Bangladesh J. Bot. **47**(3): 711-718, 2018 (September) Special

IDENTIFICATION OF USEFUL ALLELE OF *ZmRCCR* FOR CHLOROPHYLL COMPOUNDS DURING MATURATION IN MAIZE

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Keywords: Maize, Chlorophyll, *ZmRCCR*, Association analysis

Abstract

Candidate association mapping for red chlorophyll catabolite reductase gene (*ZmRCCR*) were analyzed for chlorophyll content in 141 maize inbred lines. Finally, 18 polymorphic sites were identified, which were significantly associated with the chlorophyll-related traits. The InDel S-53 from 5'UTR (5' untranslated region) decided 9.81% of phenotypic variation for chlorophyll a at T5 in Yangling, and also explained 10.05% phenotypic variation for chlorophyll b at T5 in Yulin. Excitingly, another In Del S278 in the exon 1 was associated with six traits, and the phenotypic interpretation rate range from 10.67 to 24.99%. These results indicated that *ZmRCCR* plays an important role in chlorophyll compounds content during maturation with more than one independent locus. Furthermore, these findings improve the understanding of the genetic basis of dynamic chlorophyll metabolism in late mature maize and provide information for the development of functional markers based on *ZmRCCR*.

Introduction

Chlorophyll is one of the most important photosynthetic pigments in the biosphere, which is mainly chlorophyll a (Chl a) and chlorophyll b (Chl b).Chlorophylls form light system and light system reaction takes place by absorbing light energy and converting photon energy into chemical energy in the form of excitation (Mathis and Burkey 1989, Agostiano *et al.* 1990), which plays a central role in photosynthesis (Tanaka *et al.* 1991).The chlorophyll content is an important physiological index to measure leaf senescence and is an important factor restricting the further improvement of maize yield (Liu 1983).

The study of chlorophyll metabolism has been developing very fast because of its important role. Until 2003, chlorophyll metabolisms had been descripted clearly in model plant - *Arabidopsis* by forward and reverse genetics. And the schematic of chlorophyll metabolism has been expounded plainly including chlorophyll synthesis, chlorophyll cycle and chlorophyll degradation (Hortensteiner and Krautler 2011). Chlorophyll breakdown begins with the chlorophyllase which catalyzed the formation of chlorophyllide, and then a metal chelate to remove magnesium ions to form pheophorbide a, the macrocyclic ring of pheide a is then opened by pheophorbide an oxygenase (PAO), chlorophyllase (RCC). The RCC is further catabolized into primary fluorescence fluorescent catabolite (pFCC) by RCCR and finally transported to vacuole after several modifications and is transformed into Non-fluorescent chlorophyll catabolite (NCCs) (Hortensteiner and Krautler 2011).

Red chlorophyll catabolite reductase (RCCR) relies on the reduced ferredoxin (fd) to provide electrons for the reduction of double strands between C_{20}/C_1 in RCC, converting RCC to pFCC (Pruzinska *et al.* 2007). At present, *RCCR* gene has been cloned successfully in *Arabidopsis* and

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barley (Mach *et al.* 2001, Wüthrich *et al.* 2010). The overexpression of ACD2 protein can alleviate the symptoms and cell death of *Arabidopsis* caused by *Pseudomonas syringes* (Mach *et al.* 2001). These results indicate that chlorophyll degradation is closely related to plant green cell death and RCCR plays a key role in the whole process (Greenberg *et al.* 1994).

Therefore, identification of the functional alleles will not only improve the understanding of the genetic mechanism that controls variation in chlorophyll content but also provide invaluable information that may be utilized by crop improvement programs. According to previous research and method (Hortensteiner 2013, Chan *et al.* 2017a), an association analysis of chlorophyll compounds at the late maturity stage of RCCR gene and 141 maize inbred lines at normal levels was carried out. The objective of the present study was to explore the chlorophyll metabolism and the variability of the chlorophyll gene in the late maturity stage of maize.

Materials and Methods

An association mapping group consisting of 141 maize inbred lines (AM141) was selected from Shaan A and Shaan B breeding basic population by Northwest A & F University. In 2017, all materials were planted with randomized blocks design with two replications in Yulin and Yangling which are the two important maize producing areas in Shaanxi province, China. After 20 days flowering (T1), 10 circle pieces with 6 mm diameter were cut from the middle part of ear leaf in 2 ml tubes and immediately merged in 1 ml 95% ethanol. Chlorophyll compounds were determined for 7 times with six days interval. The chlorophyll compound was extracted over 48 hrs in 4ºC under dark. After centrifuging the leaching liquor the supernatant containing chlorophyll were determined with a UV-vis spectrophotometer (Bio-RAD, Shanghai, China) at 645 and 663 nm and the absorbance were expressed as A645 and A663, respectively. Chl a and chl b were calculated according to Arnon (1949). All traits data were analyzed using the Microsoft Excel program.

To find the *ZmRCCR* gene in maize (http://www.maizesequence.org/, AGPv4), *RCCR* gene sequence was cloned in *Arabidopsis*, using the relevant protein sequence. In maize, a single copy gene (*ZM00001D030549*) encoding the red chlorophyll catabolite reductase was detected. Genomic DNA of AM141 was extracted from fresh leaves when six leaves were expanded fully by using CTAB method (Murray and Thompson 1980). The primers of *ZmRCCR* were designed using the corresponding gene sequence from B73 reference genome sequence by Primer Premier 5.0 software. Multiple sequence alignments were performed using MUSCLE (http://www.ebi.ac.uk/Tools/msa/musc) and BioEdit for the gene sequencing results.

Population structure and kinship were evaluated base on high density SNPs. Moreover, TASSEL (Bradbury *et al.* 2007) was used to extract SNPs and InDels from the RCCR genes of 141 maize lines with a minor allele frequency (MAF) of ≥ 0.05. TASSEL was also used to calculate the Linkage disequilibrium (LD) between each two sites, and the sites that showed strong linkage ($R^2 > 0.2$) were seem as the linked sites and the site with the most significant traits were selected (Hill and Robertson 1968). Mixture linear model (MLM) analysis in TASSEL (Yu *et al.* 2006) was used to incorporate population structure and kinship and to estimate individual polymorphisms to phenotypic variation explanation for chlorophyll-related traits.

Results and Discussion

According to the different climate conditions in the two experiment locations, the chlorophyll data performed differentially in the two locations. For total chlorophyll, the maximum content from Yulin was 4.58 mg/l at T4 stage with minimum of 3.09 mg/l in T7 stage. While in Yangling location, the maximum total chlorophyll content was 4.52 mg/l at T5 stage, and the least content was 3.05 mg/l in T2 stage (Table 1). The results also showed that the chl a content of maize was

generally lower than that of chl b in the later stage of maturation, and the total chlorophyll content was also decreasing at the later stage. This observation indicated that the rapid transformation of chl a to chl b was taking place during senescence at maturity.

Table 1. General statistical description of chlorophyll content.

Place	Time [a]	Chl a (mg/ml)		Chl b (mg/ml)		Total[b](mg/ml)		Ratio[c]	
		Mean ± Sd	Range	Mean ± Sd	Range	Mean ± Sd	Range	Mean ± Sd	Range
Yulin	T1	0.68±0.18	0.40-1.45	2.66±0.37	2.16-4.62	3.34±0.52	2.59-0.08	0.25±0.05	0.16-0.35
	T2	0.95±0.23	0.66-2.00	2.81±0.42	2.32-4.98	3.76±0.64	2.98-6.98	0.33±0.04	0.28-0.40
	T3	1.15±0.18	0.86-1.69	3.07±0.29	2.61-4.23	4.23±0.47	3.46-5.93	0.37±0.03	0.32-0.42
	T4	1.27±0.23	0.72-1.80	3.31±0.29	2.62-4.32	4.58±0.51	3.34-6.06	0.38±0.04	0.27-0.45
	T5	0.73±0.29	0.01-1.31	2.83±0.33	2.28-4.13	3.58±0.51	2.39-5.02	0.26±0.09	0.00-0.38
	T6	0.52±0.20	0.15-1.07	2.89±0.42	2.30-4.36	3.41±0.60	2.48-5.40	0.17±0.05	0.06-0.31
	T7	0.67±0.18	0.39-1.43	2.45±0.31	1.35-3.89	3.09±0.52	0.00-5.31	0.27±0.07	0.18-0.63
Yang ling	T1	0.44±0.21	0.08-1.12	3.17±0.39	2.53-4.61	3.63±0.58	2.69-5.72	0.13±0.05	0.03-0.31
	T2	0.37±0.28	0.01-1.65	2.63±0.41	1.66-4.48	3.05±0.63	2.19-5.64	0.13±0.09	0.01-0.44
	T3	0.55±0.17	0.27-0.94	2.70±0.25	2.07-3.41	3.25±0.42	2.45-4.35	0.20±0.05	0.12-0.27
	T4	1.33±0.22	1.00-2.19	2.84±0.54	1.14-5.15	4.17±0.68	2.49-7.34	0.48±0.12	0.37-1.18
	T5	1.35±0.23	0.97-2.08	3.17±0.43	2.52-4.87	4.52±0.65	3.56-6.95	0.43±0.03	0.37-0.63
	T6	1.32±0.19	0.99-1.99	3.15±0.37	2.30-4.66	4.46±0.54	3.39-6.65	0.42±0.03	0.37-0.62
	T7	1.11±0.18	0.81-1.53	2.71±0.33	1.95-3.64	3.82±0.50	2.83-5.18	0.41±0.04	0.35-0.56

[a] T1 indicates the period of 20 days of pollination (Others are same a argument). [b]Total value indicates the contents of Chl a and Chl b. [c]Ratio value indicate ratio of Chl a to Chl b (unit : 1).

After implementing TBLASTN in maize genome database by using protein sequence in *Arabidopsis* and rice, a single copy gene (*ZM00001D030549*) designated as red chlorophyll catabolite reductase chloroplastic. Here, it was named as *ZmRCCR*. which consists of 2 exons spanning 2267bp with 1521bp CDS length encoding a 332 amino acid protein sequence. After screening the primmer, three pairs of primers (Table 2) with a product size within the range of 800 - 1500 bp were left and used for re-sequencing the gene in AM141. Finally, a total of 54 polymorphic sites including 36 SNPs and 18 InDels were detected after multiple sequence alignment. In another word, each 40 bp genomic sequence can find one polymorphic site. Nucleotide polymorphisms were not evenly distributed in different parts of *ZmRCCR* gene and the highest level of nucleotide diversity was observed in exon 1 (Table 3).

For association analysis, 18 (11 SNPs and 7 InDels) distinct polymorphic sites identified in the gene were significantly ($p < 0.05$) associated with chlorophyll compounds after LD analysis. There were 12 associated sites associated with two or more traits (Fig.1). Among the 18 sites, two InDels were in UTR, as one in intron region and the rest were located in exon region with amino acid changes and frame shift (Table 4).

In Yulin , 10 unique associated events were discovered for chlorophyll compounds at T1, T2, T4, T5 and T6. In addition, they were assembled at related trait at T4 stage and T5 stage. The top two significant sites were the SNP site at S241 (G/T, $p = 1.26 \times 10^{-3}$) and the InDel site at S278 ($p = 1.04 \times 10^{-3}$) which explained 15.99 and 14.73% of the phenotypic variation for Chl b, respectively. The favorite alleles were T (S241) and 1 bp insertion (S278) for improving the

Table 2. Primers used in this study.

Primer name	Primer sequence (5' - 3')
RCCR_1F	GGTCCAAGACACTCCTTCACA
RCCR_1R	CCATAACCGTATTACGCACAG
RCCR_15F	TGACTCGGAATCGGCACA
RCCR_15R	GCCTCCTGAATGACCTTTGC
RCCR_6F	CGACGGACGCACCACATT
RCCR_6R	TCGCCACAGTTTCGCTTG

Table 3. Variation site distribution of *ZmRCCR*.

Position	5'UTR	Exon1	Intron1	Exon2	3'UTR
SNPs (nonsynonymous mutation)	4	25(16)	1	2(2)	4
Indels (frame shift)	1	14(14)	2	0	1

UTR un-translated region

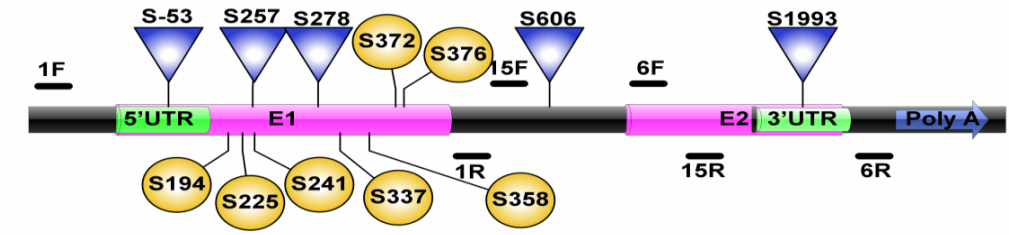

Fig. 1. Gene structure of the RCCR gene. UTR un-translated region, E exon, 1F/1R (15F/15R, 6F/6R) show primer in the corresponding position. Blue color is marked as insertion missing site (InDels). Brown color is marked as a variation site (SNPs).

chlorophyll compounds, total content and ratio. In Yangling, 17 unique associated events were identified mainly for chlorophyll compounds at five stages except T4. The most significant site was InDel S278 with $p = 9.7 \times 10^{-5}$ which explained up to 24.99% phenotypic variation for chl a at T2 stage. Meanwhile, this In Del S278 also had 13.14% phenotypic effects of chl b with $p = 3.52 \times 10^{-3}$ and can explain 10.67% phenotypic variation for ratio with $P = 1.293 \times 10^{-2}$. Here, 1 bp insert is also the favorite allele for higher phenotype. Interesting, there were only one InDel at 5'UTR with 10 bp insert associated with chlb with $p = 5 \times 10^{-3}$ at Yulin and decreased chla with $p = 7 \times 10^{-3}$ at Yangling at the same stage. Furthermore, the InDel S278 with 1 bp deletion with frame shift was not only associated with three related traits at T2 stage in Yangling but also associated with three traits at T4 stage in Yulin. Moreover, the favorite allele was consistent for different trait of different stages (Table 4). This allele can be used for molecular breeding for modifying the chlorophyll contents and ratio.

The association analysis method first introduced into the study of maize gene Dwarf8 (Thornsberry *et al.* 2001), the relationship between alleles of a large number of genes and phenotypic traits has been clarified in maize (Li *et al.* 2012, Chan *et al.* 2017a). Association analysis was used for dissecting complex quantitative traits as one main technology. However,

Table 4. Association results for chlorophyll compounds in seven periods in maturation stage.

Polymorphic site[a]	Region	Alleles	Amino acid change	Frequencies	Favorable allele[b]	Trait	p value*	R^2(%)[c]
S-53	5'UTR	0/10	-	73/4	10	Yang-chla-t5	7.00×10^{-3}	9.81
				73/4	0	Yu-chlb-t5	5.00×10^{-3}	10.05
				59/5	0	Yu-total-t4	3.68×10^{-2}	7.31
S194	E1	C/T	S-F	69/8	T	Yang-chla-t3	7.88×10^{-3}	9.75
				69/8	T	Yang-chlb-t3	1.04×10^{-3}	8.91
				54/6	C	Yu-total-t5	2.89×10^{-2}	8.81
				54/6	C	Yu-ratio-t5	1.04×10^{-3}	8.05
				59/7	C	Yang-ratio-t6	5.13×10^{-3}	13.22
S210	E1	C/G	-	6/70	G	Yang-chla-t5	6.29×10^{-3}	10.43
S225	E1	A/G	-	33/31	A	Yang-ratio-t6	7.91×10^{-3}	11.90
				33/30	G	Yang-ratio-t3	9.59×10^{-3}	12.08
				33/30	G	Yang-total-t3	1.68×10^{-2}	11.50
				30/27	G	Yang-total-t7	4.67×10^{-2}	7.81
S226	E1	1/0	Frame shift	14/60	1	Yang-chlb-t2	6.68×10^{-3}	11.28
S241	E1	G/T	V-L	69/6	T	Yu-chlb-t4	1.26×10^{-3}	15.99
				69/6	T	Yu-chla-t4	1.96×10^{-3}	14.26
				67/6	T	Yang-chlb-t2	6.78×10^{-3}	11.24
				58/5	T	Yu-total-t4	7.60×10^{-3}	14.07
				58/5	T	Yu-ratio-t1	9.94×10^{-3}	10.86
				53/5	T	Yang-ratio-t2	3.31×10^{-2}	7.78
S257	E1	1/0	Frame shift	59/4	1	Yu-ratio-t6	7.10×10^{-3}	9.66
				60/4	1	Yang-ratio-t7	1.47×10^{-2}	10.99
				54/3	1	Yang-ratio-t5	3.53×10^{-2}	7.82
S278	E1	1/0	Frame shift	5/71	1	Yu-chlb-t4	1.04×10^{-3}	14.73
				5/71	1	Yu-chla-t4	1.69×10^{-3}	13.94
				59/14	1	Yang-chlb-t2	3.52×10^{-3}	13.14
				5/65	1	Yang-chla-t2	9.70×10^{-5}	24.99
				4/60	1	Yu-total-t4	2.41×10^{-3}	16.34
				4/55	1	Yang-ratio-t2	1.29×10^{-2}	10.67
S292	E1	C/G	A-P	10/67	G	Yang-chlb-t1	9.84×10^{-3}	8.85
S334	E1	1/0	Frame shift	14/58	0	Yang-chla-t1	8.82×10^{-3}	10.07

(Contd.)

Polymorphic site[a]	Region	Alleles	Amino acid change	Frequencies	Favorable allele[b]	Trait	p value*	R^2(%)[c]
S337	E1	C/G	P-A	8/54	C	Yu-total-t1	4.04×10^{-3}	14.86
				8/54	C	Yu-ratio-t1	1.02×10^{-2}	10.57
				8/52	G	Yang-total-t1	3.53×10^{-2}	8.11
S358	E1	C/G	Q-E	70/4	G	Yu-chlb-t1	3.51×10^{-3}	13.05
				59/4	G	Yu-total-t1	2.34×10^{-3}	17.17
S366	E1	C/T	G-G	59/10	T	Yang-chla-t2	6.34×10^{-3}	11.66
S370	E1	C/T	P-S	67/8	T	Yang-chlb-t2	9.45×10^{-3}	10.25
S372	E1	C/T	P-P	6/70	C	Yu-chlb-t4	8.53×10^{-3}	9.26
				5/59	C	Yu-total-t4	6.28×10^{-3}	13.14
				5/60	C	Yang-ratio-t6	6.18×10^{-3}	12.67
S376	E1	C/T	P-S	11/46	T	Yu-ratio-t5	1.60×10^{-3}	19.20
				8/47	T	Yang-total-t2	8.27×10^{-3}	13.39
				8/47	T	Yang-ratio-t2	2.57×10^{-2}	8.89
				11/49	T	Yang-total-t3	3.42×10^{-2}	7.97
S606	I1	1/0	-	19/74	1	Yu-chla-t1	3.40×10^{-2}	5.03
				16/69	0	Yang-total-t6	4.51×10^{-3}	10.81
				16/69	0	Yang-ratio-t6	4.15×10^{-2}	5.36
				16/66	0	Yang-ratio-t1	4.20×10^{-2}	5.34
				16/61	1	Yu-total-t5	3.43×10^{-2}	5.91
				16/67	1	Yu-total-t1	4.09×10^{-2}	5.39
S1993	3'UTR	0/124	-	92/7	124	Yang-chlb-t7	1.60×10^{-2}	6.00
				87/7	124	Yu-ratio-t2	4.58×10^{-2}	4.74
				79/7	0	Yang-total-t7	4.02×10^{-2}	5.42

UTR un-translated region, E exon, I intron, Yu expressed the material came from Yulin. Yang expressed the material came from Yangling, t1 represents the first sampling(Others are same argument). [a]Only polymorphic sites significantly associated with chlorophyll-related traits are shown. Sites were named in accordance with the distance from the start code (ATG) of the gene. [b]Favorable alleles associated with higher chlorophyll related traits. [c]R2 from ANOVA shows the percentage of explainable phenotypic variation. [*]p values for association analysis are calculated using a mixed model in TASSEL that incorporates population structure and kinship.

there are a few reports about dissecting functional alleles of chlorophyll metabolism related genes, even if the pathway were clearly illuminated in *Arabidopsis.* A large number of studies were initial QTL mapping which identified some putative QTLs located in big genome interval or physical experiment to response the environment adversity (Harpazsaad *et al.* 2007, Meguro *et al.* 2011, Nakajima and Tanaka 2012, Sakuraba *et al.* 2013 , Simic *et al.* 2014, Chan *et al.* 2017b).

Similarly as *ZmNYC1* several associated sites were also found in *ZmRCCR* (Chan *et al.* 2017a). Most of the above sites are located in exon and UTR region, these changes would lead

variant of gene construction or expression. These sites play an important role in the regulation of RCCR gene. Even though, previous experiments suggested that RCCR activity remains almost unchanged at every stage of leaf development (Pruzinska *et al.* 2005). It can adjust the chlorophyll contents by protein change induced by DNA structure change, such as S194 and S278.

In addition, grain yield were also analyzed on the association with the identified polymorphism locus, because of the important role of chlorophyll in photosynthesis. However, no association site was detected. The reason could be that yield was very complex trait, although chlorophyll metabolism regulates the synthesis of carbohydrates, the effect of single gene on yield is not significant. Meanwhile, RCCR gene plays a major role in the final stage of chlorophyll degradation (Hortensteiner 2013). During the actual production, grain filling has been finished and dehydration has entered the final stage during the senescence of maize leaves, the yield was basically finalized, so the gene failed to detect the yield-related sites (data not shown).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31701438), and Project supported by Chinese Universities Scientific Fund (Grant No.2452015008).

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(Manuscript received on 27 July, 2018; revised on 3 September, 2018)