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Research article

24-Epibrassinolide alters DNA cytosine methylation of *Linum usitatissimum* L. under salinity stress

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ABSTRACT

Salinity is a common environmental challenge limiting worldwide agricultural crop yield. Plants employ epigenetic regulatory strategies, such as DNA methylation which relatively allows rapid adaptation to new conditions in response to environmental stresses. Brassinosteroids (BRs) are a novel group of phytohormones recognized as transcription and translation regulators which are able to mitigate the impact of environmental stresses on the plants. In the current investigation, the influence of salinity and 24-epibrassinolide (24-epiBL) was investigated on the extent and pattern of cytosine DNA methylation using methylation-sensitive amplified polymorphisms (MSAP) technique in flax. Upon NaCl (150 mM) exposure, total methylation of CCGG sequences was decreased in comparison to control plants, while 24-epiBL (10^{-8} M) induced total methylation under salinity stress. Sequencing and analysis of six randomly selected MSAP fragments detected genes involved in various biological and molecular processes such as vitamine B1 biosynthesis, protein targeting and localization, post-translational modification and gene regulation. In conclusion, 24-epiBL seed priming could play critical role in regulation of cellular and biological processes in response to salt stress by epigenetic modification and induction of methylation.

1. Introduction

Genetic and epigenetic information are required to build up an organism. In recent years, plant epigenetics has received notable attention by researchers (Hashemi et al., 2012; Karan et al., 2012). Epigenetics refers to heritable variation in gene regulation that can determine the functional state of cells and tissues during differentiation and developmental stages. Genetic variation is necessary for improving crop productivity as well as adaptation to environmental stresses (Hashemi et al., 2012; Karan et al., 2012). Epigenetic changes can be introduced by cytosine methylation of DNA and nucleosomal histone modifications (Tan, 2010). Cytosine methylation process is a universal DNA modification involved in many important biological processes, including transposon proliferation, genomic imprinting, genome plasticity, gene silencing and regulation (Hashemi et al., 2012; Karan et al., 2012).

MSAP is based on digestion with methylation-sensitive restriction endonucleases (*Hpa*II and *Msp*I) followed by amplification of restriction fragments which has been frequently used for the detection of differential sensitivity of DNA cytosine methylation in plants and animals (Hashemi et al., 2012; Fulneček and Kovařík, 2014, 2014). It is a modified AFLP (amplified fragment length polymorphism) technique which *Msp*I and *Hpa*II enzymes are employed as 'frequent-cutters'. These two restriction enzymes recognize the same restriction site (5′—CCGG-3′), but display differential sensitivity to DNA methylation (Shan et al., 2013; Fulneček and Kovařík, 2014).

Plants, as a sessile organism, are often exposed to various biotic (i.e. pathogen infection and insect herbivory) and abiotic stresses (i.e. extreme temperature, drought and salinity), which adversely affect the crop production and yield (Xia et al., 2009; Rattan et al., 2012). However, plants have developed strategies to cope with abiotic challenges and biotic stresses to enhance crop adaptation facing environmental stresses (Chung et al., 2014). In response to abiotic adverse conditions, plants activate several molecular networks via triggering secondary stress signals and induction of epigenetic alteration (Pandey et al., 2017). Recent studies demonstrated the effect of abiotic stresses on epigenetic modifications such as reversible DNA methylation, chromatin remodelling, histone modification and alteration of gene expression leading to both metabolic and stress-responsive pathways linked to plant adaptation mechanisms (Tan, 2010; Shan et al., 2013; Pandey et al., 2017). Although, DNA methylation plays an important

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role to enhance the crop adaptation against stresses, but there is a little information about the role of methylation alteration and its association with gene expression in response to stress (Tan, 2010).

Salinity is considered as one of the important environmental challenges in the world limiting plant growth and productivity (El-Bassiouny and Sadak, 2015). NaCl is the major salt causing salinization with two primary effects on plants (osmotic and ionic) impacting the physiology both at the cellular as well as whole plant (Sh, 2014; Ahmad et al., 2016). Imposition of salinty induces the stress signals in plants which ultimately alters the expression of transcription factor genes (Pandey et al., 2017).

Brassinosteroids (BRs), known as sixth class of phytohormones, have been associated with a wide range of plant physiological and molecular responses (Hu et al., 2016; Wani et al., 2017). Exogenous application of BRs elevates tolerance of plants confronting stresses including drought, salinity and heat (Todorova et al., 2016; Tiwari et al., 2017). BRs are considered ubiquitous in plant kingdom and recognized as regulators of transcription and translation, thereby improving the level of total proteins and enzymes (El-Mashad and Mohamed, 2012; Vardhini and Anjum, 2015) and conferring resistance against biotic and abiotic stresses (El-Mashad and Mohamed, 2012).

Flax (*Linum usitatissimum* L.), one of over 270 species within the family Linaceae, is commercially cultivated as a multipurpose crop due to high amount of protein, linolenic-rich oil, lignans and fiber over the world (Yu et al., 2014). Flax oil is one of the richest sources of essential fatty acids, omega-3 fatty acid (α-linolenic) and omega-6 fatty acid (linoleic) (Sh, 2014). The increasing demand for production of flax has led researchers' interest to investigate physiology and molecular biology of this important industrial crop in response to salinity (Yu et al., 2014).

To the best of our knowledge based on literature review, no research has been conducted on the epigenetic role of BRs in plants under salinity stress so far. In this study, the extent and pattern of cytosine DNA methylation was investigated under salinity exposure and 24-epiBL seed priming using MSAP technique in flax.

2. Materials and methods

2.1. Plant materials and stress treatment

The seeds of *L. usitatissimum* cultivar, TN-97-106 (Var. humil, Meshgin Shahr, Ardabil) were obtained from Agricultural Research Center of West Azerbaijan Province, Urmia, Iran. Based on initial screening, TN-97-106 was identified as a salt tolerant cultivar (data not show). This cultivar had been identified as a cold tolerant variety as well (Ghoreishi et al., 2017). The experiment was designed in a growth chamber at Biotechnology Institute of Urmia University. The growth chamber was set at 250 μmol m^{-2} S^{-1} light intensity, photoperiod of 16-h light and 8-h darkness and temperature of 23 ± 1 °C with 80–90% relative humidity. The surface of the seeds was sterilized in 75% ethanol for 2 min and 5% sodium hypochlorite for 5–10 min, and washed with sterile distilled water 4–6 times. Seeds were soaked either in aqueous solution of 24-epiBL (10^{-8} M) (Sigma Alderich) or distilled water for 8 h. The effective concentration of 24-epiBL was selected based on previous reportes (Rattan et al., 2012; Sharma et al., 2013; Derevyanchuk et al., 2014). After germination in Petri dish, seedlings were cultivated in 15 cm × 15 cm pots in peat moss and perlite 3-1 (v/v). After three weeks, flax plants were divided into four groups and subjected to two salinity levels for 21 days: 1- watered with NaCl (0 mM) as control groups, 2- watered with NaCl (150 mM), 3- seed primed with 24-epiBL (10^{-8} M) and watered with NaCl (0 mM), 4- seed primed with 24-epiBL (10^{-8} M) and watered with NaCl (150 mM). At the end of treatment, electrical conductivity (EC) of 1.0 and 17 (dS m^{-1}) was calculated for drainage water of pots treated with NaCl (0 mM) and NaCl (150 mM), respectively. At the end of the experiment, shoots were collected and frozen in liquid nitrogen and transferred to −80 °C until analyses.

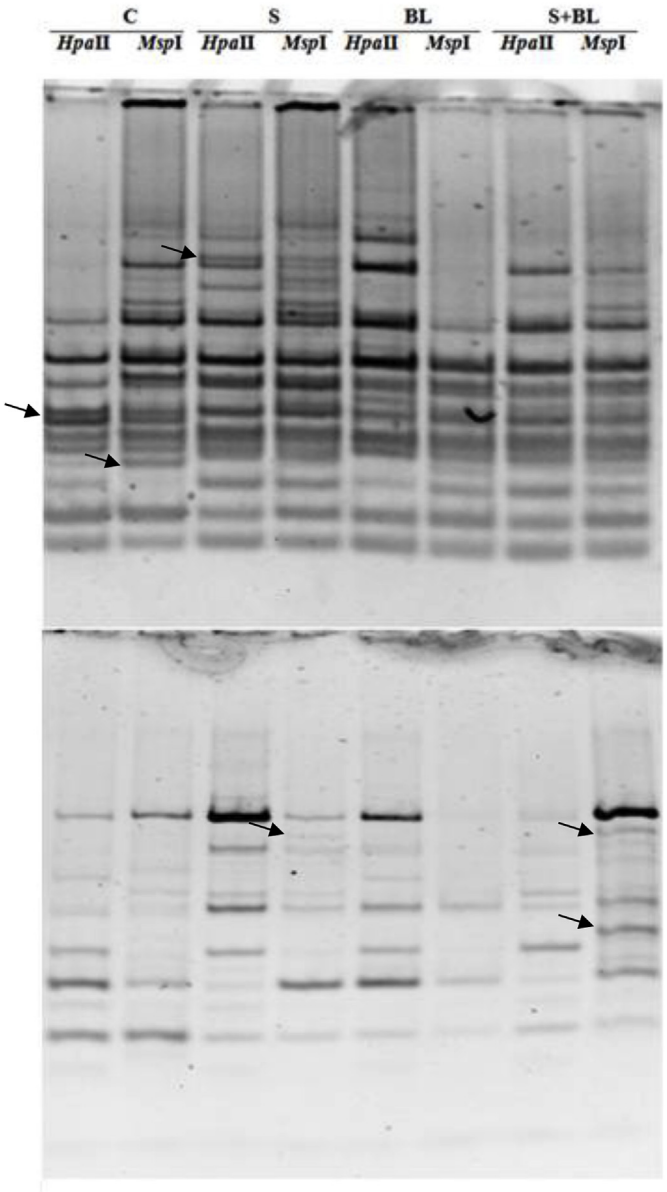

Fig. 1. MSAP patterns detected in *Linum usitatissimum* L. Control (C), NaCl (150 mM) (S), 24-epiBL (10^{-8} M) (BL) and NaCl (150 mM) + 24-epiBL (10^{-8} M) (S + BL).

2.2. MSAP analysis

Total genomic DNA was extracted using the improved CTAB method. The MSAP analysis was performed (Baurens et al., 2008) with minor modifications using a pair of methylation-sensitive restriction enzymes, *Msp*I and *Hpa*II in combination with *Eco*RI. The adapter, pre amplification, and selective amplification primers were listed (Supplementary Table S1). Pre-amplified PCR products were resolved on 1% agarose (Supplementary Fig. S1) and the selective PCR products separated on 6% sequencing gels (Fig. 1) followed by visualization via ethidium bromide staining. The MSAP patterns for displaying the DNA fragments, resulting from the digestions with the *Msp*I and *Hpa*II restriction enzymes, were divided into the following four types: type I represented the presence of bands in both enzyme combinations *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I; type II displayed bands only in *Eco*RI/*Hpa*II digestion but not in the *Eco*RI/*Msp*I, indicating a hemi-methylation profile

Table 1
MSAP-based cytosine methylation levels in *Linum usitatissimum* L. under different treatments Control (C), NaCl (150 mM) (S), 24-epiBL (10^{-8} M) (BL) and NaCl (150 mM) + 24-epiBL (10^{-8} M) (S + BL).

MSAP band type	C	S	BL	S + BL
I	107	124	95	102
II	51	44	36	57
III	57	32	46	28
IV	53	68	91	81
Total sites	268	268	268	268
Total amplified bands	215	200	177	187
Total methylated bands	161	144	173	166
[a] MSAP (%)	60.07	53.73	64.55	61.94
Fully methylated bands	110	100	137	109
[b]Fully methylated ratio(%)	41.04	37.13	51.11	40.67
[c]Hemi-methylated ratio(%)	19.02	16.41	13.43	21.26
[d]Non-methylated ratio(%)	39.92	46.26	35.44	38.05

[a] MSAP (%) = [(II + III + IV)/(I + II + III + IV)] × 100.
[b] Fully methylated ratio (%) = [(III + IV)/(I + II + III + IV)] × 100.
[c] Hemi-methylated ratio (%) = [(II)/(I + II + III + IV)] × 100.
[d] Non-methylated ratio (%) = [(I)/(I + II + III + IV)] × 100.

of the external cytosine (^{HMe}CCGG); type III generated bands in *EcoRI/MspI* digestion but not in the *EcoRI/Hpa*II, showing a complete methylation profile or hemi-methylation of the internal cytosine (C ^{Me}CGG/C ^{HMe}CGG); and type IV revealed the absent bands in both enzyme combinations, demonstrating complete methylation of the external cytosine (^{Me}CCGG), complete methylation of both cytosines (^{Me}C^{Me}CGG), hemi-methylation of both cytosines (^{HMe}C^{HMe}CGG) or polymorphism at the restriction site (Karan et al., 2012; Shan et al., 2013; Monja-Mio et al., 2018). The hemi methylated state of DNA is due to methylation of one DNA strand but not its complementary strand. Percentage of polymorphic MSAP bands was calculated using the following formula (Table 1):

MSAP (%) = [(II + III + IV)/ (I + II + III + IV)] × 100

2.3. Isolation and characterization of amplified fragments

DNA bands showing appropriate polymorphism were excised from agarose gel using Gel and PCR purification mini kit (Favorgen, Taiwan). Eluted bands were sequenced (Macrogene, South Korea) and analyzed by BLAST tool (www.ncbi.nlm.nih.gov). The gene structure prediction was performed by FGENESH (www.softberry.com).

2.4. Real-time PCR analysis

Total RNA was isolated from shoots of flax plants using CTAB method with little modification (Gambino et al., 2008). The integrity and quantity of RNA were determined using 1% agarose gel electrophoresis and spectrophotometer, respectively. The cDNAs were synthesized using RevertAid™First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's instructions. Negative control reactions including no reverse transcriptase (-RT) and no template control (NTC) were considered during cDNA synthesis to assess genomic DNA and reagent contamination, respectively. Specific primers were designed using FastPCR 4.0 and Gen runner 3.05 software (Supplementary Table S2). Real time PCRs were performed in a volume of 12.5 µl in Rotor-Gene Q (QIAGEN, USA) using Maxima SYBER Green/Fluorescein qPCR Master Mix (Fermentas, Germany) according to the manufacturer's recommendations. Temperature conditions were as follows; holding for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 58–60 °C for 30 s and 72 °C for 40 s. The experiment was carried out in three biological replicates (each contains two samples) and three technical repeats. The specificity of the amplicons was verified by melting curve analysis performed from 45 °C to 95 °C with rising by 0.5 °C each step and 1.8% agarose gel electrophoresis. *ELFα* gene was used as a reference gene. Data was analyzed by relative calculation of gene expression using the $2^{-\Delta\Delta ct}$ approach.

2.5. Statistical analysis

The experimental design was based on completely randomized factorial. Data was subjected to analysis of variance using SAS 9.2 software. The mean separations were carried out by Duncan's multiple range tests with the same software. Significance was determined at $P \leq 0.01$ level and the results were expressed as mean values and standard error (SE) of the means.

3. Results

3.1. Methylation analysis

The MSAP pattern of flax genomic DNA was performed using 16 primer combinations under four different conditions: 1- Control, 2-NaCl (150 mM), 3- 24-epiBL (10^{-8} M) and 4-NaCl (150 mM) +24-epiBL (10^{-8} M) (Supplementary Table S1). Based on the presence or absence of bands (Fig. 1), 215 (control) and 200 (salinity) fragments were detected. The 24-epiBL and NaCl + 24-epiBL treatments produced 177 and 187 clear and reproducible DNA bands, respectively (Table 1). Under control condition, total methylation of CCGG sequences was detected 60.07% while 64.55% of total methylation observed in 24-epiBL-treated plants. The lowest percentage of total methylation of CCGG sequences was obtained under NaCl treatment (53.73%) which raised to 61.94% in NaCl + 24-epiBL-treated plants (Table 1). The fully methylated loci were always more than the hemi-methylated loci. Salt stress reduced the number of fully methylated bands while 24-epiBL increased it under salinity stress. The maximum number of fully methylated bands was observed in 24-epiBL treated plants (Table 1).

3.2. Analysis of differentially methylated DNA sequences

All possible banding patterns between control and salinity stress and between salinity and salinity +24-epiBL were compared to identify alterations in cytosine methylation patterns. The differential banding pattern was considered to analyze the methylation and demethylation status of 5′—CCGG-3′ sites (Table 2). The pattern A-D represented monomorphic class in which methylation pattern was similar under different treatments. The patterns E–J indicated cytosine demethylation patterns whereas possible cytosine methylation events induced by salt stress (comparing the control group with salinity) or 24-epiBL (comparing the salinity group with salinity + 24-epiBL) were represented by the K—P patterns. Around 54.85% and 53.73% of the CCGG sites remained unchanged under salinity and 24-epiBL, respectively (Table 2). The percentage of demethylated bands was 23.13% under salt stress which decreased to 21.64% by 24-epiBL seed priming application. On the other hand, 24-epiBL increased the percentage of methylated bands from 22.01% to 24.62% (Table 2).

3.3. Analysis of polymorphic fragment sequences

The generated polymorphic fragments representing methylation/demethylation events were 121 for salinity and 124 for 24-epiBL (Table 2). The size of polymorphic DNA fragments ranged from 130 to 350 bp. Out of the 10 randomly sequenced fragments, six was found to be either within the gene body or within the 1 Kb upstream/downstream regions of protein coding regions. The fragments were distributed on the *Linum* chromosomes 2, 3, 7, 12, 13 and 15 (Table 3). The BLASTX analysis of sequenced fragments revealed three sequences which were homologous to the F-box/FBD/LRR-repeat protein At1g13570-like (F-box), thiamine biosynthetic bi-functional enzyme (TH1) and signal recognition particle receptor subunit alpha-like (SRα)

Table 2
Analysis of DNA methylation patterns under salinity with respect to control condition and salinity + 24-epiBL with respect to salinity condition in *Linum usitatissimum* L. Control (C), NaCl (150 mM) (S), 24-epiBL (10^{-8} M) (BL) and NaCl (150 mM) + 24-epiBL (10^{-8} M) (S + BL).

		Banding Pattern									
Description of Pattern	Class	C		S			S		S + BL		
		*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	Number of bands	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	Number of bands
No change	A	1	1	1	1	61	1	1	1	1	54
	B	1	0	1	0	13	1	0	1	0	11
	C	0	1	0	1	14	0	1	0	1	9
	D	0	0	0	0	59	0	0	0	0	70
	Total					147 (54.85%)					144 (53.73%)
Demethylation	E	1	0	1	1	14	1	0	1	1	12
	F	0	1	1	1	20	0	1	1	1	2
	G	0	0	1	1	6	0	0	1	1	22
	H	0	1	1	0	10	0	1	1	0	3
	I	0	0	1	0	7	0	0	1	0	7
	J	0	0	0	1	5	0	0	0	1	12
	Total					62 (23.13%)					58 (21.64%)
Methylation	K	1	1	1	0	10	1	1	1	0	26
	L	1	1	0	1	9	1	1	0	1	6
	M	1	0	0	1	2	1	0	0	1	1
	N	1	1	0	0	8	1	1	0	0	15
	O	1	0	0	0	17	1	0	0	0	11
	P	0	1	0	0	13	0	1	0	0	7
	Total					59 (22.01%)					66 (24.62%)

A score of 1 and 0 represents presence and absence of bands, respectively. Values in parentheses indicate percentage of bands in each pattern which was determined by dividing the number of bands in each pattern by total number of bands in all three patterns.

proteins located on chromosomes 3, 7 and 13, respectively.

3.4. Expression analysis of polymorphic fragments

In total, three genes (F1, F3 and F5) (Table 3) with methylation changes within the gene body (exon) were subjected to qRT-PCR analysis (Fig. 2). The *F-box* gene expression, methylated in salt treated-plants, was significantly upregulated (19 fold) by salinity stress, and decreased by 24-epiBL (33%) under NaCl treatment (Fig. 2). The expression level of *TH1*, demethylated in salt-treated plants, was significantly declined (98%) under salinity stress, while 24-epiBL enhanced its mRNA level (163 fold) under imposition of NaCl (Fig. 2). The expression of *SRα* gene, methylated in NaCl treated plants, approximately remained constant under salt stress, whereas 24-epiBL significantly upregulated its transcript (14 fold) under saline condition (Fig. 2).

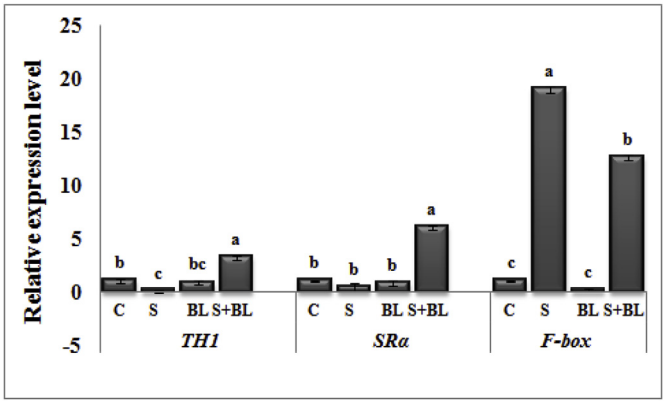

Fig. 2. Relative expression of *TH1*, *SRα* and *F-box* genes in *Linum usitatissimum* L. Control (C), NaCl (150 mM) (S), 24-epiBL (10^{-8} M) (BL) and NaCl (150 mM) + 24-epiBL (10^{-8} M) (S + BL). Means followed by the different letter are significantly different according to Duncan's multiple range test ($P \leq 0.01$).

4. Discussion

Overally, all stresses elicit complex molecular responses in plants, begin with perception of stress and initiation of signal transduction, which ultimately is manifested at cellular, physiological, and developmental levels (Lata et al., 2015). The plant genome is regulated via rearrangement and modification of DNA sequences through epigenetic strategies (Pandey et al., 2017). In the last decade, modulating gene expression through epigenetic modification has been demonstrated in plant stress responses (Chinnusamy and Zhu, 2009; Kumar, 2018; Miao

Table 3
List of selected MSAP polymorphic fragments and their location on flax genome.

Fragment	Methylation status	Description	Accession	*E*-value	Position
F1	Methylated[a]	F-box/FBD/LRR-repeat protein At1g13570-like	XP_021670892.1	1e-150	exon
F2	Demethylated[b]	GPN-loop GTPase 3	OAY76832.1	3e-47	5′-UTR
F3	Demethylated[a]	thiamine biosynthetic bifunctional enzyme TH1	XP_021658964.1	8e-56	exon
F4	Demethylated[b]	zinc finger, CCHC-type	PWA89720.1	1e-93	3′-UTR
F5	Methylated[a]	signal recognition particle receptor subunit alpha-like	XP_021669033.1	6e-139	exon
F6	Demethylated[a]	protein ABHD17C-like isoform X2	XP_008362229.1	7e-72	intron

[a] Methylation and demethylation status of 5′—CCGG-3′ sites with NaCl (Comparing the control group with salinity).
[b] Methylation and demethylation status of 5′—CCGG-3′ sites with 24-epiBL (Comparing the salinity group with salinity + 24-epiBL).

et al., 2018). Several environmental stimuli are known to alter cytosine methylation patterns throughout the genome at specific loci for stress adaptation through regulation of gene expression (Karan et al., 2012; Shan et al., 2013). In this regard, phytohormones such as BRs, as plant growth regulators, are involved in varieties of biological processes as center of stress signaling pathways (Tiwari et al., 2017).

In the present study, the MSAP technique was applied to assess the extent and pattern of DNA methylation in response to salt stress. Moreover, the temporal changes in methylation status of 5′—CCGG-3′ tetranucleotide were analyzed in response to 24-epiBL seed priming under salinity stress. Six genes with cytosine methylation alteration pattern were detected under either salinity or 24-epiBL treatments (Table 3). The F2 and F4 fragments, representing 24-epiBL demethylation, were found to be homologous with sequences of GPN-loop GTPase 3 and zinc finger (CCHC-type proteins), respectively. The four remaining fragments (F1, F3, F5 and F6), representing salt de/methylation, showed high homology with sequences of F-box/FBD/LRR-repeat protein At1g13570-like (F-box), thiamine biosynthetic bi-functional enzyme (TH1), signal recognition particle receptor subunit alpha-like (SRα) and protein ABHD17C-like isoform X2 proteins, respectively (Table 3). These genes are involved in different biological processes and molecular functions such as vitamin B1 biosynthesis, protein targeting and localization, post-translational modification and gene regulation. In this study, the altered DNA methylation pattern in various genes provided clear evidences that epigenetics might play an important role in plant adaptation to adverse environment.

GPN-loop GTPase (GPN), a conserved subfamily of GTPases, has recently been described in archaea and eukaryotes. The GPN functions in a wide-range of cellular processes in the cell such as sister chromatid cohesion, DNA replication, double-strand DNA repair and transcriptional regulation (Hinds, 2011; Alonso et al., 2013). Zinc fingers (ZFs) such as CCHC-type ZFs are among the most structurally diverse protein domains in eukaryotes. They are frequently found in transcription factors and DNA repair enzymes, mediating DNA–protein and protein–protein binding to facilitate multitude biological processes (Hartwig, 2001). The ZFs function is affected by post-translational modifications including phosphorylation, acetylation, methylation or nitrosylation (Kluska et al., 2018). Considering these data, it is proposed that 24-epiBL can participate in regulation of a wide-range of cellular and biological processes in response to salt stress by epigenetic modification.

The S-acylation plays critical roles in cellular physiology such as signaling across membranes and regulating ion, hormone and metabolite transport through membranes (Hemsley, 2017). S-acylation of Ras regulates trafficking between the golgi apparatus and the plasma membrane (Lin and Conibear, 2015). The novel proteins deacylase a/b-hydrolase domain-containing protein 17 members A, B, and C (ABHD17A-C) deacylate N-Ras proteins and promote its re-localization to internal membranes (Lin and Conibear, 2015; Beck et al., 2017; Zaballa and van der Goot, 2018).

Three homologues MSAP loci were selected to study the transcriptional changes: *F-box*, *TH1* and *SRα* genes. The *F-box* genes form one of the largest multigene super families which highly widespread in plants and generally identified as SCF components. However, some F-box proteins function in non-SCF complexes as well, or possess enzyme activity (Kuroda et al., 2002; Stefanowicz et al., 2015). F-box proteins can recognize a wide array of substrates and regulate many important biological processes such as hormonal responses, lateral root formation, branching, senescence, light signaling, circadian clock regulation, floral development, self-incompatibility, and responses to abiotic and biotic stresses (Jia et al., 2017). In our investigation, the expression of *F-box* gene was significantly increased in response to salt stress while 24-epiBL decreased its expression under salinity compared to NaCl-treated plants (Fig. 2). Jia et al. (2017) reported a total of 51 *GmFBXs* genes in soybean which were differentially expressed in response to salt stress according to the RNA-seq data (Jia et al., 2017). Moreover, an F-box protein called TaFBA1 has recently been identified in wheat which is upregulated upon water and salt stresses as well as ABA treatment (Zhou et al., 2014).

Thiamine, also known as vitamin B1, is required for key metabolic processes, for example carbohydrate catabolism, NADPH and ATP synthesis and in the formation of nucleic acids in cellular organisms. However, thiamine is prone to destruction under stress conditions. The thiazole and pyrimidine moieties are joined together by the bi-functional enzyme (TH1) with both HMP-P kinase and TMP synthase activities to form thiamine monophosphate (TMP). Then, thiamine monophosphate phosphatase (TH2) dephosphorylates TMP to form thiamine (Rapala-Kozik et al., 2012; Kamarudin et al., 2017). In this study, the expression of *TH1* was declined under salinity conditions and 24-epiBL induced its transcripts compared to NaCl—treated plants (Fig. 2). Rapala-Kozik et al. (2012) showed higher expression of thiamine biosynthesis genes, such as *TH1* during early response of Arabidopsis seedlings to oxidative, salt and osmotic stresses. Interestingly, decline in gene expression was reported under salt stress over a longer time which suggests generation of new stimuli and adaptation processes (Rapala-Kozik et al., 2012).

All living organisms and eukaryotic organelles like chloroplasts possess protein targeting. The SR, a heterodimeric membrane protein, consists of a 70 kD peripheral membrane protein (SRα) and 30 kDa integral membrane protein (SRβ) (Jadhav et al., 2015). In response to salt stress, no significant change was observed in the expression of *SRα* gene, while 24-epiBL upregulated its transcript upon salinity condition in comparison to NaCl-treated plants (Fig. 2). It might be concluded that 24-epiBL seed priming had a positive impact on targeting and insertion of membrane proteins in flax under salinity.

In our analysis, the *F-box* and *SRα* genes were found to be methylated and the *TH1* gene was demethylated in gene body regions under NaCl exposure. The high induction was observed for *F-box* mRNA level in response to NaCl imposition while, the level of *TH1* transcript was reduced upon salt stress. Moreover, despite to the belief that DNA methylation is associated with inhibition of transcription, weak relationship has been reported between the hypermethylation status and transcript level (Vaillant et al., 2006). Similar to our data, gene expression level was inversely associated with cytosine methylation in rice under saline condition (Karan et al., 2012). Shukla et al. (2011) demonstrated a possible mechanistic link between methylation in gene body region, especially in the intronic elements, and alternative pre-mRNA splicing. The DNA methylation of coding regions (Body methylation) has been found to be conserved across eukaryotes with functionality of aberrant expression prevention from intragenic promoters or enhancement of the accuracy of splicing. Body methylation may even be important than unmethylation (Takuno and Gaut, 2011). Bisulfite sequencing and qRT-PCR results, obtained by Kumar et al. (2016) also revealed correlation between gene body methylation and gene expression. Moreover, Shan et al. (2013) showed elevation in transposon gene expression level after the occurrence of demethylation in *Zea mays* plants in response to cold stress.

Our results suggest that epigenetic changes in the flax genome may be considered as alternative regulatory mechanism to sense and respond to salt stress through modification of responsive genes. The epigenetic alterations might cause restricted functionality during exposure to adverse conditions and consequently lead to abnormal phenotype (Pandey et al., 2017). In addition, MSAP analysis detected a few changes in methylation in presence to 24-epiBL. A few changes in DNA methylation has also been reported under 0.5 μmol/L Homobrassinolide (HBL) application based on MSRF analysis (Temel and Gozukirmizi, 2012). Increased tolerance to diverse abiotic stresses has been shown under exposing plants to BR during early seedling development too (Kagale et al., 2007). The BES1 and BZR1, as the major BR-related transcription factors, are known in repression of BR biosynthetic genes, light signaling components and chloroplast development-related genes by epigenetic reprogramming of gene expression through DNA

methylation, histone modification and chromatin remodelling (Jaenisch and Bird, 2003; Ryu et al., 2014).

5. Conclusion

To our best of knowledge, this is the first report on the effects of 24-epiBL on DNA methylation in flax upon salinity exposure. Analysis of the methylation changes demonstrated that 24-epiBL slightly reduced demethylation and induced methylation under saline condition. Moreover, expression of selected gene body de/methylated genes showed alteration under different treatments. Thus, our results demonstrate that epigenetic modification through cytosine methylation, especially in gene body region, might be an important regulatory mechanism in flax plants under salinity stress. Based on the findings of this study, 24-epiBL seed priming could play critical role in flax adaptation against salinity stress via epigenetic modification and alteration of gene expression. However, a much more extensive analyses will be required to confirm this.

Author contribution statement

FR designed and conducted the research; LA performed the research and analyzed the data; LA and FR wrote the paper; BAM helped in data analysis. All authors have read and approved the final manuscript.

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Appendix A. Supplementary data

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References

Ahmad, P., Abdel Latef, A.A., Hashem, A., Abd_Allah, E.F., Gucel, S., Tran, L.S.P., 2016. Nitric oxide mitigates salt stress by regulating levels of osmolytes and antioxidant enzymes in chickpea. Front. Plant Sci. 7, 347.

Alonso, B., Beraud, C., Meguellati, S., Chen, S.W., Pellequer, J.L., Armengaud, J., Godon, C., 2013. Eukaryotic GPN-loop GTPases paralogs use a dimeric assembly reminiscent of archeal GPN. Cell Cycle 12, 463–472.

Baurens, F.C., Causse, S., Legavre, T., 2008. Methylation-sensitive amplification polymorphism (MSAP) protocol to assess CpG and CpNpG methylation in the banana genome. Fruits 63, 117–123.

Beck, M.W., Kathayat, R.S., Cham, C.M., Chang, E.B., Dickinson, B.C., 2017. Michael addition-based probes for ratiometric fluorescence imaging of protein S-depalmitoylases in live cells and tissues. Chem. Sci. 8, 7588–7592.

Chinnusamy, V., Zhu, J.K., 2009. Epigenetic regulation of stress responses in plants. Curr. Opin. Plant Biol. 12, 133–139.

Chung, Y., Kwon, S.I., Choe, S., 2014. Antagonistic regulation of Arabidopsis growth by brassinosteroids and abiotic stresses. Mol. Cell. 37, 795.

Derevyanchuk, M.V., Grabelnyh, O.I., Litvinovskaya, R.P., Voinikov, V.K., Sauchuk, A.L., Khripach, V.A., Kravets, V.S., 2014. Influence of brassinosteroids on plant cell alternative respiration pathway and antioxidant systems activity under abiotic stress conditions. Biopolym. Cell 30, 436–442.

El-Bassiouny, H., Sadak, M.S., 2015. Impact of foliar application of ascorbic acid and α-tocopherol on antioxidant activity and some biochemical aspects of flax cultivars under salinity stress. Acta Biol. Colomb. 20, 209–222.

El-Mashad, A.A.A., Mohamed, H.I., 2012. Brassinolide alleviates salt stress and increases antioxidant activity of cowpea plants (*Vigna sinensis*). Protoplasma 249, 625–635.

Fulneček, J., Kovařík, A., 2014. How to interpret methylation sensitive amplified polymorphism (MSAP) profiles? BMC Genet. 15, 2.

Gambino, G., Perrone, I., Gribaudo, I., 2008. A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. Phytochem. Anal. 19, 520–525.

Ghoreishi, M., Rahmani, F., Mandoulakani, B.A., Gorttapeh, A.H., 2017. Impact of variety on resistance to cold stress at physiological levels in '*Linum usitatissimum*'. Plant Omics 10, 269.

Hartwig, A., 2001. Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antioxidants Redox Signal. 3, 625–634.

Hashemi, S.H., Nematzadeh, G., Askari, H., Ghasemi, Y., 2012. Pattern of DNA cytosine methylation in *Aeluropus littoralis* during temperature stress. J. Plant Mol. Breed. 1, 16–24.

Hemsley, P.A., 2017. An outlook on protein S-acylation in plants: what are the next steps? J. Exp. Bot. 68, 3155–3164.

Hinds, P.W., 2011. Unbearable stress: collapse of the SSeCKS/AKAP12 scaffold leads to senescence and transformation. Cell Cycle 10 2836-2835.

Hu, Y., Xia, S., Su, Y., Wang, H., Luo, W., Su, S., Xiao, L., 2016. Brassinolide increases potato root growth in vitro in a dose-dependent way and alleviates salinity stress. BioMed Res. Int. 3, 1–11.

Jadhav, B., McKenna, M., Johnson, N., High, S., Sinning, I., Pool, M.R., 2015. Mammalian SRP receptor switches the Sec61 translocase from Sec62 to SRP-dependent translocation. Nat. Commun. 6, 10133.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. 33, 245.

Jia, Q., Xiao, Z.X., Wong, F.L., Sun, S., Liang, K.J., Lam, H.M., 2017. Genome-wide analyses of the soybean F-box gene family in response to salt stress. Int. J. Mol. Sci. 18, 818.

Kagale, S., Divi, U.K., Krochko, J.E., Keller, W.A., Krishna, P., 2007. Brassinosteroid confers tolerance in Arabidopsis thaliana and Brassica napus to a range of abiotic stresses. Planta 225, 353–364.

Kamarudin, A.N., Lai, K.S., Lamasudin, D.U., Idris, A.S., Yusof, B., Zetty, N., 2017. Enhancement of thiamine biosynthesis in oil palm seedlings by colonization of endophytic fungus Hendersonia toruloidea. Front. Plant Sci. 8, 1799.

Karan, R., DeLeon, T., Biradar, H., Subudhi, P.K., 2012. Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes. PLoS One 7, e40203.

Kluska, K., Adamczyk, J., Krężel, A., 2018. Metal binding properties, stability and reactivity of zinc fingers. Coord. Chem. Rev. 367, 18–64.

Kumar, G., Rattan, U.K., Singh, A.K., 2016. Chilling-mediated DNA methylation changes during dormancy and its release reveal the importance of epigenetic regulation during winter dormancy in apple (*Malus* x *domestica* Borkh. PLoS One 11, e0149934.

Kumar, S., 2018. Epigenetic memory of stress responses in plants. J. Phytochem. Biochem. 2 e102.

Kuroda, H., Takahashi, N., Shimada, H., Seki, M., Shinozaki, K., Matsui, M., 2002. Classification and expression analysis of Arabidopsis F-box-containing protein genes. Plant Cell Physiol. 43, 1073–1085.

Lata, C., Muthamilarasan, M., Prasad, M., 2015. Drought stress responses and signal transduction in plants. In: Pandey, G.K. (Ed.), Elucidation of Abiotic Stress Signaling in Plants. Springer, New York, NY, pp. 195–225.

Lin, D.T.S., Conibear, E., 2015. ABHD17 proteins are novel protein depalmitoylases that regulate N-Ras palmitate turnover and subcellular localization. Elife 4, e11306.

Miao, R., Wang, M., Yuan, W., Ren, Y., Li, Y., Zhang, N., Zhang, J., Kronzucker, H.J., Xu, W., 2018. Comparative analysis of Arabidopsis ecotypes reveals a role for brassinosteroids in root hydrotropism. Plant Physiol. 176, 2720–2736.

Monja-Mio, K.M., Quiroz-Moreno, A., Herrera-Herrera, G., Montero-Muñoz, J.L., Sánchez-Teyer, F., Robert, M.L., 2018. Analysis of two clonal lines (embryogenic and non-embryogenic) of *Agave fourcroydes* using AFLP and MSAP. Am. J. Plant Sci. 9, 745.

Pandey, G., Yadav, C.B., Sahu, P.P., Muthamilarasan, M., Prasad, M., 2017. Salinity induced differential methylation patterns in contrasting cultivars of foxtail millet (*Setaria italica* L.). Plant Cell Rep. 36, 759–772.

Rapala-Kozik, M., Wolak, N., Kujda, M., Banas, A.K., 2012. The upregulation of thiamine (vitamin B 1) biosynthesis in Arabidopsis thaliana seedlings under salt and osmotic stress conditions is mediated by abscisic acid at the early stages of this stress response. BMC Plant Biol. 12, 2.

Rattan, A., Kapoor, N., Bhardwaj, R., 2012. Role of brassinosteroids in osmolytes accumulation under salinity stress in Zea mays plants. Int. J. Sci. Res. 3, 1822–1827.

Ryu, H., Cho, H., Bae, W., Hwang, I., 2014. Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of ABI3. Nat. Commun. 5, 4138.

Sh, S.M., 2014. Role of ascorbic acid and α tocopherol in alleviating salinity stress on flax plant (*Linum usitatissimum* L.). J. Stress Physiol. Biochem. 10, 93–111.

Shan, X., Wang, X., Yang, G., Wu, Y., Su, S., Li, S., Liu, H., Yuan, Y., 2013. Analysis of the DNA methylation of maize (*Zea mays* L.) in response to cold stress based on methylation-sensitive amplified polymorphisms. J. Plant Biol. 56, 32–38.

Sharma, I., Ching, E., Saini, S., Bhardwaj, R., Pati, P.K., 2013. Exogenous application of brassinosteroid offers tolerance to salinity by altering stress responses in rice variety Pusa Basmati-1. Plant Physiol. Biochem. 69, 17–26.

Shukla, S., Kavak, E., Gregory, M., Imashimizu, M., Shutinoski, B., Kashlev, M., Oberdoerffer, P., Sandberg, R., Oberdoerffer, S., 2011. CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. Nature 479, 74.

Stefanowicz, K., Lannoo, N., Van Damme, E.J., 2015. Plant F-box proteins–judges between life and death. Crit. Rev. Plant Sci. 34, 523–552.

Takuno, S., Gaut, B.S., 2011. Body-methylated genes in Arabidopsis thaliana are functionally important and evolve slowly. Mol. Biol. Evol. 29, 219–227.

Tan, M.P., 2010. Analysis of DNA methylation of maize in response to osmotic and salt stress based on methylation-sensitive amplified polymorphism. Plant Physiol. Biochem. 48, 21–26.

Temel, A., Gozukirmizi, N., 2012. Effects of homobrassinolide in barley callus culture. Plant Soil Environ. 58, 441–445.

Tiwari, S., Lata, C., Singh Chauhan, P., Prasad, V., Prasad, M., 2017. A functional genomic perspective on drought signalling and its crosstalk with phytohormone-mediated

signalling pathways in plants. Curr. Genom. 18, 469–482.
Todorova, D., Talaat, N.B., Katerova, Z., Alexieva, V., Shawky, B.T., 2016. Polyamines and brassinosteroids in drought stress responses and tolerance in plants. In: In: Ahmad, P. (Ed.), Water Stress and Crop Plants: A Sustainable Approach, vol. 2. First Edition, pp. 608–627.
Vaillant, I., Schubert, I., Tourmente, S., Mathieu, O., 2006. MOM1 mediates DNA-methylation-independent silencing of repetitive sequences in Arabidopsis. EMBO Rep. 7, 1273–1278.
Vardhini, B.V., Anjum, N.A., 2015. Brassinosteroids make plant life easier under abiotic stresses mainly by modulating major components of antioxidant defense system. Front. Environ. Sci. 2, 67.
Wani, A.S., Hayat, S., Ahmad, A., Tahir, I., 2017. Efficacy of brassinosteroid analogues in the mitigation of toxic effects of salt stress in *Brassica juncea* plants. J. Environ. Biol. 38, 27.
Xia, X.J., Wang, Y.J., Zhou, Y.H., Tao, Y., Mao, W.H., Shi, K., Asami, T., Chen, Z., Yu, J.Q., 2009. Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol. 150, 801–814.
Yu, Y., Huang, W., Chen, H., Wu, G., Yuan, H., Song, X., Kang, Q., Zhao, D., Jiang, W., Liu, Y., Wu, J., 2014. Identification of differentially expressed genes in flax (*Linum usitatissimum* L.) under saline–alkaline stress by digital gene expression. Gene 549, 113–122.
Zaballa, M.E., van der Goot, F.G., 2018. The molecular era of protein S-acylation: spotlight on structure, mechanisms, and dynamics. Crit. Rev. Biochem. Mol. Biol. 53, 420–451.
Zhou, S., Sun, X., Yin, S., Kong, X., Zhou, S., Xu, Y., Luo, Y., Wang, W., 2014. The role of the F-box gene TaFBA1 from wheat (*Triticum aestivum* L.) in drought tolerance. Plant Physiol. Biochem. 84, 213–223.