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Research Article

The contrasting response to drought and waterlogging is underpinned by divergent DNA methylation programs associated with transcript accumulation in sesame

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ABSTRACT

DNA methylation is a heritable epigenetic mechanism that participates in gene regulation under abiotic stresses in plants. Sesame (*Sesamum indicum*) is typically considered a drought-tolerant crop but highly susceptible to waterlogging, probably because of its origin in Africa or India. Understanding DNA methylation patterns under drought and waterlogging conditions can provide insights into the regulatory mechanisms underlying sesame contrasting responses to these abiotic stresses. We combined Methylation-Sensitive Amplified Polymorphism and transcriptome analyses to profile cytosine methylation patterns, transcript accumulation, and their interplay in drought-tolerant and waterlogging-tolerant sesame genotypes. Drought stress strongly induced *de novo* methylation (DNM) whereas most of the loci were demethylated (DM) during the recovery phase. In contrast, waterlogging stress decreased the level of methylation but during the recovery phase, both DM and DNM were concomitantly deployed. In both stresses, the levels of the differentially accumulated transcripts (DATs) highly correlated with the methylation patterns. We observed that DM was associated with an increase of DAT levels while DNM was correlated with a decrease of DAT levels. Altogether, sesame has divergent epigenetic programs that respond to drought and waterlogging stresses and an interplay among DNA methylation and transcript accumulation may partly modulate the contrasting responses to these stresses.

1. Introduction

Sesame (*Sesamum indicum* L.) is a traditional oilseed crop widely grown in tropical areas [1]. Over the past few years, growing attention has been paid to the crop because of the discovery of the health-promoting effects of its oil [2]. On the other hand, as a hardy crop able to survive in extreme climatic conditions, sesame production provides an opportunity to valorize marginal lands and represents an important source of income for small-scale farmers in developing countries. Globally, sesame production is increasing and the growing area is markedly expanding. Nonetheless, in the different growing regions, sesame has a very weak productivity and low yield, mainly due to the negative effects of abiotic stresses. Therefore, understanding the mechanisms of abiotic stress tolerance for improvement towards higher productivity and yield has become a hot topic in current sesame research [3]. Two principal abiotic stresses including drought and waterlogging affect sesame productivity [4]. Drought stress is mainly significant in the arid and semi-arid areas of Africa, America and Asia, but, in south and East Asia, waterlogging represents the major threat for the sesame production. Sesame is typically considered a drought tolerant crop [5]. However, intense and prolonged drought stress limits sesame plant growth, impairs flower production, reduces the formation of capsule and seed and ultimately, affects seed yield [6–9]. When it occurs at the seedling stage, prolonged drought stress can result in increased plant mortality. Conversely, sesame is highly susceptible to waterlogging stress [10]. While the crop can sustain drought stress for

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several days, few hours of flooding leads to catastrophic plant mortality and yield loss even in the case of waterlogging-tolerant varieties. The contrasting responses to drought and waterlogging suggest the existence of different underlying molecular mechanisms in sesame. It is speculated that being cultivated for millennia in drought-prone environments, sesame may have been genetically shaped with a sophisticated and heritable molecular mechanism for drought adaptation.

One of the molecular mechanisms worth investigating relates to environmentally induced epigenetic modifications. In fact, methylation of the DNA is the most stable and heritable epigenetic modifications which has been associated with regulation of gene expression and response to environmental stresses in plants [11–13]. It results in the covalent addition of a methyl group to the fifth position of the aromatic ring in cytosine. The cytosine base may be methylated by DNA methyltransferases (DNMTs) [14], or demethylated by REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), and DEMETER like-proteins (DME 2,-3) [15] in conjunction with environmental and developmental cues [16] (Baulcombe and Dean, 2014). In plants, DNA methylation occurs commonly within three sequence contexts: CG, CHG and CHH (where H is A, C, or T); however, it varies depending on the level and pattern found within different genomic regions [17]. Among these three cytosine contexts, CpG dinucleotides are typically clustered around the regulatory region of a gene, especially in the promoter and first exon, which can impact its transcriptional regulation [18].

Under stress, DNA methylation patterns depend on the plant species, the tissues and the specific type of stress. For example, total methylation increases under salt stress in alfafa [19] but decreases in salt-sensitive rapeseed [20]. It has been reported that drought stress induces both demethylation and *de novo* methylation of DNA throughout the genome of barley [21] and ryegrass [22]. Under drought treatment, a decrease of DNA methylation was observed in leaf tissues of two faba bean genotypes [23]. Moreover, Bednarek et al. [24] observed an elevated demethylation in both non-tolerant and tolerant plants, with *de novo* methylation occurring less frequently than demethylation under Aluminum stress in triticale lines.

The methylation-sensitive amplified polymorphism (MSAP) technique developed by Reyna-López et al. [25] is an adaptation of amplified fragment length polymorphism analysis and has proven to be a powerful tool for analyzing DNA methylation. The MSAP technique has been applied to study CpG methylation in the genome of plants, somaclonal epigenetic variation, cytosine methylation during various developmental stages and resistance to biotic and abiotic stresses [22–24,26–31]. Different approaches have been proposed to interpret MSAP outputs [32,33], but none takes into account the multiple events reflecting various methylation patterns that must take place simultaneously to explain the differences in individual digestion patterns between control and stressed materials. Recently, Bednarek et al. [24] introduced an efficient theoretical model for the quantification of cytosine methylation patterns at restriction sites of the isoschizomers *Hpa*II and *Msp*I which evaluates demethylation, *de novo* methylation, and preservation of methylation status between control and stressed samples.

While the contribution of DNA methylation to plant performance under abiotic stress has been well studied in other major crops, no study has been done in sesame. Previously, our group sequenced the whole transcriptome under drought [34,35] and waterlogging [4] at different time points. These data represent an important resource to get insight into the interplay among DNA methylation and transcript level regulation in sesame. The main objective of the present investigation is to test the hypothesis that drought and waterlogging stress induce divergent DNA methylation patterns as part of the mechanisms leading to the contrasting responses of sesame.

2. Materials and methods

2.1. Plant materials and stress treatment

Two genotypes of sesame (*Sesamum indicum* L.) were obtained from the China National Genebank, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences and used in this experiment. The genotype ZZM0635 displays a tolerance to drought stress [34,35] while the genotype Zhongzhi No.13 is relatively waterlogging tolerant [4]. The seeds were sown in pots (25 cm across and 30 cm deep) containing 6 kg of loam soil mixed with 10% vermiculite. The experiment was conducted under shelter in natural conditions with the mean temperature of 31/27 °C day/night. A completely randomized blocking design with 4 replicates was employed and plants were watered normally under the optimum soil volumetric water content (vwc) of 35%. The soil moisture was measured manually in each pot using a Moisture Meter *Takeme* over the entire experiment. For the drought experiment, the genotype ZZM0635 was used. At the flowering stage, the irrigation was suspended for 11 days (DS) in 1/3 of the pots with the soil volumetric water content falling from 35% to 6%. The plants displayed heavy wilting signs, thereafter, they were allowed to recover for 4 days (DR) by re-supplying irrigation to reach the optimum soil volumetric water content (35%) according to the experimental descriptions of Dossa et al. [35] (Fig. 1). For the waterlogging application, the genotype Zhongzhi No.13 was used. 1/3 of the pots were flooded by standing in a plastic bucket filled with tap water to 3 cm above the soil surface and maintained for 9 h (WS) according to the experimental descriptions of Wang et al. [4]. Under stress, plants showed moderate wilting signs as presented in Fig. 1. Then, water was drained from the pots to allow the plants to recover for 20 h (WR). Meanwhile, the control plants (D-CK and W-CK) were kept under normal irrigation condition (35% vwc) throughout the whole experiment. The roots of 3 stressed plants were harvested individually under stress application and during recovery and those of the control plants were sampled at the same periods.

2.2. DNA extraction and MSAP epigenotyping

DNA was extracted from the root samples using the hexadecyltrimethylammonium bromide CTAB method [36]. The quality of the DNAs was checked on 1% agarose gel and the quantity was evaluated on the ultraviolet spectrophotometer. Aliquots were diluted to the final concentration of 300 ng.μl^{-1}. The extracted DNAs were subjected to a Methylation-Sensitive Amplified Polymorphism (MSAP) analysis following the method of Xiong et al. [37] with slight modifications. The technique is based on the use of the isoschizomers *Hpa*II and *Msp*I that differ in their sensitivity to methylation of their recognition sequences. Both enzymes recognize the tetranucleotide sequence 5'-CCGG-3', but their action is affected by the methylation state of the external or internal cytosine residues. 2 µL of each DNA sample (300 ng) was digested with 0.5 µL *Eco*RI-10 U with 13.5 µL deionized H_2O and 4 µL 10× Tango buffer (Thermo, USA) at 37 °C for 2 h, before deactivation by heating at 65 °C for 20 min. Then, the digested DNA fragments were subjected to *Hpa*II-10 U and M*sp*I-10 U digestion into two separated series at 37 °C overnight. The restriction enzymes were deactivated by heating at 80 °C for 15 min. The ligation was performed in a final volume of 10 µL including 5 µL enzyme-digested products, 1 µL of 5 pmol *Eco*RI adapter (Table S1), 1 µL of 50 pmol *Hpa*II/*Msp*I adapter (Table S1), 3 µL deionized H_2O, 1 µL 10 × T4 ligase buffer (Promega, USA) and 0.5 µL T4 DNA ligase (5U.μL^{-1}) and 1 µL ATP incubated at 37 °C overnight.

Pre-selective amplification was performed in a 20 µL reaction volume with 0.5 µl *Eco*RI primer (10 mM) and 0.5 µl *Msp*I/*Hpa*II primer (10 mM), 2.5 µL restriction-ligation DNA, 8 µL of 2× Reaction Mix (Tiangen Biotech, Beijing, China) supplied together with the dNTPs and $MgCl_2$, 8 µL deionized H_2O and 0.5 µL of 1 U Taq polymerase. The pre-

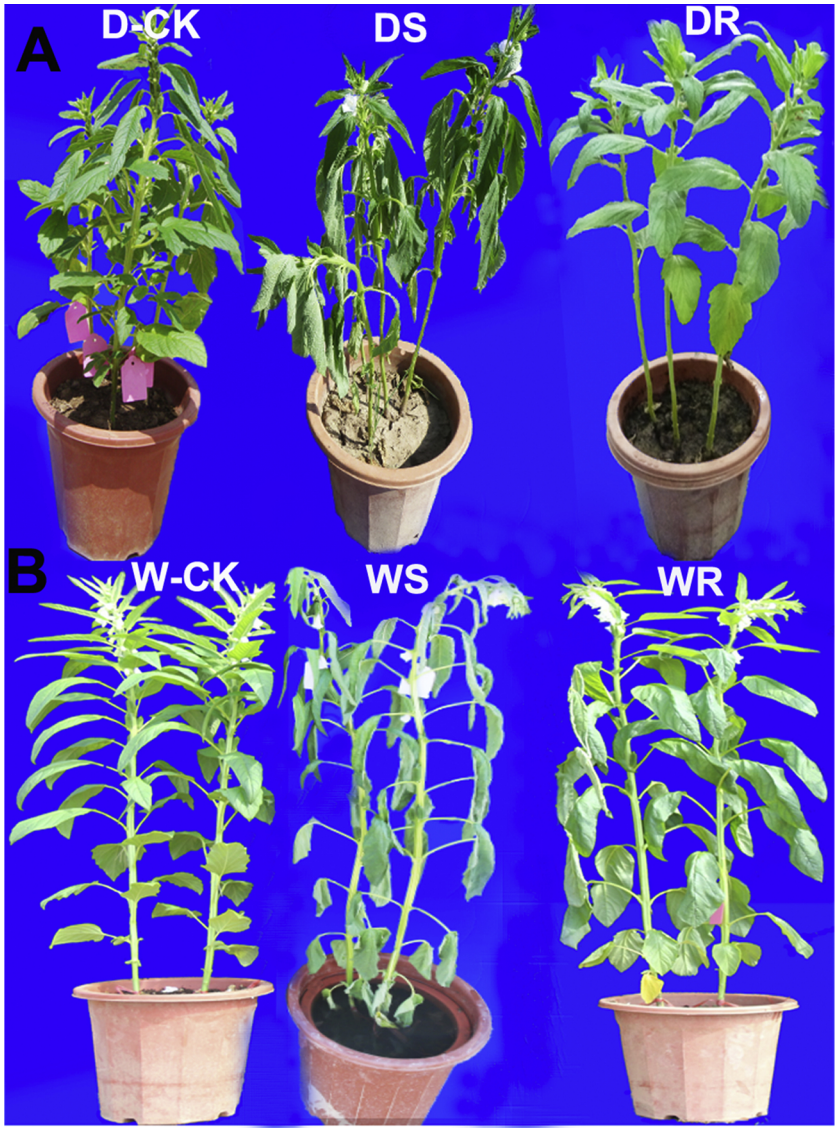

Fig. 1. Phenotype characteristics of the sesame genotypes under control and stress conditions. A. Drought tolerant ZZM0635 under control (D−CK), drought stress (DS) and drought recovery (DR); B. waterlogging tolerant Zhongzhi No.13 under control (W−CK), waterlogging stress (WS) and waterlogging recovery (WR).

selective amplification was conducted with the following temperature cycling conditions: 1 cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 15 s, and finally one cycle at 72 °C for 7 min prior to 30 min incubation at 60 °C.

Selective amplification was conducted in a 20 μL volume including 3 μL of 10-fold diluted pre-amplified PCR products, 8 μL of 2× Reaction Mix (Tiangen Biotech, Beijing, China) supplied together with the dNTPs and $MgCl_2$, 0.5 μL of primers (10 mM), 7 μL deionized H_2O and 1 μL of 1 U Taq polymerase. A total of 60 primer combinations were tested on 4 DNA samples and 25 primer combinations (E_n/HM_n) displaying clear PCR profiles were finally retained for MSAP epigenotyping (Table S1). The PCR amplification reactions were performed using touch-down cycles under the following conditions: 94 °C for 5 min; 13 touch-down cycles of 94 °C for 30 s, 65 °C (subsequently reduced each cycle by 0.7 °C) for 30 s and 72 °C for 30 s; 23 continued cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 15 s, and finally extension at 72 °C for 7 min. The PCR products of selective amplifications were separated using 6% polyacrylamide gel electrophoresis at 50 W for 1 h 30 min. Gels were silver stained using the method described by Benbouza et al. [38]. MSAP analysis was performed twice for each primer combination.

2.3. Data scoring and analysis of MSAP profiles

The MSAP profiles showing reproducible results between replicates were transformed into a binary matrix, with 1 as a presence of band and 0 the absence of band from either *EcoR*I/*Msp*I or *EcoR*I/*Hpa*II digestion and analyzed according to the protocol developed by Bednarek et al. [24]. In general, these fragments could be divided into four types representing four types of DNA methylation status of the restriction sites (5′-CCGG-3′): unmethylated (Type I, presence of the band in both enzyme combinations), hemi-methylated at the outer cytosine in one DNA strand (Type II, presence of the band only in digestion with *EcoR*I/*Hpa*II), fully-methylated at the internal cytosine in both DNA strands (Type III, presence of the band only in digestion with *EcoR*I/*Msp*I), and hyper-methylated with outer methylation at both DNA strands (Type IV, absence of band in both enzyme combinations) [33]. Only 100-bp or longer PCR products were considered for analysis. Also, two main treatment comparisons were done in this study: Stress vs Control and Recovery vs Stress so as to understand epigenetic changes during stress and recovery, respectively. The resultant code of the binary matrix, expressed as 4 binary digits, describes the presence/absence of each fragment in the *EcoR*I/*Hpa*II and *EcoR*I/*Msp*I digests of DNA from 2 compared treatments. Theoretically, 16 permutations are possible and

Table 1
Global DNA methylation patterns in root of sesame genotypes under control (CK), stress (DS and WS) and recovery (DR and WR) treatments.

MSAP band type	ZZM0635 (CK)	ZZM0635 (DS)	ZZM0635(DR)	Zhongzhi No.13 (CK)	Zhongzhi No.13 (WS)	Zhongzhi No.13 (WR)
I	266	202	278	248	274	258
II	105	68	66	83	64	165
III	172	47	92	140	181	60
IV	16	242	123	62	14	50
Total amplified bands	559	559	559	533	533	533
Total methylated bands	293	357	281	285	259	275
MSAP (%)	52.42	63.86	50.27	53.47	48.59	51.59
Fully-methylated (%)	33.63	51.70	38.46	37.90	36.59	20.64
Hemi-methylated (%)	18.79	12.16	11.81	15.57	12.00	30.95

Type II = hemi methylated bands and types III + IV are full methylated bands. Total methylated bands = II + III + IV.

could be classified into demethylation (DM), *de novo* methylation (DNM), preservation of methylated sites (MSP), and preservation of non-methylated sites (NMSP) which are the 4 events that were investigated simultaneously in this study. We assume that these events are equally probable, then, the multiplication of the number of individual events participating in the explanation of a given 4-digit code (Table S2) by the number of MSAP profiles depicted by that code, followed by summation of events of the same kind and normalization of the data (expressed as percentages), correspond to the relative quantitative characteristics of these 4 events [24].

2.4. Sequencing of polymorphic MSAP fragments

The polymorphic MSAP fragments are relative to demethylation (DM) and *de novo* methylation (DNM). A total of 80 polymorphic bands (42 drought and 38 waterlogging MSAP bands) were excised from fresh gel and transferred into 0.6 mL tube. The excised gels were crushed and dissolved in 50 μL deionized H_2O prior incubation at 50 °C overnight. These bands were re-amplified with the appropriate selective primer combinations. Sizes of the PCR products were checked by agarose gel electrophoresis and 57 positive PCR products were sent for sequencing at Tsingke (www.tsingke.net). Homology analysis with the reference sesame genome sequence was performed via the BLASTn search program with a cut off E-value $\leq 1 \times 10^{-40}$ on Sinbase (http://ocri-genomics.org/Sinbase/index.html) [39].

2.5. RNA extraction and qRT-PCR analysis

Total RNAs from root samples were extracted with the Easy Spin RNA kit (Aidlab, Beijing, China) following descriptions by Dossa et al. [35]. The quantity and quality of RNA samples were assessed by 1% agarose gel electrophoresis and on the ultraviolet spectrophotometer measurement of the A260/A280 ratio. For cDNA synthesis, 1.5 μg of RNA was reverse transcribed using the Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. We designed primers to amplify the differentially methylated genes using the Primer Premier 5.0 software [40] (Table S3). The qRT-PCR was conducted on a Roche Lightcyler® 480 instrument using the SYBR Green Master Mix (Vazyme), according to the manufacturer's protocol. Each reaction was performed using a 20 μL mixture containing 10 μL of 2× ChamQ SYBR qPCR Master Mix, 6 μL of nuclease-free water, 1 μL of each primer (10 mM), and 2 μL of 4-fold diluted cDNA. All of the reactions were run in 96-well plates and each cDNA was analyzed in triplicate. The following cycling profile was used: 95 °C for 30 s, followed by 40 cycles of 95 °C/10 s, 60 °C/30 s. Each reaction was conducted in biological triplicates and the sesame *Histone H3.3* gene (*SIN_1004293*) was used as the endogenous control gene [41]. The transcript level of the *Histone H3.3* gene was very stable in both genotypes under the normal and the stressful conditions. The method developed by Livak and Schmittgen [42] was employed for the data analysis.

2.6. RNA-seq data analysis

Raw data of drought and waterlogging RNAseq experiments were retrieved from GeneBank Short Read Archive (SRA) with the accession numbers SAMN06130606 and SRR2886790, respectively. The sequencing reads containing low-quality were cleaned with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The clean reads were mapped to the sesame reference genome v2.0 [43] using HISAT2 [44]; the program StringTie [45] was used for transcript assembly. The Cufflinks 2.0 software [46] was used to calculate the transcripts accumulation level for each sample expressed as fragments per kilobase of transcript per million fragments mapped (FPKM). The differentially accumulated trancripts (DATs) were detected as described by Tarazona et al. [47] based on the parameters: Fold change > = 2.00 and Probability > = 0.8. The DATS were identified by comparing: DSvsCK, DRvsDS for drought stress and WSvsCK, WRvsWS for waterlogging stress. The $\log_2$ transformed FPKM values were used to construct heatmap using the MEV software [48].

3. Results

3.1. Global DNA methylation levels under control, drought, waterlogging and recovery treatments in sesame

Cytosine methylation patterns in the root of drought-tolerant and waterlogging sesame genotypes under control (CK), stress (DS, WS) and recovery (DR, WR) conditions were assessed using 25 primer combinations (Table S1). Similar numbers of clear and reproducible bands were successfully revealed in drought (559) and waterlogging (533) conditions. Furthermore, most of the CCGG sites were shown to be largely methylated with the values ranging between 63.86% and 48.59% (Table 1). In the control treatment, we observed slight variations in the numbers of methylated sites between the drought- and waterlogging-tolerant genotypes. However, in the stress treatment, drought strikingly increased the methylation level while it was decreased under waterlogging stress when levels of DNA methylation were compared with those in the respective unstressed control. At the recovery stage, the levels of methylation tended to reach those observed under control conditions. Further analyses showed that fully-methylated bands were more predominant that the hemi-methylated ones. However at WR, the percent of fully-methylated bands tended to decrease as compared to the control. The opposite is observed for the hemi-methylated ones.

3.2. DNA methylation alteration under stress and recovery treatments

To evaluate the impact of stress and recovery treatments on DNA methylation in sesame, the differentially methylated DNA bands between DSvsCK, DSvsDR, WSvsCK and WRvsWS were classified into 4 events including demethylation (DM), *de novo* methylation (DNM), preservation of methylated sites (MSP), and preservation of non-

Table 2
Profiles reflecting given MSAP 4-bit binary code evaluated among compared treatments.

4-bit Code/events	CKvsDS	DSvsDR	CKvsWS	WSvsWR
0001	25	80	81	104
0010	46	51	40	25
0011	25	42	33	13
0100	147	32	90	63
0101	13	4	18	29
0110	7	5	9	4
0111	5	7	13	2
1000	87	50	2	39
1001	4	4	10	12
1010	11	7	16	10
1011	3	6	13	9
1100	48	12	1	33
1101	5	4	2	9
1110	4	3	1	14
1111	9	23	10	13
Total	439	330	390	379

Table 3
Summary of the relative quantification of the DNA methylation patterns evaluated based on MSAP data.

Cases (events)	DSvsCK	DRvsDS	WSvsCK	WRvsWS
DM	1060 (22.03)	1326 (39.29)	1474 (35.00)	1383 (31.40)
DNM	2020 (41.97)	804 (23.82)	1140 (27.07)	1336 (30.32)
MSP	858 (17.83)	565 (16.74)	753 (17.88)	784 (17.80)
NMSP	874 (18.17)	680 (20.15)	845 (20.05)	902 (20.48)
DNM-CHG	1488 (30.92)	380 (11.26)	747 (17.74)	725 (16.46)
DNM-CG	532 (11.06)	424 (12.56)	393 (9.33)	611 (13.87)
DM-CHG	403 (8.37)	924 (27.38)	893 (21.20)	967 (21.95)
DM-CG	657 (13.65)	402 (11.91)	581 (13.79)	416 (9.44)
Total	4812	3375	4212	4405

Data in bracket are percentage. Demethylation (DM), *de novo* methylation (DNM), preservation of methylated sites (MSP), and preservation of non-methylated sites (NMSP).

methylated sites (NMSP) according to the method developed by Bednarek et al. [24]. A total of fifteen 4-bit codes representing the pattern changes at a given cytosine between compared treatments, were identified in this study (Table 2). In fact, the code "0000" was not taken into account because this pattern cannot be easily recognized. Interestingly, the most frequent codes in drought stress compared with the control condition were 0100, 1000 which were totally different from the predominant pattern changes from drought to recovery phase (0001 and 0010). Conversely, the most induced methylation alteration under waterlogging stress and the recovery phase are the cases corresponding to the codes 0100 and 0001 (Table 2). Table 3 presents the converted codes into events of the corresponding type (based on Table S2), and their relative quantification. The results indicated that the imposition of drought stress induced principally DNM (41.97%) and to a lesser extent DM (22.03%) in the sesame genotype. In addition, DNM at the CHG sites was the most frequent (30.92%) while DM affected predominantly the CG sites (13.65%). About 36% of loci were not affected by drought stress at the epigenetic level. From DS to DR, an opposite trend was observed. Most of loci were demethylated with the majority harboring a CHG site. Moreover, few sites underwent DNM and about 37% of loci remained unchanged. These results suggest a divergent and drastic reprogramming of the methylation pattern from drought stress to the recovery stage in sesame. In the case of waterlogging, DM occurred at a higher proportion than DNM under stress. However, at the recovery phase, both DNM and DM were proportionally activated as epigenetic responses. In the two treatments, DNM-CHG and DM-CHG were the most represented types of methylation and demethylation, respectively (Table 3). Similarly as in drought conditions, 37% of loci were not affected by waterlogging and recovery treatments.

3.3. Comparative analysis of DNA methylation patterns and alteration of the levels of transcript under stress and recovery treatments

The relationship between DNA methylation variation and alteration of the levels of transcript under drought and waterlogging stresses in sesame was assessed. Under drought stress, the analysis of the differentially accumulated transcripts (DATs) showed that the levels of 77% of DATs were decreased whereas at the recovery stage, more than the levels of 80% of DATs were increased (Fig. 2a, b). These patterns of transcripts level alteration correlate well with the DNA methylation changes (Fig. 2c). It implies that the high DNM observed under drought stress may induce a decreased of the level of a large number of drought-responsive transcripts while the high DM observed at the recovery stage allowed for the resumption of drought-responsive transcript accumulation. An inverse trend was observed in the waterlogging treatments with the levels of 60% of DATs increased under stress while, the levels of comparable numbers of DATs were decreased and increased during the recovery stage (Fig. 2d,e). A similar reasoning about the correlation of the observed DNA methylation patterns and alteration of the levels of transcripts under waterlogging suggests that a high DM under stress was favorable for the up-accumulation of waterlogging-responsive transcripts in sesame. We further scrutinized the common DATs between DSvsCK and DRvsDS and found that 79% of DATs which levels decreased under stress were increased during the recovery from drought damage while only 20% of DATs experienced the opposite scenario (Fig. 3a). By analyzing the common DATs between WSvsCK and WRvsWS, we observed that 37% of common DATs which levels decreased under stress were up-accumulated during the recovery stage while 58% of the common DATs showed the opposite regulation pattern (Fig. 3b).

Moreover, we compared the DATs between waterlogging and drought under stress (WSvsDS) and at the recovery (WRvsDR) stages. We identified 1526 and 1739 common DATs for WSvsDS and WRvsDR, respectively. Within the common DATs for WSvsDS, the majority (1087) displayed contrasting regulation patterns with most of these transcripts up-accumulated in waterlogging stress while they were down-accumulated under drought stress (Fig. 3c). In regard to the common DATs at the recovery phases (WRvsDR), the majority was also conversely regulated between drought and waterlogging. In this case, however, most of these transcripts were down-accumulated during the waterlogging recovery whereas they were found up-accumulated during the drought recovery (Fig. 3d). Overall, these results indicate that drought and waterlogging induce a divergent transcript accumulation scenario in sesame which correlates with the DNA methylation patterns.

3.4. Analysis of polymorphic MSAP fragments and identification of differentially methylated genes

Out of the 57 sequenced polymorphic MSAP fragments, 44 bands including 24 for drought and 20 for waterlogging showed high similarities (97–100%) with the sesame genomic regions. The size of the excised bands ranged from 115 to 600 bp. The sequence analysis indicated that these successfully sequenced fragment termini have the CCGG site. Additionally, 17 fragments have one or more internal CCGG sites suggesting that the relative total methylation levels in sesame may be underestimated by the MSAP technique. Table 4 presents the methylation events (4-digit codes) of all the sequenced fragments and their associated genes. Interestingly, 40 differentially methylated genes (DMGs) were enlisted within the DATs from drought and waterlogging treatments. In addition, 5 fragments overlapped with gene coding sequences (CDS) while the remaining fragments were located in the promoter region (UTR_5) of the associated genes. Functional annotation of the DMGs demonstrated that various classes of genes are methylated in response to drought and waterlogging stresses in sesame. The transcript accumulation patterns of these DMGs were investigated using the

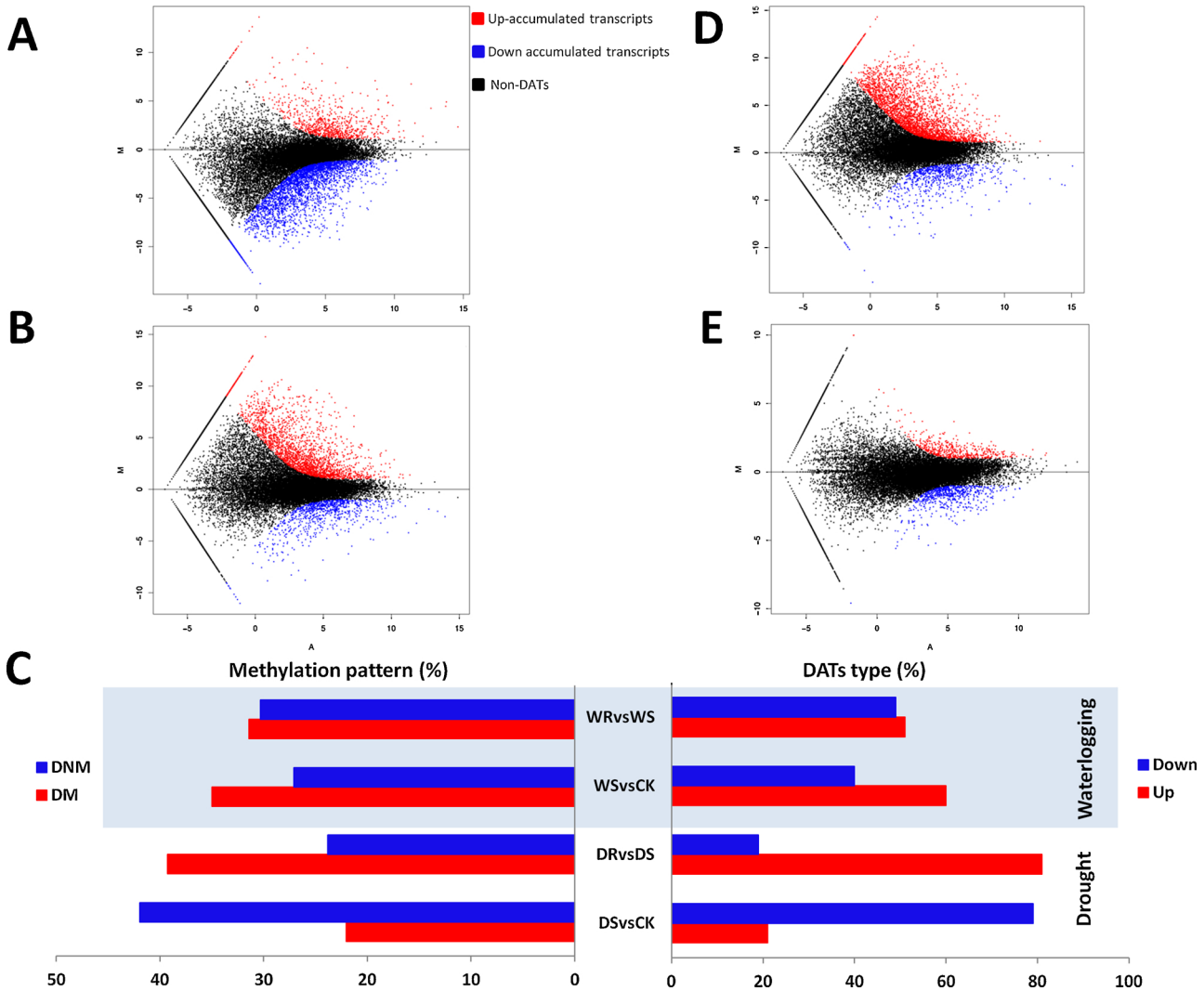

Fig. 2. Transcript level regulation under drought and waterlogging and its correlation with DNA methylation patterns. A. MA plot showing the differentially accumulated transcripts (DATs) between drought stress (DS) and the control (CK) treatments, B. MA plot showing the DEGs between drought recovery (DR) and drought stress (DS) treatments, C. Positive correlation between DNA methylation patterns (*de novo* methylation (DNM) and demethylation (DM)) with transcript level regulation (up-accumulated and down-accumulated) in waterlogging and drought conditions, D. MA plot showing the DATs between waterlogging stress (WS) and the control (CK) treatments, E. MA plot showing the DATs between waterlogging recovery (WR) and waterlogging stress (WS) treatments.

transcriptome data in control, stress and recovery conditions (Fig. 4). The transcript levels of all the DMGs changed from one treatment to another. In drought condition, the transcript levels of most of the DMGs decreased from the control to the stress treatment, subsequently increasing from the stress to the recovery treatment (Fig. 4a). Meanwhile, in waterlogging condition, the transcript levels of most the DMGs were mainly induced during stress and at the recovery stage (Fig. 4b). Importantly, for drought and waterlogging DMGs, the patterns of transcript accumulation matched well the methylation events represented by the 4-digit codes in Table 4. It is obvious that for most of the DMGs that experienced DNM, the levels of transcript accumulation were decreased while DM principally leads to the up-accumulation of the transcripts level. We selected 16 and 14 DMGs from drought and waterlogging conditions, respectively, to further validate the alteration in their transcript accumulation using qRT-PCR. As shown in Fig. 4c,d, the qRT-PCR results corroborated well the transcriptome quantification of the transcript accumulation of these DMGs. These results demonstrate that the MSAP technique is an efficient approach to isolate stress-responsive genes. Altogether, we highlighted an intimate correlation between DNA methylation patterns and changes in transcript accumulation under drought and waterlogging in sesame.

4. Discussion

As sessile organisms, plants respond to abiotic stresses by adjusting their physiological and developmental machinery through differentially regulated gene expression [49]. Mechanisms such as DNA methylation and demethylation of cytosine have been demonstrated to play a key role in this adjustment [50]. In the present study, cytosine methylation analysis of sesame (~350 Mb) using the MSAP approach revealed that the level of methylation in the normal growth condition of two different genotypes was similar (52–53%). This level of methylation is close to those of ryegrass (57%), rapessed (46%), faba bean (41%) but obviously higher than those of rice (31%), *Arabidopsis thaliana* (30%), maize (35%) [22,51–55]. Using the MSAP sequencing technique, Pan et al. [56] reported that the overall level of DNA methylation was about 70% in *Triticum aestivum*. Hence, these observations suggested that DNA methylation is a direct function of the plant species, particularly, the genome size and structure.

Under stress, significant alteration of DNA methylation was observed in waterlogging and drought conditions. Importantly, drought stress increased the global methylation level through an active *de novo* methylation while under waterlogging stress, the opposite scenario was

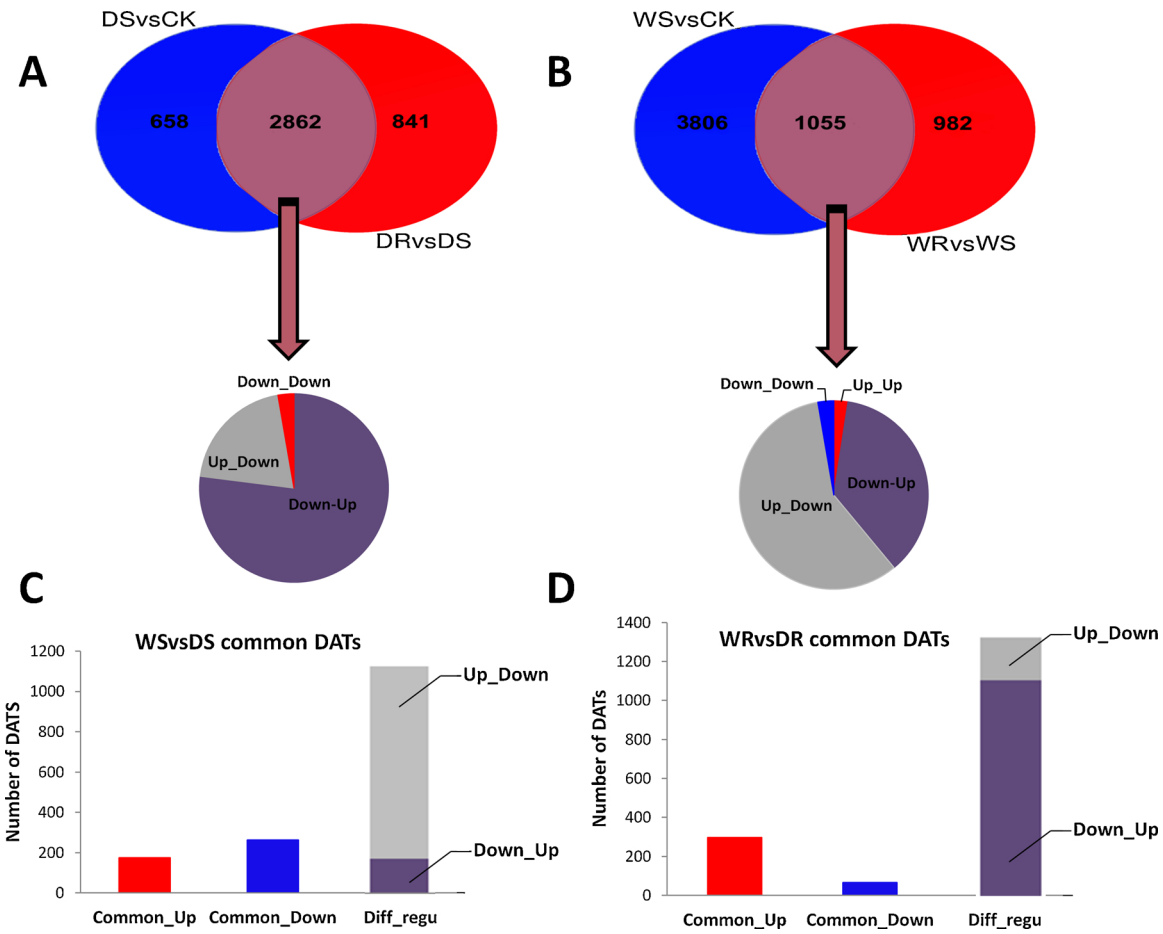

Fig. 3. Regulation patterns of the common differentially accumulated transcripts (DATs) between treatments and stresses. A. Shared and unique DATs between drought stress (DSvsCK) and recovery (DRvsDS) and change in regulation status of the shared DATs. Down_Down relates to DATs constitutively down-regulated under drought stress and at the recovery phase; Up_Down relates to DATs which were up-accumulated under drought stress and down-accumulated during the recovery phase; Down_Up relates to DATs which were down-accumulated under drought stress and up-accumulated during the recovery phase. C. Comparison of DATs between waterlogging and drought under stress (WSvsDS). Down_Down relates to DATs constitutively down-accumulated under drought stress and waterlogging stress; Up_Up relates to DATs constitutively up-accumulated under drought stress and waterlogging stress; diff_regu relates to DATs that displayed contrasting regulation status between drought and waterlogging under stress. D. Comparison of DATs between waterlogging and drought at the recovery stage (WRvsDR). Down_Down relates to DATs constitutively down- accumulated under drought recovery and waterlogging recovery; Up_Up relates to DATs constitutively up- accumulated under drought recovery and waterlogging recovery; diff_regu relates to DATs that displayed contrasting regulation status between drought and waterlogging during recovery.

observed. Drought stress has been investigated in various plants species at the epigenetic level. Our report is in agreement with the study of Labra et al. [57] who showed a hypermethylation in pea root tip under water deficit. In contrast to our results, drought has been reported to decrease the level of total DNA methylation by 12.1% and 10.28% in rice and ryegrass, respectively [22,58]. Similarly, a quantification of the genome wide cytosine methylation polymorphism using the MSAP analysis showed a predominant hypomethylation in a tolerant rice accession under drought stress [59]. Furthermore, Abid et al. [23] recently noticed that drought stress reduces the methylation level in two faba bean genotypes, irrespective to their tolerance level. These contrasting reports imply that different methylation mechanisms for drought tolerance exist in plant species. Similar conclusions were drawn from the epigenetic responses of clover and hemp which decreased the methylation level under chromium stress but an active *de novo* synthesis of methylated cytosine was found to positively correlate with the intensity of the stress in the rape genome [60,61]. Nonetheless, we deduced that hypermethylation under drought stress is a tolerance strategy in sesame and it would be valuable to compare in a future experiment the epigenetic alterations of (1) several sesame genotypes with contrasting levels of drought tolerance to understand the intra-species variation [23], (2) various plant species exhibiting contrasting responses to drought to uncover the inter-species variation [60].

Various abiotic stress have been investigated using MSAP markers including cold, drought, salt, aluminum stresses, however, waterlogging stress has been scarcely studied. Here, we showed that waterlogging stress in contrast to drought decreased the global methylation level in sesame. As a typical waterlogging-susceptible and drought-tolerant crop, we were expecting a contrasting epigenetic response of sesame to these abiotic stresses, hypothesis which has been confirmed in the present study. Is it possible that mimicking the effective drought epigenetic response by imposing a strong directed *de novo* DNA methylation during waterlogging stress will lead to stress tolerance? Unfortunately the mechanism underpinning the contrasting DNA methylation alteration under drought and waterlogging in sesame remains unclear. We suspected that DNA methyltransferases and DNA glucolsylase demethylase genes may be the master players in this mechanism [62]. In this case, the identification and manipulation of the key enzymes that control the level of methylation may assist in efforts to develop stress-tolerant sesame plants.

In nature, environmental stresses are rarely permanent and the ability of plants to fully recover after stress relief is an important component of stress resistance mechanisms [63]. Unfortunately, many studies overlooked the methylation patterns during the recovery phase.

Table 4
Blast results and methylation patterns of randomly selected polymorphic MSAP fragments.

Fragment code	Primer	Corresponding gene	Nucleotide Identity (%)	Methylated region	Gene annotation	CKvsDS	DSvsDR	CKvsWS	WSvsWR
D1	E3/HM2	SIN_1004951	100	UTR_5	Rho protein GDP-dissociation inhibitor	0100			
D2	E3/HM3	SIN_1009148	99	UTR_5	uncharacterized protein	0100			
D3	E5/HM3	SIN_1003402	98	UTR_5	uncharacterized protein		0010		
D4	E2/HM5	SIN_1005766	100	UTR_5	uncharacterized protein	1000			
D5	E2/HM5	SIN_1005766	100	UTR_5	uncharacterized protein		0010		
D6	E4/HM3	SIN_1004132	100	CDS	uncharacterized protein	0100			
D7	E4/HM5	SIN_1023920	100	CDS	protein ECERIFERUM 3-like	1100			
D8	E1/HM5	SIN_1026612	99	UTR_5	multidrug resistance, MATE	0010			
D9	E2/HM3	SIN_1012446	100	UTR_5	EID1-like F-box		0001		
D10	E3/HM5	SIN_1025394	100	UTR_5	uncharacterized protein	1000			
D11	E2/HM1	SIN_1006002	100	UTR_5	mini zinc finger	0100			
D12	E2/HM5	SIN_1018961	100	UTR_5	chalcone synthase		0001		
D13	E1/HM4	SIN_1011169	100	UTR_5	uncharacterized protein	1000			
D14	E2/HM3	SIN_1024581	100	UTR_5	uncharacterized protein	1000			
D15	E5/HM2	SIN_1015755	100	UTR_5	uncharacterized protein		0010		
D16	E5/HM5	SIN_1025937	98	UTR_5	miraculin-like		0010		
D17	E4/HM3	SIN_1003652	99	UTR_5	leucine-rich repeat extensin	0100			
D18	E1/HM1	SIN_1006002	99	UTR_5	mini zinc finger		1000		
D19	E3/HM2	SIN_1011228	100	CDS	uncharacterized protein	1001			
D20	E5/HM4	SIN_1021189	98	UTR_5	ENHANCER OF AG-4		0100		
D21	E1/HM4	SIN_1010035	97	UTR_5	uncharacterized protein		0001		
D22	E3/HM5	SIN_1016502	100	UTR_5	uncharacterized protein	1001			
D23	E5/HM2	SIN_1005077	100	UTR_5	gibberellin 3-beta-dioxygenase	0100			
D24	E5/HM3	SIN_1018472	100	UTR_5	proteinase inhibitor-like	0100			
D25	E/HM	SIN_1000140	100	UTR_5	protein DEK-like		1000		
W1	E2/HM2	SIN_1026309	100	UTR_5	PGR5, chloroplastic				0001
W2	E1/HM5	SIN_1026612	98	UTR_5	protein TRANSPARENT TESTA				1100
W3	E5/HM3	SIN_1010093	100	CDS	pentatricopeptide repeat-containing			0010	
W4	E4/HM2	SIN_1014786	100	UTR_5	Organ specific protein			0011	
W5	E4/HM5	SIN_1021358	100	UTR_5	chaperone protein dnaJ 6			0011	
W6	E3/HM1	SIN_1012764	99	UTR_5	exopolygalacturonase clone GBGE184				1101
W7	E2/HM2	SIN_1023017	100	UTR_5	early nodulin-93			0110	
W8	E1/HM4	SIN_1021693	100	UTR_5	protein NUCLEAR FUSION DEFECTIVE 4			0001	
W9	E3/HM1	SIN_1001866	100	CDS	putative auxin efflux carrier component 8			0010	
W10	E4/HM1	SIN_1024058	100	UTR_5	GDSL esterase/lipase				0110
W11	E1/HM3	SIN_1004028	99	UTR_5	pectate lyase				0010
W12	E3/HM2	SIN_1015574	99	UTR_5	heat stress transcription factor B-2b			1000	
W13	E5/HM5	SIN_1017332	100	UTR_5	laccase-1			1000	
W14	E3/HM4	SIN_1022082	100	UTR_5	purple acid phosphatase 7 isoform X1			0011	
W15	E1/HM2	SIN_1023017	100	UTR_5	early nodulin-93				0110
W16	E2/HM5	SIN_1004928	100	UTR_5	uncharacterized protein			0110	
W17	E3/HM3	SIN_1012088	100	UTR_5	uncharacterized protein			0110	
W18	E4/HM1	SIN_1024768	100	UTR_5	acidic endochitinase			0100	
W19	E5/HM2	SIN_1026139	100	UTR_5	pathogenesis-related leaf protein 6				0110
W20	E3/HM5	SIN_1015706	100	UTR_5	blue copper protein			0110	

D1-D24 and W1-W20 represent the fragments from drought and waterlogging, respectively.

In this study, our data demonstrated a difference in the epigenetic behavior as a result of the recovery from drought and waterlogging damages. We showed that after recovery from drought, a reversion of the methylated sites took place and tended to reach the level under a stress-free condition. Similar to our observations, it was found that about 70% of the sites which exhibited drought-induced epigenetic methylation were demethylated when rice plants were allowed to recover [58]. Hence, sesame plants were able to promptly recover an optimal physiological status after stress relief. However, during the waterlogging recovery phase, both demethylation and *de novo* methylation concomitantly occur in the sesame root. We inferred from that observation that waterlogging stress causes extensive damages which require prolonged physiological and morpho-anatomical adjustments to recover as compared to the drought recovery. Altogether, our MSAP analysis indicated that sesame has divergent epigenetic programs to respond to drought and waterlogging, which may explain its contrasting response to these major abiotic stresses.

DNA methylation and demethylation have been proven to be associated with transcript acumulation leading to adaptive physiological and morphological responses to abiotic stress [18,64]. Here, our comparative analysis of MSAP profiles and transcriptome data revealed a strong correlation between DNA methylation pattern and the regulation of the responsive-transcripts to drought and waterlogging in sesame. In particular, we deduced that *de novo* DNA methylation participates in the down-accumulation of transcripts while DNA demethylation results in the up-accumulation of trancripts. In addition, 90% of the sequenced polymorphic MSAP fragments corresponded to significantly and differentially accumulated transcripts (DATs) between treatments. Also, they were predominantly located in the promoter region of the protein-coding genes, which implies that DNA methylation and demethylation lead to the activation and inactivation of the transcriptional processes for specific genes related to drought and waterlogging response in sesame. In accordance with our results, Meng et al. [65] by combining transcriptome and DNA methylation data from *Arabidopsis thaliana* concluded to a significant epigenetic contribution to gene expression regulation. Moreover, similar conclusions were drawn by Vining et al. [64] who reported a negative correlation between methylation and transcription of gene at the genome wide level in *Populus trichocarpa*.

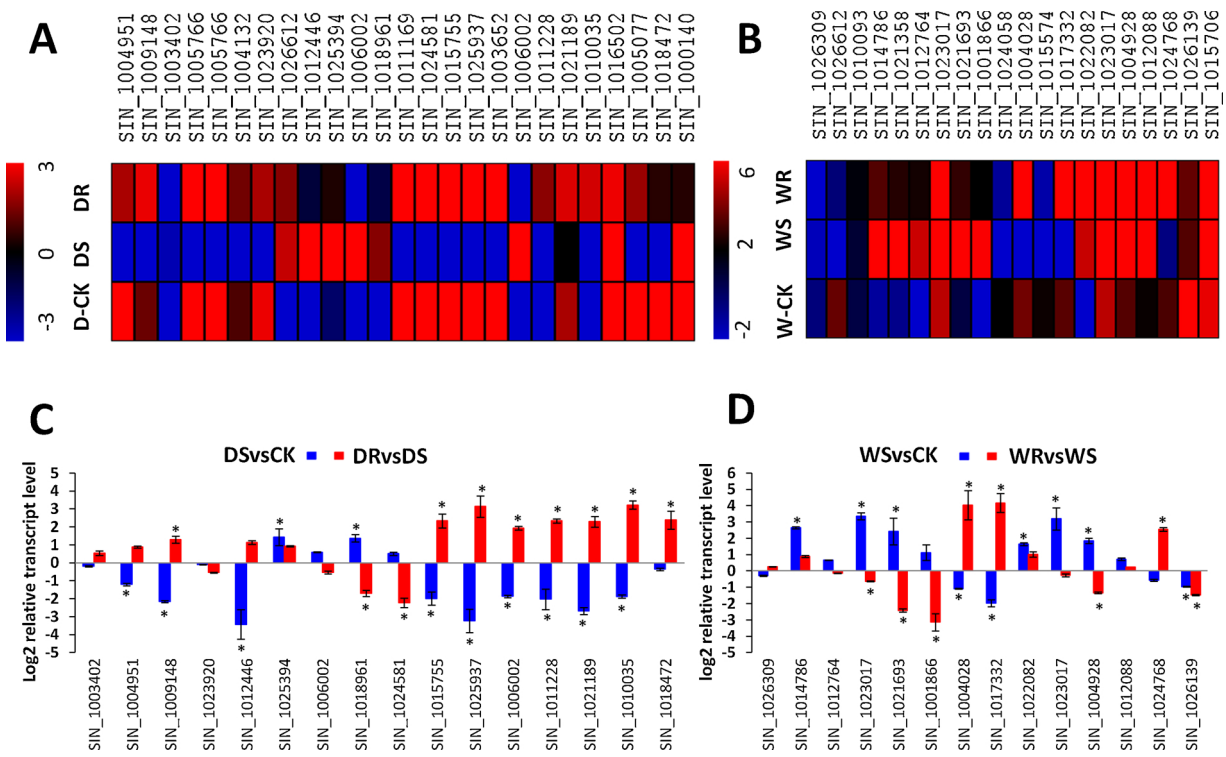

Fig. 4. Transcript level analysis of the differentially methylated genes. A. a heatmap displaying the transcript levels of 24 differentially methylated genes under control (D−CK), drought stress (DS) and drought recovery (DR) based on RNAseq data. B. a heatmap displaying the transcript levels of 20 differentially methylated genes under control (W−CK), waterlogging stress (WS) and waterlogging recovery (WR) based on RNAseq data. The blue color depicts the weakly accumulated transcripts while the red color depicts the highly accumulated transcripts. C,D. qRT-PCR validation of transcript levels of 16 and 14 selected gene under drought and waterlogging conditions, respectively. The blue bars correspond to the relative transcript levels of the genes under stress compared with the control whereas the red bars correspond to the relative the transcript levels of the genes during the recovery phase compared with the stress. The sesame *Histone H3.3* gene (*SIN_1004293*) was used as the internal reference. The error bar indicates the standard error of the mean. The mean values issued from three independent biological replicates were analysed for significance using the statistical *t*-test (p value < 0.05). An asterisk (*) indicates a significant change in transcript level between the two compared groups of samples (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Another interesting finding in this study concerns the quasi contrasting regulation of the shared DATs between drought and waterlogging under stress and recovery stages. This important observation further shed light on the opposite molecular responses to drought and waterlogging treatments, which may be partly directed by divergent epigenetic factors such as DNA methylation.

5. Conclusion

For the first time, the epigenetic responses of sesame to drought and waterlogging were revealed using the MSAP approach. Drought stress increased the methylation level while waterlogging induced a high demethylation, indicating an opposite epigenetic responsive program. At the recovery stage, drought-stressed plants promptly readjusted the methylation level through a strong demethylation. However, waterlogging stressed-plants required a coordinated methylation and demethylation activity during the recovery. A high degree of correlation was also observed between DNA methylation pattern and level of transcript accumulation with a high methylation associated with decrease of transcript levels while a strong demethylation was correlated with the up-accumulation of transcript. In summary, drought and waterlogging stress induce divergent DNA methylation patterns which may influence transcript accumulation levels and lead to a contrasting response of sesame to these two abiotic stresses. Further investigations are required to better understand the key modulators of the DNA methylation in the sesame genome so as to modify epigenetic cascades and improve sesame productivity and yield under abiotic stress.

Author's contributions statement

Conception and design: KD, NC, DD, XZ; Production of the data: KD, MAM, QZ, MY, WL, RZ; Analysis and interpretation of the data: KD, LW, MAM; Drafting of the article: KD, MAM; Final approval of the article: KD, MAM, RZ, QZ, MY, NC, DD, LW, XZ.

Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

Ethical standards

The authors declare that the experiments comply with the current laws of the countries in which the experiments were performed.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.plantsci.2018.09.012.

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