Global DNA methylation variations after short-term heat shock treatment in cultured microspores of *Brassica napus* cv. Topas

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Heat stress can induce the cultured microspores into embryogenesis. In this study, whole genome bisulphite sequencing was employed to study global DNA methylation variations after short-term heat shock (STHS) treatments in cultured microspores of *Brassica napus* cv. Topas. Our results indicated that treatment on cultured Topas microspores at 32 °C for 6 h triggered DNA hypomethylation, particularly in the CG and CHG contexts. And the total number of T32 (Topas 32 °C for 6 h) vs. T0 (Topas 0 h) differentially methylated region-related genes (DRGs) was approximately two-fold higher than that of T18 (Topas 18 °C for 6 h) vs. T0 DRGs, which suggested that 32 °C might be a more intense external stimulus than 18 °C resulting in more changes in the DNA methylation status of cultured microspores. Additionally, 32 °C treatment for 6 h led to increased CHG differential methylations of transposons (DMTs), which were mainly constituted by overlaps between the hypomethylated differentially methylated regions (hypo-DMRs) and transposon elements (TEs). Further analysis demonstrated that the DRGs and their paralogs exhibited differential methylated/demethylated patterns. To summarize, the present study is the first methylome analysis of cultured microspores in response to STHS and may provide valuable information on the roles of DNA methylation in heat response.

Plants have evolved complicated genetic and epigenetic regulatory systems to respond quickly to unfavorable environmental conditions. The alteration of growth patterns, through the adjustment of cell division and expansion, is a characteristic response of plants to environmental stress. Plant reproduction, in particular pollen development, is the most stress-sensitive process in the life cycle of the organism. Especially, developmental stages around the meiotic and mitotic divisions are the most vulnerable. In angiosperms, microspores are generated by microsporocytes after meiosis and give rise to mature pollen after mitosis. Increasing evidences showed that microspore as a specific cell type can deviate from the original gamete-producing pathway and enter into the embryogenesis after a short severe heat shock treatment. And this heat treatment is often performed at 33–37 °C for a duration that varies from several hours to several days. In *Brassica napus*, the most efficient temperature for microspore fate changing is obtained by increasing the culture temperature to 32 °C. Using ultrastructural analysis, Telmer et al. demonstrated that the pollen differentiation pathway of the cultured microspores of *B. napus* cv. Topas is disrupted after short-term heat shock (STHS) treatment (within 6 h of 32.5 °C treatment). Apart from this, culture of isolated microspores of *B. napus* at 18 °C has been proposed as an ideal system to study the gametophytic development *in vitro*. However, Prem et al. demonstrated that microspore embryogenesis (ME) induction in Topas could also be achieved at 18 °C with a longer duration, which should be extended to several weeks.

Along with its essential role in the maintenance of genome integrity, DNA methylation participates in regulation of genes which are significant for plant development and stress response. Actually, external heat stimulation causes DNA methylation changes in plants. A recent study has also demonstrated that DNA methylation was...
involved in the control of cell growth during heat stress in tobacco BY-2 cells. Although heat pre-treatment is one of the significant stresses for ME induction, few studies have examined whether DNA methylation changes in cultured microspores after STHS treatment. Here, we tried to employ genome-wide bisulphite sequencing (GWBS) to decipher global DNA methylation variations after 32°C and 18°C treatments for 6 h in cultured microspores of *B. napus* cv. Topas at single-base resolution. And our results revealed that 32°C heat treatment for 6 h was sufficient to induce global DNA hypomethylation in cultured Topas microspores. And 32°C might be a more intense external stimulus than 18°C generating more changes in the DNA methylation status of cultured microspores. To summarize, the present study is the first methylome analysis of cultured microspores in response to STHS and may provide valuable information on the roles of DNA methylation in heat response.

**Results**

**Microspore collection and culture.** A highly embryogenic cultivar Topas of *B. napus* was chosen for the analysis based on previous studies. We attempted to collect only late uninucleate microspores as the initial materials for *in vitro* heat treatment and culture by bud selection and mesh screening (Fig. 1A). The mean diameter of these isolated microspores was 19.58 ± 1.09 μm (Fig. 1D). Then 32°C and 18°C treatments on the isolated microspores for 6 h were adopted in our experiments. Results showed that many enlarged microspores were formed after 6 h under 32°C treatment rather than 18°C treatment (Fig. 1B,C). The mean diameter and the frequency of the swollen microspores following the 32°C treatment for 6 h were 26.22 ± 1.90 μm and 57.50 ± 7.50%, respectively (Fig. 1D,E). However, the mean diameter of the unswollen microspores under this same heat treatment condition was 20.02 ± 1.62 μm, which was almost identical to the size of the initial microspores (Fig. 1D). In order to decipher the global DNA methylation variations after treatments at 32°C and 18°C for 6 h in cultured microspores of *B. napus* cv. Topas, sample without treatment (Fig. 1A) was chosen as a control for DNA methylation comparisons.

**The DNA methylation landscapes of baseline and STHS-treated microspores.** Genomic DNA extracted from the three collected samples (T0, Topas 0h; T18, Topas 18°C for 6 h; T32, Topas 32°C for 6 h) was...
treated with sodium bisulphite and then sequenced at ~26x coverage (Supplementary Table S1). The paired-end sequence files were subjected to multiple filtration steps and then aligned and deduplicated with Bismark (Supplementary Methods). The generated alignment reports indicated that the unique mapping efficiencies varied from 47.8% to 49.4% and the average proportions of methylation in three contexts (CG, CHG, and CHH) were 53.0%, 16.4%, and 3.5%, respectively (Supplementary Table S2). The level of symmetrical methylation was much higher compared with non-symmetrical methylation. Subsequent descriptive statistics by methylKit were shown in Supplementary Methods. Additionally, we depicted the identified methylated cytosines of the three samples on each chromosome (Fig. 2A). And the amount of identified methylated cytosines of the three samples distributed on the C genome was obviously higher than that distributed on the A genome (Fig. 2A).

**Identification of differentially methylated regions (DMRs).** To identify regions of the genome subjected to differential methylation, we used methylKit to calculate DMRs in a pairwise fashion (Fig. 2B and figure 3).
Supplementary Data S1). Then we randomly selected two DMRs for pyrosequencing validation, and the results confirmed the methylation status detected by GWBS (Supplementary Fig. S3). Deeper analysis indicated that more DMRs were identified in T32 vs. T0 than those in T18 vs. T0, which was mainly due to more CG and CHG

Figure 4. % of hyper and hypo methylated regions per chromosome.

Figure 5. The analysis on the overlaps between DMRs and TEs.
DMRs were induced by 32 °C culture (Fig. 3A). Besides, more total DMRs were observed in the C genome than in the A genome in all pairwise samples (Fig. 3B and Supplementary Data S1), particularly in the T32 vs. T0 comparison (Fig. 3B). In T32 vs. T0 and T32 vs. T18 comparisons, there were more hypomethylated DMRs (hypo-DMRs) than hypermethylated DMRs (hyper-DMRs) (Fig. 3C and Supplementary Data S1). And these T32 vs. T0 and T32 vs. T18 hypo-DMRs were mainly occupied by CG and CHG DMRs (Fig. 3C and Supplementary Data S1). Moreover, 222 and 116 T32 vs. T0 hypo-DMRs were distributed on the C and A genomes, respectively (Fig. 3D).

Analysis of the percentages of hyper- and hypo-methylated regions per chromosome indicated that the 32 °C treatment on the cultured Topas microspores for 6 h resulted in a significant proportion of hypomethylation in the symmetrical CG and CHG contexts (Fig. 4).

Differential methylation in transposons. Cytosine methylation is chiefly targeted towards transposon element (TE) silencing. Stress-induced transposon activation has been confirmed by molecular data in many different hosts. Therefore, it was necessary to investigate the overlapping information between the identified DMRs and TEs. Although Chalhoub et al. previously analysed the TEs identified in the released B. napus genome, their position information is not available in the public database. We firstly employed RepeatScout and RepeatMasker to de novo identify TEs in the whole B. napus genome. A total of 146,998 TEs (43,083 in the A genome and 103,915 in the C genome) were identified. Of them, 102,624 were retrotransposons and 44,374 were DNA transposons. Then, the differential methylations of transposons (DMTs) were searched based on the position information of TEs and DMRs (Supplementary Data S2). Further analysis showed that the amount of CHG DMTs was higher than the CG and CHH DMTs in T32 vs. T0 and T32 vs. T18 comparisons; the greater number of CHG DMTs was attributed to an increased overlap between the TEs and hypo-DMRs rather than hyper-DMRs (Fig. 5A, Supplementary Data S2, and Supplementary Table S4). Additionally, the differential methylations of retrotransposons (DMRTs) in all contexts in the three pairwise samples were more than the differential methylations of DNA transposons (DMDTs); LINE, LTR/Copia, and LTR/Gypsy were the most abundant retrotransposable elements (Fig. 5B and Supplementary Table S5). Subsequently, the distributions of DMTs on each chromosome were analysed. Results demonstrated that the numbers of DMTs located on A and C genomes were unequal (Supplementary Table S6).

Identification of DMR-related genes (DRGs). We employed the methylKit package and an in-house R script to identify DRGs (Supplementary Data S3). Then the distribution of DRGs on each chromosome of B. napus was investigated (excluding the DRGs located on unassembled scaffolds) (Supplementary Table S7). The total number of DRGs in T32 vs. T0 (96) was approximately two-fold higher than T18 vs. T0 (52), mainly because of the increase in CG and CHG DRGs in T32 vs. T0 (Fig. 6 and Supplementary Table S8). T32 vs. T0 DRGs could also be divided into 69 hypomethylated DRGs (hypo-DRGs) and 27 hypermethylated DRGs (hyper-DRGs), respectively. However, these two types of DRGs in T18 vs. T0 were almost equal (Table 1). Further analysis showed that only four common CG DRGs were identified between T32 vs. T0 and T18 vs. T0 (Fig. 6 and Table 2). They exhibited similar tendencies of methylation change in both comparisons (Table 2). Functional annotation analysis indicated that BnaA03g36810D was similar to AT3G22840, which encodes an early light-inducible protein (ELIP) (Table 2) and transiently accumulates in response to environmental stress. Moreover, the mortality rates of plants lacking ELIPs are sometimes higher. The in vitro culture itself was an environmental stress independent
Did the culture rather than temperature effects induce the similar methylation changes of the common CG DRGs identified between T32 vs. T0 and T18 vs. T0?

To elucidate the differences of cultured microspores under different temperatures, subsequent analyses were mainly focused on the T32 vs. T18 DRGs (Table 2, Table 3 and Supplementary Data S3). Among the total 77 T32 vs. T18 DRGs, 47 were hypo-DRGs and 30 were hyper-DRGs (Table 1). In addition, five common CG and two common CHH DRGs were sought between T32 vs. T18 and T18 vs. T0, respectively (Fig. 6 and Table 2). Amazingly, the methylation/demethylation tendencies of these DRGs were totally opposite in two comparisons.

| Comparisons | TD* | DRG Locus | From | MDD* | DDT* | $S^\dagger$ | SAL* | Description (TIGR) |
|-------------|-----|-----------|------|------|------|--------|------|-------------------|
| T32 vs. T18 & T32 vs. T0 | CG hypo-DRGs | BnaA02g21500D | T32 vs. T18 | −31.31 | 0 | + | AT4G22758 | unknown protein |
| | | T32 vs. T0 | −30.94 | | | | | |
| | | BnaA03g39290D | T32 vs. T18 | −37.09 | −138 | + | AT2G16740 | ubiquitin-conjugating enzyme 29 (UBC29) |
| | | T32 vs. T0 | −36.11 | | | | | |
| | | BnaA03g54520D | T32 vs. T18 | −30.61 | 0 | − | AT4G04090 | BTB/POZ domain-containing protein |
| | | T32 vs. T0 | −27.20 | | | | | |
| | | BnaA04g24700D | T32 vs. T18 | −39.49 | −1873 | − | AT2G42840 | protodermal factor 1 (PDF1) |
| | | T32 vs. T0 | −35.25 | | | | | |
| | | BnaC03g39290D | T32 vs. T18 | −36.65 | −110 | − | ATCG00190 | RNA polymerase subunit beta, RPOB |
| | | T32 vs. T0 | −28.17 | | | | | |
| | | BnaC08g29000D | T32 vs. T18 | −47.18 | −1363 | − | AT3G58120 | BZIP transcription factor 61 (BZIP61) |
| | | T32 vs. T0 | −38.19 | | | | | |
| | | BnaC09g13630D | T32 vs. T18 | −30.31 | 0 | − | NA | NA |
| | | T32 vs. T0 | −25.65 | | | | | |
| | CHG hypo-DRGs | BnaA02g16440D | T32 vs. T18 | −26.83 | −1324 | + | NA | NA |
| | | T32 vs. T0 | −27.68 | | | | | |
| | | BnaA06g03030D | T32 vs. T18 | −27.65 | −975 | − | AT1G49710 | fucosyltransferase 12 (FUT12) |
| | | T32 vs. T0 | −33.45 | | | | | |
| | | BnaC01g24140D | T32 vs. T18 | −34.33 | −1673 | − | AT3G45040 | dolichol kinase 1 (DOK1) |
| | | T32 vs. T0 | −28.92 | | | | | |
| | | BnaC03g31070D | T32 vs. T18 | −38.24 | −1693 | − | NA | NA |
| | | T32 vs. T0 | −30.31 | | | | | |
| | | BnaC06g31320D | T32 vs. T18 | −27.14 | −1585 | + | AT1G70230 | altered xyloglucan 4 (AXY4) |
| | | T32 vs. T0 | −29.29 | | | | | |
| T32 vs. T0 & T18 vs. T0 | CG hypo-DRGs | BnaA02g21210D | T18 vs. T0 | −49.26 | 0 | − | AT4G04090 | ribosomal protein L36e family protein |
| | | T32 vs. T0 | −48.31 | | | | | |
| | | BnaA03g36810D | T18 vs. T0 | −29.68 | 9 | − | AT3G22840 | early light-inducible protein 1 (ELIP1) |
| | | T32 vs. T0 | −33.45 | | | | | |
| | | BnaC03g43830D | T18 vs. T0 | −31.55 | 383 | − | NA | NA |
| | | T32 vs. T0 | −29.22 | | | | | |
| | CG hyper-DRGs | BnaC03g57230D | T18 vs. T0 | 35.03 | 1399 | − | AT3G43610 | Spc97/Spc98 family of spindle pole body (SBP) component |
| | | T32 vs. T0 | 37.62 | | | | | |
| T32 vs. T18 & T18 vs. T0 | CG hypo-DRGs | BnaA03g17310D | T32 vs. T18 | 32.38 | 51 | − | AT2G37600 | ribosomal protein L36e family protein |
| | | T32 vs. T0 | 37.62 | | | | | |
| | CG DRGs | BnaA03g24920D | T32 vs. T18 | −30.84 | −382 | − | AT4G11960 | proton gradient regulation 5-like 1B (PGRL1B) |
| | | T32 vs. T0 | 37.52 | | | | | |
| | | BnaA03g38010D | T32 vs. T18 | 30.91 | 0 | + | AT2G05160 | CCCH-type zinc finger family protein with RNA-binding domain |
| | | T32 vs. T0 | −32.58 | | | | | |
| | | BnaA04g16720D | T32 vs. T18 | 40.20 | −1291 | − | AT2G28830 | plant U-box 12 (PUB12) |
| | | T32 vs. T0 | −26.29 | | | | | |
| | CHH DRGs | BnaC05g34070D | T32 vs. T18 | 25.39 | 0 | − | NA | NA |
| | | T32 vs. T0 | −27.71 | | | | | |
| | | BnaC05g07550D | T32 vs. T18 | −32.10 | 640 | − | AT1G10200 | ATWLIM1 |
| | | T32 vs. T0 | 35.71 | | | | | |
| | | BnaC07g3260D | T32 vs. T18 | 64.58 | −152 | + | AT4G31340 | myosin heavy chain-related |
| | | T32 vs. T0 | −32.49 | | | | | |

Table 2. The common DRGs identified between comparisons. *TD, type of DRG. "MDD, meth.diff of the DMR. "DDT, distance from DMR to transcriptional start site. "S, strand. "SAL, similar to Arabidopsis locus. "NA, no available.

of the different temperatures. Did the culture rather than temperature effects induce the similar methylation changes of the common CG DRGs identified between T32 vs. T0 and T18 vs. T0?

To elucidate the differences of cultured microspores under different temperatures, subsequent analyses were mainly focused on the T32 vs. T18 DRGs (Table 2, Table 3 and Supplementary Data S3). Among the total 77 T32 vs. T18 DRGs, 47 were hypo-DRGs and 30 were hyper-DRGs (Table 1). In addition, five common CG and two common CHH DRGs were sought between T32 vs. T18 and T18 vs. T0, respectively (Fig. 6 and Table 2). Amazingly, the methylation/demethylation tendencies of these DRGs were totally opposite in two comparisons,
| TD* | MDD* | DRG Locus | DDT* | S* | SAL* | Description (TIGR) |
|-----|------|-----------|------|----|------|--------------------|
| −40.67 | BnaA03g39270D | 0 | + | NA | NA |
| −27.90 | BnaA09g46290D | −87 | − | AT1G34110 | Tetrapeptide repeat (TPR)-like superfamily protein |
| −31.82 | BnaC01g25370D | 0 | − | NA | NA |
| −27.24 | BnaC01g33320D | 1906 | − | NA | NA |
| −25.69 | BnaC01g35440D | 0 | + | NA | NA |
| −25.95 | BnaC03g44230D | −1048 | + | AT2G04520 | Nucleic acid-binding, OB-fold-like protein |
| −25.89 | BnaC06g00730D | −1195 | + | NA | NA |
| −25.61 | BnaC07g12960D | 1789 | − | NA | NA |
| −28.84 | BnaC08g34040D | 0 | − | NA | NA |
| −29.39 | BnaA01g13320D | 478 | − | AT4G23640 | tiny root hair 1 (TRH1) |
| −26.97 | BnaA07g06910D | 1253 | − | AT1G31320 | lob domain-containing protein 4 (LBD4) |
| −26.57 | BnaA08g02650D | 411 | + | AT1G49480 | related to vernalization 1 (RTV1) |
| −29.10 | BnaA08g02600D | 724 | − | AT1G49475 | AP2/I3-like transcriptional factor family protein |
| −34.68 | BnaA08g05750D | 1395 | + | AT1G5660 | thiorodoxin superfamily protein |
| −36.21 | BnaC04g3690D | −1177 | + | AT2G21170 | triosephosphate isomerase (TIM) |
| −36.54 | BnaC07g08220D | 310 | − | AT1G41360 | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein |

**CG hypo-DRGs**

| 34.20 | BnaA03g49000D | 183 | + | NA | NA |
| 35.27 | BnaA03g39020D | 377 | + | AT2G16365 | F-box family protein |
| 46.27 | BnaA05p10770D | 626 | + | AT2G32280 | vasculature complexity and connectivity (VCC) |
| 26.27 | BnaA05p16350D | −1480 | + | AT1G32180 | cellulose synthase-like d6 (CDSL6) |
| 28.30 | BnaA07g12210D | 1222 | − | AT5G66985 | unknown protein |
| 40.63 | BnaA09g20620D | −342 | − | AT4G04020 | plastoglobulin 35 (PGL35) |
| 29.73 | BnaC01g19080D | 0 | − | AT2G49660 | embryo defective 2782 (EMB2752) |
| 26.54 | BnaC03g25860D | 0 | + | AT2G46530 | auxin response factor 11 (ARF11) |
| 38.37 | BnaC04g03320D | −1631 | − | AT2G44090 | ankyrin repeat family protein |
| 50.37 | BnaC04g39580D | −1581 | + | AT2G28190 | copper/zinc superoxide dismutase 2 (CSD2) |
| 33.33 | BnaC05g08160D | 420 | − | AT1G10650 | SBP (S-ribonucleic binding) encoding protein family protein |
| 26.85 | BnaC06g24840D | −845 | − | AT1G70180 | Sterile alpha motif (SAM) domain-containing protein |
| 32.29 | BnaC07g08840D | 1196 | + | AT1G30550 | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein |

**CG hyper-DRGs**

| −32.48 | BnaA06g19130D | −1177 | − | AT4G12730 | FASCIICLIN-like arabinogalactan 2 (FLA2) |
| −33.36 | BnaC01g20920D | −467 | − | NA | NA |
| −43.07 | BnaC03g4740D | 879 | + | NA | NA |
| −28.40 | BnaC08g28690D | 0 | − | NA | NA |
| −26.83 | BnaA01g19320D | −1456 | + | AT4G27170 | seed storage albumin 4 (SESA4) |
| −25.67 | BnaA02g29760D | −1848 | − | AT1G7230 | scarecrow-like 13 (SCL13) |
| −42.05 | BnaC02g29760D | 1635 | + | AT5G42150 | glutathione S-transferase (GST) family protein |
| −26.87 | BnaC03g11550D | 1557 | − | AT5G03300 | cysteine proteinases superfamily protein |
| −27.27 | BnaC03g23710D | −1234 | − | AT2G24380 | BZIP transcription factor 34 (BZIP34) |
| −30.15 | BnaC03g42260D | −1429 | − | AT3G21700 | Arabidopsis thaliana G protein (AtG2P) |
| −34.65 | BnaC03g65030D | −428 | − | AT2G25270 | adenine phosphoribosyl transferase 3 (APT3) |
| −35.83 | BnaC06g12760D | −505 | − | AT5G39990 | beta-glucuronosyltransferase 14a (GLCAT14A) |
| −25 | BnaC07g42330D | 0 | − | AT4G30080 | auxin response factor 16 (ARF16) |
| −28.69 | BnaC09g29070D | −1151 | − | AT5G50330 | unknown protein |

**CHG hypo-DRGs**

| 25.59 | BnaA09g10660D | 14 | − | NA | NA |
| 43.44 | BnaA11g13520D | −1153 | + | AT4G23900 | nucleoside diphosphate kinase family protein |
| 40.28 | BnaA04g17830D | −1063 | − | AT2G30210 | laccase 3 (LAC3) |
| 26.05 | BnaA08g15210D | −115 | − | AT4G63350 | purple acid phosphatase 25 (PAP25) |
| 27.29 | BnaC02g00380D | −284 | + | AT5G65360 | histone 3.1 (H3.1) |
| 29.43 | BnaC03g19880D | −1382 | − | AT2G36020 | hv2a-like protein 3 (HVA22) |
| 32.60 | BnaC05g39140D | −1220 | − | AT3G14450 | ctc-interacting domain 9 (CID9) |

**CHH hypo-DRGs**

| −25.65 | BnaA09g32770D | −873 | + | AT3G52300 | ‘ATP synthase d chain, mitochondrial’ (ATPQ) |
| −25.15 | BnaC01g4970D | −1207 | + | AT4G33210 | slow motion (SLOMO) |
| −28.54 | BnaC06g4480D | −490 | + | AT1G51210 | UDP-Glycosyltransferase superfamily protein |

**CHH hyper-DRGs**

| 25.05 | BnaC02g26820D | −329 | − | NA | NA |
| 31.20 | BnaC01g10210D | 903 | + | AT4G17615 | calcineurin b-like protein 1 (CBL1) |
| 37.82 | BnaC05g5660D | 719 | + | AT1G07900 | lob domain-containing protein 1 (LBD1) |
| 26.88 | BnaC05g37060D | −1671 | + | AT3G13560 | O-Glycosyl hydrolases family 17 protein |
| 27.14 | BnaC09g32910D | 0 | − | AT5G57320 | villin 5 (VLN5) |

Table 3. The specific DRGs identified in T32 vs. T18. *TD, type of DRG. *MDD, meth.diff of the DMR. *DDT, distance from DMR to transcriptional start site. *S, strand. *SAL, similar to Arabidopsis locus. *NA, no available.
and only BnaA03g24920D and BnaC05g07550D were hypo-DRGs in T32 vs. T18 (Table 2). BnaA03g24920D was similar to proton gradient regulation 5-like 1B (PGRL1B). And PGRL1 has been proved to be involved in cyclic electron flow (CEF), which only generated ATP and was driven by photosystems I (PSI)\(^2\). Arabidopsis PSI CEF is abolished following thermal-stress\(^2\). We next asked whether PGRL1 maintained normal CEF functions during thermal-stress and generated sufficient energy for the survival of cultured microspores. BnaC05g07550D might encode LIM protein that regulating transcription or organizing the cytoskeleton by triggering the formation of actin bundles\(^2\). Actually, heat shock has been shown to cause changes in microtubule and cytoskeleton in cultured Topas microspores\(^2\).

All the seven common CG and five common CHG DRGs further identified between T32 vs. T18 and T32 vs. T0 had negative meth.diff values in both comparisons (Fig. 6 and Table 2). Among these DRGs, BnaA03g39290D was similar to \textit{UBC29} (ubiquitin-conjugating enzyme 29). UBCs participate in protein degradation via proteasome and may be involved in various biological processes\(^2\). As for the STHS treatment on cultured microspore can disrupt pollen differentiation\(^1\), we asked whether BnaA03g39290D functioned in sweeping the proteins that required for pollen differentiation. Another common DRG BnaA04g24700D resembled \textit{PDF1} (PROTODERMAL FACTOR 1), which was involved in the fate determination of epidermal cell\(^2\). The CHG DRG BnaA06g03030D resembled a heat stress-responsive gene \textit{FUT12} (fucosyltransferase 12)\(^2\). In addition, BnaC01g24410D was similar to \textit{AtDOK1} (\textit{A. thaliana} dolichol kinase 1), the expression of which could complement the temperature-sensitive growth and glycosylation defects of the \textit{Saccharomyces cerevisiae} sec59 mutant\(^2\). Moreover, AtDOK1 is involved in the synthesis of dolichol phosphate (Dol-P), which serves as a carrier of complex polysaccharides during protein glycosylation\(^3\). Glycoproteins are confirmed to be involved in adaptation to biotic and abiotic stresses\(^4\).

Except for the common DRGs, there were 58 specific DRGs (33 hypo-DRGs and 25 hyper-DRGs) existing in T32 vs. T18, including 29 CG, 21 CHG, and eight CHH DRGs (Table 3). These specific DRGs contained several genes that might be involved in various biological processes (Table 3). For example, BnaA08g05750D (thioredoxin superfamily protein) and BnaC02g29760D (glutathione S-transferase family protein) might function in maintaining cell redox homeostasis (Table 3). High temperature provokes the accumulation of reactive oxygen species (ROS) in plants\(^5\). Heat stress-incuced ME also generates an oxidative burst and ROS\(^6\). Therefore, sustaining cell redox homeostasis was crucial for the survival of cultured microspore during heat stress. Additionally, two putative glycosyltransferases (BnaC06g12760D and BnaC06g04480D) and a putative fasciclin-like arabinogalactanprotein 2 (FLA2) (BnaA06g19130D) were found as specific hypo-DRGs in T32 vs. T18 (Table 3). Previous study has indicated that the protein encoded by AT5G39990 (similar to BnaC06g12760D) was involved in the biosynthesis of type II arabinogalactan (AG) and cell elongation during seedling growth\(^7\). Most AGPs are O-glycosylated at hydroxyproline residues by type II AG group\(^8\). And their pivotal roles in cell wall signal transduction, plant development and stress tolerance have also been discussed\(^9\). Intriguingly, FLA2 was found to be significant in several stress-related AFGC microarray experiments\(^10\). It could be part of the auxin transporters ABCB19/PINFORMED1 (PIN1) nanodomain\(^11\). In addition, BnaC01g22680D resembled AtSGP2 (\textit{A. thaliana} G-protein) (Table 3). Arabidopsis monomeric G-proteins are implied to be markers of early and late events in cell differentiation\(^12\). Intriguingly, a specific CHG hypo-DRG BnaC01g19320D coincidently matched with napin gene \textit{SESA4} (seed storage albumin 4) (Table 3), which was identified as an early molecular marker for ME in \textit{B. napus}\(^13,14\). Another specific CHG hypo-DRG BnaC01g21110D identified from T32 vs. T18 might be a putative SCL (scarecrow-like) gene (Table 3). Joosen et al.\(^9\) previously identified a \textit{B. napus} gene, which was homologous to \textit{Arabidopsis} SCL11, as a robust marker for ME. Whether these STHS-induced DRGs are really related to ME is still

**Figure 7. Circos plot of the DRGs and their corresponding paralog genes.** The colors displayed in chromosomes represented A-X CCBs. The paralog gene pairs were connected by lines. And the red, blue, and green lines stranded for CG, CHG, and CHH DRGs, respectively. Similarly, red, blue, and green tickets along with the inner chromosomes indicated the locations of CG, CHG and CHH DRGs on chromosomes, respectively.
an open question. Besides, other gene groups related to energy metabolism, protein degradation, transcription and translation, and signal transduction (Table 3) were also included in specific T32 vs. T18 DRGs.

Chromosome localization of DRGs and their paralogs. Schranz et al. have built the A to X conserved collinear blocks (CCBs) of the reduced karyotype (n = 5) of A. thaliana, and these blocks were defined by their position in a proposed ancestral karyotype (n = 8). The genomes of Brassica rapa (AA) and its sister species Brassica oleracea (CC) were almost complete triplications of the genome of A. thaliana. B. napus (AACC) was formed by recent allopolyploidy between the ancestors of B. oleracea and B. rapa. Therefore, the CCBs of B. napus were constructed based on the position information of the A. thaliana A to X segments. Then we investigated the number of DRGs in each of the A to X CCBs of B. napus. Results indicated that the U and F blocks possessed the most DRGs in the T32 vs. T0 and T18 vs. T0 comparisons, whereas no DRGs were distributed on the G, K, and S blocks (Supplementary Table 9). Due to the polyploid properties, it was also necessary to evaluate whether differential methylated An-Cn paralog gene pairs existed in B. napus. The An-Cn paralog gene pairs of B. napus were searched according to the methods described by Liu et al. Although almost all of the T32 vs.
In plants, DNA methylation occurs frequently in all three sequence contexts: the symmetric CG and CHG contexts and the asymmetric CHH context. The relationships between DNA methylation variations and expression levels of the 16 selected DRGs. *SAL, similar to Arabidopsis locus. *SCD, sequence context of DMR. *DDT, distance from DMR to transcriptional start site. *MDD, meth.diff of the DMR. *NA, no available.

Table 4. The relationships between DNA methylation variations and expression levels of the 16 selected DRGs. *SAL, similar to Arabidopsis locus. *SCD, sequence context of DMR. *DDT, distance from DMR to transcriptional start site. *MDD, meth.diff of the DMR. *NA, no available.

T0 and T18 vs. T0 DRGs possessed one or more paralog genes, none of these paralog genes were included in the identified DRGs (Fig. 7 and Supplementary Data 4). This result suggested that distinct DNA methylation regulatory pathways might exist even for paralogs. Chang and Liao46 demonstrated that DNA methylation could “rebalance” the overall expression dosage of paralogs.

Real-time PCR to analyse DRG expression. To examine the relationships between DNA methylation and gene expression levels, 16 DRGs (13 hypo-DRGs and three hyper-DRGs) were randomly selected for real-time PCR verification based on the UniGene information (Supplementary Data S5). Results indicated that seven genes were up regulated, two genes were down regulated, and the expression levels of seven genes remained unchanged (Fig. 8). DNA demethylation and methylation are considered to be associated with elevating and suppressing gene expressions, respectively. However, the expressions of four hypo-DRGs and two hyper-DRGs did not follow this principle (Fig. 8 and Table 4). More dynamic and complex relationships between DNA methylation and expression have been illustrated in other studies46,47. Among these 16 selected genes, BnaA09g40710D was a T32 vs. T0 CG hypo-DRG and its expression was up-regulated by nearly two-fold in T32 compared with that of T0 (Fig. 8). Furthermore BnaA09g40710D was identical to Arabidopsis ELF3 (early flowering 3) (Table 4), which controlled elongation growth in response to temperature48. Similarly, another hypo-DRG BnaC05g29060D was up-regulated in the 32 °C treatment (Fig. 8). This gene resembled Arabidopsis SCAMP5 (secretory carrier membrane protein 5) (Table 4). Whether BnaC05g29060D functions in vesicles transportation and protein trafficking during heat treatment remains unknown.

Discussion

Exposure of Arabidopsis plants to heat stress results in an increased global methylation49. Nevertheless, in cotton (Gossypium hirsutum) anthers, high temperature leads to the genome-wide hypomethylation at the tetrad stage and the tapetal degradation stage50. Our results showed that the T32 vs. T0 and T32 vs. T18 DMRs were mainly comprised of hypo-DMRs rather than hyper-DMRs (Fig. 3C and Supplementary Data S1). The percentages of the hyper- and hypo-methylated regions per chromosome clearly indicated that the 32 °C treatment on cultured microspores generated a significant proportion of hypomethylation in the symmetrical CG and CHG contexts (Fig. 4). This result demonstrated that STHS initiated the demethylation process, giving rise to a decrease in global DNA methylation in cultured microspores. Actually, the percentage of methylated cytosine increased from 3 to about 11% from microspore to mature stage in pollen of G. hirsutum51. Therefore, it seems that there is no consistent trend in the changes of DNA methylation under heat in different species or in different cell types. Whether plant male germ cell tended to be global hypomethylated rather than hypermethylated after heat stress? If so, why the DNA methylation tendencies were different? Additionally, we also found that the total number of DRGs in T32 vs. T0 was approximately two-fold higher than T18 vs. T0 suggesting 32 °C may be a more intense external stimulus than 18 °C and ultimately resulted in more changes in the DNA methylation status of cultured microspores. Whether BnaC05g29060D functions in vesicles transportation and protein trafficking during heat treatment remains unknown.

In plants, DNA methylation occurs frequently in all three sequence contexts: the symmetric CG and CHG contexts and the asymmetric CHH context. Each type of DNA methylation is vital for development and responses to environmental stresses52. Here, our data revealed that the 32 °C treatment brought about increased CHG DMTs in the T32 vs. T0 and T32 vs. T18 comparisons due to abundant overlaps between the TEs and hypo-DMRs (Fig. 5A). Moreover, DMRs in all contexts in the three pairwise samples were higher than DMDTs. LINE, LTR/ Copia, and LTR/Gypsy were the most abundant retrotransposable elements (Fig. 5B and Supplementary Table S5).
Yang et al. previously observed that retrotransposons were more tightly controlled by methylation than DNA transposons during the floral development of Arabidopsis. ONSEN, an LTR/copia type retrotransposon was found activated by heat stress in Arabidopsis11,12. Deeper studies are needed to answer whether and how these DMRs function during STHS treatment in cultured microspores.

Intriguingly, some molecular markers, such as napin, G-protein, SCL and AGP, for early ME in B. napus identified via transcriptome and proteome analysis were also existed among the 58 specific T32 vs. T18 DRGs (Table 3). Additional experiments are required to decipher how DNA methylation functions as an epigenetic response to heat stress in cultured microspores, whether DNA methylation status is associated with the expression of the overlapped markers for early ME in B. napus and whether the microspores from different cultivars that with different embryogenic potentials possess different epigenetic response to heat stress.

Methods
Sample preparation, GWBS, and genome-wide cytosine methylation analysis. Plants of the B. napus inbred line ‘Topas’ were sown in an experimental field at Qinghai University (Xining, Qinghai Province, China) on April 10, 2014 (Supplementary Methods). Bud selection, microspore isolation and cultivation were performed (Supplementary Methods). Genomic DNA and total RNA were extracted for further analysis (Supplementary Methods). Qualified genomic DNA was sent to BGI (BGI Tech Solutions Co., Ltd, Shenzhen, China) for GWBS (Supplementary Methods). Genome-wide cytosine methylation analysis was carried out based on the methods described in Supplementary Methods. Two randomly selected regions that contained identified 5mC sites were used to perform pyrosequencing validation (Supplementary Methods). Additionally, we performed CG island prediction and digital expression analyses (Supplementary Methods).

Analysis of the overlaps between TEs and DMRs. First, RepeatScout (http://bix.ucsd.edu/repeatscout/) was used to construct a repeat library of the B. napus genome using an ab initio approach. Then this library was used to screen DNA sequences for interspersed repeats and low complexity DNA sequences using RepeatMasker (http://www.repeatmasker.org/). Retrotransposon and DNA transposons position information was retrieved from the out file generated by RepeatMasker. An in-house Perl script was used to calculate the overlaps between TEs and DMRs.

Quantitative RT-PCR analysis. Gene-specific primers (Supplementary Table S10) for the 16 randomly selected DRGs were designed with GeneTool. The housekeeping gene β-actin (AF111812) was used to normalize the expression of each gene in the different RNA samples. cDNA synthesis and quantitative RT-PCR analysis was performed according to the methods described by Li et al.53.

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Acknowledgements
We thank help from Professor Dezhi Du, Dr. Shaomin Guo (Institute of Spring Rapeseed, Qinghai Academy of Agriculture and Forestry Sciences) and their colleagues. We also thank Dr. Xia Wan (Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences) for polishing the language. This work was funded by the National Natural Science Fund for Distinguished Young Scholars (31100236), the National Science & Technology Pillar Program during the Twelfth Five-Year Plan Period (2013BAD01B01 and 2013BAD01B03), the Scientific and Technical Innovation Project of Chinese Academy of Agricultural Sciences, and the Wuhan Youth Science and Technology Chen Guang Program (201271031402).

Author Contributions
J.L. and X.W. conceived and designed the experiments. B.C., G.G. and K.X. conducted management of plant materials in field. J.L., Q.H. and T.Z. performed the microspore isolation, in vitro culture, microscopy observation, and sample collection. J.L. and H.L. performed DNA and RNA extractions. E.L., G.Y. and J.Q. participated in discussions. J.L., M.S. and Y.C. analyzed the data: J.L. wrote the paper.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Li, J. et al. Global DNA methylation variations after short-term heat shock treatment in cultured microspores of Brassica napus cv. Topas. Sci. Rep. 6, 38401; doi: 10.1038/srep38401 (2016).
