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Analysis of DNA methylation patterns associated with drought stress response in faba bean (*Vicia faba* L.) using methylation-sensitive amplification polymorphism (MSAP)

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Highlights:

- Study the effect of methylation on drought stress tolerance in faba bean
- Use MSAP to characterize differentially methylated regions (DMRs)
- Understand the potential role of DNA methylation changes in gene regulation

ABSTRACT:

The effects of drought on water relations, gas exchanges and epigenetic alterations were studied in two faba bean (*Vicia faba* L.) genotypes with contrasting levels of drought tolerance. The drought-tolerant Bachar genotype was less affected by water deficit in comparison with the drought-sensitive F177 in terms of photosynthetic function and water status in plants as indicated by less reduction in net photosynthesis (A), transpiration rate (E), stomatal conductance (gs) and relative water content (RWC). In this study, the methylation-sensitive amplification polymorphism (MSAP) technique was used to profile the DNA methylation patterns of Bachar and F177 under drought and control conditions. Overall, the amount of methylation was higher in leaves than in roots and the contribution of fully methylated loci was always higher than that of hemimethylated loci. Under control and drought treatment, the total methylation level in leaf tissues was 37.43% and 30.62% in Bachar, 41.23% and 38.16% in F177, indicating a decrease of 6.81% and 3.07% in Bachar and F177 respectively, due to drought exposure. The results revealed that DNA methylation in root tissues was decreased by 3.63% (from 23.43% to 19.80%) in Bachar and increased by 0.66% (from 16.53% to 17.19%) in F177 under drought stress. Differentially displayed DNA fragments in MSAP profiles were cloned and sequenced. A sequence analysis identified six potentially drought stress-related differentially methylated regions (DMRs). Five of these have high homology to previously identified or putative proteins found in other plants, including lipoxygenase (*LOX*), calcium-dependent protein kinase (*CDPK*), ABC transporter family (*ABC*), glycosyl hydrolase (*GH*) and phosphoenolpyruvate carboxylase (*PEPC*) and were chosen for further characterization. Quantitative reverse transcription PCR analysis revealed that *VfLOX*, *VfCDPK*, *VfABC* and *VfGH* varied under drought stress; the expression level of these studied genes was higher in Bachar than in F177. This could suggest their possible role in faba bean drought stress tolerance. Overall, the genome-wide epigenetic changes are probably an important regulatory mechanism for faba bean response to drought and as well to other environmental stresses.

Keywords: DNA methylation · Drought stress · Faba bean · Gene expression · Methylation-sensitive amplification polymorphism (MSAP)

1. Introduction

Food legume crops play important roles in the sustainability of the crop production system and contribute to food security in developing countries. Faba bean (*Vicia faba* L.) is the most important food legume crop in Tunisia, followed by chickpea and pea: these three crops represent about 78%, 12% and 9% of the total grain legume area (66000 ha) respectively. Faba bean is widely used for human food and animal feed due to its high protein content as well as to its important role in soil fertility through symbiotic fixation of nitrogen. In comparison with the world average yield of faba bean (1.6 t/ha, FAOSTAT, 2011), the national average yield is low (1.38 t/ha for the small seed and 1.03 t/ha for the large seed) and characterized by significant annual fluctuation (Kharrat and Ouchari, 2011). The variability of the yield is mainly due to the susceptibility of most of the varieties grown by farmers to biotic and abiotic stress. Therefore, there is an urgent need to develop new improved faba bean varieties to overcome the constraints posed by climate change. In Tunisia, this crop is grown mainly in the northern part of the country, where the annual rainfall is above 400 mm. However, over the last decade drought has become the major abiotic factor limiting faba bean production in these regions, which are known for their irregular water distribution and moderate moisture levels. Moreover, Amede and Schubert (2003) found that faba bean is more sensitive to drought than other grain legume crops such as common bean, pea and chickpea.

Plant drought tolerance is a complex trait involving multiple metabolic pathways and is controlled by multiple genes with various functions and several transcription factors involved in the regulation of drought stress-inducible genes (Rabara et al., 2014). Several recent studies have highlighted the involvement of epigenetic modification (mechanisms that regulate gene expression without changing DNA nucleotide sequence) in the plant's biotic and abiotic stress response (Liu et al., 2015a). Various processes have been described as implicated in epigenetic gene regulation, including chromatin regulation mediated by histone modifications, DNA methylation and small RNAs or longer non-coding RNAs (Meyer, 2015). However, the alteration of DNA methylation was recently confirmed to be coordinated with changes in the expression of genes involved in abiotic responses to and tolerance of stress, such as drought in rice (Wang et al., 2011; Zheng et al., 2013; Zheng et al., 2014; Garg et al., 2015), salt in *Arabidopsis* (Bilichak et al., 2012) and cold in rice (Pan et al., 2011). Interestingly, a growing number of reports have demonstrated that numerous abiotic stresses can induce potential epigenetic modifications which could be stably transmitted to subsequent generations, and which may help plants to cope with environmental constraints (Yu et al., 2013). DNA methylation is one of the best-studied epigenetic mechanisms in plants. It has been suggested

that, unlike other epigenetic mechanisms, methylation may be responsible for the stable maintenance of a particular gene expression pattern through mitotic cell division (Law and Jacobsen, 2010). DNA methylation is a process by which methyl groups are added to the C5 position of the cytosine residues ring of DNA by DNA methyltransferases (DNMTs) to form 5-methylcytosines. According to Sanchez and Mackenzie (2016), plants display cytosine methylation in three sequence contexts: CG, CHG and CHH (where H = A, T or C).

It has been reported that drought stress induces change in the expression of drought response genes through hypo- or hypermethylation of DNA throughout the genome and at specific loci in several plant species such as barley (Chwialkowska et al., 2016) and ryegrass (Tang et al., 2014a). Differences in drought tolerance among rice cultivars have been revealed to be related to particular methylation levels. According to Gayacharan and Joel (2013), the drought-sensitive rice genotypes (IR 20 and CO 43) have a higher level of methylation than drought-tolerant rice genotypes (PL and PMK 3). On the other hand, in barley vegetative and reproductive tissues, drought stress-induced expression of the *HvDME* gene encoding a DNA glycosylase was correlated with an increase in methylation within the gene coding region (Kapazoglou et al., 2013).

Many studies of DNA methylation have been carried out via three main strategies: restriction enzymes-based strategies such as restriction landmark genome scanning (RLGS), affinity-based strategies such as methylated CpG island recovery assay (MIRA), and bisulfate-based strategies such as whole genome shotgun bisulfite sequencing (WGSBS) (Olkhov-Mitsel and Bapat, 2012). Methyl-sensitive amplification polymorphism (MSAP) is a powerful technique used to study the global DNA methylation status between individual plants based on digestion with methylation-sensitive restriction endonucleases such as *Hpa*II and its methylation-insensitive isoschizomer *Msp*I (Fulneček and Kovařík, 2014).

Up to now, no research on DNA methylation in faba bean subjected to abiotic or biotic stresses has been reported. The main objective of this study was to investigate the changes in DNA methylation levels occurring in two faba bean genotypes with contrasting drought tolerance in order to better understand the potential role of DNA methylation in gene regulation and drought tolerance.

2. Material and methods

2.1. Plant material and growth conditions

The experiment was carried out under controlled conditions at the Experimental Station of the Biotechnology Center at Borj Cedria (35 km south-east of Tunis), under natural light and at air temperatures regulated between 18°C and 25°C (night/day). Faba bean (*Vicia faba* L. var. *minor*) F177 and Bachar genotypes were used for all physiological and molecular experiments. F177 genotype beans (sensitive to drought stress) were received from the International Center for Agricultural Research in the Dry Areas (ICARDA, Aleppo, Syria), while the commercial cultivar Bachar (tolerant to drought stress) was largely cultivated in different grain legume production semi-arid areas in Tunisia (Maazaoui et al., 2016). The seeds were surface-sterilized in a dilute solution of sodium hypochlorite (5%) for 5 min and then washed ten times in sterilized distilled water. Afterwards, the seeds were soaked in sterile distilled water overnight before sowing in 5-kg pots filled with a soil mix. The soil texture used in this study was clay loam. The results of the physical analyses of the soil showed 41% silt, 30.5% clay and 28.5% sand; the total organic matter was evaluated at 1.25% and the cation exchange capacity (CEC) was 413.50 ms cm^{-1} at 0~15 cm depth. The chemical analysis results of the soil were: N: 0.28 (%); P: 16 (mg kg-1); K: 120 (mg kg-1) and pH: 8.15. Half-strength Hoagland's solution (Hoagland and Arnon, 1950) was used for irrigation. The studied genotypes underwent three water treatments: 90% of field capacity (FC) as a control treatment, 50% FC (moderate stress) and 30% FC (severe stress). Water deficit treatments were chosen on the basis of preliminary results obtained on this species (Ziadi Backouchi et al., 2015), and the soil field capacity of the soil mixture was determined by weighing. Five replicates per treatment were used for each genotype. Physiological measurements and plant sampling were conducted 20 days after starting drought stress (seedling stage).

2.2. Relative water content (RWC)

RWC was determined as described by Galmés et al. (2007). The uppermost fully expanded leaf of the main stem was weighed (fresh weight, FW), and then turgid weight (TW) was obtained after submerging samples in distilled water for 24 h. The samples were then immediately dried at 70°C for 72 h and weighed (dry weight, DW). The RWC was determined according to the equation:

RWC (%) = [(FW-DW)/(TW-DW)] X 100

2.3. Leaf gas exchange

Gas exchange measurements were determined in both stressed and non-stressed plants at 10.00 a.m. in atmospheric CO_2 and full sunlight using a Portable Photosynthetic System (LCpro+, Inc., UK). The measurements were conducted on the third totally expanded leaf

from five individual plants per treatment. Transpiration rate (E), net photosynthesis (A), stomatal conductance (gs) and sub-stomatal CO_2 concentration (Ci) were recorded.

2.4. DNA extraction and methylation-sensitive amplified polymorphism (MSAP) assay

Roots and shoots (leaves only) tissues were collected separately 20 days after starting drought stress from 10 seedlings of Bachar and F177 which under stressed and controlled conditions (90% and 30% FC) and immediately frozen in liquid nitrogen and stored at -80°C in a freezer until use. Genomic DNA was extracted from tissue samples as described by Murray and Thompson (1980). The quality of the genomic DNA was checked using 0.8% (w/v) agarose gel electrophoresis prior to its use for MSAP analysis. The MSAP technique was conducted according to the protocol developed by Reyna-Lopez et al. (1997), with some modifications (Yaish et al., 2014). Two methylation-sensitive isoschizomers with different methylation sensitivity, *Msp*I and *Hpa*II, were used as frequent cutters for the MSAP analysis, and *Eco*RI was used as rare cutter. The digestion reactions were performed in a volume of 20 µl. Genomic DNA (1 µg) was digested with 5 U *Eco*RI (Thermo Fisher Scientific) at 37 °C for 6 h. After digestion, DNA was precipitated and digested with 5 U *Hpa*II or *Msp*I (Thermo Fisher Scientific) independently at the same time at 37 °C for 16 h. Digested genomic DNA (300 ng) from the two reactions was simultaneously ligated to 50 pmol of H*pa*II/M*sp*I adapter and 5 pmol of *Eco*RI adapter (Supplementary File 1) in a total reaction volume of 20 µl, comprising 2 µl 10X T4 ligase buffer and 1 U T4 DNA ligase (Promega, USA). The mixture was incubated at 15 °C for 16 h, inactivated at 65 °C for 10 min and stored at -20 °C. The ligation mixture was diluted 10-fold prior to preselective polymerase chain reaction (PCR) amplification. The preselective amplification reaction was performed in a final volume of 20 µl, containing 1 X PCR reaction buffer, 1 U *Taq* polymerase (Thermo Fisher Scientific), 5 µl of diluted DNA template, 5 pmol of each preselective primer (Supplementary File 1), 2.5 mM $MgCl_2$ and 0.2 mM dNTPs. The reaction was performed for 30 cycles (MyCycler, Bio-Rad, USA) of 30 s denaturation at 94 °C, 30 s annealing at 56 °C and 1 min extension at 72 °C, with a final extension at 72 °C for 5 min. The selective amplification was conducted in a volume of 20 µl using 1 µl preamplification product, 1 X PCR buffer, 2.5 mM $MgCl_2$ and 0.2 mM dNTPs, 1 pmol of each selective primer (Supplementary File 1) and 1 U *Taq* DNA polymerase (Thermo Fisher Scientific). The selective amplification was performed with a touchdown program of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min, decreasing the annealing temperature by 0.7 °C per cycle during 12 cycles and then 24 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, with a final extension of 5 min at 72 °C. The selective amplification products were separated by electrophoresis in 6.5% denaturing polyacrylamide

gel using an automated LI-COR DNA sequencer (LI-COR Biosciences, Lincoln, NE, USA). MSAP images were analyzed with Allelinks^{TM} analysis software.

2.5. MSAP analysis

The fingerprints showing reproducible results between replicas were scored for MSAP data analysis. MSAP bands were scored as "1" for the presence and "0" for the absence of bands from either *Eco*RI-*Msp*I or *Eco*RI-*Hpa*II digestion. 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 signal in both enzyme combinations), hemimethylated at the outer cytosine in one DNA strand (Type II, presence of the signal only in digestion with *Eco*RI/*Hpa*II), fully methylated at the internal cytosine in both DNA strands (Type III, presence of the signal only in digestion with *Eco*RI/*Msp*I), and hypermethylated with outer methylation at both DNA strands (Type IV, absence of signal in both enzyme combinations) (Fulneček and Kovařík, 2014).

2.6. Silver staining, cloning and sequencing of MSAP fragments

To isolate the differentially methylated fragments detected by MSAP, the same products of selective amplifications were separated using 6% polyacrylamide gel (acrylamide/bisacrylamide, 19:1) electrophoresis at 60 W for 3 h using the PROTEAN II xi Cell system (Bio-Rad, USA). Gels were silver stained using the method described by Benbouza et al. (2006). Fragments of interest were excised directly from the wet polyacrylamide gel using a razor blade and purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequently, the products were cloned in a pJET1.2/blunt vector using the CloneJETTM PCR Cloning Kit (Thermo Fisher Scientific) and transformed into competent Escherichia coli DH5α. DNA sequencing was carried out using the GENOMELABTM CEQ/GeXP DNA ANALYSIS SYSTEM (BECKMAN COULTER). Sequences were deposited in GenBank under accession numbers JZ948737 through JZ948742. Homology analysis with sequences in the GenBank database was performed via the BLASTX and BLASTN search programs provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

2.7. RNA isolation and cDNA synthesis

The specific organ expression of faba bean (Bachar) genes was analyzed for leaves (3-week-old plants), flowers (0 days after pollination), stems (3-week-old plants), roots (3-week-old plants), cotyledons (3-day-old seedlings) and seeds (15 days after pollination). For quantitative real-time PCR, leaf and root tissues of Bachar and F177 were collected from control (90% FC) and drought-stressed (30% FC) plants. Two hundred milligrams of samples

were ground to a fine powder using liquid nitrogen and transferred into a 2 ml centrifuge tube for total RNA extraction following the protocol described by Chang et al. (1993). Genomic DNA contamination in extracted RNA was eliminated by treatment with 5U of RNase-free DNase I (Thermo Fisher Scientific) for 30 min at 37 °C. The first-strand cDNA was synthesized from 5 µg of total RNA using 200 U of RevertAid M-MuLV reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's recommendations.

2.8. Semi-quantitative RT-PCR analysis

Based on the cloning fragment sequencing results, we selected the corresponding genes for quantitative analysis of expression. Semi-quantitative RT-PCR of *VfABC*, *VfCDPK*, *VfGH* and *VfLOX* genes was carried out using gene-specific primers (Table 1). Primer pairs were designed via Primer3 Input (version 0.4.0) software (Rozen & Skaletsky 2000) (http://frodo.wi.mit.edu/primer3/). Faba bean 18S rRNA (*Vf18S*) was used as an internal control. All PCR reactions were conducted in triplicates in a MyCycler™ Thermal Cycle (Bio-Rad, USA). The thermal cycling conditions were 3 min of initial denaturation at 95 °C followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55-60 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min.

2.9. Quantitative RT-PCR analysis

Quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems). Each reaction mix was performed in 25 µl containing 5 µl of cDNA (100 ng), 12.5 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Biomatik), and 0.3 µM of each gene-specific primer (Table 1). The reaction mix was subjected to the following program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The specificity of the PCR amplification was verified with a melt curve analysis (from 55 °C to 94 °C) following the final cycle of the PCR. Each RT-qPCR was replicated three times and the mean was used for RT-qPCR analysis. The gene coding for *Vf18S* was used as a reference gene for data normalization. The relative expression of the studied genes was calculated according to the $2^{-\Delta CT}$ method (Livak & Schmittgen 2001)

2.10. Statistical analyses

Statistical analyses were performed using a one-way analysis of variance (ANOVA, $P < 0.05$), followed by Tukey's test in the SYSTAT 8·0 software. All physiological and RT-qPCR data are reported as mean ± SD (standard deviation).

3. Results

3.1. Leaf relative water content and gas exchange

A significant variation ($P \leq 0.05$) was found between the Bachar and F177 genotypes for the RWC and leaf gas exchange parameters (Table 2). All parameters are higher for the Bachar genotype than for F177. The relative water content (RWC), internal concentration of CO_2 (Ci), net photosynthesis (A), transpiration rate (E) and stomatal conductance (gs) declined in both studied genotypes under moderate and severe drought stress as compared to control conditions.

Bachar had higher (A) than F177 in both control and stress conditions, suggesting a high ability of photosynthetic machinery in Bachar under different drought stress levels. The transpiration rate (E) was also affected by the water deficit treatment. The transpiration rate was 18.33% and 27.50% lower in Bachar under moderate and severe water deficit stress respectively. However, the highest reduction was observed in F177 (40.77%) under severe drought stress. The reduction in (E) data suggested that Bachar conserved more water than F177.

Water deficit significantly reduced RWC of Bachar and F177 genotypes; the RWC of Bachar remained the highest under moderate and severe drought stress.

3.2. Global DNA methylation patterns in faba bean leaves and roots under control conditions and drought stress

Cytosine methylation patterns in the root and leaf tissues of drought-stressed and control plants of Bachar and F177 faba bean genotypes were assessed by MSAP (Table 3). Using 16 MSAP primer pairs, a total of 764 and 913 bands were revealed in leaves and roots respectively (Fig. 1). Under control conditions in leaf tissues, total methylation of CCGG sequences was 37.43% in Bachar and 41.23% in F177, while in root tissues, total methylation ranged between 16.53% and 23.43% in F177 and Bachar respectively (Table 3). Moreover, the overall methylation level in leaves of both studied faba bean genotypes was consistently higher than in roots under all water conditions. Among these methylated loci, fully methylated loci (Types III and IV) were more common than hemimethylated loci. The total DNA methylation level decreased under drought stress treatment in leaves (-6.81%) and roots (-3.63%) of Bachar. Similar data were obtained for leaves of F177 (-3.14%), while the percentage of methylated bands slightly increased in roots (+0.66%) compared to the control condition.

To evaluate the impact of drought stress and find out the relationship between DNA methylation variations and drought stress tolerance in faba bean, differentially methylated DNA bands were classified into 16 classes (Table 4). Classes A, B, C and D referred to stable bands. Classes E-J indicated cytosine demethylation patterns. The percentage of demethylated

bands under drought stress was 19.89% and 21.85% in leaf tissues and 8.21% and 7.22% in root tissues of Bachar and F177 respectively, indicating relatively more DNA demethylation events in drought-stressed leaf than in the root tissues of both studied faba bean genotypes (Table 4). However, possible cytosine methylation events induced by drought stress were represented by Classes K-P (15.85% and 22.26% of the CCGG sites in leaf of Bachar and F177 respectively, and 7.90% and 4.94% in root tissues). The data obtained showed that drought stress induces methylation and demethylation events in both studied genotypes: the rate of modified amplified bands varies according to organ and genotype, with 35.74% and 44.11% of modified amplified bands in leaves and 16.11% and 12.16% of modified amplified bands in roots for Bachar and F177 respectively.

Principal component analysis (PCA) was performed to produce a visual representation of the relative distance of each genotype, the genetic and DNA methylation state (Fig. 2). The first, second and third dimensions contributed 67.25%, 13.21% and 7.62% respectively (accounting for 88.08% of the total variance in the status of methylation of the MSAP loci) of the relationship in the multivariate space. The PCA scores for the MSAP profiles indicated a clear differentiation between control and drought faba bean genotypes and also separated the samples (leaves and roots) within each group by drought treatment.

3.3. Analysis of the differentially methylated regions (DMRs)

To investigate the effects of change in DNA methylation on regulation of gene expression, a random set of 10 bands (ranging from 216 to 340 bp) were methylated or demethylated in response to drought stress, were excised from acrylamide gels, cloned and sequenced (Table 5). Searches for similarities with known genes as well as with genome sequences were undertaken by investigations of the faba bean genomes database and the genomes databases of model plants such as *Medicago truncatula* using the BLAST alignment tool. Six sequences showed at least one significant alignment with a cutoff value of e^{-4} in the *Vicia faba*, *Medicago truncatula*, *Arachis hypogaea* and *Camellia sinensis* genome. Among these DMRs, there were five overlaps of coding sequences including lipoxygenase (*LOX*), calcium-dependent protein kinase (*CDPK*), ABC transporter family (*ABC*), glycosyl hydrolase (*GH*) and phosphoenolpyruvate carboxylase (*PEPC*) (Table 5). Moreover, the VfMSAP4 (JZ94874) sequence similar to previously identified *Vicia faba* sequence (AGC78838.1) encoded an unknown protein.

3.4. Tissue-specific expression of selected differentially methylated regions (DMRs) in faba bean

To determine tissue-specific expression of the selected faba bean ESTs, RNA was isolated from leaves, flowers, stems, roots, cotyledons and seeds, and RT-PCR was carried out with specific primers for *VfABC*, *VfGH*, *VfLOX* and *VfCDPK* genes (Fig. 3). This analysis revealed the presence of these gene transcripts in all tested faba bean organs at different expression levels, confirming that the four studied genes were expressed in various tissues.

3.5. Expression analysis of selected DMRs in leaves and roots of faba bean genotypes under control and drought stress conditions

To investigate the expression patterns of differentially methylated sequences, transcript accumulations of *VfCDPK*, *VfLOX*, *VfABC* and *VfGH* under drought stress were evaluated by real-time quantitative RT-PCR in leaf and root tissues from both studied genotypes under control and water deficit stress conditions in order to determine the effect of methylation changes (Fig. 4). All studied genes were differentially expressed under the applied water stress treatments.

The expression profiles of *VfABC* (Fig. 4A and B) demethylated in the leaf tissues of Bachar and of *VfCDPK* (Fig. 4C and D) demethylated in the leaf tissues of F177 globally increased under drought stress conditions in leaves and roots of both genotypes. Moreover, this increase was higher in Bachar than in F177 genotype. On the other hand, the expression of *VfGH* gene methylated in the root of Bachar, slightly increased at 50% (FC) in Bachar (Fig. 4E and F) and then globally decreased under severe drought stress for both genotypes. However, at 30% (FC) the expression of *VfGH* remains higher in Bachar than in F177. The expression of *VfLOX* methylated in the leaf tissues of F177 remains stable in the leaf tissues of Bachar and F177 genotype under water deficit stress (Fig. 4G). However, at 30% (FC), the expression level of *VfLOX* was significantly decreased or increased in the root tissues of Bachar and F177 respectively (Fig. 4H).

4. Discussion

Water deficit resulted in a marked reduction in RWC and leaf gas exchange parameters. In fact, there was gradual reduction in leaf RWC and physiological parameters (gs, A, E and Ci) in faba bean genotypes as stress severity increased. Our result is in agreement with the results of Siddiqui et al. (2015), in which all morphological, physiological and biochemical parameters were reduced in 10 faba bean genotypes grown under water deficit stress. Interestingly, of the two faba bean genotypes tested in this study, Bachar showed less inhibition of physiological parameters and RWC. This may suggest that it has better adaptive potential under water deficit conditions than F177. Physiological parameters such as RWC,

electrolyte leakage (EL), A, gs, E, and water use efficiency (WUE) have been widely used as markers for evaluating drought stress tolerance and in screening adapted cultivars of various plant species, including switchgrass and faba bean, under drought stress (Liu et al., 2015b; Siddiqui et al., 2015). Under drought stress, the tolerant genotypes maintained higher rates of RWC, A and Ci than susceptible genotypes in chickpea (Rahbarian et al., 2011), soybean (Hossain et al., 2014) and sugarcane (Silva et al., 2007). Taken together, based on physiological parameter values, these results indicate that Bachar is more tolerant to water deficit than F177.

DNA methylation is an epigenetic mechanism which plays a key role in tuning gene expression in response to environmental stresses causing phenotypic variations that are not correlated with genetic variation in crop plants (Chen, 2007). The correlation between epigenetic changes in plants and abiotic stress tolerance has been discussed in detail in previous research (Kim et al., 2015). Moreover, several differentially methylated regions among rice cultivars which showed different response to drought and salt stress have been identified, and some of these have been correlated with differential expression of key genes involved in abiotic stress response and tolerance (Garg et al., 2015).

In the present study, the MSAP technique was applied to investigate the variation in DNA methylation in leaves and roots of faba bean in response to drought stress. Our results are consistent with previous reports showing that drought could induce changes in DNA methylation/demethylation across the plant genome in species such as rice (Wang et al., 2011; Zheng et al., 2013; Zheng et al., 2014), ryegrass (Tang et al., 2014a) and tall fescue (Tang et al., 2014b).

Faba bean is a diploid species with 2n = 2x = 12 chromosomes and a huge genome size (13,000 Mb) with a high proportion of repetitive DNA elements (Johnston et al., 1999). Comparative DNA methylation analysis of faba bean leaves and roots under drought stress conditions revealed that leaves and roots may differ greatly in the level and pattern of DNA methylation, with many more changes occurring in the leaves than in the roots (Table 3). This indicates specific biological processes performed by each tissue which may play a role in the response to drought stress. Overall, the DNA methylation level of Bachar ranged from 37.43% to 30.62% in leaves and from 23.43% to 19.80% in roots under the control and water deficit conditions respectively. On the other hand, the DNA methylation level of F177 ranged from 41.23% to 38.16% in leaves under control and water deficit conditions respectively. However, the total genomic methylation level in roots of F177 ranged from 16.53% to 17.19% under control and stress conditions respectively. This suggests a high level of DNA

methylation in comparison with other plants (Du and Wang, 2011; Karan et al., 2012; Shan et al., 2013) such as *Arabidopsis thaliana* (25%), *Oryza sativa* (20-30%) and *Zea mays* (35%). Using the MSAP sequencing (MSAP-Seq) technique, Pan et al. (2012) reported that the overall level of DNA methylation was about 70% in *Triticum aestivum*. These comparisons are consistent with those reported by Alonso et al. (2015) suggesting a positive correlation between the global DNA methylation level and the genome size.

Differential DNA methylation between the two studied genotypes was revealed in this work; this finding is consistent with studies in rice (Wang et al., 2011; Zheng et al., 2013). Interestingly, the results of our experiments (Table 3) revealed that drought stress slightly enhanced (by 0.66%) the methylation rate in the susceptible genotype (F177). However, the tolerant genotype (Bachar) showed demethylation (-3.63%) which in turn could lead to the transcriptional activation of drought-related genes. Comparison of DNA methylation patterns between the considered genotypes (Table 3) revealed that Bachar leaf tissues exhibited a higher level of hypomethylation than F177 (-6.81% and -3.14% respectively). Our results are in agreement with those reported by Gayacharan and Joel (2013) in rice under drought stress. These authors reported that hypomethylation was more abundant in tolerant genotypes, whereas methylations were more abundant in sensitive ones. Moreover, drought stress causes oxidative stress (Niu et al., 2013), which induces DNA demethylation according to Cerda and Weitzman (1997).

DNA methylation in rice and jatropha has been found to be genotype, tissue and developmental stage-specific (Wang et al., 2011; Mastan et al., 2012). This is consistent with our results for faba bean, which reveal methylation patterns specific to drought-tolerant or drought-susceptible genotypes in roots or leaves under drought stress. In the present study, drought induced differential DNA methylation/demethylation alteration in the whole genome of Bachar and F177. This may lead to the activation and inactivation of the transcriptional processes for specific genes related to drought tolerance, and hence to improved faba bean adaptation to drought. A total of six MSAP fragments (Table 5) showing a pattern modification under drought stress were cloned and sequenced. Homology analysis showed that four of them, *VfABC*, *VfGH*, *VfLOX* and *VfCDPK*, could be involved in a wide range of functions, including response to abiotic stress and other functions related to plant development (Opassiri et al., 2006; Das and Pandey, 2010; Yang et al., 2012; Tian et al., 2017). The four genes (*VfCDPK*, *VfLOX*, *VfABC* and *VfGH*) putatively involved in drought stress response were selected. Their expression pattern was analyzed by quantitative PCR

under various drought conditions in roots and leaves of Bachar and F177 genotypes in order to throw light on their potential role in drought stress response and tolerance.

Quantitative RT-PCR analysis showed that all studied genes were differentially expressed in the roots and leaves of both genotypes under water deficit, suggesting that these genes may play a critical role in faba bean tolerance to drought.

The plant ATP-binding cassette (ABC) transporters are encoded by large gene families and play an important role in plant growth and development, detoxification processes and response to abiotic stresses. According to Kuromori et al. (2010) and Kuromori and Shinozaki (2010), *AtABCG25* and *AtABCG40* were shown to be responsible for ABA transport and guard cell responses respectively under drought stress in *Arabidopsis thaliana*. Moreover, Kuromori et al. (2011) found an important function of *AtABCG22* in stomatal regulation. These results suggested that ABC transporters mediate ABA uptake and are important for rapid responses to environmental stress in *Arabidopsis thaliana*. Analysis of *VfABC* gene expression by qRT-PCR demonstrated that this gene is up-regulated in root and leaf tissue of faba bean genotypes under drought stress. Interestingly, the level of expression of *VfABC* in Bachar was higher than in F177. These results were in agreement with those reported by Silveira et al. (2015), which showed that the level of ABC transporter in a drought-tolerant rice cultivar (Douradão) was higher than in the sensitive cultivar (Primavera).

Plant glycosyl hydrolases (GHs) participate in various aspects of normal plant growth and development, including cell wall metabolism, disease resistance and abiotic stress tolerance. In *Arabidopsis thaliana*, the *HOT2* gene encoding GH plays a key role in tolerance to abiotic stresses, including heat, salts and drought stress (Kwon et al., 2007). Whole-genome oligonucleotide microarrays revealed a decrease in the expression of this gene in members of Arabidopsis glycosyl hydrolase family 1 under drought stress. This could be associated with decreased cell expansion, or related to reducing turgor pressure associated with stress according to Huang et al. (2008). The expression of *VfGH* was increased in the roots and leaves of Bachar at 50% FC and significantly decreased at 30% FC compared to the control condition. Drought decreases *VfGH* transcripts in root and leaf tissue of F177 under drought stress conditions. This result was consistent with previous reports showing that the rice GH gene was differentially expressed under different forms of abiotic stress (drought, salt and low temperature) through qPCR analysis (Guo et al., 2013).

Drought stress induces abscisic acid (ABA) accumulation which among other effects could promote the activity of some classes of calcium sensor proteins, including calcium-dependent protein kinases (CDPKs). Some *Arabidopsis* CDPKs have been reported to play a role in

abiotic stress responses and abscisic acid (ABA) signaling pathways (Zhu et al., 2007). Interestingly, several CDPKs such as *Solanum tuberosum* CDPK4 and CDPK5 (Kobayashi et al., 2007), *Arabidopsis thaliana* CPK5/CPK6 and CPK4/CPK11 (Boudsocq et al., 2010) and *Oryza sativa* CPK12 (Asano et al., 2012) have been shown to positively or negatively regulate abiotic stress tolerance by modulating ABA signaling and reducing the accumulation of reactive oxygen species (ROS). More recently, analyses of over- and down-expressed OsCPK9 in rice mutants have revealed that OsCPK9 plays a positive role in drought stress tolerance by enhancing stomatal closure and by improving the osmotic adjustment ability of the plant (Wei et al., 2014). *VfCDPK* expression was examined under conditions of drought and was found to be substantially induced in drought-treated seedlings, especially in the Bachar genotype. These results are in agreement with those obtained by Liu et al. (2006) in faba bean. According to these authors, *VfCPK1* was highly expressed in faba bean leaves subjected to drought stress and may play important roles in stomatal movement.

In plants, lipoxygenases (LOXs) have long been considered to be associated with various physiological processes, including fruit ripening and senescence (Zhang et al., 2006). Many other studies have also demonstrated that the expression of *LOX* genes could be induced by abiotic stresses and could play a positive role in plant tolerance to drought and osmotic stress (Lim et al., 2015). According to these authors, overexpression of pepper (*Capsicum annuum* L.) *LOX1* in transgenic *Arabidopsis thaliana* enhances tolerance to drought and salt stress. This occurs through a rapid scavenging reactive oxygen species (ROS) and the induction of drought- and ABA-responsive genes including *DREB2A*, *RD20*, *RD29A* and *RD29B*. Similar results were obtained in transgenic tomato and *Arabidopsis* plants overexpressing persimmon (*Diospyros kaki* L.) *LOX3* (Hou et al., 2015). Moreover, the early induction of *Panax ginseng* Meyer *LOX3* (*PgLOX3*) in seedlings subjected to drought stress could correspond to adaptive response to drought-stress according to Bae et al. (2016). *VfLOX* did not express differentially in the leaves of the two genotypes subjected to drought stress, but was downexpressed in Bachar roots. On the other hand, *VfLOX* gene was induced in the root tissues of F177 at 30% FC. Taken together, *VfLOX* showed different expression patterns which may suggest different specific tissue functions in response to abiotic stresses.

Several studies showed that DNA methylation and demethylation are associated with either transcriptional repression or activation (Choi and Sano, 2007; Tang et al., 2014a). As indicated by quantitative real-time PCR, two demethylated fragments (VfMSAP3 and VfMSAP5) revealed in leaf tissue of F177 and Bachar under drought stress respectively, thought to be a calcium-dependent protein kinase (*VfCDPC*) and ABC transporter family

(*VfABC*), exhibited up-regulated expression. In particular, the expression of *VfABC* increased significantly when the methylation status changed from methylation to demethylation in leaf tissue of Bachar under drought stress. In the other hand, another fragment (VfMSAP1), which was shown undergo drought-induced methylation, encoded to glycosyl hydrolase (*VfGH*). In the other hand, another fragment (VfMSAP1) revealed in root tissue of bachar encodes a glycosyl hydrolase (*VfGH*), which was shown undergo drought-induced methylation showed their expression decreased (Fig. 4). All of these results suggested that demethylation up-regulated related gene expression, and the extent of methylation was negatively correlated with expression. These results are similar to those in previous reports on drought-treated of perennial ryegrass (*Lolium perenne*) (Tang et al., 2014a).

5. Conclusion

In summary, genome-wide differences in DNA methylation between the drought-tolerant (Bachar) and drought-sensitive (F177) faba bean genotypes under drought and control conditions were revealed using the MSAP approach. Drought stress decreased the total DNA methylation level in the Bachar and F177 genomes. The level of demethylation was higher in Bachar than in F177. Thus, altered methylation in response to drought stress was probably involved in environmental stress acclimation.

DNA methylation polymorphic fragments were isolated, cloned and sequenced. The analysis of DNA methylation modifications showed a high degree of correlation between changes in DNA methylation and expression of *VfCDPK*, *VfLOX*, *VfABC* and *VfGH* in Bachar and F177 roots and leaves under control and water deficit conditions. This suggests that DNA methylation status influences the expression pattern of drought stress response genes, suggesting a possible role of DNA methylation in faba bean drought response and tolerance.

This study may also provide a basis for further studies into the role of epigenetic regulation of faba bean responses to drought stress and other environmental stresses.

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Fig. 1. Examples of DNA MSAP patterns in leaf (L) and root (R) of faba bean genotypes Bachar and F177 using the primer combination *Eco*RI-AG/*Msp*I-AG (A) and *Eco*RI-AG/*Msp*I-AC (B). EH and EM refer to digestion DNA with *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I, respectively. RC: root control; RS: root stress; LC: leaf control; LS: leaf stress. The *arrows* indicate differential MSAP products resulting from methylation (M), demethylation (D) or no change (NO).

Fig. 2. Principal component analysis (PCA) scores plot of DNA methylation data. Multivariate analysis of the methylation status data set for each locus of faba bean plants sampled under control and drought conditions (unmethylated, hemimethylated, fully methylated or hypermethylated).

Fig. 3. Expression patterns of selected genes in various tissues by RT-PCR. L: leaves; F: flowers; St: stems; R: roots; C: cotyledons; Se: seeds.

Fig. 4. Expression profile analyses of 4 selected drought stress-related genes (*VfCDPK*, *VfLOX*, *VfABC* and *VfGH*) by real-time quantitative RT-PCR in the leaf (A, C, E and G) and root (B, D, F and H) tissues of Bachar and F177 genotypes under drought stress conditions. Levels of expression for each gene were normalized according to *Vf18S* amplifications. Data shown represent mean values obtained from three independent amplification reactions and the errors bars indicate standard deviation. Different letters denote significant differences (Tukey's HSD, $P<0.05$).

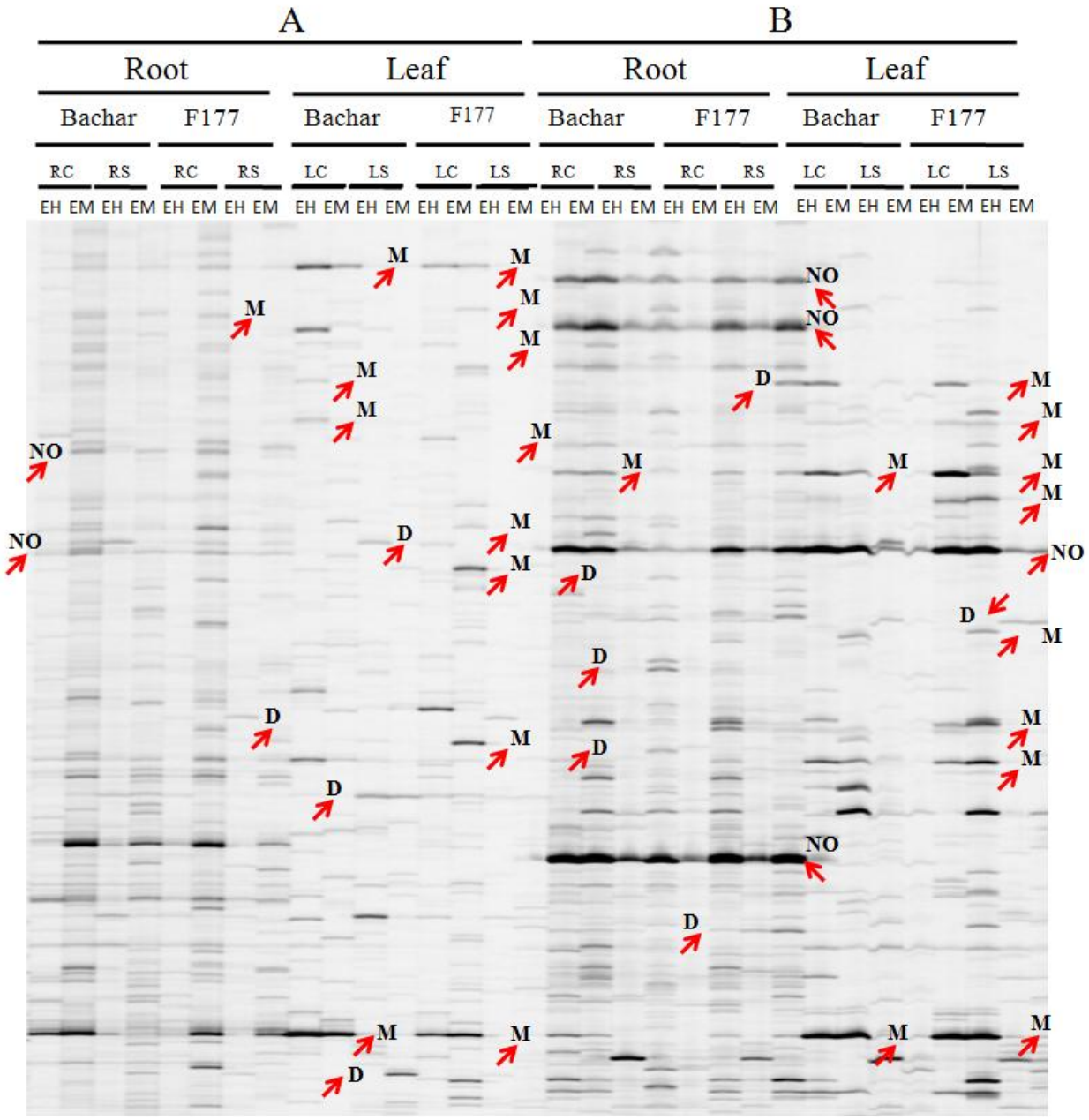

Fig. 1

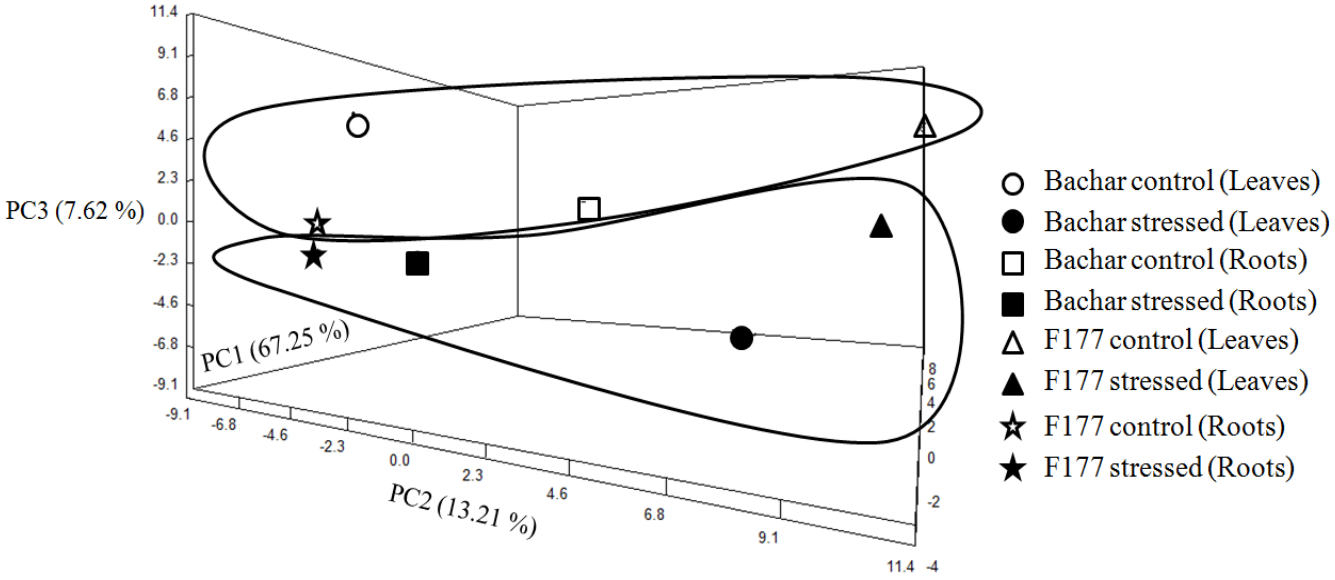

Fig. 2

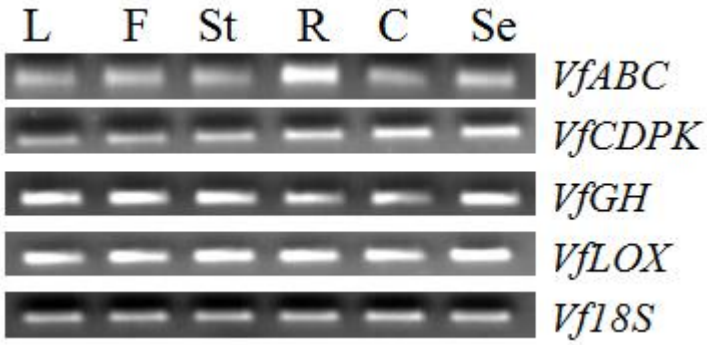

Fig. 3

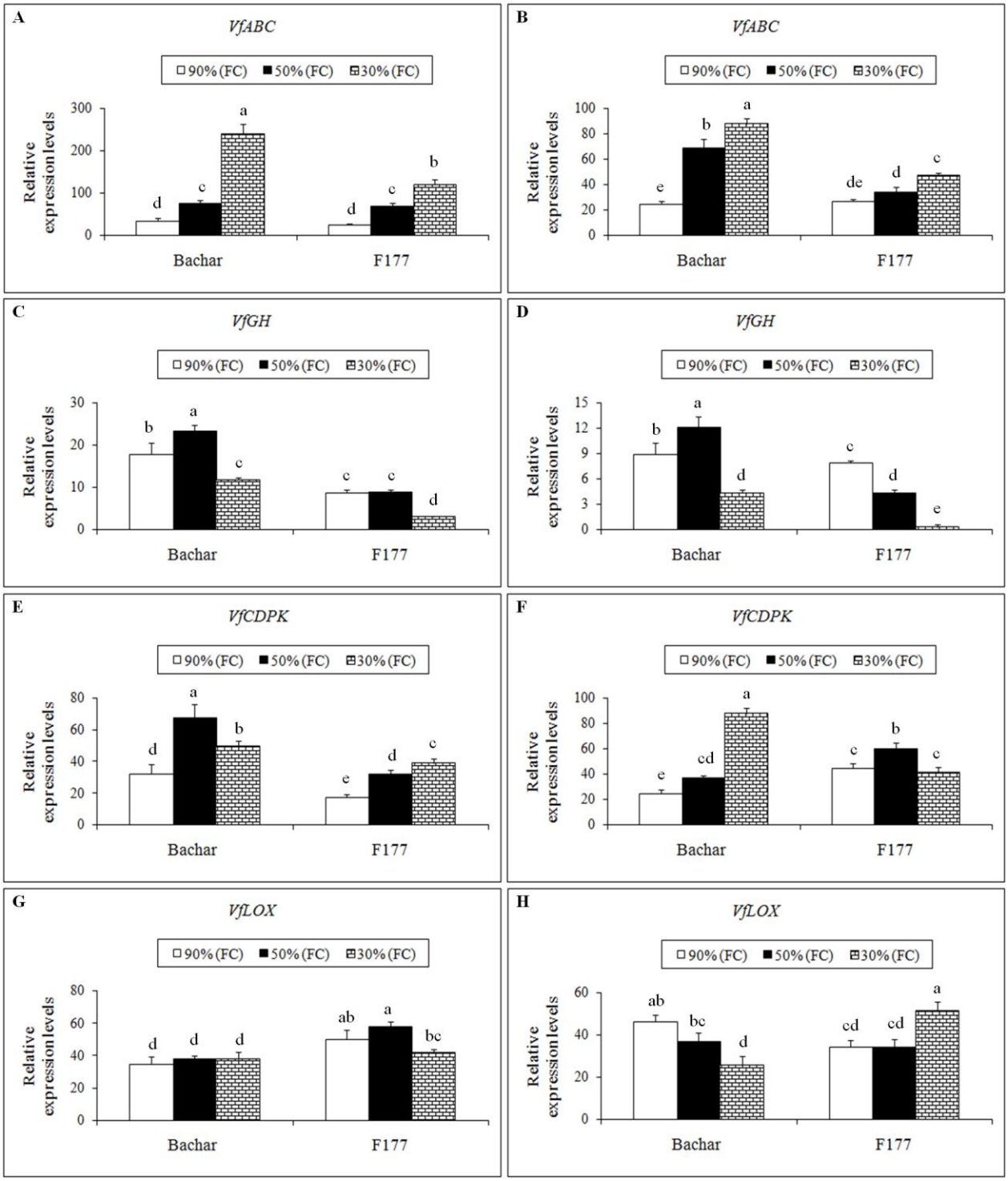

Fig. 4

Table 1 Primers used for semi-quantitative RT-PCR and quantitative real time PCR assays.

Transcript	Sequence	Tm (°C)
VfCDPK	F: 5'-CAAGGTGGTCCTTGCTGATT-3' R: 5'-AAGTCCAGTAGCCGAAAGCA-3'	60
VfLOX	F: 5'-GCAGCTTTCAAGAGGTTTGG-3' R: 5'-ACTGGCCCTACTCGGTTTCT-3'	58
VfABC	F: 5'-AGACCATCCGCGTCTTCAT-3' F: 5'-CGGGGAACAGGAGTAGCAT-3'	59
VfGH	F: 5'-GTGGTTGGATACCGATGGTC-3' R: 5'-TCTTGTGAGCGTGGTAGGTG-3'	59
Vf18S	F: 5'-GCAACAAACCCCGACTTCTG-3' R: 5'-TGCGATCCGTCGAGTTATCA-3'	58

Table 2 Effect of moderate (50% FC) and severe (30% FC) drought stress on relative water content (RWC), internal concentration of CO_2 (Ci), net photosynthesis (A), transpiration (E) and stomatal conductance (gs) in faba bean genotypes (Data are mean of 5 measurements). ***Different letters denote significant differences*** **(*Tukey's*** HSD, ***P<0.05).***

Faba bean genotypes	**Treatments**	**RWC (%)**	**Ci (µmol·mol^{-1})**	**A (µmolCO2 m^{-2}·S^{-1})**	**E (mmol·m^{-2}·S^{-1})**	**gs (mol H_2O m^{-2}·S^{-1})**
Bachar	Control	82.03±2.70^{a}	325.50±2.12^{a}	9.17±0.30^{a}	2.40±0.04^{a}	0.18±0.01^{a}
	Moderate	72.51±2.78^{bc}	311.50±0.70^{ab}	6.49±0.02^{c}	1.96±0.19^{ab}	0.13±0.03^{b}
	Severe	53.39±2.25^{d}	281.50±12.72^{cd}	4.03±0.84^{d}	1.74±0.32^{b}	0.10±0.01^{bc}
F177	Control	79.33±2.09^{ab}	301.50±9.19^{b}	7.79±0.79^{b}	2.06±0.30^{ab}	0.14±0.04^{b}
	Moderate	65.23±2.99^{c}	294.50±3.53^{bc}	4.93±0.29^{d}	1.76±0.05^{b}	0.11±0.01^{bc}
	Severe	46.74±3.01^{e}	269.50±5.65^{d}	3.14±0.09^{e}	1.22±0.02^{c}	0.07±0.01^{c}

Table 3 Global DNA methylation patterns and levels in shoot and root of Bachar and F177 under control and drought treatments.

[a]Fully methylated bands (%) = [(III + IV)/ (I + II + III + IV)] × 100

[b]Hemi-methylated bands (%) = [(II)/ (I + II + III + IV)] × 100

[c]Total methylated bands (%) = [(II + III + IV)/ (I + II + III + IV)] × 100

MSAP band types	Bachar		F177	
	Control	Drought	Control	Drought
Leaves				
I (Unmethylated)	478	530	449	473
II (Hemi-methylated)	132	111	135	143
III (Methylated)	63	49	74	56
IV (Hyper methylated)	91	74	106	93
Total amplified bands	**764**	**764**	**764**	**764**
Full-methylated bands (%)[a]	20.15	16.09	23.56	19.47
Hemi-methylated bands (%)[b]	17.27	14.52	17.67	18.69
Total methylated bands (%)[c]	37.43	30.62 (-6.81)	41.23	38.16 (-3.14)
Roots				
I	699	733	762	756
II	77	86	44	55
III	31	27	22	34
IV	106	68	85	68
Total amplified bands	**913**	**913**	**913**	**913**
Full-methylated bands (%)[a]	15.00	10.39	11.71	11.17
Hemi-methylated bands (%)[b]	8.43	9.40	4.81	6.02
Total methylated bands (%)[c]	23.43	19.80 (-3.63)	16.53	17.19 (0.66)

Table 4 Summary of DNA methylation pattern changes in Bachar and F177 under drought stress conditions. (+) band present, (-) band absent.

Pattern	Class	Control		Drought		Leaves		Roots	
		***Hpa* II**	***Msp* I**	***Hpa* II**	***Msp* I**	**Bachar**	**F177**	**Bachar**	**F177**
No change	A	+	-	+	-	23	49	38	12

	B	-	+	-	+	71	37	31	26
	C	+	+	+	+	328	285	614	720
	D	-	-	-	-	69	56	83	44
	Total					**491** (64.26%)	**427** (55.89%)	**766** (83.89%)	**802** (87.84%)
Demethylation	E	+	-	+	+	18	25	12	4
	F	-	+	+	+	45	34	7	16
	G	-	-	+	+	27	41	31	28
	H	-	+	+	-	12	23	4	8
	I	-	-	+	-	14	15	9	2
	J	-	-	-	+	36	29	12	8
	Total					**152** (19.89%)	**167** (21.85%)	**75** (8.21%)	**66** (7.22%)
Methylation	K	+	+	+	-	12	4	7	4
	L	+	+	-	+	25	11	6	5
	M	+	+	-	-	41	33	24	7
	N	+	-	-	+	16	14	10	2
	O	+	-	-	-	9	57	14	9
	P	-	+	-	-	18	51	11	18
	Total					**121** (15.85%)	**170** (22.26%)	**72** (7.90%)	**45** (4.94%)

Table 5 BLAST result of six randomly selected differentially methylated MSAP fragments.

MSAP fragments		**Methylation status under stress**	**Tissue type**	**Genotype**	**Size (bp)**	**Accession no in Genbank**	**Homology analysis**	**Sequence homology**	**Identities (aa/aa or bp/bp)**	**E-value**
Name	**Primer combination**									
VfMSAP1	*Eco*RI-CG/*Msp*I-AC	Methylated	Root	Bachar	225	JZ948737	BLASTx	Glycosyl hydrolase *Medicago truncatula* (XP_013453468.1	60/70 (86%)	3E-320

VfMSAP2	*Eco*RI-GG/*Msp*I-AC	Methylated	Root	F177	258	JZ948873	BLASTn	Lipoxygenase *Vicia faba* (Z73498.1)	242/258 (94%)	9E-140
VfMSAP3	*Eco*RI-CA/*Msp*I-AT	Demethylated	Shoot	F177	340	JZ948739	BLASTx	Calcium-dependent protein kinase *Arachis hypogaea* (AHA61365.1)	59/63 (94%)	2E-320
VfMSAP4	*Eco*RI-AG/*Msp*I-AA	Demethylated	Shoot	Bachar	337	JZ948874	BLASTx	Hypothetical protein *Vicia faba* (AGC78838.1)	74/95 (77%)	8E-460
VfMSAP5	*Eco*RI-AG/*Msp*I-AG	Demethylated	Shoot	Bachar	219	JZ948741	BLASTx	ABC transporter family *Medicago truncatula* (XP_003625677.2)	54/56 (96%)	4E-280
VfMSAP6	*Eco*RI-GG/*Msp*I-AA	Demethylated	Shoot	F177	216	JZ948742	BLASTx	Phosphoenolpyruvate carboxylase *Camellia sinensis* ACU68931.1	61/72 (84%)	5E-320

Table 5