Breeding Science 64: 125–133 (2014)
doi:10.1270/jsbbs.64.125

Comparison of the heat stress induced variations in DNA methylation between heat-tolerant and heat-sensitive rapeseed seedlings

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DNA methylation is responsive to various biotic and abiotic stresses. Heat stress is a serious threat to crop growth and development worldwide. Heat stress results in an array of morphological, physiological and biochemical changes in plants. The relationship between DNA methylation and heat stress in crops is relatively unknown. We investigated the differences in methylation levels and changes in the cytosine methylation patterns in seedlings of two rapeseed genotypes (heat-sensitive and heat-tolerant) under heat stress. Our results revealed that the methylation levels were different between a heat-tolerant genotype and a heat-sensitive one under control conditions. Under heat treatment, methylation increased more in the heat-sensitive genotype than in the heat-tolerant genotype. More DNA demethylation events occurred in the heat-tolerant genotype, while more DNA methylation occurred in the heat-sensitive genotype. A large and diverse set of genes were affected by heat stress via cytosine methylation changes, suggesting that these genes likely play important roles in the response and adaption to heat stress in *Brassica napus* L. This study indicated that the changes in DNA methylation differed between heat-tolerant and heat-sensitive genotypes of *B. napus* in response to heat stress, which further illuminates the molecular mechanisms of the adaption to heat stress in *B. napus*.

Key Words: rapeseed, heat stress, methylation, methylation-sensitive amplification polymorphism (MSAP).

Introduction

Global temperatures are rising and extreme temperature events have been observed in some areas of the world (Easterling *et al.* 2000). Extensive agricultural losses worldwide are attributed to heat, often in combination with drought (Mittler 2006). Heat stress disturbs cellular homeostasis and can lead to leaf etiolation, severe retardation in plant growth and development, and even death (Yu *et al.* 2012). *B. napus* is an important winter oilseed crop; early sowing of *B. napus* has several important advantages, such as the avoidance of disease infestation and aphid attacks during the flowering stage by early harvest and forced mature by high temperatures during the maturation period (Kaur *et al.* 2009). However, this crop encounters heat stress during the early sowing period and the maturation period, which affects plant growth and development and, ultimately, causes great losses in yield (Angadi *et al.* 2000).

Increasing evidence indicates that epigenetic mechanisms, such as DNA methylation and histone modification, play crucial roles in the regulation of gene expression in the plant response to environmental stresses, such as salinity, drought, extreme temperatures, heavy metals, and so on (Choi and Sano 2007, Dyachenko *et al.* 2006, Kou *et al.* 2011, Lukens and Zhan 2007, Wada *et al.* 2004). DNA methylation, an important epigenetic modification, may lead to environmentally induced phenotypic variations by regulating gene expression (Angers *et al.* 2010, Zhang *et al.* 2010). The levels of DNA methylation vary in different species. It has been reported that approximately 30–50% of all cytosine residues in the nuclear DNA are methylated in higher plants (Chan *et al.* 2005). Environmental stress can cause an increase or a decrease in the cytosine methylation levels throughout the genome and at specific loci (Aina *et al.* 2004, Labra *et al.* 2002, Li *et al.* 2009). In *Brassicas*, cadmium stress stimulated demethylation at specific loci (Filek *et al.* 2008); potassium dichromate induced extensive methylation changes in CCGG-sequences as revealed by the methylation-sensitive amplification polymorphism (MSAP) approach (Labra *et al.* 2004).

Several studies have shown that MSAP is an efficient and convenient method to detect DNA methylation (Ashikawa 2001, Cerveraet *et al.* 2002, Xiong *et al.* 1999). MSAP profiling is an AFLP based method for the detection of DNA methylation (Xu *et al.* 2000) and has been applied to studies of drought-induced site-specific DNA methylation and its association with drought tolerance in rice (*Oryza sativa* L.) (Wang *et al.* 2011), DNA methylation in *Arabidopsis thaliana* (Cervera *et al.* 2002), the association between the up-regulation of stress-responsive genes and the hypomethylation of genomic DNA in tobacco (Wada *et al.* 2004), epigenetic changes in cotton (Li *et al.* 2009) and

Communicated by T. Nishio
Received September 30, 2013. Accepted February 16, 2014.
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DNA-methylation changes induced by salt stress in wheat (*Triticum aestivum*) (Zhong *et al.* 2009). MSAP has become one of the important methods for the investigation of methylation levels and pattern in the genome (Portis *et al.* 2004).

As sessile organisms, plants manifest numerous adaptive changes in response to heat stress. These responses include the induction of signaling cascades, the profound changes in the expression of specific genes (Xu *et al.* 2006) and the expression of heat shock proteins (HSPs) (Queitsch *et al.* 2000). However, little is known about the general pattern of DNA methylation in rapeseed under heat stress. The relationship between epigenetics and the regulation of gene expression under heat stress in different genotypes in rapeseed is also relatively unknown. In a previous study, we demonstrated that heat tolerance in different genotypes of *B. napus* were dissimilar; the genotypes Huyou 2 and Fengyou 1 were identified as a heat-tolerant genotype and a heat-sensitive genotype, respectively (Gao *et al.* 2010). Our goal was to investigate whether thermotolerance in seedlings was similar to that in seeds and whether DNA methylation was induced during seedling heat stress. We employed an electrolyte leakage analysis to determine the thermotolerance of *B. napus* seedlings. The MSAP technique was used to investigate the differences in methylation levels and changes in cytosine methylation patterns in seedlings of two rapeseed genotypes (heat-sensitive and heat-tolerant) under heat stress. The results obtained in this study revealed the effects of heat stress on DNA methylation in the *B. napus* genome; understanding these effects is important for the further analysis of the molecular mechanism of the adaptation to heat stress in rapeseed.

Materials and Methods

Plant material and treatment conditions

Two rapeseed genotypes (Huyou 2 and Fengyou 1) obtained from the Chinese National Mid-term Seed GeneBank of Oil Crops were used in this study. Previous studies demonstrated that the seeds of Huyou 2 were tolerant to high temperature and the seeds of Fengyou 1 were heat-sensitive (Gao *et al.* 2010). The seeds of both genotypes were germinated and grown in controlled growth conditions (21°C/23°C, 16 h/8 h light/dark photoperiod). Seedlings at the five-leaf stage were treated with a heat pretreatment at 37°C for 2 h and then heat stress treatment at 45°C for 3 h. The leaves were sampled and immediately frozen under liquid nitrogen for DNA and RNA extraction.

Electrolyte leakage analysis

An electrolyte leakage (EC) analysis was carried out using a modified version of the procedure described by March *et al.* (1982). Leaf discs (1.4 cm) were cut using cork borers, placed in 15-mL polypropylene tubes with 5.0 mL of distilled water and incubated in 37°C and 45°C water baths. The EC was measured using a conductivity meter (METTLER TOLEDO FE30) every 30 min (EC1). The total leakage (EC2) was determined after the samples were frozen and thawed. The ratio of EC1/EC2 × 100 was used to determine relative damage by heat stress. The experiment was performed three times.

DNA isolation

Genomic DNA was extracted using a modified hexadecyl trimethyl ammonium bromide (CTAB) protocol. DNA quality was verified by separation on 0.8% agar gels and analysis with an UV-1800 spectrophotometer (SHIMADZU, JAPAN). The DNA concentration and purity were determined spectrophotometrically. Aliquots were diluted to a final concentration of 10–15 ng/μl.

MSAP analysis

The MSAP marker was used to assess the stability/alteration in the cytosine methylation patterns at the 5′-CCGG sites. MSAP analysis was performed as described by Xiong *et al.* (1999), with minor modifications; a pair of methylation-sensitive restriction enzymes, *Msp*I and *Hpa*II, were used in combination with *Eco*RI. *Hpa*II and *Msp*I are isoschizomers that recognize the same restriction site (5-CCGG) with a differential sensitivity to methylation modifications at the two cytosines. *Hpa*II will not cut if either of the cytosines is fully methylated (double-strand), whereas *Msp*I will not cut if the external cytosine is hemi-methylated (Dong *et al.* 2006).

The DNA was digested with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I at 37°C for 4 h. Digested aliquots were ligated with *Eco*RI-and *Msp*I-or *Hpa*II-specific adopters at 20°C for 5 h. After the ligation reaction, the samples were incubated for 10 min at 60°C to inactivate the enzymes. The ligated DNA was diluted 1 : 10 and pre-amplified. The pre-amplified product was diluted 1 : 10 and amplified using different combinations of *Eco*RI and *Msp*I or *Hpa*II primers. Each primer contained three selective nucleotides at the 5′ and 3′ ends. The sequences of the adapters and the primers used for pre-amplification and selective amplification are provided in Supplemental Tables 1, 2. The MSAP PCR products were separated by 6% polyacrylamide gels electrophoresis (PAGE) and visualized after silver staining.

The MSAP patterns of the DNA fragments resulting from the digestion with the isoschizomers were divided into the following four types. Type I: two bands presenting for both enzyme combinations indicated that the corresponding CCGG site is unmethylated. Type II: One band representing only *Eco*RI/*Hpa*II indicated a hemimethylation state of DNA due to the methylation of one DNA strand but not its complementary strand. Type III: one band appearing for *Eco*RI/*Msp*I reflects the case of a full CG (internal cytosine) methylation. Type IV: the absence of bands for both enzyme combinations indicating that full methylation occurred at both cytosines (McClelland *et al.* 1994). The MSAP bands were scored "1" or "0" to indicate the presence or absence, respectively, of a band at a particular position.

Isolation and characterization of the amplified fragments

The polymorphic fragments were excised from the gel,

hydrated in 50 µl of water, and incubated in boiling water for 5 min. The supernatant was recovered by centrifugation and used for re-amplification using the same primers under the conditions described above for the selective amplification. The re-amplified DNA fragments were purified and cloned into a T-vector (TransGen Biotech, China) for sequencing. The sequences obtained were analyzed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BRASSICA (http://brassicadb.org/brad/blastPage.php) search algorithms.

Quantitative RT-PCR analysis

To test the effect of heat stress, seedlings of Huyou 2 and Fengyou 1 were treated at 45°C for 3 h. Seedlings grown in normal conditions were used as control. The leaves were sampled and immediately frozen under liquid nitrogen for RNA extraction. We used RNeasy Plant Mini Kit (QIAGEN, Germany) to extract the total RNA from each sample. Quantitative RT-PCR analysis was performed by LightCycler® 480 SYBR Green I Master using a LightCycler®480II real-time PCR machine (Roche, http://www.roche-applied-science.com). Each sample was analyzed at least three times and three replicates were used in each case. The method of reverse transcription-polymerase chain reaction (RT-PCR) was performed as described by Li *et al.* (2013). The relative expression levels were analyzed as described by Yuan *et al.* (2008). Primers were designed to amplify the genes that were homologous to M7, M9 and M16, which are genes differentially methylated, using GeneSript (https://www.genscript.com/ssl-bin/app/primer). The primers used in this study are listed in Supplemental Table 3.

Results

Relative thermotolerance in two rapeseed genotypes

To examine the heat stress response in rapeseed seedlings, we analyzed two genotypes. In response to high temperature storage, the seeds of the first genotype (Huyou 2) have been described as heat-tolerant and the seeds of the second genotype (Fengyou 1) have been described as heat-sensitive (Gao *et al.* 2010). Electrolyte leakage analysis has been shown to be a reliable, quantitative and reproducible assay to predict thermotolerance in a variety of plants (Coria *et al.* 1998, Keeler *et al.* 2000). To observe thermotolerance in the green leaf tissue of seedlings, we measured electrolyte leakage from leaf discs as an indicator of heat injury. The leaves of Fengyou 1 seedlings reached 82% injury; this observed injury was greater than observed in Huyou 2 after exposure to a test temperature of 45°C for 90 min (Fig. 1). When seedlings were pretreated at 37°C for 2 h before the 45°C heat treatment, the thermotolerance of both Huyou 2 and Fengyou 1 increased. These results strongly suggest that pretreatment at 37°C increases the thermotolerance of rapeseed seedlings. The Fengyou 1 seedlings were more sensitive to heat stress than Huyou 2 seedlings; these results correlate with those observed in seeds.

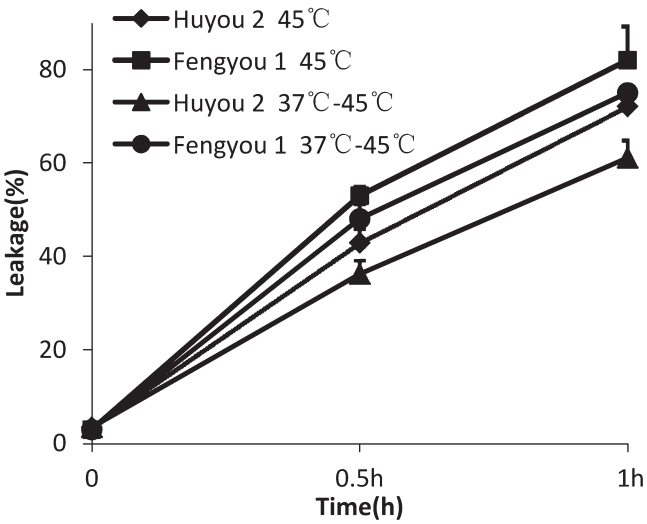

Fig. 1. Electrolyte leakage of heat-tolerance and heat-sensitive rapeseed under heat stress.

The dynamic level of DNA methylation induced in heat-tolerant and heat-sensitive genotypes by heat stress

A total of 47 primer pair combinations (listed in Supplemental Table 2) were used to assess changes in the methylation of DNA and the polymorphisms in methylated DNA from the leaves of seedlings of two rapeseed genotypes (Huyou 2 and Fengyou 1). The seedlings were subjected to different levels of heat stress. Between 675 and 794 clear bands were amplified in seedlings of both genotypes under control conditions and high temperature conditions. Of these bands, 196–297 (1/0 and 0/1) were methylated (hemi- or full-methylation), of which 138–223 were full-methylated. The percentages of the total methylated bands and the full-methylated bands were 26.8%–37.4% and 18.5%–28.3%, respectively. Overall, the number of methylated DNA bands observed in Huyou 2 (199) was lower than that observed in Fengyou 1 (295) after a 45°C heat stress treatment preceded by either a 37°C pretreatment or no pretreatment (Table 3).

Under control conditions, the overall methylation levels in the DNA isolated from the leaves of Huyou 2 were 2.2% greater than that observed in the leaves of Fengyou 1 (Table 1). Compared with the control conditions, heat stress induced a greater number of observed methylated bands (both hemi- and full-methylation) at the expense of unmethylated bands in Fengyou 1. However, no obvious changes in the overall methylation level were observed in the Huyou 2 seedlings following heat stress. However, the overall level of DNA methylation was approximately 26.8% in the leaves of Fengyou 1 under the control condition and 37.4% after the 45°C heat treatment; The percentage of full-methylated bands increased from 18.5% to 28.3%. This result indicated that there was a great difference in the overall level of genomic DNA methylation in Fengyou 1 under control conditions and heat stress. These results also indicated that in both rapeseed genotypes, no obvious changes were detected

Table 1. DNA methylation changes in leaves of Huyou 2 and Fengyou 1 under control and heat stress

MSAP band type	H	M	Huyou 2			Fengyou 1		
			CK	45°C	37–45°C	CK	45°C	37–45°C
Type I	1	1	479	479	479	574	494	497
Type II	1	0	58	61	60	65	72	74
Type III	0	1	138	138	140	145	223	223
Total amplified bands			675	678	679	784	789	794
Total methylated bands			196	199	200	210	295	297
MSAP (%)			29.0	29.4	29.5	26.8	37.4	37.4
Fully methylated ratio (%)			20.4	20.4	20.6	18.5	28.3	28.1

CK indicates the control check.
A score of 1 and 0 represents the presence and absence of bands, respectively.
H and M represent digestion with *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI, respectively.

Table 2. Changes of DNA methylation pattern under control and heat stress in Huyou 2 and Fengyou 1

Banding pattern	CK		T		Methylation status		45°C		37–45°C	
	H	M	H	M			Huyou 2	Fengyou 1	Huyou 2	Fengyou 1
A1	0	0	1	1	C<u>C</u>GG GG<u>C</u>C	CCGG GGCC	2	5	4	2
A2	0	1	1	1	<u>CC</u>GG GGCC	CCGG GGCC	1	0	0	1
A3	1	0	1	1	<u>CC</u>GG GG<u>CC</u>	<u>CC</u>GG GGCC	0	0	2	0
A4	0	0	1	0	<u>CC</u>GG GG<u>CC</u>	C<u>C</u>GG GG<u>C</u>C	2	6	2	9
A5	0	0	0	1	CCGG GGCC	<u>CC</u>GG GGCC	1	4	3	4
Demethylation							6 (0.9%)	15 (1.9%)	11 (1.6%)	16 (2.0%)
B1	1	1	1	0	CCGG GGCC	C<u>C</u>GG GG<u>C</u>C	1	1	1	0
B2	1	1	0	1	CCGG GGCC	<u>CC</u>GG GG<u>CC</u>	1	74	0	75
B3	1	1	0	0	C<u>C</u>GG GG<u>C</u>C	<u>CC</u>GG GG<u>CC</u>	1	11	2	12
B4	0	1	0	0	C<u>C</u>GG GG<u>C</u>C	<u>CC</u>GG GG<u>CC</u>	1	0	1	0
Methylation							4 (0.6%)	86 (10.7%)	4 (0.6%)	87 (10.9%)
C1	1	1	1	1	CCGG GGCC	CCGG GGCC	476	489	476	488
C2	1	0	1	0	<u>CC</u>GG GGCC	<u>CC</u>GG GGCC	58	66	57	65
C3	0	1	0	1	C<u>C</u>GG GG<u>C</u>C	C<u>C</u>GG GG<u>C</u>C	136	145	137	145
No change							670 (98.5%)	700 (87.4%)	670 (97.8%)	698 (87.1%)

CK and T indicate the control and heat stress conditions, respectively. Methylated cytosine is underlined, A score of 1 and 0 represents the presence and absence of bands, respectively. H and M represent digestion with *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI, respectively.

when the seedlings underwent pretreatment at 37°C before direct heat stress.

Differences in the DNA methylation patterns in heat-tolerant and heat-sensitive genotypes under heat stress

Twelve methylation patterns, organized into polymorphism and monomorphism groups, were observed in the products of the MSAP assay (Table 2 and Fig. 2). In the polymorphism group, the CCGG/GGCC sites were methylated differently between the control and the heat stress treatments; such a result indicates that the methylation patterns of the genomic DNA changed under heat stress. The methylation banding patterns were divided into the following three major types: A, B and C (Table 2). Type A indicates cytosine demethylation patterns under heat stress, showing that demethylation can occur in both internal cytosines (A2) and external cytosines (A3). Contrary to type A patterns, type B indicates cytosine methylated patterns in the treatment that corresponds with the control treatment; heat induced methylation can occur in both the external cytosine (B1) and internal cytosine (B2). Type C indicates patterns of a monomorphic classification; the same CCGG sites were detected following both the heat stress treatment and the control treatment (Fig. 2).

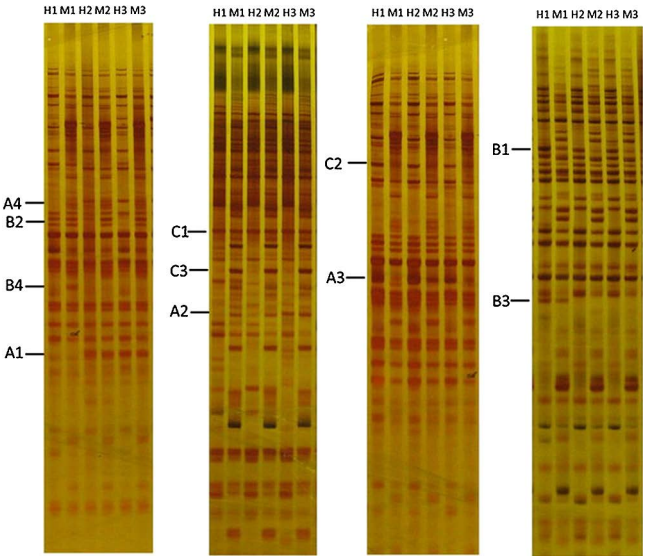

Fig. 2. MSAP fingerprints of rapeseed seedling between control and heat stress. Lanes H1 and M1 are MSAP patterns of control, lanes H2 and M2 are MSAP of 45°C heat stress, lanes H3 and M3 are MSAP of 45°C heat stress followed 37°C pretreatment. H represent digestion with *Eco*RI/*Hpa*II, M represent digestion with *Eco*RI/*Msp*I. Band patterns of A, B, and C can be seen in Table 2.

By using 23 pairs of primer combinations which illustrated polymorphisms in the MSAP analysis, banding patterns were detected between control conditions and the heat stress treatments in the two genotypes. Approximately 87.1%–97.8% of the CCGG sites in the two genotypes remain unchanged under heat stress (Table 2). The percentage of demethylated bands in Huyou 2 were 0.9% and 1.6% for the 45°C treatment without pretreatment and the 45°C treatment with a 37°C pretreatment, respectively. In Fengyou 1, the percentage of demethylated bands were 1.9% and 2.0% for the 45°C treatment without pretreatment and the 45°C treatment with a 37°C pretreatment, respectively. Under heat stress, the percentage of methylated bands was 0.6% in Huyou 2 and 10.7% in Fengyou 1. These results indicate that more DNA demethylation events occurred in Huyou 2 than methylation and relatively more DNA methylation occurred in Fengyou 1 than demethylation under heat stress. In general, DNA methylation and demethylation events caused by heat stress were different in the two genotypes, which may be related to the genotype-specific differences in thermotolerance.

Cloning and sequence analysis of the methylated DNA bands

Of the polymorphic fragments described above, a random set of 17 were cloned and sequenced to identify the nature of the DNA sequences involved in methylation and demethylation. The resulting sequences were subjected to BLAST searches against the NCBI and Brassica databases. The length of the cloned bands varied between 137 to 437 bp, with an average length of 258 bp (Table 3). Among these 17 DNA sequences, seven sequences (M11–M17) differed between genotypes under non-stress conditions. These seven sequences were homologous to a WD-40 repeat family protein/beige-related; an *A. thaliana* mRNA for a hypothetical protein; ECR1; and four *Brassica rapa* subsp. pekinensis clones (Table 3). These results indicate an epigenetic difference in these sequences between the two genotypes.

Three sequences were demethylated in both lines in response to heat stress (M6–M8); these were found to be homologous to a calcium-transporting ATPase, an AFLP marker M5 sequence of *B. napus* mitochondrial DNA, and a galactoside 2-alpha-L-fucosyltransferase. Five sequences were methylated or demethylated in response to heat stress in Fengyou 1 (M1–M3, M9–M10). Two sequences were demethylated under heat stress in Huyou 2 (M4–M5) (Table 3). These results indicate that heat stress affects a large number of diverse genes in rapeseed via DNA-specific changes in cytosine methylation.

Expression analysis of polymorphic fragments

The expression analysis of three MSAP polymorphic fragments was performed using quantitative RT-PCR. The expression results of heat treated leaves and untreated leaves were showed in Fig. 3. M7 was demethylated in heat treated leaves contrasted to untreated leaves, M9 was demethylated in Fengyou 1 and M16 was methylated in Fengyou 1 but the two genes were not changed in Huyou 2 under heat stress. The expression level of the M7 demethylated gene was up-regulated obviously, but the M9 was up-regulated slightly, and the M16 methylated gene was down-regulated in heat treated leaves.

Discussion

Many studies have indicated that primary DNA methylation plays important roles in the responses to environment stresses and may contribute to environmentally inducing phenotypic variation by modifying gene expression (Angers *et al.* 2010, Pecinka *et al.* 2010). In this study, we analyzed the relative thermotolerance of seedlings in two genotypes of *B. napus* using electrolyte leakage analysis. The seeds of these two genotypes were previously considered to be heat-sensitive (Fengyou 1) or heat-tolerant (Huyou 2) during seed preservation at high temperatures (Gao *et al.* 2010). We found that the response to heat stress in seeds of both genotypes correlated with that of the seedlings. Furthermore, our results demonstrated that high temperature (45°C) affects leaf membrane permeability and a pretreatment at 37°C increases leaf membrane permeability. Our results are consistent with previous reports that demonstrated that pretreatment at 37°C results in an observable decrease in leaf cell damage at 45°C (Hikosaka *et al.* 2006, Keeler *et al.* 2000, Larkindale *et al.* 2005, Senthil-Kumar *et al.* 2007).

Some studies have revealed that environmental stimuli can result in an increase or decrease in cytosine methylation

Table 3. Blast results of 17 polymorphic methylated DNA fragments

MSAP fragment	Size (bp)	Accession No./locus	E value	Nuclear identity (%)	Score	chromosome Position	Gene Position	Sequence homology
M1	243	Bol030657	4.00E-71	90	268	C03	Exon	CSDP1; CSDP1 (cold shock domain protein 1)
M2	338	Bra016894	6.00E-11	95	68	A04	Exon	POLD2; DNA binding/DNA-directed DNA polymerase
M3	245	Bra013050	2.00E-19	98	96	A04	Exon	GTP binding / GTPase
M4	153	FR715249.1	1.00E-55	98	224	mitochondria	Intergenic regions	*Brassica napus* complete mitochondrial genome
M5	137	AP006444.1	2.00E-15	98	92	mitochondria	Intergenic regions	*Brassica napus* mitochondrial DNA
M6	222	DQ321812.1	5.00E-94	99	352	mitochondria	Intergenic regions	*Brassica napus* AFLP marker M5 sequence
M7	287	Bol023401	1.00E-139	98	496	C08	Exon	calcium-transporting ATPase
M8	256	Bol029195	1.00E-124	99	448	C08	Promotor	FUT8 (FUCOSYLTRANSFERASE 8)
M9	256	Bra002340	1.00E-111	97	400	A10	Exon	protein kinase family protein
M10	180	Bra036388	3.00E-45	96	180	A07	Exon	zinc ion binding
M11	433	AC241103.1	6.00E-34	78	154	A06	Intergenic regions	*Brassica rapa* subsp. pekinensis clone KBrH015L02
M12	437	Bol030652	0.00E + 00	97	761	C03	Intron	WD-40 repeat family protein/beige-related
M13	299	AK228893.1	2.00E-18	70	102	C02	Intergenic regions	*Arabidopsis thaliana* mRNA for hypothetical protein
M14	300	AC189501.2	6.00E-57	79	230	A10	Intergenic regions	*Brassica rapa* subsp. pekinensis clone KBrB013O20
M15	246	AC189495.2	2.00E-05	78	99	C09	Intergenic regions	*Brassica rapa* subsp. pekinensis clone KBrB085G17
M16	225	Bra002219	7.00E-99	97	359	A10	Exon	ECR1; ECR1 (E1 C-terminal related 1)
M17	169	AC189233.2	9.00E-14	74	86	A03	Intergenic regions	*Brassica rapa* subsp. pekinensis clone KBrB013O20

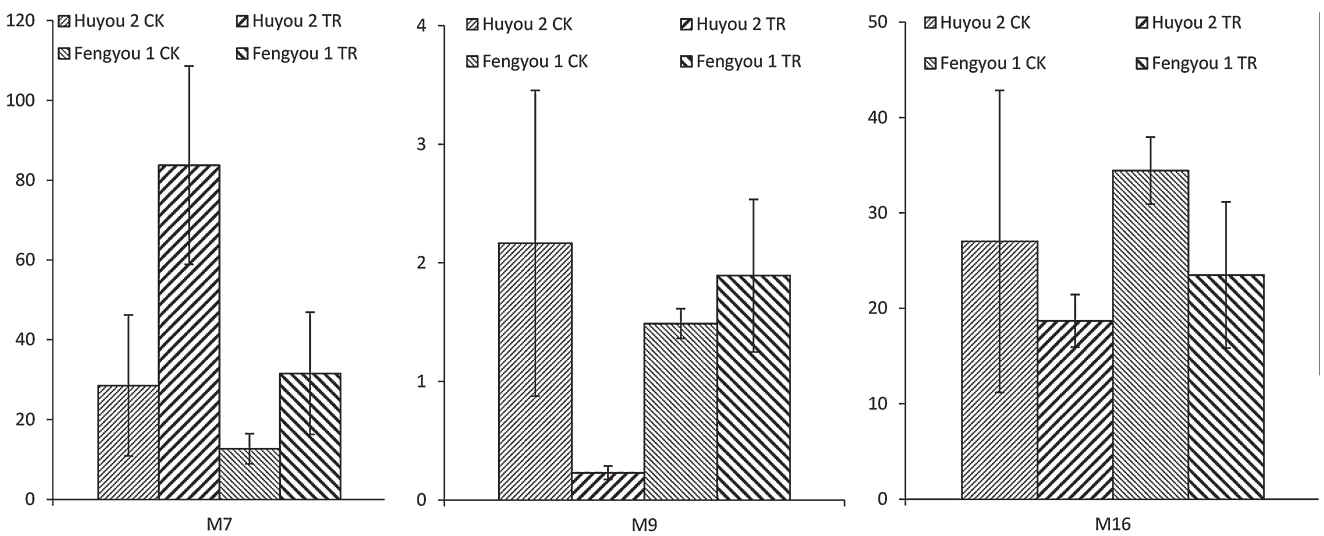

Fig. 3. Expression of genes homologous to M7, M9 and M16 in leaves of rapeseed under control and heat stress condition. CK and TR indicate the control and heat stress conditions, respectively.

throughout the genome and at specific loci (Lukens and Zhan 2007, Mastan *et al.* 2012, Tan 2010, Wang *et al.* 2011). In this study, we found that the percentage of MSAP sites in the Huyou 2 and Fengyou 1 genotypes were approximately 29.0% and 26.8%, respectively; these percentages were lower than those observed in *A. thaliana* (35–43%)

(Cervera *et al.* 2002). Our results indicated that differential DNA methylation occurs among distinct genotypes; this finding is consistent with studies in rice (Karan *et al.* 2012, Takata *et al.* 2005). In addition, heat stress tended to increase the overall DNA methylation levels in the leaves of seedlings of both rapeseed genotypes. Similar results were observed in rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) under heavy metal stress (Ge *et al.* 2002), in pea (*Pisum sativum* L.) under water deficit stress (Labra *et al.* 2002), and in Arabidopsis and *Pinus silvestris* in response to irradiation (Kovalchuk *et al.* 2003, 2004). DNA methylation was found to be specific to the genotype, the tissue and the developmental stage (Mastan *et al.* 2012, Tan 2010, Wang *et al.* 2011). Further studies are needed to identify the tissue and developmental specificity of these epigenetic changes in the rapeseed genome under heat stress. According to our study, the heat-sensitive genotype Fengyou 1 showed a greater increase in cytosine methylation under heat stress compared to the heat-tolerant genotype Huyou 2. Rapp and Wendel (2005) stated that methylation changes may generate nonspecific (random) differences between individuals, which may have adaptive significance during times of stress; such differences in methylation patterns increase the range of variation that natural selection can act upon (Verhoeven *et al.* 2010). Several studies have also suggested that crop genotypes that avoid cytosine methylation may be agriculturally superior to genotypes that are sensitive to methylation (Guo *et al.* 2006). A study by Tani *et al.* (2005) supported the hypothesis that hybrids perform better than inbred lines because they resist alterations in methylation under stress. A greater number of methylation/demethylation loci were observed in the heat-sensitive genotype compared to the heat-tolerant genotype. Our results are in agreement with previous global gene expression analyses revealing that the expressions of a strikingly large number of genes are induced by salinity stress in sensitive genotypes (Walia *et al.* 2005, 2007). The methylation and demethylation of DNA cytosines in specific regions of these genes, their promoters or in the neighboring sequences play an important role in regulating gene expression during plant development (Filek *et al.* 2008, Portis *et al.* 2004).

Heat stress induced extensive alterations at multiple genomic loci. In total, 17 MSAP fragments that were methylated or demethylated were identified and represent both responses to stress and genotype-specific methylation patterns. Our results indicate that changes in DNA methylation occur throughout the entire genome in response to abiotic stress, as previously observed in rice under salt stress (Wang *et al.* 2011). Recent genomic studies in *A. thaliana* revealed that many endogenous genes are methylated either within their promoters or within their transcribed regions and that gene methylation is highly correlated with the transcription level (Cokus *et al.* 2008). A close correlation exists between methylation and gene expression in response to abiotic stress (Choi and Sano 2007). In rapeseed seedlings, an increase in the Cd concentration in the medium stimulated demethylation at specific loci (Filek *et al.* 2008). In tobacco, viral infections (Wada *et al.* 2004) as well as several abiotic stresses (Choi and Sano 2007) resulted in demethylation and the associated up-regulation of stress-related genes (Verhoeven *et al.* 2010). In this study, we found that demethylated M7 was homologous to calcium-transporting ATPase. Through RT-PCR analysis, the expression of the gene was up-regulated in two genotypes under heat stress. Generally, the plasma membrane and ER of plant cells have been shown to possess primary calcium-transporting ATPase that use ATP hydrolysis to drive the direct transport of calcium ions (Thomson *et al.* 1993), and it has been suggested that a stress-induced change in $[Ca^{2+}]$cyt might be one of the primary transduction mechanisms whereby gene expression and biochemical events are altered to adapt plant cells to environmental stresses under the influence of various stress signals (Gong *et al.* 1998). Our results suggest that genotype-specific epigenetic changes may be an important regulatory mechanism in response to heat stress through the modification of the expression network of responsive genes. In sum, the changes in DNA methylation patterns induced by heat stress were different in two rapeseed genotypes. In addition, the methylation/demethylation induced by heat stress involved a wide range of genes. Our results help to clarify the molecular mechanisms of the adaption to heat stress in rapeseed.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (31100236), Chen Guang Program for Young Scientists of Wuhan Municipal Government (201271031402, 2013070104010031), and National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2011BAD35B09), Strategic Japanese-Chinese Cooperative Program on "Climate Change" (2012DFG90290).

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