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# Analysis of DNA methylation during the germination of wheat seeds

F.-R. MENG[1], Y.-C. LI[2,3]*, J. YIN[2,3], H. LIU[1], X.-J. CHEN[1], Z.-F. NI[4] and Q.-X. SUN[4]

*College of Life Science[1], National Engineering Research Centre for Wheat[2] and State Key Laboratory Cultivation Base of Crop Physiological Ecology and Genetic Improvement in Henan Province[3], Henan Agricultural University, Zhengzhou 450002, P.R. China*
*Department of Plant Genetics and Breeding, State Key Lab for Agro-Biotechnology, China Agricultural University, Beijing 100094, P.R. China[4]*

## Abstract

DNA methylation is known to play a crucial role in regulating plant development and organ or tissue differentiation. Here, we focused on the DNA methylation dynamics during the germination of wheat seeds using the adapted AFLP technique so called methylation-sensitive amplified polymorphism (MSAP). The MSAP profiles of genomic DNA in embryo and endosperm tissues of germinating seeds, as well as dry seeds were characterized and notable changes of cytosine methylation were detected. Comparisons of MSAP profiles in different tissues tested showed that the methylation level in dry seeds is the highest. The alteration analysis of cytosine methylation displayed that the number of demethylation events were three times higher than that of *de novo* methylation, which indicated that the demethylation was predominant in germinating wheat seeds, though the methylation events occurred as well. Sixteen differentially displayed DNA fragments in MSAP profiles were cloned and the sequencing analysis confirmed that nine of them contained CCGG sites. The further *BLAST* search showed that four of the cloned sequences were located in coding regions. Interestingly, three of the sixteen candidates were homologous to retrotransposons, which indicated that switches between DNA methylation and demethylation occurred in retrotransposon elements along with the germination of wheat seeds.

*Additional key words*: embryo, endosperm, cloning, sequence analysis.

## Introduction

DNA methylation is a common phenomenon in eukaryotes, which has both epigenetic and mutagenic effects on various cellular activities (Portis *et al.* 2004). In the genome of higher plants, up to 30 % of the total cytosine is methylated (Matassi *et al.* 1992). Cytosine methylation is mainly restricted to the symmetrical sequences 5′-CpG-3′ or 5′-CpNpG-3′, but also can occur in a nonsymmetrical context (Meyer *et al.* 1994, Finnegan *et al.* 1998b, Fulnecek *et al*. 2002, Cokus *et al*. 2008). Although the physiological functions of DNA methylation in plants are not fully understood, it has been implicated that DNA methylation has been associated with numerous biological processes, including genomic imprinting, transcriptional regulation of gene expression, suppressing the mobility of transposable elements, and gene silencing (Hafiz *et al.* 2001, Martienssen and Colot 2001, Paszkowski and Whitham 2001).

Recent research has demonstrated that DNA methylation plays an integral role in regulating the growth and development of plant, although the evidences were fragmentary (Finnegan *et al.* 2000). Significant changes of cytosine methylation have been observed among different organs in species such as tomato (Messeguer *et al.* 1991), rice (Xiong *et al.* 1999) and *Silene latifolia* (Zluvova *et al.* 2001), and among different developmental phases in *Pinus radiata* (Fraga *et al.* 2002) and *Prunus*

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*Abbreviations*: MSAP - methylation-sensitive amplification polymorphism; AFLP - amplified fragment length polymorphism; PCR - polymerase chain reaction.
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* Corresponding author; fax: (+86) 37163558202, e-mail: yongchunli71@henau.edu.cn

*persica* (Bitonti *et al.* 2002). The stability analysis of DNA methylation throughout *Arabidopsis thaliana* development showed a progressive methylating trend from cotyledons to vegetative organs to reproductive organs (Ruiz-García *et al.* 2005). Furthermore, the genome-wide demethylation caused abnormal development (Finnegan *et al.* 1998a, 2000) and also the vernalization mediated earlier flowering was associated with the reduced levels of DNA methylation in *Arabidopsis thaliana* (Burn *et al.* 1993, Finnegan *et al.* 1998a).

Wheat is one of the major crops worldwide and the epigenetic regulation mechanisms involved in DNA methylation have been proved to play important roles during the wheat development (Drozhdeniuk *et al.* 1976, Sherman and Talbert 2002), while the study on DNA methylation changes during the germination of wheat seeds is limited. In this study, we characterized the patterns and extent of cytosine methylation in embryo and endosperm tissues from germinating wheat seeds, as well as in dry seeds. Sixteen differentially displayed DNA fragments detected by MSAP profiling were cloned and sequenced, and their possible roles in the developmental regulation were discussed.

## Materials and methods

**Plants:** Mature seeds of wheat (*Triticum aestivum* L.) cv. Zhengmai 9023 were surface-sterilized in 5 % sodium hypochlorite for 15 min and washed 4 times (2 min each) with sterilized water, then imbibed on the moist filter paper in Petri dishes with water in a cultivation chamber (16-h photoperiod, irradiance of 150 μmol $m^{-2}$ $s^{-1}$ and temperature of at 25 °C). Embryo and endosperm tissues isolated from the germinating seeds at the time point of 6, 12, 18 and 24 h after wetting were collected. Radicle and plumule were harvested from seeds imbibed in water for 48 h. Samples were frozen in liquid nitrogen immediately and then stored at -70 °C for genomic DNA extraction. Every treatment included 30 seeds and was repeated in triplicate, DNAs from three repeats were pooled and then used for methylation-sensitive amplification polymorphism analysis (MSAP).

**MSAP assay:** The original AFLP technique was modified to incorporate the use of methylation-sensitive restriction enzymes. The protocol involved use of the isoschizomers *Hpa*II and *Msp*I instead of *Mse*I as 'frequent cutter' enzymes. The adapter and primer for the 'rare-cutter' enzyme *Eco*RI was the same as that used in standard AFLP analysis (Vos *et al.* 1995), while the *Hpa*II/*Msp*I adapter was designed according to Xiong *et al*. (1999) as listed in Table 1.

To detect MSAP, restriction and ligation were conducted concurrently and two sets of digestion/ligation reactions were carried out simultaneously. In the first reaction, 5 $mm^3$ of the extracted DNA (400 - 500 ng DNA) was added to 45 $mm^3$ buffer (10 mM Tris-HCl, pH 7.5, 10 mM $Mg(Ac)_2$, 50 mM KAc) containing 10 units *Eco*RI, 20 units *Hpa*II (*Promega*, Madison, WI, USA), 2 units $T_4$ DNA ligase (*Promega*), 5 pmol *Eco*RI adapter, 50 pmol *Hpa*II/*Msp*I adapter and 0.2 mM ATP. The mixture was then incubated at 37 ºC for 6 h. The reaction was stopped by incubating at 65 ºC for 10 min and diluting 10 times in 0.1× TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8) for PCR amplification. The second digestion/ligation reaction was carried out in the same way, except that *Msp*I was used in place of *Hpa*II.

Table 1. Sequences of MSAP primers and adapters.

| Primers/adapters | Sequences (5´- 3´) |
|---|---|
| *Eco*RI adapter | CTCGTAGACTGCGTACC |
| | AATTGGTACGCAGTCTAC |
| E + 1 primer | GACTGCGTACCAATTC+A |
| E + 3 primers | + AAC (E1) |
| | + ACG (E2) |
| | + ACT (E3) |
| | + AGT (E4) |
| *Hpa*II/*Msp*I adapter | GATCATGAGTCCTGCT |
| | CGAGCAGGACTCATGA |
| HM + 1 primer | ATCATGAGTCCTGCTCGG+T |
| HM + 3 primers | +TAA (HM1) |
| | +TCC (HM2) |
| | +TTC (HM3) |

We used two consecutive PCRs to selectively amplify the *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I DNA fragments. The pre-selective amplification (first PCR) was performed using 5 $mm^3$ of the above mentioned diluted mixture added to a 15 $mm^3$ mixture giving a final concentration of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 40 ng of *Eco*RI and *Hpa*II/*Msp*I adapter-directed primer, each possessing a single selective base (E + 1; HM + 1) as listed in Table 1 and 1 unit of *Taq* polymerase (*Promega*). PCR reactions were performed with the following profile: 94 ºC for 60 s, 25 cycles of 30 s denaturing at 94 ºC, 30 s annealing at 55 ºC and 60 s extension at 72 ºC, ending with 10 min at 72 ºC to complete extension. After checking for the presence of a smear of fragments (100 - 1000 bp in length) by agarose electrophoresis, the amplification product was diluted 40 times in 0.1× TE.

Selective amplification (second PCR) of the diluted pre-amplification products was carried out using a total of 12 primer combinations obtained with four *Eco*RI primers in combination with three *Hpa*II/*Msp*I primers with three selective bases each (E + 3, HM + 3) as listed in Table 1. Selective PCR reactions were performed with

the following profile: 94 ºC for 60 s, 36 cycles of 30 s denaturing at 94 ºC, 30 s annealing and 60 s extension at 72 ºC, ending with 10 min at 72 ºC to complete extension. Annealing was initiated at a temperature of 65 ºC, which was then reduced by 0.7 ºC for the next 12 cycles and maintained at 56 ºC for the subsequent 23 cycles. The second PCR products were mixed with 15 $mm^3$ of formamide dye (98 % formamide, 10 mM EDTA, 0.01 % bromophenol blue and 0.01 % xylene cyanol), denatured at 95 ºC for 4 min.

PCR products were separated on 0.4 mm thick, 4 % denaturing polyacrylamide sequencing gels in a temperature-regulated sequencing system *Bio-Rad* (Hercules, CA, USA) at 45 ºC. Gels were silver-stained and photographed.

**Cloning and sequence analysis of differentially displayed fragment:** Bands that showed DNA methylation differences during the seed germination were excised from the gel and re-amplified using the selective amplification PCR conditions. The amplified fragments were ligated into *pGEM-T* easy vector (*Promega*) and sequenced. Homology search were performed at the public database *NCBI* (http://www.ncbi.nlm.nih.gov).

## Results

**DNA methylation profiling:** MSAP profiles were generated from 11 samples, including dry seeds, embryo and endosperm tissues of germinating seeds imbibed in water after 6, 12, 18, 24 h, plumules and radicles. 12 primer combinations were used and 423 fragments were amplified for each sample in average, in which 114 (27.0 %) were differentially amplified in the case of at least one of the samples (Table 2). Among these differentially amplified fragments, 73 of them were displayed due to hemimethylation of the external cytosines, which permits cleavage by *Hpa*II but not by *Msp*I, while 41 due to full methylation of the internal cytosines, which allows cleavage by *Msp*I but not by *Hpa*II (Table 2). Comparisons of MSAP profiles generated from different samples showed that the methylation level in dry seeds is higher than that in other tissues tested. When compared to dry seeds, the DNA methylation levels in endosperm, embryo, plumule and radicle tissues were decreased by 10, 22, 26 and 28 %, respectively (Table 3).

Table 2. MSAP profiles during the seed germination of wheat. Embryo and endosperm 1 - 4 tissues were isolated from germinating seeds 6, 12, 18 and 24 h after wetting, respectively; radicles and plumules were harvested from germinating seeds imbibed in water for 48 h.

| Samples | Number of amplified bands | *Hpa*II$^+$/*Msp*I$^-$ | *Hpa*II$^-$/*Msp*I$^+$ | Number of methylation events |
|---|---|---|---|---|
| Dry seeds | 475 | 101 | 52 | 153 |
| Embryo 1 | 423 | 66 | 39 | 105 |
| Embryo 2 | 352 | 46 | 31 | 77 |
| Embryo 3 | 409 | 68 | 38 | 106 |
| Embryo 4 | 431 | 77 | 43 | 120 |
| Endosperm 1 | 406 | 74 | 42 | 116 |
| Endosperm 2 | 454 | 79 | 49 | 128 |
| Endosperm 3 | 373 | 66 | 41 | 107 |
| Endosperm 4 | 436 | 86 | 48 | 134 |
| Plumules | 417 | 62 | 36 | 98 |
| Radicles | 482 | 74 | 37 | 111 |
| Average | 423 | 73 | 41 | 114 |

Table 3. Relative levels of cytosine methylation in different tissues of germinating seeds [%]. The average methyl-cytosine ($^{m}$C) levels were calculated as the percentage of methyl-cytosine of total cytosine showed in MSAP profiles; the relative methyl-cytosine levels in different tissues were compared to that in dry seeds. Means ± SE, *n* = 3.

| Tissues | Average $^{m}$C | Relative methylation | Reduction of methylation |
|---|---|---|---|
| Dry seed | 32.2 ± 0.12 | 100 | -- |
| Embryo | 25.1 ± 1.22 | 78 | 22 |
| Endosperm | 28.9 ± 0.63 | 90 | 10 |
| Plumule | 23.7 ± 0.26 | 74 | 26 |
| Radicle | 23.1 ± 0.82 | 72 | 28 |

**Alterations of cytosine methylation during the seed germination:** Comparisons of the MSAP profiles revealed two main classes of differentially displayed

bands during the seed germination. Class I: bands appeared after digestion with *Hpa*II but not with *Msp*I or *vice versa* in all detected samples (83 bands were included in this group), which were generated due to the DNA methylation at the CCGG sites and did not switched during the seed germination (Fig. 1*a*,*b*). Class II: bands showing polymorphism at different stages of germination (56 bands were included in this group), which were from changes of DNA methylation status during germination (Fig. 1*c*-*j*). The class II can be sorted into 4 categories: II-1, bands present in dry seeds when digested with *Hpa*II or *Msp*I, but no longer detected in germinating seeds (Fig. 1*c*,*d*); II-2, bands detected in dry seeds as well as in endosperm (Fig. 1*e*,*f*) or embryo of

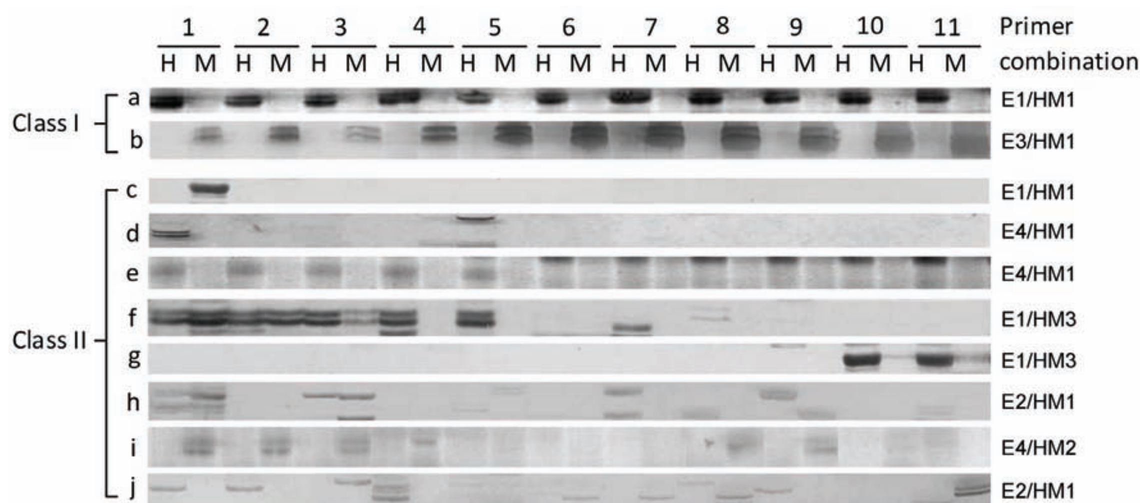


Fig. 1. The patterns of differentially displayed bands showed by MSAP analysis during wheat seed germination. *Eco*RI-*Hpa*II (H) and *Eco*RI–*Msp*I (M) were used for digestion in MSAP analysis. The tissues detected including dry seeds (1), endosperm (2 - 5) and embryo (6 - 9) issues of germinating seeds imbibed in water for 6, 12, 18, and 24 h, respectively, as well as radicles (10) and plumules (11) harvested from seeds imbibed in water for 48 h. *a* and *b* - bands with no methylation changes during the seed germination; *c* and *d* - bands detected in dry seeds while disappeared in germinating seeds; *e* and *f* - bands only observed in dry seeds and endosperm tissues of germinating seeds; *g* - bands only appeared in radicle and plumule tissues; *h* to *j* - bands appeared in one or more tissues and fluctuated .

Table 4. Sequence analysis of cloned fragments differentially methylated during germination. A - bands only appeared in dry seeds; B - bands not detected in dry seeds but found in germinating seeds; C - bands detected in dry seeds as well as in endosperm tissues of germinating seeds; D - bands appeared in dry seeds and embryos of germinating seeds; E - bands detected in one or more stages of germinating seeds; F - bands only appeared in radicles; G - bands only appeared in plumules.

| Fragment name | Primer combination | Length [bp] | Methylation pattern | Sequence homology |
|---|---|---|---|---|
| M1 | E4/HM1 | 298 | A | CJ688630: mRNA sequence [*Triticum aestivum*] |
| M2 | E1/HM1 | 229 | A | AY663391: genomic DNA [*Triticum turgidum*] |
| M3 | E3/HM3 | 248 | A | AB008772: retrotransposon Tar1 DNA [*Triticum aestivum*] |
| M4 | E2/HM1 | 451 | A | AY569610: phosphate transporter mRNA [*Oryza sativa*] |
| M5 | E3/HM1 | 297 | B | unknown DNA sequence |
| M6 | E1/HM3 | 306 | C | XM_001798616: mRNA sequence [*Phaeosphaeria nodorum*] |
| M7 | E1/HM3 | 225 | C | AP008214: chromosome 8 genomic DNA [*Oryza sativa*] |
| M8 | E4/HM1 | 546 | C | AP008211: chromosome 5 genomic DNA [*Oryza sativa*] |
| M9 | E4/HM1 | 516 | D | AK252044:mRNA sequence [*Hordeum vulgare*] |
| M10 | E4/HM1 | 340 | E | AP004531: chromosome 3 genomic DNA [*Lotus japonicus*] |
| M11 | E2/HM2 | 347 | E | EF560592: retrotransposon Copia WIS-1 [*Triticum turgidum*] |
| M12 | E4/HM1 | 215 | E | AY951944: genomic sequence [*Triticum monococcum*] |
| M13 | E4/HM2 | 175 | E | AY641412: retrotransposon Sandra5 [*Hordeum vulgare*] |
| M14 | E4/HM2 | 173 | E | AF497474: leucine-rich-like protein gene [*Aegilops tauschii*] |
| M15 | E2/HM1 | 214 | F | AC120496: genomic sequence [*Sorghum bicolor*] |
| M16 | E2/HM1 | 312 | G | EF081030: genomic sequence [*Triticum urartu*] |

germinating seeds; II-3, bands appeared in radicle and plumule tissues (Fig. 1*g*) or either of them, which indicated the *de novo* methylation; II-4, those appeared in some stages of germination and vibrated (Fig. 1*h-j*). There are 17, 10, 8 and 21 bands were included in the II-1 to II-4 groups, respectively. The further analysis showed that the demethylation events were 3 times higher than that of *de novo* methylation during seed germination. These results indicated that the demethylation was predominant in germinating wheat seeds, though the methylation events occurred as well.

**Cloning of differentially displayed fragments in MSAP profiles:** To obtain more information of those differentially displayed fragments which showed polymorphism during the seed germination, sixteen candidate bands, numbered M1-16 as showed in Table 4, were eluted from the polyacrylamide gels and cloned into T-vectors. Sequencing analysis showed that the 16 cloned fragments were relatively short in length (173 - 546 bp), and nine of them (M1-3, M7-8, M11-13 and M16) contained the internal 5′-CCGG-3′ sites. *BLAST* search showed that four cloned sequences (corresponding to band M1, M6, M9 and M14) were highly homologous to the coding regions of protein coding genes, three (M3, M11, M13) fragments were homologous to retro-transposons (Table 4), which implied that switches between DNA methylation and demethylation occurred in retrotransposon elements along with the germinating of wheat seeds, while other fragments were annotated to the genomic regions which roles were still remained unknown.

## Discussion

The regulatory mechanisms involved in the DNA methylation in wheat have been concerned 30 years ago (Drozhdeniuk *et al.* 1976, Sulimova *et al.* 1978), and it has been proved that cytosine methylation plays an important role during the wheat development (Sherman and Talbert 2002). In this paper, the DNA methylation patterns during the wheat seed germination were estimated by MSAP profiling and the results showed that the methylation level in germinating wheat seeds was about 27 % on average. This value was significantly higher than that of 16.3 % methylation level in flag leaves of rice (Xiong *et al.* 1999) and 15.7 % in germinating seeds of rapeseed (Lu *et al.* 2006), while significantly lower than that of 35 – 43 % in seedlings of *Arabidopsis* (Cervera *et al.* 2002). These differences of DNA methylation level may result either from different type of tissues tested or from genetic control of different species, and also it has been reported that both genetic and epigenetic mechanisms were responsible for the variation of the methylation levels in *Arabidopsis* (Riddle and Richards 2002).

MSAP analysis using double digestion with *Eco*RI and either *Hpa*II or *Msp*I isoschizomers allowed identification of methylation-sensitive CCGG polymorphisms at genome level (Cervera *et al.* 2002). *Msp*I cleaves $C^{5m}CGG$ but not $^{5m}CCGG$ sequences, *Hpa*II is inactive if one or both cytosines are fully methylated while cleaves the hemimethylated sequences (Korch and Hagblom 1986, McClelland *et al.* 1994). In this study, comparison of MSAP profiles showed that the methylation in dry seeds is the highest, and it was relatively reduced by 10, 22, 26 and 28 % in endosperm, embryo, plumule and radicle tissues from the germinating wheat seeds, respectively, when compared with that in dry seeds, and this result agreed with another report (Drozhdeniuk *et al.* 1976), in which the amount of 5-methylcytosine was higher in dormant wheat seeds than that in germinating ones. In tomato (Messeguer *et al.* 1991) and rapeseed (Lu *et al.* 2006), it was also reported that the DNA methylation was the highest in seeds. Such variation of DNA methylation level is certainly expected based on current knowledge of role of methylation as one of the regulatory mechanisms of gene expression during plant development and differentiation (Siroky *et al.* 1998), although the details of regulatory mechanisms involved in the DNA methylation are still not clear.

In eukaryotes, the distribution and number of 5-methylcytosines (5mC) along the DNA is heritable but can also change with the developmental state of the cell (Koukalova *et al*. 2005). DNA methylation probably has a number of functions and scientific interest has focused on its gene silencing effect (Grunau *et al.* 2001). It was reported that the cytosine methylation in coding or promoter regions can block the expression of the target genes, while the artificial demethylation lead to the reactivation of gene expression, which indicated that the DNA demethylation might be one of necessary steps toward transcriptional activation in eukaryotic genome (Finnegan *et al.* 2000, Kovarik *et al*. 2000, Lu *et al.* 2006). In *Capsicum annuum*, notable changes in MSAP profiles of genomic DNA obtained from embryo tissues of dry seeds and germinating seeds were also detected, and the results indicated that the demethylation events appeared to be necessary for transcriptional activation during the germination (Portis *et al*. 2004). In this study, the number of demethylation events was three times higher than that of *de novo* methylation in germinating wheat seeds, which was in agreement with the requirement of activating large scale genes during germination (Koornneef *et al.* 2002, Fait *et al.* 2006). The results implied that the programmed gene expression was controlled, at least in part, by the epigenetic mechanisms depend on the DNA methylation or demethylation during the wheat seed germination. In order to further confirm the observed changes in methylation pattern and decide

whether hypomethylation or *de novo* methylation is the reason of those *de novo* appearing bands during the seed germination, Southern blot hybridization with appropriate probe or *Msp*I/*Hpa*II digestion of AFLP product should be performed. However, it is not easy to conduct Southern blotting as independent control of methylation alterations because wheat is allohexaploid with huge and highly complex genome.

Interestingly, three candidate fragments, with methylation switches during the wheat seed germination, were annotated to retrotransposons. As a ubiquitous component of the genomic DNA in plants, retrotransposons play a major role in plant gene and genome evolution (Kumar and Bennetzen 1999). Retrotransposons can generate mutations by inserting within or near functional genes, and these elements may provide regulatory sequences for gene expression and then alter the expression of the adjacent genes (Fedoroff 2000, Kashkush *et al.* 2003). Because of the potential harmful effects, the expression of most transposable elements in the genome is suppressed so that, even if whole and capable of autonomous transposition, most transposons remain silent throughout the plant's life cycle (Slotkin and Martienssen 2007). The transposons can be silenced both at transcription and after transcription through epigenetic mechanisms, which involving DNA methylations and histone modifications (Slotkin and Martienssen 2007, Cantu *et al.* 2010). Transcription of most of the active plant transposable elements is largely quiescent during development but the induction of some elements had been observed under biotic and abiotic stresses (Wendel and Wessler 2000, Petit *et al.* 2010). In this study, three putative retrotransposons were detected by MSAP profiling during the wheat seed germination, which indicated that methylation switches occurred in some retrotransposon elements and their functions and roles will be a new point for further study in wheat germination.

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