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Full Length Article

Analysis of DNA Methylation of Wheat in Response to Low Nitrogen Stress Based on Mathylation-Sensitive Amplified Polymorphisms

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Abstract

Abiotic stress can induce considerable changes in the composition and quality of wheat grain and cause genomic DNA methylation. Here, methylation-sensitive amplified polymorphism (MSAP) was used to detect the effect of low nitrogen stress on the extent and patterns of DNA methylation in wheat grain at 5, 8, 11, 14, 16 and 21 days post-anthesis (DPA). First, our results showed that low nitrogen stress could reduce grain N content, sodium dodecylsulfate sedimentation (SDS) volume, the amounts of high-molecular-weight glutenin subunits (HMW-GS 2, 12 and 20) and total protein. Then, at 8, 11 and 21 DPA, the overall level of DNA methylation in low nitrogen group was higher than in control group with the maximum difference of 14.1% at 21 DPA. And, at 8 DPA, methylation / demethylation revealed the highest level under low nitrogen conditions, about 17.07% –30.99% of the CCGG sites. Thus, thirteen differentially displayed DNA fragments in MSAP profiles in samples at 8 DPA were further cloned for sequencing analysis. Homology analysis for these DNA fragments showed that most of them were involved in storage matter (protein, fatty acid), some engaged in stress signaling and physiological response regulation. Moreover, the relative expression levels revealed that the expression of a demethylated fragment was increased but the expressions of two other methylated fragments decreased. In conclusion, our results suggested that low nitrogen stress could decrease SDS volume, HMW-GS amount and total protein which might be associated with DNA methylation.

Keywords: DNA methylation/demethylation; Gene expression; Low nitrogen; MSAP; Wheat grain

Introduction

DNA methylation affects gene expression and plays an important role in plant's adaptation to adverse environments (Choi and Sano, 2007; Choi and Kim, 2008; Iqbal *et al*., 2011; Hua *et al.*, 2017). Previous study indicated that salt stress can induce DNA sites demethylation, methylation and hypermethylation, with the net result being genome-wide hypomethylation. And the CCGG sequences of the salt-tolerant wheat variety were more methylated than the salt-sensitive varieties (Zhong *et al*., 2009; Wang *et al*., 2014). Thus, methylation could be a mechanism for the adaptation of plant adaptation under salt stress. Moreover, the total methylation level could be decreased in wheat under drought stress. Recent research demonstrated that the endophytic fungus SMCD 2206 improved drought tolerance in wheat co-cultured with SMCD 2206 which were associated with DNA methylation (Hubbard *et al*., 2014). In addition, DNA methylation in the regulatory region of the Ppd-B1 alleles improves photoperiod insensitivity and develops adaptation of wheat to a wider geographical range during breeding (Sun *et al*., 2014). These studies suggested that abiotic stress could induce DNA methylation in wheat.

Abiotic stress can also induce considerable changes in the composition and quality of wheat grain. For example, drought stress could significantly reduce the sodium dodecyl sulfate (SDS) sedimentation volume and carbohydrate contents including sucrose and starch (Jenner, 1994; Gooding *et al*., 2003; Barnabas *et al*., 2008). Zheng *et al*. (2009a, b) reported that salt stress could increase grain protein in cultivars with different levels of salt tolerance and waterlogging reduce the protein and starch content in the grains. A moderately high temperature during the grain filling stage was also reported to enhance the flour protein content (Stone, 2001). Nitrogen-deficient fertilizers also affect wheat quality, such as flour protein, dough stability, and relative concentration of glutenin, unextractable polymeric protein, and relative amounts of high/low-molecular-weight glutenin subunits (HMW-GS/LMW-GS) (Park *et al*., 2014). However, whether the effects of nitrogen-deficient stress on wheat grain were associated with DNA methylation remain unclear.

Therefore, in the study, first, we studied the effects of nitrogen-deficiency stress on SDS volume, the amounts of HMW-GS and the total protein in wheat grain. Then, methylation-sensitive amplified polymorphism (MSAP) was used to detect the effect of nitrogen-deficiency on the extent

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and patterns of DNA methylation in wheat grain at 5, 8, 11, 14, 16 and 21 days post-anthesis (DPA). Moreover, differentially displayed DNA fragments in MSAP profiles were cloned and sequenced, and their possible roles in nitrogen-deficient stress were analyzed. In addition, the relationship between DNA methylation and relative gene expression levels was analyzed by quantitative real-time PCR. These results provided preliminary but global insight into the DNA methylation of wheat exposed to nitrogen-deficient stress, and provide a foundation for further studies focused on revealing the epigenetic regulation mechanisms of the stress response in plants.

Materials and Methods

Plant Materials and Treatment

A nitrogen fertilization experiment was conducted with common wheat, *T. aestivum*. Shannong981A of HMW-GS 2, 12, and 20 associated with bad dough quality，but with a high SDS-sedimentation volume. Wheat was sown in 1.5 L pots containing farm soil mixed evenly and grown in a greenhouse in October 2013 in Chengdu (30.67°N, 104.06°E), Sichuan Province China (natural photoperiod and irradiance). Wheat was divided into two groups, low nitrogen and control group. The basic soil total N content was 1.2 g/kg. Wheat in low nitrogen group was treated with no nitrogen fertilizer, while in control group were treated with 31 mg N per plant as ammonium $(NH_4)_2SO_4$ to simulate the initial soil N availability under field conditions (Fuertes-Mendizabal *et al*., 2013), 24 mg N per plant as ammonium $(NH_4)_2SO_4$ at beginning of tillering (GS20), 29 mg N per plant at beginning of stem elongation (GS30) and 24 mg N per plant as ammonium $(NH_4)_2SO_4$ at beginning of the flag leaf stage (GS37) according to Zadoks scale (Zadoks *et al*., 1974) (Table 1), respectively. All experiments were repeated ten times with three plants per pot. An increase in expression of genes associated with wheat storage product synthesis in the endosperm starting at 6 days, leveling off after 10 days and declining from 21 days post-anthesis (DPA) was observed (Wan *et al*., 2008). Thus, the developing grain samples were collected from wheat Shannong981A at 5, 8, 11, 14, 16, and 21 DPA and stored at -80^{o}C for further use. Gain N concentration was determined by combustion with an elemental analyser (Thermo Finnigan, Waltham, MA, USA) (Fuertes-Mendizabal *et al*., 2013). Total genomic DNA was extracted using a routine CTAB procedure as described by Murray and Thompson (1980) and stored at −20ºC. The mature wheat seeds were obtained at 49 DPA for quality tests.

MSAP Assay

MSAP analysis was performed as described (Tang *et al*., 2014). Two enzyme combinations were used, namely: HpaII/EcoRI and MspI/EcoRI (New England Biolabs, Beverly, MA, USA). The sequence information for the adapters and primers of pre-amplification and selective amplification is provided in Table S1. The MSAP PCR products were separated on 6% denaturing polyarylamide gel (PAGE, 6% polyacrylamide, 8 *M* urea) and visualized by silver staining.

Cloning and Sequence Analysis of Differentially Displayed Fragments

Differentially expressed bands in DNA methylation were excised from the gel and re-amplified using selective amplification PCR conditions. The amplified fragments were ligated into pGEM-T easy vector (TransGen) and transformed into competent *Escherichia coli* DH5α. The recombinants were screened for sequencing according to the Sanger method. A homology search was performed at the public database NCBI (http://www.ncbi.nlm.nih.gov).

Real-time Quantitative RT-PCR (qRT-PCR)

Total RNA from the wheat kernel at 8 DPA was extracted using Trizol (Omega Total RNA Kit) and first-strand cDNA was synthesized using the Super Script TM First-Strand Synthesis System (Transgen Biotech, China). The primers used for qRT-PCR analyses were designed using Primer 5.0 software (Table S2), and two normalizing housekeeping genes: actin and 18S rRNA (Altenbach *et al*., 2002). QRT-PCR was performed using SYBR Premix ExTaq (Bio-Rad, USA) in the Bio-Rad fluorescence RT- PCR amplifier CFX96 (AmCell, USA). The PCR conditions consisted of an initial denaturation step at 95ºC for 20 s, followed by 39 cycles of 10 s at 95ºC, 30 s at 60ºC and 30 s at 72ºC. The melting curve was generated from 60ºC to 95ºC, read every 0.5ºC, and held every 5 s. The $2^{-\Delta\Delta CT}$ method was used to normalize and calibrate transcript values relative to the endogenous actin control. A total 18 independent samples with triplicate repeats were analyzed.

Quality Tests

The assay procedure of the SDS (Sodium dodecyl sulfate) sedimentation volume of whole meal was conducted according to the improved AACC method 56-61A (Takata *et al*., 1999). Subunit composition and quantitative determination of HMW were analyzed by SDS-PAGE and densitometry procedures (Lookhart *et al*., 1993). The total protein content of the grain were determined by Near Infrared Spectroscopy (NIR).

Results

Decreased Grain N Content Induced by Low Nitrogen Stress

The grain N content at 5, 8, 11, 14, 16 and 21 DPA in control and low nitrogen stress determined showed that grain N contents in low nitrogen group were

Table 1: N source and splitting of the different N fertilization treatments at stage GS20, GS30 and GS37 in each group

Group	mg N added per plant		
	GS20	GS30	GS37
Control	24	29	24
Low nitrogen	0	0	0

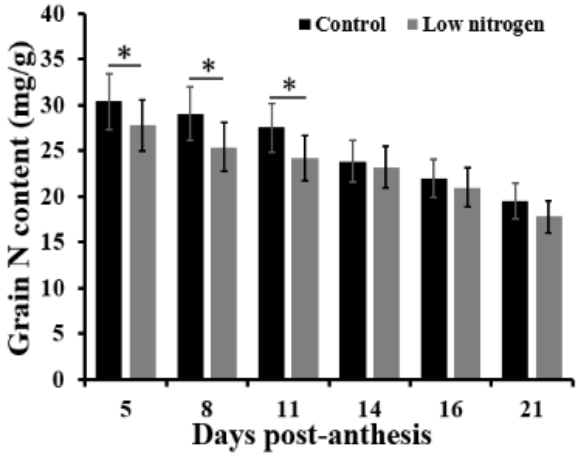

Fig. 1: Gain N content at different days post-anthesis in control and low nitrogen groups. *$P < 0.05$

significantly lower than in control group at 5, 8 and 11 DPA (Fig. 1). These results suggested that low nitrogen could reduce the grain N accumulation to some extent.

Effect of Low Nitrogen Stress on the Quality of Wheat

The quality index of the wheat under low nitrogen stress was significantly different from the control. The SDS sedimentation volume of the wheat flour in the low nitrogen group was significantly lower than in the control group. The proteins were 17.58% in the control but 14.90% in the low nitrogen group. The gel electrophoresis map showed that the amounts of HMW-GS 2, 12 and 20 in the low nitrogen group were significantly lower than those in the control. The concentration of HMW-GS 2, 12 and 20 in the low nitrogen group were 13.75, 8.77 and 16.09, but those in the control were 19.16, 10.16 and 20.32 (Fig. 2). These results indicated that low nitrogen could affect the SDS sedimentation, total protein and the accumulation of HMW-GS.

Methylation differences between wheat under low nitrogen stress and control were detected by MSAP. A total of 539 5′-CCGG-3′ site bands were amplified in both low nitrogen and control at 5, 8, 11, 14, 16 and 21 DPA. (Table 2) Both in low nitrogen and control, the number of methylated (types II + III + IV) DNA bands were determined. A total of 338-414 bands were polymorphic (types II + III + IV), which accounted for 62.71–76.81% of all bands. The overall DNA methylation level in the low nitrogen was not always lower than in the control during the six stages of grain development. At 8, 11 and 21 DPA, the

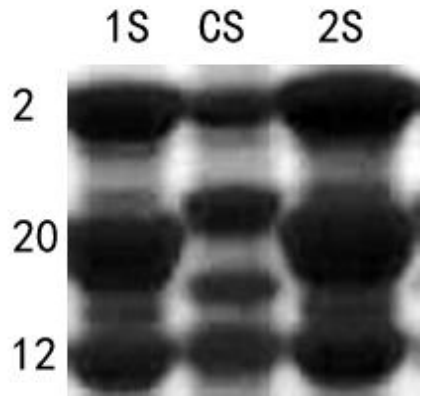

Fig. 2: The gel electrophoresis map of HMW-GS. 2, 20, 12, the composition HMW-GS of Shannong 981 A. CS, *Triticum aestivum* "Chinese Spring"; 1S, the low nitrogen; 2S, the control

Global DNA methylation profiles in wheat grain

overall DNA methylation level in the low nitrogen group was higher than in the control group, with the maximum difference of 14.1% at 21 DPA; however, this pattern was opposite at 5, 14, and 16 DPA with the maximum difference of 2.79% at 16 DPA. At 8 and 21 DPA, the DNA hemi-methylation level in the low nitrogen group was higher than in the control, with the maximum difference of 10.67% at 21 DPA. This pattern was also opposite at 5, 11, 14 and 16 DPA with the maximum difference of 3.53% at 14DPA. Of all the four types in the low nitrogen group, only the type IV bands were more than in the control at six stages. Thus, the type IV fragment was the main source of the changes in DNA methylation banding patterns induced by low nitrogen stress.

Changes in Methylation/Demethylation induced by Low Nitrogen Stress

All possible banding patterns between control and low nitrogen groups in the six time-points were compared to investigate the changes in cytosine methylation patterns under low nitrogen stress. The changes in cytosine methylation/demethylation bands were divided into three major patterns: no change, demethylation, and methylation, which include 16 classes (Table 3). The first pattern, no change, was monomorphic with no cytosine methylation alteration and included classes A, B, C and D, indicating that the same 5′-CCGG-3′ sites were detected both in the control and low nitrogen groups. The second pattern was demethylation, which included classes E-J, and showed that cytosine was demethylated to different extents in the low nitrogen group compared to the control group. The third pattern was methylation and included classes K-P, representing the cytosine methylation events induced by low nitrogen stress. Approximately 17.07–30.99% of the CCGG sites changed under low nitrogen stress, with the highest at 8

Table 2: Different types of MSAP cytosine methylation levels under low nitrogen and control

Type	5 DPA	8 DPA	11 DPA	14 DPA	16 DPA	21 DPA
	LN Control	LN Control	LN Control	LN Control	LN Control	LN Control
I (11)	161 151	132 146	134 135	157 147	154 139	125 201
II (10)	156 168	198 189	183 193	168 187	192 210	219 161
III (01)	127 127	104 111	100 104	104 110	88 96	93 106
IV (00)	95 93	105 93	122 107	110 95	105 94	102 71
Tas	539 539	539 539	539 539	539 539	539 539	539 539
Tms	378 388	407 393	405 404	382 392	385 400	414 338
Fms	222 220	209 204	222 211	214 205	193 190	195 177
Tmr	70.13 71.9	75.51 72.91	75.14 74.95	70.87 72.72	71.42 74.21	76.81 62.71
Fmr	41.19 40.82	38.78 37.84	41.19 39.15	39.70 38.03	35.81 35.25	36.18 32.84
Hmr	28.94 31.17	36.73 35.06	33.95 35.81	31.17 34.70	35.62 38.96	40.63 29.87

LN: Low nitrogen; DPA: Days post-anthesis
A score of 1 or 0 represents the presence or absence of bands, respectively
I:1 (HpaII)1 (MspI); II:1 (HpaII) 0 (MspI); III:0 (HpaII)1(MspI); IV:0 (HpaII) 0 (MspI);
Type I is unmethylated bands; Type II is hemi-methylated bands; and types III+IV are fully methylated bands. Total methylated bands=II+III+IV
Tas: Total amplified sites; Tms: Total methylated sites; Fms: Fully methylated sites
Tmr: Total methylated ratio (%); Fmr: Fully methylated ratio (%); Hmr: Hemi-methylated ratio(%)
Total methylated ratio = [(II+III+IV)/(I+II+III+IV)]×100%
Fully methylated ratio = [(III+IV)/(I+II+III+IV)]×100%
Hemi-methylated ratio = [(II)/(I+II+III+IV)]×100%

Table 3: The different patterns of DNA methylation changes induced by low nitrogen

Classes	No. of sites (methylation changes) and Frequency					
	5DPA	8DPA	11DPA	14DPA	16DPA	21DPA
A	133	103	110	125	120	112
B	134	135	159	145	166	139
C	108	80	85	89	77	76
D	72	54	93	79	75	60
Total	447(82.93%)	372 (69.01%)	447 (82.93%)	438 (81.26%)	438 (81.26%)	387 (71.80%)
E	20	16	17	24	24	8
F	7	11	7	7	10	5
G	1	2	0	1	0	0
H	9	21	9	11	14	7
I	11	16	5	4	5	4
J	2	6	1	1	0	6
Total	50(9.28%)	72 (13.36%)	39 (7.24%)	48 (8.91%)	53 (9.83%)	30 (5.57%)
K	11	36	14	11	12	67
L	7	4	10	9	5	12
M	0	3	1	2	2	10
N	13	34	17	16	19	13
O	10	14	11	13	9	19
P	1	4	0	2	1	1
Total	42(7.79%)	95 (17.63%)	53 (9.83%)	53 (9.83%)	48 (8.91%)	122 (22.63%)

No change: A (ItoI), B (II to II), C (III to III), D (IV to IV)
Demethylation: E (II to I), F (III to I), G (IV to I), H (IV to II), I (IV to III), J (III to II)
Methylation: K (Ito II), L(Ito III), M (Ito IV), N (II to IV), O (III to IV), P (II to III)

PDA and lowest at 11 PDA.

The percentage of the demethylated bands under low nitrogen stress was 9.28%, 13.36%, 7.24%, 8.91%, 9.83% and 5.57%, whereas the percentage of methylated bands was 7.79%, 17.63%, 9.83%, 9.83% 8.91% and 22.63% in low nitrogen group at 5, 8, 11, 14, 16 and 21 DPA, respectively. These results demonstrated that low nitrogen mainly induced DNA methylation in grains at 8 and 21 DPA. In general, low nitrogen induced more DNA methylation than DNA demethylation in wheat grain.

Sequence Analysis of Polymorphic Fragments

As described above, changes in methylation fluctuates during wheat grain development, with the highest found at 8 DPA. To annotate the changed bands representing methylation changes, 13 differentially amplified bands in samples at 8 DPA, numbered Ta1–Ta13, were purified, cloned, and sequenced (Table 4). Among these 13 differentially methylated DNA bands, five fragments were methylated, and eight demethylated under low nitrogen (Fig. 3). The length of these 15 sequences ranged from 134 bp to 564 bp. The resulting sequences were BLAST searched against the databases at NCBI (http://blast.ncbi.nlm.nih.gov). Of the five methylated fragments (Ta1–Ta5), Ta1 was homologous to the retro-transposons of *T. aestivum*, whereas Ta1, Ta2, Ta3, Ta4 and Ta5 were homologous to genes involved in the metabolism of storage substance, namely, *T. turgidum* HMW glutenin, *T. aestivum*, storage protein activator (spa), *A.*

Table 4: BLAST results of the 13 polymorphic differentially MSAP DNA fragments

MSAP fragments		Accession No. (E-value)	Methylation changes	Sequence homology
Name (size)	Primer combination			
Ta1 (142 bp)	E38Msp39	AY368673.1 (6e-18)	II to IV	HMW-glutenin retrotransposon
Ta2 (247 bp)	E41Msp41	FM242575.1 (1e-05)	II to IV	storage protein activator
Ta3 (321 bp)	E38Msp39	JX295577.2(3e-121)	III to IV	prolamin gene regulation regions
Ta4 (134 bp)	E46Msp42	JX295577.2(1e-13)	III to IV	LMW-glutenin regulation regions
Ta5 (246 bp)	E40Msp40	EU660903.1 (9e-13)	II to IV	acetyl-CoA carboxylase
Ta6 (230 bp)	E44MSP41	GU985444.1 (5e-92)	IV to II	mitochondrion, complete genome
Ta7 (105 bp)	E46Msp42	FN564430.1 (5e-04)	III to I	contig ctg0464b Repeat region
Ta8 (564 bp)	E40Msp40	XM_010242360.1 (9e-18)	IV to II	auxin response factor
Ta9 (238 bp)	E40Msp40	FN564432.1(5e-16)	IV to II	contig ctg0616b retrotransposon
Ta10 (120 bp)	E41Msp41	AF453674.1(2e-29)	IV to II	TAEST Sukkula retrotransposon
Ta11 (287 bp)	E54Msp42	AY494981.1(9e-08)	III to II	HMW glutenin Retrotransposon
Ta12 (172 bp)	E40Msp40	XM_010242282.1(2e-07)	III to I	uncharacterized, mRNA
Ta13 (140 bp)	E44Msp41	HG670306.1(3e-35)	IV to II	chromosome 3B genomic scaffold retrotransposon

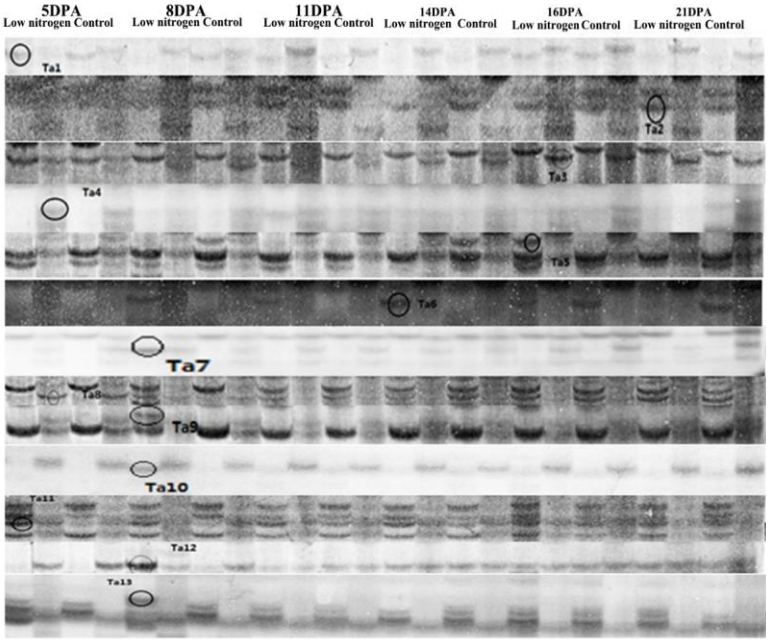

Fig. 3: The different patterns of DNA methylation changes induced by low nitrogen

tauschii prolamin/LMW glutenin, and *T. aestivum* cytosolic acetyl-CoA carboxylase (fatty acid synthesis). Both Ta3 and Ta4 shared low homology with the sequence located at the different loci of JX295577.2. Of the eight demethylated fragments (Ta6–Ta13), Ta6, Ta8, Ta10 and Ta11 were homologous to genes of *T. aestivum* mitochondrion (respiration), *B. distachyon* auxin response factor (hormone regulation), *T. aestivum* isolate TAEST Sukkula retrotransposon, and the retrotransposon of *T. turgidum* HMW glutenin, respectively. The methylated fragments of Ta1 showed low homology with the demethylated fragments of Ta11 but both were homologous to the retrotransposon of gene *T. turgidum* HMW glutenin AY368673.1 and AY494981.1, respectively. In addition, Ta3, Ta4, Ta5, Ta6, Ta9 and Ta13 were located in the non-coding regions of the homologous gene. Thus, under nitrogen-deficient treatment, both coding and non-coding regions could be involved in methylation or demethylation changes at the cytosine nucleotide.

Expression Analysis of Polymorphic Fragments in Response to Low Nitrogen Stress

The expression analysis of three MSAP polymorphic genes was performed using qRT-PCR. Ta1 was methylated at 8 DPA where as Ta8 and Ta11 were demethylated at the same development time (Fig. 4). On the one hand, low nitrogen stress slightly up-regulated the expression levels of the demethylated genes of Ta8 and Ta11 compared with the control group. On the other hand, it significantly down-regulated the expression levels of the methylated genes of Ta1 compared with that in the control group at 8 DPA.

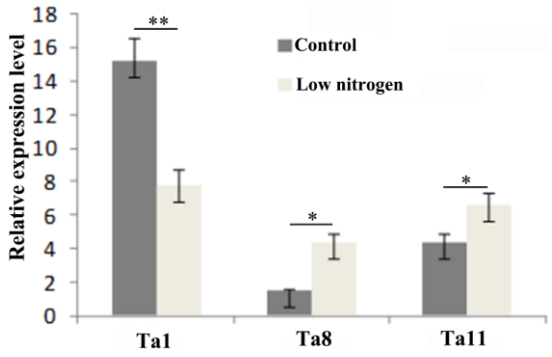

Fig. 4: Expression changes of differential MSAP fragments (Ta1, Ta8, Ta11) under low nitrogen. $^{**}P < 0.01$; $^{*}P < 0.05$

Discussion

This research showed that the total genomic methylation level was relatively high in wheat grain, accounting for an average of 71.57 and 73.13% in control and low nitrogen treatment at six development phases, respectively. The resulting total genomic methylation level in this study was significantly higher than in perennial ryegrass (57.67%), maize (33%) and even in the germinating seed of another wheat variety (Meng *et al*., 2012; Shan *et al*., 2013; Tang *et al*., 2014). Thus, the methylation status differs among various plants and varieties (Yang and Li, 2016. Even in different parts of same tissue, methylation levels vary. For example, during seed development, in rice, DNA in the endosperm is hypomethylated compared with in the embryo (Zemach *et al*., 2010).

Evident differences in SDS sedimentation volume, content of total protein and HMW-GS of wheat flour were observed between low nitrogen and control groups. SDS sedimentation volume gives a good indication of the end-product quality of wheat and is closely associated with protein content and protein composition, specially HMW-GS, LMW-GS and prolamin (Blackman and Gill, 1980; Carter *et al*., 1999). Nitrogen-deficient stress affected protein production which affected the SDS sedimentation volume. Methylation analysis results confirmed this. Of these differentially amplified bands, the homologous genes of Ta1, Ta2, Ta3, Ta4 and Ta11, were directly related to nitrogen metabolism. Among them, Ta1and Ta11 were homologous to the retro-transposon of *T. turgidum* HMW glutenin. Ta2, Ta3 and Ta4 were homologous to the regulation of *T. aestivum* storage protein activator (spa), the prolamin regulation regions of *Aegilops tauschii* chromosome 1Ds, and the LMW-GS regulation regions of *Aegilops tauschii* chromosome 1Ds. Besides, Ta8 was homologous to the *B. distachyon* auxin response factor related to the abiotic stress-response, Simultaneously, Ta8 involved in the regulation of wheat quality (Narayan and Nair, 1990; Sekhon and Singh, 1994).

Of all the 13 differentially amplified bands, Ta1 and Ta11 were explicitly involved in HMW glutenin synthesis. Ta1 was methylated, whereas Ta11 were demethylated. Ta1 and Ta11 were homologous to AY368673.1 and AY494981.1, respectively. Both were the retro-transposon of *T. turgidum* A genome HMW glutenin A gene locus which could affect the expression of HMW-GS. That the decrease of the SDS sedimentation volume and the reduction in the accumulation of HMW-GS 2, 12 and 20 in this study confirmed the content of HMW–GS are directly linked to wheat quality (Johansson *et al*., 1999; Deng *et al*., 2006, 2007).

Three homologues of MSAP loci were selected to study transcriptional changes. Of the three genes- Ta1 and Ta11 were closely related to HMW whereas Ta8 was closely related to the abiotic stress-response. Ta1 was methylated (10 to 00) at 8 DPA; Ta8 was demethylated (00 to 10) at 8 DPA; Ta11 was demethylated at 8 DPA (01 to 10). The results showed that nitrogen-deficiency increased the expression of Ta8 and Ta11 (Fig. 4). By contrast, the expression of Ta1 was reduced by nitrogen-deficiency. Thus, nitrogen-deficiency triggered both cytosine methylation (Ta1), and cytosine demethylation in genes (Ta8 and Ta11). Nitrogen-deficiency may affect gene expression by changing the methylation status in its cytosine nucleotide as in other stresses (Choi and Sano, 2007; Shan *et al*., 2013; Tang *et al*., 2014).

DNA methylation and demethylation are associated with either transcriptional repression or activation (Choi and Sano, 2007). Though, the methylated DNA levels at 8 DPA increased slightly by 2.6% after nitrogen-deficient stress, methylation or demethylation occurred at 167 sites (30.98%). It was demonstrated that nitrogen-deficient stress had a great impact on genomic methylation alterations in wheat grain and then affected the expression of many gene. In our study, these affected genes might to be involved in storage matter (protein, fatty acid), stress signaling and physiological response regulation. Ta8 was homologous to the *B.distachyon* auxin response factor related to the abiotic stress-response. The gene was demethylated under nitrogen deficient conditions to improve adaptation to nitrogen-deficient stress in wheat (Narayan and Nair, 1990; Sekhon and Singh, 1994).

In this study, the fact that nitrogen-deficient stress increased the total DNA methylation level in wheat grain contradicted that many stress such as drought stress could decrease the total DNA methylation level in plant (Zhong *et al*., 2009; Wang *et al*., 2011; Fan *et al*., 2012). Homology analysis showed that most of these fragments were predicted to be involved in storage matter (protein, fatty acid) and very few fragments were predicted to be involved in stress signaling. These results indicated that though the extent of the nitrogen deficiencies in this study was so small not to affect the normal growth and development of wheat plants and the protein quality of some wheat lines (data not shown).

The 21 DPA was also an important time in this study. At 8, 11 and 21 DPA, the overall DNA methylation level in

the treated samples was higher than in the control samples, with the maximum difference of 14.1% at 21 DPA. The overall DNA methylation level in the control at 21 DPA (74.21%) was significantly less than at 16 DPA (62.71%). Based on the theory that demethylation are associated with transcriptional activation，there was another expression peak of gene about wheat storage product at 21 DPA. This finding was not consistent with the previous research results that expression of genes associated with wheat storage product synthesis in the endosperm decline from 21 DPA (Wan *et al*., 2008). That may be because the seeds of special wheat varieties Shannong981A were obviously bigger than other wheat lines and the gene expression peak at 21 DPA had a drastic effect on the formation and accumulation of storage substance. But the overall DNA methylation level in the treated samples at 21 DPA (76.81%) was somewhat higher than at 16 DPA (71.42%).The genes expression was inhibited by the nitrogen-deficient stress and stopped the synthesis of storage substance prematurely.

The methylation/demethylation of transposons can affect programmed genome epigenetic regulation and the expressions of downstream genes in plants, which can be altered by environmental stresses (Yaish, 2013). In this study, of the 13 fragments that were sequenced in wheat, these five fragments (Ta1, Ta9, Ta10, Ta11 and Ta13) were predicted to be retro-transposons. Ta1 was methylated under stress. Transposon inactivity is necessary for the stable maintenance of the genome and these three methylated retrotransposons could avoid DNA sequence changed by adverse environmental conditions (Mann *et al*., 2015).

The BLAST results and transcriptional changes in the MSAP fragments indicated that epigenetic changes such as the methylation states in the wheat grain genome are likely very important regulatory mechanisms for wheat adaptation to low nitrogen stress. Thus the methylation states have an important effect on the wheat gain quality. Our study provides valuable information for further studies focusing on the epigenetic regulation of responses to low nitrogen in wheat grain.

Conclusion

Alterations in DNA methylation patterns play an integral role in regulating growth and development of plant. The epigenetic changes in the wheat genome regulated the grain's development to adapt to changes in the environment, induced changes in grain composition, and eventually affected wheat quality.

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