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Short communication

CLONING AND SEQUENCE ANALYSIS OF A PUTATIVE CCT-MOTIF GENE IN TEN FOXTAIL MILLET CULTIVARS

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ABSTRACT

CCT-motif genes are key genes participating in photoperiod-control plant flowering, and proved to be closely related to crop production. In this study, a putative foxtail millet CCT-motif gene was cloned in ten cultivars, and seven agronomic traits of these cultivars were investigated. The results showed that totally 34 mutation sites including 33 SNPs and 1 InDel (insertion-deletion) were detected in coding region of CCT-motif gene among the ten cultivars, which led to sixteen aa substitution and one aa insertion/deletion among CCT-motif gene protein sequences of 10 foxtail millet cultivars. According to the mutation types of CCT-motif gene protein sequences, ten foxtail millet cultivars could be divided into seven genotypes. The nucleotide polymorphism value (θ) of CCT-motif gene coding sequence was 0.017, indicating the low diversity level of this gene in selected ten foxtail millet cultivars. The Tajima'D value was 0.998, greater than zero, indicating the CCT-motif gene subject to natural selection during evolutionary process. LD analysis showed that a large LD structure including twenty mutation sites was existed in coding region. All of the results above gave valuable information for launching association analysis study between the CCT-motif gene and main agronomic traits in foxtail millet. The mutation information in coding region of CCT-motif gene provided valuable clue for further discovering function altering mechanism of this gene. Seven agronomic traits, days to heading (DH), plant height (PH), panicle length (PL), ear diameter (ED), branch number per panicle (BNP), grain number per branch (GNB) and 1000-grain weight (1000-GW), ranged from 38 to 74, from 56.8 to 128.7, from 6.8 to 21.6, from 3.0 to 7.6, from 58 to 113, from 7 to 72, from 0.7 to 2.8 respectively, indicating abundant genetic variation existed among the ten selected cultivars. Association analysis showed that three mutation sites, locus 109, locus 161, locus 424 were associated with 1000-grain weight (1000-GW), one mutation site, locus 588, was associated with panicle length (PL). Conclusion: cloned a putative CCT-motif gene in foxtail millet, this gene showed low diversity level and subject to natural selection during evolutionary process. Four mutation sites in CCT-motif gene coding region associated with1000-GW and PL.

Key words: Foxtail millet, CCT-motif gene, SNPs, LD analysis, Association analysis.

INTRODUCTION

CONSTANS (*CO*) was the first cloned gene that participated in photoperiod-control flowering pathway in Arabidopsis (Putterill *et al*., 1995). In carboxy terminal region of CO protein, a nuclear localization sequence including 43-45 aa was found, and the further studies proved that this nuclear localization sequence widely existed in CONSTANS-type genes and TOC1 gene, so which was named CCT motif (Griffiths et a1., 2003). Besides nuclear localization function, the CCT motif was also related to flowering in long day condition, as some mutations in CCT motif could lead to lost of promoting flowering function in long day (Onouchi *et al*., 2000).

Foxtail millet is an important crop in a long history period in China. In recent years, the balance nutrition composition, the smaller genome suitable for an ideal research model of C4 crops and energy crops make foxtail millet paid more attention by researchers of domestic and overseas. The complete genome sequence of foxtail millet was determined in 2012 by China and America respectively (Zhang *et al*., 2012; Bennetzen *et al*., 2012). While as a short-day and warm-like crop, foxtail millet was sensitive to photoperiod, which limited its plant region. CCT-motif genes were proved to be the key genes participating in photoperiod-control flowering pathway in Oryza sativa, Zea mays, Sorghum bicolor and so on (Xue *et al*., 2008; Miller *et al*., 2008; Murphy *et al*., 2014). The natural variation of CCT-motif genes was closely related to ecological adaptability (Hung *et al*., 2012; Murphy *et al*., 2014). So the aim of this study is to clone a putative CCT-motif gene in foxtail millet by homologous cloning method, analysis of gene sequence variation in ten foxtail millet cultivars, preliminarily

explore the relationship between CCT-motif gene variation and seven agronomic traits.

MATERIALS AND METHODS

Ten foxtail millet cultivars including Jigu1, Xianzihui, 71za30-2, Baimi1, An04-5014, Jiyecong4, Longgu26, Jigu27, Fagu28-81, Yugu1 were kindly provided by professor Lu ping (Institute of Crop Science, Chinese Academy of Agricultural Sciences). The foxtail millet seeds were sown in experimental field of Henan University of Science and Technology on May 8, 2014, harvested during September, 2014. The planting pattern: two rows per cultivar, row spacing was 30 cm, plant spacing was 5 cm, 20 plants per row. The traits investigated including days to heading (DH), plant height (PH), panicle length (PL), ear diameter (ED), branch number per panicle (BNP), grain number per branch (GNB) and 1000-grain weight (1000-GW).

We used a Sorghum bicolor CCT-domain protein (Ghd7) mRNA (Accession number: JX448388.1) to blast search foxtail millet genome database deposited in phytozome10.3 (http://phytozome.jgi.doe.gov /pz/portal.html), and a CCT-motif gene mRNA sequence coding 216 aa was found (Accession number: Si011920m), which reached a 74% identity rate with Sorghum bicolor CCT-domain protein. The genomic sequence of this foxtail millet CCT-motif gene was also found by blast search (Accession number: gi|513046637). Two pairs of primer sequences were designed to amplify about 2470 bp genomic sequence of putative CCT-motif gene. The primer pairs were: F1-CCTAGCATCTTGTTCCTCCT, R1-GCTCCCACAGAATGACATC and F2-GTGGGAGCACATTCACTG, R2-TTGGAAAGTTGATGCACTG. PCR reactions were performed in 25µl volumes containing 50 ng of genomic DNA, 200 µM dNTPs, 2.5 µl 10×PCR buffer, 0.5 µM each of forward and reverse primer, 1.25 U Taq DNA polymerase (Tiangen, Beijing, China). The PCR profile included an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 40 s at 94°C , 40 s at 55°C and 1 min at 72°C . Finally, an extension of 72°C for 5 min was followed. The PCR production was cloned to pMD-18 vector (Takara, Dalian, China), then transformed to Top10 competent cells (Tiangen, Beijing, China). The positive clones were sent to Sunbiotech Company (Beijing, China) for sequencing. The Clustal 1.8 software was used to perform multiple sequence alignment and the Tassel 2.1 software was used to perform gene diversity, LD analysis, preliminary association of CCT-motif gene mutation sites and seven agronomic traits.

RESULTS AND DISCUSSION

Table 1 gave the traits measured value of ten foxtail millet cultivars. According to the first trait “days to heading”, ten foxtail millet cultivars could be divided into three groups: long days to heading group, which including An04-5014, Jigu1, Baimi1 and Fagu28-81, middle days to heading group, which including Yugu1, Jiyecong4, 71za30-2, Xianzihui, short days to heading group, which including Jigu27 and Longgu26. The cultivars with longer DH showed longer PL. The cultivars with the shortest DH such as Longgu26 and Jigu27 showed the smallest PL, ED, and BNP.

Two pairs of primers produced about 1713bp and 764bp fragments respectively after PCR amplifying genomic DNA of ten foxtail millet cultivars (Fig. 1). After sequencing, the two amplification fragments were assembled into an about 2471 bp fragment which contained the complete CCT-motif gene coding sequence. The CCT-motif gene included two exons and one intron.

After multiple sequence alignment, 158 mutation sites were detected among CCT-motif gene sequences of ten foxtail millet cultivars, while only 34 sites existed in the coding region. The 34 mutation sites included 33 SNPs and 1 InDel (insertion-deletion), 79 percent of which existed in exon 1, only 21 percent of which existed in exon 2, indicating the high conservative property of CCT-motif as this conservative motif lied in exon 2 (Table 2). Of the 33 SNPs sites in coding region, sixteen were C/T type substitution, twelve were G/A type substitution, the remaining five were G/C and A/C types substitution. According to the putative CCT-motif gene mRNA sequence of Yugu1 (Accession number: Si011920m), we predicted the CCT-motif gene protein sequences of ten foxtail millet cultivars and performed the multiple sequence alignment. Among the ten protein sequences, sixteen aa substitution sites and one aa insertion/delete site were detected (Fig. 2), which could divided the ten foxtail millet cultivars in to seven genotypes: I type included An04-5014 and Baimi1, showing long days to heading phenotype, II type included Yugu1 and Fagu28-81, showing middle or long days to heading phenotype, III type included Jiyecong4 and Jigu27, showing middle or short days to heading phenotype. The remaining four cultivars, Longgu26, 71za30-2, Jigu1, Xianzihui, formed IV, V, VI, VII genotypes respectively. IV showed short days to heading phenotype, V, VII showed middle days to heading phenotype, VI showed long days to heading phenotype.

The nucleotide polymorphism value (θ) of CCT-motif gene coding sequence was 0.017, indicating the low diversity level of this gene in selected ten foxtail millet cultivars. The Tajima’D value was 0.998, greater than zero, indicating the CCT-motif gene was subjected to natural selection during evolutionary process. LD

analysis showed that the mutation sites in coding region existed some large LD structures (Fig3), the largest LD structure included twenty mutation sites, they were locus 60, locus 116, locus 149, locus 152, locus 155, locus 173, locus 192, locus 245, locus 257, locus 264, locus 266, locus281, locus310, locus 338, locus 367, locus 383, locus 407, locus 441, locus 457, locus 483. The association analysis between 34 mutation sites in CCT-motif gene coding region and seven agronomic traits was done. The results showed that only four sites were associated with two of the seven agronomic traits ($p<0.05$).Three sites, locus 109, locus 161, locus 424 were associated with1000-GW, one site, locus 588, was associated with PL (Table 3). No sites associated with DH were found.

In this study, we found a very interesting thing. Though many mutation sites (158) were detected among the CCT-motif genomic sequences of ten foxtail millet cultivars, two foxtail millet cultivars, Yugu1 and Fagu28-81, had a 100% identity rate through whole genomic sequence of CCT-motif gene. In the other word, Yugu1 had the same CCT-motif genomic sequence as Fagu28-81. As Yugu1 was a wide-ecological adaptation cultivar, insensitive to photoperiod and temperature, its genomic sequence has been determined (Bennetzen *et al*., 2012).The CCT-motif genes were proved to be related to photoperiod-control flowering pathway (Onouchiet a1. 2000). The result in this study seemingly supported that Yugu1 contained the genetic component from Fagu28-81, but whether the genetic component from Fagu28-81 played some role in Yugu1's insensitivity to photoperiod and temperature should be further investigated.

In rice, the CCT-motif gene Ghd7 was proved to be closely related to yield. Some mutation sites of Ghd7 gene associated with plant height, grain number and heading date (Xue *et al*., 2008). In this study, the CCT-motif gene of foxtail millet was also related to yield, as four mutation sites of this gene were associated with two important yield-related traits, panicle length and 1000-grain weight. But no mutation sites associated with plant height, grain number and heading date were found, which may be attributed to the fewer cultivars selected or the difference between Ghd7 and the CCT-motif gene in this study, as the CCT-motif type genes included many family members. For example, the hd1 gene in rice was proven to have effects on plant height and 1000-grain weight (Chen *et al*., 2013). Though hd1 gene belong to CCT-motif family, the agronomic traits it affected different from that of Ghd7 and the CCT-motif gene in this study.

Table 1. Seven agronomic traits measured values of ten foxtail millet cultivars.

Cultivarname	Origin geography	Traits investigated						
		DH	PH	PL	ED	BNP	GNB	1000-GW
Jigu1	Hebei province	58	112.0	17.7	7.2	85.9	44.5	2.5
Xianzihui	Inner mongolia	50	90.4	14.3	5.1	70.5	13.5	2.2
71za30-2	Hebei province	51	110	12.4	5.9	61.6	49.3	2.5
Baimi1	Hebei province	58	78.8	15.7	5.4	92	31.1	2.1
An04-5014	Henan province	55	122.5	21.6	7.6	102	72	2.8
Jiyecong4	Shandong province	51	106.4	13.3	4.2	113	19.8	2.0
Longgu26	Heilongjiang province	38	63.1	6.8	3.0	59	10	0.7
Jigu27	Hebei province	39	56.8	7.5	3.2	58	7	2.1
Fagu28-81	France	74	128.7	21.2	6.7	95	60.2	2.3
Yugu1	Henan province	53	118.2	18.6	7.1	112	35.5	2.1

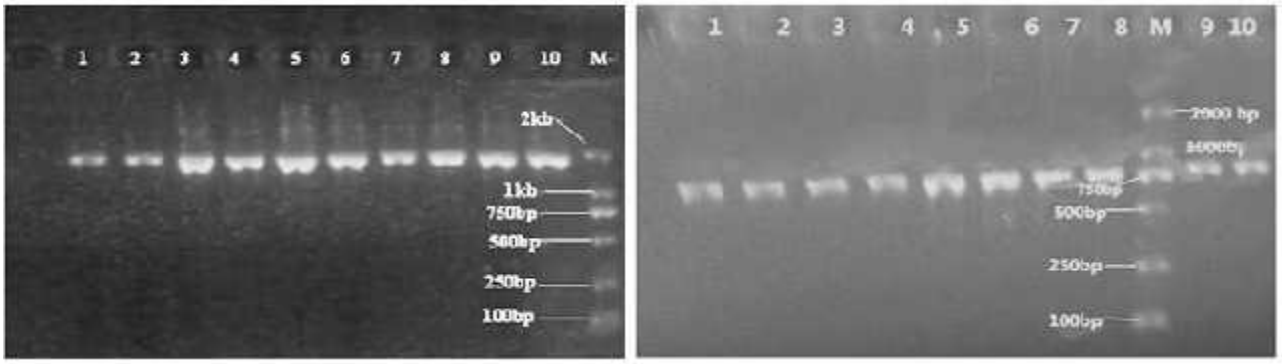

Fig. 1 Amplification of two CCT-motif gene fragments in ten foxtail millet cultivars (left: products of F1 primer pair, right: products of F2 primer pair)

Table 2. 34 mutation sites detected in exon1 and exon 2 of foxtail millet CCT-motif gene.

	upstream of initiation codon			Exon 1																											
Cultivar	20	30	60	77	109	116	148	149	152	155	161	173	192	245	257	264	266	281	306	313	338	347	363	369	407	424	451	476	513	546	583
71za30-2	G	C	8	G	G	G	A	G	T	T	C	T	G	C	T	G	T	C	G	C	A	T	A	C	C	T	G	G	A	G	
An04-5014	G	T	8	G	G	G	A	G	T	T	C	T	G	C	T	G	T	C	G	C	A	T	A	T	C	T	G	G	A	G	
Baimi1	C	T	8	G	G	G	A	G	T	T	C	T	G	C	T	G	T	C	G	C	A	T	A	T	C	T	G	G	G	G	
Fagu28-81	C	T	0	G	G	A	G	A	C	C	C	C	C	A	C	A	C	T	A	T	G	C	G	T	G	T	C	C	A	A	
Jigu1	G	T	8	G	G	G	G	G	T	T	C	T	G	C	T	G	T	C	G	C	A	T	A	T	C	T	G	G	A	G	
Jigu27	C	T	0	A	G	A	G	A	C	C	C	C	C	A	C	A	C	T	A	C	G	C	G	T	G	T	C	C	A	A	
Jiyecong4	C	T	0	G	G	A	G	A	C	C	C	C	C	A	C	A	C	T	A	C	G	C	G	T	G	T	C	C	A	A	
Longgu26	C	T	0	G	A	A	G	A	C	C	T	C	C	A	C	A	C	T	A	C	G	C	G	T	G	C	C	C	A	A	
Xianzihui	G	T	8	G	G	G	A	G	T	T	C	T	G	C	T	G	T	C	G	C	A	T	A	T	C	T	G	G	A	G	
Yugu1	C	T	0	G	G	A	G	A	C	C	C	C	C	A	C	A	C	T	A	T	G	C	G	T	G	T	C	C	A	A	

Table2 (Continued)

	Exon 2						
Cultivar	547	553	588	621	642	647	683
71za30-2	T	T	0	A	C	T	T
An04-5014	T	T	3	A	C	T	T
Baimi1	T	T	3	A	C	T	C
Fagu28-81	T	T	3	A	C	T	T
Jigu1	C	T	0	G	C	T	T
Jigu27	T	T	0	A	C	T	T
Jiyecong4	T	T	0	A	C	C	T
Longgu26	T	T	0	A	C	T	T
Xianzihui	T	C	0	A	T	C	T
Yugu1	T	T	3	A	C	T	T

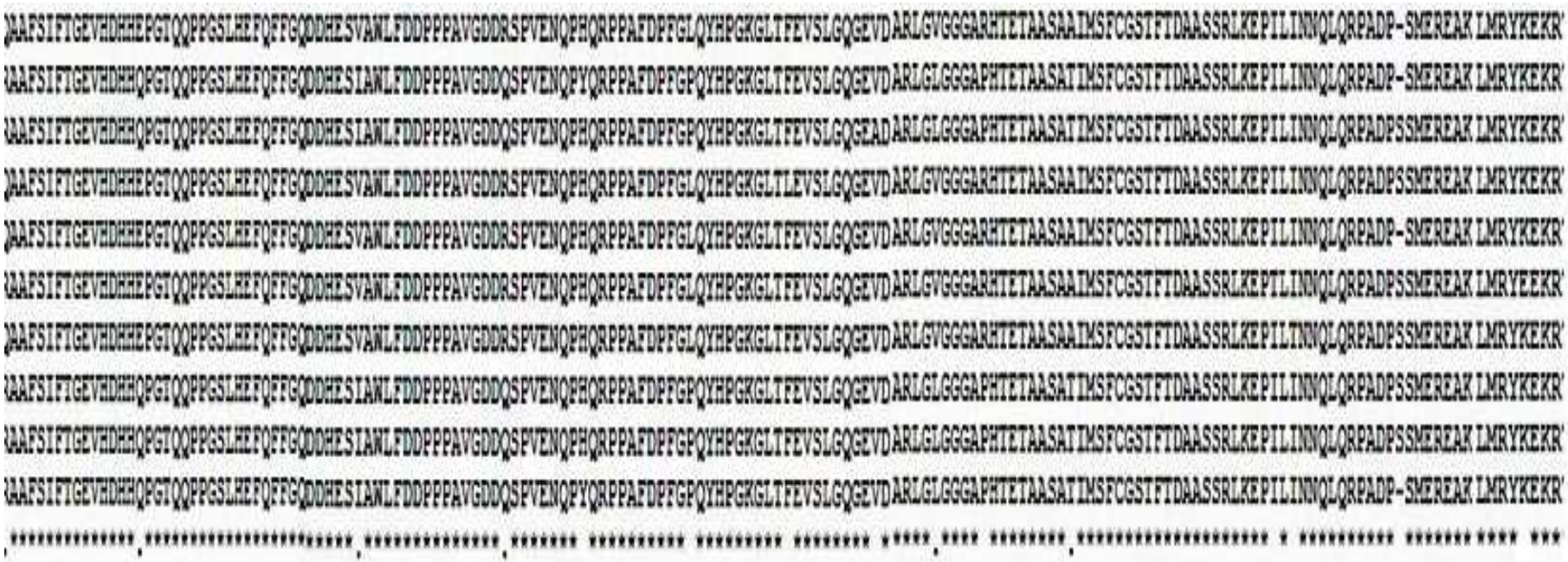

Fig. 2 Multiple sequence alignment of putative CCT-motif gene protein sequences among ten foxtail millet cultivars (the mutation sites marked with red-dotted lines)

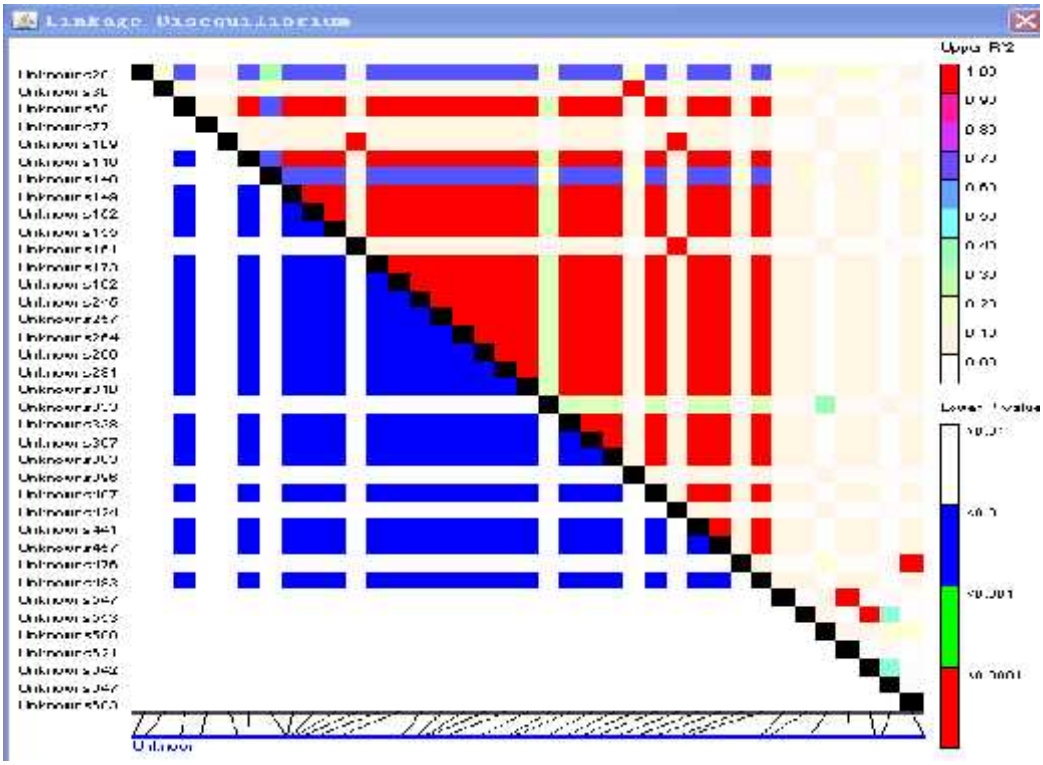

Fig. 3 LD analysis of 34 mutation sites in CCT-motif gene coding region

Table 3. Four mutation sites associated with 1000-GW and PL

Trait	Site	F_Marker	p_Marker	Rsq_Model	Rsq_Marker
1000-GW	109	33.1158	4.27E-04	0.8054	0.8054
1000-GW	161	33.1158	4.27E-04	0.8054	0.8054
1000-GW	424	33.1158	4.27E-04	0.8054	0.8054
PL	588	9.2999	0.0158	0.5376	0.5376

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