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Quantitative Genetic Analysis of Chlorophyll during Dark-induced Senescence at Seedling Stage in Recombinant Inbred Line Population of Maize

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Abstract

Chlorophyll is an important pigment that is involved in the process of photosynthesis in plants and some algae. Different photoperiods and chlorophyll contents affect grain yield and plant health. In the present study, one recombinant inbred line (RIL) population constructed by using Zong3 and Yu87-1 was used for quantitative genetics analysis of chlorophyll content before and after dark treatment at the seedling stage. Chlorophyll index of the first and second leaves of six plants from each line were measured with SPAD-502 before and after dark treatment. A total of 19 putative quantitative trait loci (QTLs) were identified with each accounted for 6.87–15.9% of the observed phenotypic variation. Moreover, eight major putative QTLs that explained more than 10% of the observed phenotypic variation were identified. In addition, one QTL located on chromosome 3, which affected DF (SPAD value at the first leaf after dark treatment) and LF (SPAD value at the first leaf before dark treatment) was co-localized with a candidate gene (*GRMZM2G170013* translated into chlorophyll b reductase) of the chlorophyll pathway. These results help better understand the genetic background of chlorophyll during dark-induced senescence and photoperiodic variations.

Keywords: Maize; Chlorophyll; Dark; QTL

Abbreviation: LF: SPAD value at the first leaf before dark treatment; LS: SPAD value at the second leaf before dark treatment; DF: SPAD value at the first leaf after dark treatment; DS: SPAD value at the first leaf before dark treatment; Diff_F: the decrement of the average for the first leaf before and after dark; Diff_S: the decrement of the average for the second leaf before and after dark; RIL: Recombinant Inbred Line

Introduction

Chlorophyll the most abundant pigment on earth, plays an important role in harvesting solar energy and drives electron transport (Green and Durnford, 1996; Fromme *et al.*, 2003). Chlorophyll is actively synthesized from glutamate during leaf development for the formation of photosystems, degraded and converted to safety molecules of non-fluorescence chlorophyll catabolites during leaf senescence and fruit ripening (Hörtensteiner, 2006; Tanaka and Tanaka, 2007; Hörtensteiner and Krautler, 2011; Liu *et al.*, 2016).

Chlorophyll degradation is an initial symptom of leaf senescence, which includes natural and induced senescence. Chlorophyll metabolism has been clearly elucidated in the plant model *Arabidopsis* and most of the key genes involved in this pathway have been cloned and characterized (Hörtensteiner and Krautler, 2011; Hörtensteiner, 2013). Chlorophyll metabolism involves three major processes, namely, chlorophyll synthesis (Willows, 2003; Grossman *et al.*, 2004) chlorophyll cycle (Rüdiger, 2002) and chlorophyll degradation (also called PAO pathway) (Takamiya *et al.*, 2000; Eckhardt *et al.*, 2004). The free form of chlorophyll from excessive chlorophyll synthesis produces reactive oxygen species, which in turn results in cell death (Op den Camp *et al.*, 2003). Furthermore, chlorophyll degradation is not finely regulated and intermediate chlorophyll degradation molecules such as pheophorbide a accumulate and induce cell death in both light-dependent (Palaisa *et al.*, 2003; Tanaka *et al.*, 2003) and light-independent manner (Hirashima *et al.*, 2009). Thus the metabolism of chlorophyll is highly regulated during plant development to maintain healthy growth (Masuda and Fujita, 2008). In addition, the chlorophyll cycle which involves the inter-conversion between chlorophyll a and chlorophyll b is thought to be important for the adjustment of the ratio of chlorophyll a to chlorophyll b ratio in various physiological

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conditions (Ito and Tanaka, 1996; Rüdiger, 2002) and plays a crucial role in the processes of greening acclimation to light intensity, seed formation and senescence.

Chlorophyll is an important biochemical attribute of crop growth and plant biological research (Lamb *et al.*, 2002). Higher chlorophyll content with longer period in the reproductive stage is essential for increasing crop production (Guo and Li, 1996; Guo *et al.*, 2008). Moreover, it provides a better physiological foundation for the absorption of more light energy for higher-yield rice varieties. On the other hand decline in chlorophyll content is a good indicator of leaf senescence and an important parameter of physiological reaction under various stressful environments.

Chlorophyll content based on the chlorophyll index is measured by using a SPAD machine and chlorophyll-related parameters are detected by a photosynthetic apparatus. QTL mapping has been extensively performed and a large number of QTLs have been identified on 10 maize chromosomes using different mapping populations (Jompuk *et al.*, 2005; Messmer, 2006; Trachsel *et al.*, 2010; Šimić *et al.*, 2014; Zaidi *et al.*, 2015). In addition a number of studies involving quantitative genetic analysis of chlorophyll-related traits in a wide range of species have been conducted under various stress conditions (Jompuk *et al.*, 2005; Cai *et al.*, 2012; Zaidi *et al.*, 2015). Differential QTLs identified under normal and stress statuses suggest that the regulatory mechanism for chlorophyll production varies with different environments. Here, we report quantitative genetic analysis of the chlorophyll index in a RIL population derived from a cross between Zong3 and Yu87-1 under controlled conditions of normal light and short-day dark-induced senescence. In addition, a putative QTL was co-localized with a candidate gene involved in the chlorophyll metabolic pathway. The findings of the present study are likely to increase our understanding of the genetic basis of chlorophyll regulation under an abnormal light environment.

Materials and Methods

Plant Materials, Experimental Conditions and Treatments

The Zong3/Yu87-1 recombinant inbred lines (RIL) population consisting of 192 family lines and the parents were provided by the National Maize Improvement Centre of China. Seeds were germinated in Petri dishes for 4 days and two replicates of uniformly germinated seedlings with ~2 cm long shoots were transplanted into pots (top and bottom were 15 and 7.5 cm in diameter, respectively and 7 cm high) with enriched soil (light nutritional soil: vermiculite = 1:1). Experiments were conducted in a growth chamber under the following conditions: light/dark: 10 h/14 h; temperature: 25°C and white light: 400 $\mu mol \cdot m^2 \cdot s^1$. When the second leaf was fully expanded the chlorophyll content of the first and second leaves of six plants in each line in the RIL population was determined by using a SPAD502 chlorophyll meter (Minolta Camera Co., Osaka, Japan). Then, the plants were moved to the dark for 4 days and the chlorophyll content was again measured using a SPAD502 chlorophyll meter.

Data Analysis

All the RILs and the corresponding parents were genotyped by using an Illumina MaizeSNP50 BeadChip (Ganal *et al.*, 2011). The final linkage map with an average interval (0.81 cM) and 1698.4 cM total length were constructed using the R program by Pan *et al.* (2012) Phenotypic data from the RIL populations and parents were analyzed using SAS version 9.1. Descriptive statistics was performed to calculate the mean and standard error (SE) of the parental lines as well as the range mean SE for the RIL population. Then, Pearson correlation coefficients were calculated to explore the relationship between SPAD reading of the first and second leaves.

QTL Mapping

Windows QTL Cartographer 2.5 (WinQTL Cart 2.5; Basten *et al.*, 2005) was used for QTL detection with average values of the two replications using a linkage map created by Pan *et al.* (2012). The whole genome scan was performed using composite interval mapping (CIM) and the relevant parameters were set with a 0.5 cM scanning interval between markers and a window size of 5 cM. Model 6 of the *Zmapqtl* module was selected for detecting QTL and estimating their effects (Basten *et al.*, 2005) and forward–backward stepwise regression with five controlling markers was used to control the background from flanking makers. After 1,000 permutations the threshold LOD value was determined at a significance level of $P<0.05$. The confidence interval of the QTL position was determined with the left marker and the right marker of the threshold LOD.

Annotation of Candidate Genes

Based on the large data on the metabolomics in PMN (www.plantcyc.org), chlorophyll pathway genes in *Arabidopsis* as well as protein sequences were collected (www.arabidopsis.org). The amino acid and nucleic acid sequences were used in tBLASTn and BLAST analyses (Altschul *et al.*, 1997) of maize homologs based on the information available in the Maize Sequence database (E value <1.0 E-10, http://ensembl.gramene.org/Zea_mays/Info/Index). The successful protein matches were analyzed by Interproscan (Quevillon *et al.*, 2005) to identify their functional motifs. When their motifs were identical the corresponding maize and *Arabidopsis* genes were then annotated as homologous genes.

Results

Phenotypic Variation of Chlorophyll Index during Dark Treatment

The Zong3 parental line showed a higher SPAD reading than the Yu87-1 parental line under both treatments and the SPAD reading of the first leaf was higher than that of the second leaf with the same treatment. However, the difference in readings between the first and second leaves of the two parents was observed under same environments with that in Zong3 showing a significant difference compared to that in Yu87-1. For Diff_F and Diff_S in Zong3 the former was significantly lower than the latter (3.92 and 8.54, respectively) but nearly the same in Yu87-1 (8.04 and 8.00, respectively). In the RIL population the average level of phenotypic variation in DF varied from 14.03 to 44.09 was 32.16, which was less than the LF (38.17) but more than the DS (20.44) and LS (29.67). Comparison of chlorophyll content before and after dark treatment showed that the Diff_F (6.92) was smaller than the Diff_S (9.85). A wider range of variation in SPAD readings was observed in the first leaf compared to that in the second leaf before or after dark treatment also wider for Diff_F than Diff_S (Table 1).

The Pearson correlation coefficients for each pair of traits indicated a significant positive correlation between DF and DS, LF and LS and Diff_F and Diff_S at $P < 0.01$ level. Furthermore, Diff_F showed a significant negative association with DF and DS at $P < 0.01$ level but no significant association with LF and LS. However, Diff_S showed a significant negative correlation with DF and DS at the $P < 0.01$ level and little positive correlation with LF and LS (Table 2). The observed high correlation between DF and DS ($p = 0.71$), LF and LS ($p = 0.88$) and Diff_F and Diff_S ($p = 0.64$) was indicative of a regulatory mechanism for chlorophyll between the first and the second leaves of the seedling.

QTL Analysis

A total of 19 putative QTLs were distributed across five chromosomes of the RIL population namely chromosomes 1, 2, 3, 7 and 10, were identified and were apparently associated with chlorophyll before and after dark treatment. These QTLs were located in 14 intervals based on five pairs that were distributed in the same region on the chromosome. However, no putative QTLs were identified for DS and Diff_S. In addition, eight major putative QTLs that accounted for >10% of the observed chlorophyll index variation were identified, which included LF7_2, LS2_3, DF1_1, DF1_2, DF7_1, DF7_2, DF10_2 and Diff_F3_2 (Table 3).

Five QTLs on chromosomes 2, 3 and 7 were identified for LF that accounted for 42.73% of the observed total phenotypic variance. Single QTLs could explain 6.87 to 10.90% of the observed phenotypic variation. The favorable alleles that increase chlorophyll index were contributed by the female parent Zong3 and located on chromosomes 3 and 7 whereas that from the male parent Yu87-1 was located on chromosome 2. In addition, five QTLs on chromosomes 2 and 3 were identified for LS, which together explained 45.16% of the observed phenotypic variation and each QTL contributed 7.79% to 10.59% of the observed phenotypic variation. Among the five QTLs the favorable alleles for improving chlorophyll index on chromosome 3 were contributed by the female parent Zong3, whereas those from Yu87-1 were on chromosome 2 (Table 3).

Six QTLs for DF explaining 69.58% of the observed total phenotypic variance was identified on chromosomes 1, 7 and 10 whereas a single QTL was responsible for 8.58 to 15.90% of the observed phenotypic variation. Among these the favorable alleles with higher chlorophyll index under dark conditions on chromosome 10 were derived from the male parent Yu87-1, whereas those on chromosomes 1 and 7 originated from the female parent Zong3. Moreover, three QTLs for Diff_F were detected on chromosomes 3 and 10 that accounted for 31.32% of the observed total phenotypic variance and a single QTL was responsible for 8.9 to 12.87% of the observed phenotypic variation. All these favorable alleles responsible for the higher response to dark conditions were derived from male parent Zong3 (Table 3).

Candidate Genes Involved in the Chlorophyll Metabolism

To obtain the homologous genes involved in chlorophyll metabolism in maize comparative genome analysis was conducted based on the large sequence data of the maize genome and the mechanisms involved in the chlorophyll metabolic pathway of *Arabidopsis* and rice. Here, seven key genes that encode enzymes that were involved in the chlorophyll metabolic pathway, including *CBR* (translated into chlorophyll b reductase), *CLH* (translated into chlorophyllase), *HCAR* (translated into hydroxymethyl chlorophyll a reductase), *CHLG* (translated into chlorophyll synthase), *CAO* (translated into chlorophyll a oxygenase), *PAO* (translated into pheophorbide a oxygenase) and *RCCR* (translated into red chlorophyll catabolite reductase) were used for colocalization analysis. The putative genes in maize were identified based on similarities in the amino acid and nucleic acid sequences of *Arabidopsis* and rice with E values of <1.0 E-10 after performing tBLASTn and BLAST (Altschul *et al.*, 1997) and the same functional motifs by Interproscan (Quevillon *et al.*, 2005). The two enzymes, CBR and CLH had two copies, whereas the others had only one copy in *Arabidopsis*. However, one copy of these genes performed differently in maize because of the existence of a special ancient tetraploid genome. Only one copy of the *HCAR* (chromosome 1), *CHLG* (chromosome 6) and *RCCR* (chromosome 1) genes were observed two copies of the *CBR* (chromosomes 3 and 5) and *PAO* (both chromosome 1) genes

Table 1: Descriptive statistics of SPAD reading in RIL population

Trait	Taxon	DF	DS	LF	LS	Diff_F[a]	Diff_S[a]
Zong3	Mean±SE	34.30±1.36	26.92±1.38	38.28±0.90	35.46±0.85	3.98±0.34	8.54±0.51
Yu87-1	Mean±SE	26.87±1.42	17.27±0.03	34.91±0.60	25.27±0.44	8.04±0.45	8.00±0.37
RIL population	N	182	180	184	184	179	175
	Range	14.03–44.09	5.27–31.02	16.52–48.99	12.43–39.07	0.97–27.88	0.86–23.56
	Mean±SE	32.16±0.54	20.44±0.4	38.17±0.47	29.67±0.37	6.92±0.42	9.85±0.34

[a]these data were decrease in the average for the first and second leaves after dark treatment

Table 2: Pearson Correlation Coefficients of SPAD reading in RIL population

Trait	DF	DS	LF	LS	Diff_F
DS	0.71**				
LF	0.64**	0.43**			
LS	0.58**	0.58**	0.88**		
Diff_F	-0.69**	-0.55**	0.11	0.08	
Diff_S	-0.29**	-0.62**	0.29**	0.28**	0.64**

**Significant probability at 0.01 level

Table 3: QTLs-associated with the chlorophyll content in Zong3/Yu87-1 RIL populations

QTL	Chr[a]	Peak Marker	Genetic position[b] (cM)	Physical Position[c] (Mb)	Interval (cM)	LOD	Add	R^2
LS3_1	3	SYN9021	16.3	2.29	14.9–22.8	4.63	1.50	9.01
LS3_2	3	SYN28626	23.9	2.81	22.8–25.8	3.88	1.41	7.79
LS2_1	2	PZE-102175453	265.3	21.78	263.8–266.9	4.11	-1.46	8.38
LS2_2	2	PZE-102177131	270	219.78	266.9–271.8	4.78	-1.58	9.39
LS2_3	2	PZE-102177929	277.1	220.65	271.8–279.3	5.42	-1.67	10.59
LF7_1	7	PZE-107076499	125.6	131.65	124.1–126.5	3.81	1.70	7.46
LF7_2	7	PZE-107082484	134.5	137.49	128.5–141.6	5.81	2.03	10.90
LF7_3	7	PZE-107084584	143.5	140.26	143.3–144.5	3.52	1.62	6.87
LF3_1	3	PZE-103003116	17.9	2.38	14.7–27.4	4.75	1.93	9.13
LF2_1	2	PZE-102188267	305.6	232.36	302.8–311.8	4.65	-1.91	8.37
Diff_F10_1	10	PZE-110044162	87.1	84.07	85.8–87.8	4.05	-1.60	8.90
Diff_F3_1	3	PZE-103011348	41.0	5.99	38.9–44.3	4.10	-1.90	9.55
Diff_F3_2	3	SYN14580	48.6	7.41	45.6–52.4	5.80	-2.27	12.87
DF10_1	10	PZE-110040221	82.0	77.27	79.4–85.1	4.52	-2.13	8.58
DF10_2	10	PZE-110044398	87.5	84.24	85.1–94.4	6.32	-2.48	11.44
DF7_1	7	PZE-107076499	126.0	131.65	122.9–128.2	6.38	2.39	12.38
DF7_2	7	SYN22398	132.0	134.13	128.2–141.8	8.46	2.76	15.90
DF1_1	1	PZE-101014266	40.3	8.10	39.5–45.7	5.41	2.33	10.61
DF1_2	1	SYN372	50.3	12.36	45.7–53.0	5.89	2.30	10.67

Note: Marker: closest marker to the QTL position. Peak: position of the LOD peak on the genetic linkage map in centiMorgans. Interval: support interval on the linkage map in which the LOD decreases by half. Add: additive genetic effect of the Zong3 allele on trait expression (the effect of the alleles assumes that the favorable allele came from Zong3). R^2 value (coefficient of determination): the percentage of phenotypic variance explained by marker genotypes at the locus

were detected and three copies of the *CAO* (chromosomes 3, 6 and 8) and *CLH* (two located on chromosome 1 and one on chromosome 7) genes were identified.

Colocalization of QTLs with Candidate Genes

To colocalize QTLs with candidate genes involved in chlorophyll metabolism, the physical position of the peak marker was defined and used. The physical map was published in the maize database (Table 3 http://ensembl.gramene.org/Zea_mays/Info/Index). Here, only one QTL interval located on chromosome 3 (LF_3 and LS_3_1) and peak position between genomic positions 2,291,828 and 2,383,657, which affected LS and LF were co-localized with a candidate gene that encoded chlorophyll b reductase (*GRMZM2G170013*) which is involved in the chlorophyll metabolic pathway (Table 4).

Discussion

Chlorophyll is one of the most important physiological traits for leaf photosynthesis. Several studies on QTL mapping for chlorophyll have been reported and a large number of QTLs have been identified on 10 maize chromosomes using different mapping populations including subjected to normal and adverse environments (Hund *et al.*, 2004; Jompuk *et al.*, 2005; Messmer, 2006; Trachsel *et al.*, 2010; Zaidi *et al.*, 2015). Hund (2004) conducted genetic mapping in $F_{2:4}$ from a Lo964 × Lo1016 cross under cold stress and discovered seven QTLs for SPAD value at chromosomes 1, 3, 4, 5 and 10. Jompuk *et al.* (2005) also reported 10 QTLs that were responsible for SPAD values of the third leaf of maize seedlings under different times of sowing in one $F_{2:3}$ population and three of these on chromosomes 1, 3 and 10

Table 4: Information of chlorophyll-related genes in *Arabidopsis* and maize

Gene	Gene accession number in *Arabidopsis*	Maize		References
		Gene accession number	Location	
CBR	AT4G13250(*NYC1*)	GRMZM2G170013(*ZmCBR1*)	3:2.1Mb	(Horie *et al*., 2009; Nakajima *et al.*, 2012)
	AT5G04900(*NOL*)	GRMZM2G065194(*ZmCBR2*)	5:15.7Mb	
HCAR	AT1G04620	GRMZM2G056686(*ZmHCAR*)	1:201.9 Mb	(Ito and Tanaka, 1996; Meguro *et al.*, 2011; Sakuraba *et al.*, 2012b)
CHLG	AT3G51820	GRMZM2G162672(*ZmCHLG*)	6:140.5 Mb	(Gaubier *et al.*, 1995)
CAO	AT1G44446	GRMZM2G425647(*ZmCAO1*)	6:114.7 Mb	(Tanaka *et al*., 1998; Tanaka *et al*., 2001)
		GRMZM2G171390(*ZmCAO2*)	3:146.6 Mb	
		GRMZM2G038487(*ZmCAO3*)	8:156.7 Mb	
CLH	AT5G43860 AT1G19670	GRMZM2G127421(*ZmCLH1*)	1:239.9 Mb	(Tsuchiya *et al*., 1999; Kariola *et al.*, 2005; Harpaz-Saad *et al*., 2007)
		GRMZM2G170734(*ZmCLH2*)	7:62.1 Mb	
		GRMZM2G103197(*ZmCLH3*)	1:204.5 Mb	
PAO	AT3G44880(*ACD1*)	GRMZM2G339563(*ZmPAO1*)	1:10.1 Mb	(Gray *et al.*, 2002)
		GRMZM2G349062(*ZmPAO2*)	1:290.2 Mb	
RCCR	AT4G37000(*ACD2*)	GRMZM2G458824(*ZmRCCR*)	1:141.0Mb	(Mach *et al*., 2000)

were common QTLs that were associated with early and late sowing times. Trachsel *et al.* (2010) detected three QTLs on chromosomes 1 and 7 at the seedling stage of the second leaf that explained 7–11% of the observed phenotypic variation in a RIL maize population. Zaidi *et al.* (2015) used a RIL population to analyze waterlogging tolerance and one major QTL on the short arm of chromosome 2 explained 13.6% of the observed phenotypic variation in the SPAD value of the flag leaf. Another two major QTLs on chromosomes 2 and 10 were identified at the flowering stage in temperate $F_{2:3}$ maize lines (Messmer, 2006). In the present study QTL mapping for chlorophyll content was performed by using one RIL population that was subjected to two conditions: before and after dark treatments. A total of 19 QTLs including eight major QTLs with>10% phenotypic variation that were located on chromosomes 1, 2, 3, 7 and 10 were associated with SPAD values that were determined at two leaves under two treatments. Among of these, LF7_3, DF10_1, DF10_2 and Diff_F10 shared the same physical interval as that described in previous reports (Jompuk *et al.* 2005; Trachsel *et al.* 2010; Zaidi *et al.* 2015). However, other QTLs were localized to a new interval, which may be due to differences in genetic background of the segregating population, stages and treatment. The findings of the present study suggested that a number of major and minor QTLs contribute to phenotypic variation in chlorophyll content in maize, thus reflecting the complex genetic nature of chlorophyll pigments.

Among the 19 putative QTLs, one common QTL was responsible for LF and LS two common QTLs were identified for LF and DF and one common QTL explained DF and Diff_F. However, other QTLs such as those on chromosomes 1 and 10 affected DF were responsible for a given trait after by two specific conditions. These findings indicate that some specific genes modulate chlorophyll under different photoperiods particularly those involving normal illumination (before dark treatment) and extreme photoperiodism (after dark treatment). No QTL was associated with DS and Diff_S, which varied between the first and the second leaves. For Diff_S a nearly phenotype value in the parents and the narrow range of phenotypic variation in the population may explain the absence of a putative QTL. The same phenomenon was observed for DS despite significant differences in the DS between parents and the population variant (Table 1). It is possible that extensive regulation occurs among different parts of the leaves and the chlorophyll pigment of the first leaf may have been transported to the second leaf to maintain healthy growth (Masuda and Fujita, 2008). Most of the QTLs for same traits such as LS and DF were also clustered with a common chromosomal region (Table 3) suggesting that these regions may harbor more than one gene or are involved in various interactions involving different functional genes.

Several biosynthetic genes regulate chlorophyll metabolism of which seven are related to chlorophyll a and chlorophyll b in *Arabidopsis*, including *CBR*, *HCAR*, *CHLG*, *CAO*, *CLH*, *PAO* and *RCCR* (Hörtensteiner and Krautler, 2011; Hörtensteiner, 2013). Maize homologous genes were identified based on nucleic acid and protein sequence analysis with some genes having more than one copy in maize and the physical position, chromosome location and gene accession number of these candidate genes in B73 are shown in Table 4. Here, only one candidate gene (*GRMZM2G170013*, which is translated into chlorophyll b reductase) which is involved in the chlorophyll pathway was colocalized to the QTL (LF3 and LS3_1) on chromosome 3 thus indicating that this gene influences the chlorophyll content of different part of leaves at the seedling stage. A similar phenomenon was observed for tocopherol in which only the candidate genes *VTE4* and *HPPD5* could not be collocated to the identified QTLs (Xu *et al.*, 2012). These findings may be due to the complex genetic mechanisms of these quantitative traits and their genotype may have influenced their agronomic traits during natural and human selection.

The key role of *CBR* is to convert chlorophyll a to chlorophyll b to adjust its ratio and to degrade LHCII during

leaf senescence (Horie *et al.*, 2009; Sato *et al.*, 2009; Nakajima *et al.*, 2012). Based on the function of this gene in the chlorophyll pathway it may influence chlorophyll in the first and second leaves by regulating the transfer and conversation between chlorophyll a and chlorophyll b. Furthermore, candidate associated analysis were conducted in one associated panel and some alleles from this gene showed an association with chlorophyll compounds and related degradation traits to some extent (Aye *et al.*, 2017). However, further and deeper investigation on this gene such as fine mapping and functional validation is necessary to confirm of results.

Based on the importance of chlorophyll, which affected grain yield and may apparently be used as a physiological index of senescence and resistant to adversity the new QTLs identified in this experiment under light and dark conditions could provide complementary information for better understanding the genetic basis of chlorophyll content and highlight a new research field in relation to photoperiodism. Also, in relation to the development and the high efficient of marker-assisted selection (Huang *et al.*, 1997; Collard and Mackill, 2008; Zhao *et al.*, 2012) the major QTLs with higher phenotypic variations could be used as molecular markers for stay-green breeding in maize especially for silage corn.

Conclusion

Genetic analysis of chlorophyll genes in the Zong3 and Yu87-1 RIL population showed that chlorophyll from first and second leaves at the seedling stage (V2) were controlled by major and minor QTLs each explaining 7.79–15.9% of the observed variations under both before and after dark treatment suggesting that regulation for chlorophyll at different leaf positions in different environments was general as well as specific In addition, four major QTLs were observed on chromosomes 1 and 10 during after dark treatment, which was indicative of the important role of these chromosomes in chlorophyll content during senescence. Of the detected QTLs one QTL on chromosome 3 was colocalized with one candidate gene (*GRMZM2G170013,* which encodes for chlorophyll b reductase) that is involved in the chlorophyll pathway indicating that this gene affects chlorophyll content at the seedling stage by adjusting the chlorophyll ratio. Further investigations on this gene are thus warranted.

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