs of chlorophyll content in the autumn of 2008 and spring of 2009, respectively.

and other crops (Wang et al, 2003; Yang et al., 2003; Tong et al., 2006; Shen et al., 2007; Wang et al., 2008; Shen et al., 2005).

The chlorophyll content of plants is regulated by many factors such as the genotype and the environment, where the genotype is a major component involved in the mechanisms regulating chlorophyll content. Li et al. (2009) showed that the total chlorophyll content is controlled by a mini-polygene in cucumber that is influenced by environmental changes. Dennijs and Smeets (1987) also reported that chl.a, chl.b and total chlorophyll content were controlled by a mini-polygene in cucumber; Yang et al., (2006) supported this hypothesis in rice. Moreover, gene expression is influenced by other factors, including differences in species and growth conditions. For example, 2 QTLs (ant2.1 and ant5.1) were detected in the gene regulating

cucumber flower development; however, ant1.1 had the opposite effect in the other test environments (Fazio et al., 2003). Both genotype and environment had significant effects on all 3 traits and different environments (different low-light intensities) played an important role in the expression of the genes for cucumber low-light tolerance.

In our study, 7, 4 and 6 QTLs were identified for chl.a, chl.b, and chl.a+b content, respectively, thereby supporting the hypothesis that chlorophyll content is controlled by a mini-polygene in a genetic analysis of cucumber seedlings under low light stress (Li et al., 2009). The additive effect values for other QTLs were positive or negative, indicating the additive and subtractive effects of the genes. For chl.b, 6 locations were detected in different environments, of which, 2 were identical with the same flanking loci interval in each season; therefore, 4 QTLs were finally identified (chlb2.1, chlb2.2, chlb3.1 and chlb3.2). Six chl.a+b QTLs (chla+b2.1, chla+b2.2, chla+b4.1, chla+b4.2, chla+b7.1 and chla+b7.2) were identified with R^2 values ranging from 5.8 to 17.3%, with chla+b2.1 and chla+b2.2 identified as additive effect genes and the other 4 as subtractive effect genes. One of the QTL musters controlling chl.a and chl.a+b content was detected in the interval ME1EM8-ME2EM8 on LG2, proving the multi-effect hypothesis for the QTLs, since ch1.a and ch1.a+b were controlled by the same interval. Until now, only Zhang Haiying et al. (2004) reported 5 QTLs for leaf area growth of cucumber seedlings under low-light stress on LGs 1, 7 and 9. Although, there have been no recent reports on QTLs for chlorophyll content in cucumbers, many studies have been conducted on the chlorophyll content in other crops. For example, Yang et al. (2003) reported 5 QTLs for the leaf chlorophyll content in rice on the basis of 98 BC_1F_9 lines and obtained a major QTL between the RFLP markers C86 and C813. Six QTLs for chl.a, chl.b, other chlorophyll (chl.c) and total chlorophyll (chl.z) content were identified using 189 F_2 lines (Wang et al., 2008).

Four QTLs (chla2.1, chlb2.2, chlb3.1, and chla+b2.2) were identified as important locations that contributed similarly to phenotypic variation and additive effect direction between seasons. Additionally, the QTL chlb3.1 was located close to the SSR marker SSR15698 on LG3 in each season. Therefore, we presumed that these QTLs, which were detected in 2 seasons, would probably be the genes for the traits of interest under low-light stress. The SRAP markers can now be closely linked to the 4 QTLs and can be transformed into SCAR markers to facilitate breeding of plants with the low-light resistance trait.

Several factors for cucumber low-light tolerant traits were analyzed and QTL mapping was performed. On the basis of these factors, MAS could aggregate the QTLs for chlorophyll content traits from different cucumber lines into a single line. Different environments affected chlorophyll content and the selection of this trait by phenotype was not feasible. Using MAS, seedlings were selected on the basis of molecular markers linked to chlorophyll content traits. This action can shorten the breeding process and increase breeding efficiency. Thus, we believe that all the results obtained in this study can contribute to efficient cucumber breeding in the future.

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