The Dynamic Changes of DNA Methylation and Histone Modifications of Salt Responsive Transcription Factor Genes in Soybean

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Abstract

Epigenetic modification contributes to the regulation of gene expression and plant development under salinity stress. Here we describe the identification of 49 soybean transcription factors by microarray analysis as being inducible by salinity stress. A semi-quantitative RT-PCR-based expression assay confirmed the salinity stress inducibility of 45 of these 49 transcription factors, and showed that ten of them were up-regulated when seedlings were exposed to the demethylation agent 5-aza-2-deoxycytidine. Salinity stress was shown to affect the methylation status of four of these ten transcription factors (one MYB, one b-ZIP and two AP2/DREB family members) using a combination of bisulfite sequencing and DNA methylation-sensitive DNA gel blot analysis. ChiP analysis indicated that the activation of three of the four DNA methylated transcription factors was correlated with an increased level of histone H3K4 trimethylation and H3K9 acetylation, and/or a reduced level of H3K9 demethylation in various parts of the promoter or coding regions. Our results suggest a critical role for some transcription factors’ activation/repression by DNA methylation and/or histone modifications in soybean tolerance to salinity stress.

Introduction

Soybean (Glycine max (L.) Merr.) is an important source of protein and oil in both the human and domestic animal diet. As for most crop species, its productivity is significantly compromised by soil salinity [1], but, like most plants, it has evolved a variety of mechanisms to aid its survival under environmental stress. The expression of many plant genes is altered by salinity stress; some of these encode aspects of cellular metabolism and stress tolerance, while others are regulatory in nature [1,2]. Transcription factors (TFs), which belong to the latter class, have been classified into a number of families on the basis of their sequence, and some members of the MYB, NAC, b-ZIP and AP2-DREB families have been shown to be intimately involved in the stress response [3,4,5,6,7,8]. Such as, the heterologous expression of three soybean MYB and three b-ZIP TFs in Arabidopsis thaliana improved its response to salinity and freezing stress [9,10]. Similarly the heterologous expression of GmDREB2 was able to enhance the drought and salinity tolerance of tobacco [11], as did the over-expression of either GmNAC11 or GmNAC20 for soybean [12].

Once a plant detects the onset of stress, TFs characteristically respond by inducing the expression of a cascade of downstream targets. However, their activation is in part also dependent on their chromatin structure, which is largely determined by epigenetic means [13,14,15,16]. Cytosine methylation within the promoter sequence has been shown to underlie numerous cases of gene down-regulation or silencing [17,18,19,20,21]. DNA methylation in the plant genome mostly at CG dinucleotides and CNG trinucleotides, but also at an asymmetrical sequence contexts CNN (N is any nucleotide but G) [22,23,24]. The N terminus of the histone molecule can be acetylated, phosphorylated, methylated, ubiquitinated or ribosylated [25]. The presence of the trimethylated form of histone H3K4 and of the acetylated form of H3K9 in the promoter region have been frequently associated with transcriptional activation, while that of the dimethylated form of H3K9 represses it [26,27,28]. Sometimes, H3K9 methylation can trigger cytosine methylation in both Neurospora crassa [29] and A. thaliana [30], while cytosine methylation at the CNG trinucleotide appears to be partially dependent on the activity of a histone methyltransferase [29,31,32].

A number of examples where epigenetic modification has contributed to the regulation of gene expression during periods of environmental stress have been presented. In particular, the low temperature induced expression of the maize gene ZmMII has been correlated with a reduction of DNA methylation in its nucleosome core [33]. In tobacco, several stress agents are known to promote demethylation in the NtGPDL coding sequence, leading to alterations in its level of expression [34]. The submergence of rice seedlings reduces histone H3K4 trimethylation and acetylation in genes encoding both alcohol dehydrogenase and pyruvate decarboxylase, leading to their up-regulation [35]. In A. thaliana, the drought-induced expression of a number of stress-responsive
genes has been associated with an increase in H3K4 trimethylation and H3K9 acetylation [36]. Here, we set out to document the induction by salinity stress of DNA methylation and histone modification in a number of salinity responsive soybean TFs, and to identify what relationship there is, if any, between the expression of a TF and the epigenetic status of its promoter sequence. In addition, since to date no systematic attempt has been made to investigate the dynamics and reversibility of both DNA and histone modification over the course of a stress episode, we have explored this feature focusing on four salinity stress inducible soybean TFs.

**Materials and Methods**

**Exposure of soybean seedlings to salinity stress and 5-ADC treatment**

Seedlings of the soybean cultivar Williams 82 were grown in vermiculite under a 16h photoperiod at 25°C for 14 days before being exposed to stress treatment. Once the seedlings had been removed from the vermiculite and their roots rinsed in water, they were then treated with either 150 mM NaCl for 1h, 3h, 6h, 12h or 24h, or with 50 μM 5-aza-2-deoxycytidine (5-ADC) for 12h, 24h, 48h or 72h. RNA and DNA was extracted from snap-frozen plants both before the stress treatment had begun and then at each time interval. Mock treatments (ddH2O only) were included as a control. RNA was prepared from 0.2 g plant material using the TRizol (Invitrogen) reagent, following the manufacturer’s protocol, and DNA was extracted from 1 g plant material using a DNeasy Plant Mini kit (Qiagen).

**Microarray analysis**

RNA were isolated from the mock (M0, M1, M3, M6, M12, M24) and salinity treated (S0, S1, S3, S6, S12, S24) seedlings. 0.5 μg RNA that extracted from each time point of the mock and salinity-stressed seedlings were mixed respectively to obtain the mock and salinity-stressed RNA pools, and then they were used to synthesize the cDNA. The cDNA was labeled with biotin, and then hybridized to an Affymetrix soybean Genome Array according to the manufacturer’s instructions (15h in a rotating hybridization ovenset at 45°C and 60 rpm). After the hybridization, the microarrays were scanned using a GeneChip® Scanner 3000 (Affymetrix, P/N 00-00212). Then the scaling factor, background, noise, and percentage presence were calculated according to the Affymetrix Data Mining Tool protocols (Affymetrix). All resulting datasets were filtered using the absolute call metric (present or absent) implemented within Microsoft Access (Microsoft Corporation, Redmond, WA) and the microarray data were processed in an R (v2.7.0) environment, using the LIMMA package [37]. Quantile normalization was performed. A single repeat microarray analysis for each group was performed.
Semi-quantitative RT-PCR (sqRT-PCR) and quantitative real-time RT-PCR (qRT-PCR) were employed to quantify transcript levels more precisely. RNA was extracted from 0.2 g of seedling material ground in liquid nitrogen by the addition of 1 ml TRIZOL reagent (Invitrogen) and treated with RNase-free DNase I. A 3 μg aliquot of total RNA was used to generate the first cDNA strand with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. A 1 μl aliquot of this cDNA was used as the template for a 22–34 cycle sqRT-PCR, where the cycling regime was 94°C/30 s, 55°C/30 s, 72°C/30 s.

A fragment of the soybean TUBULIN gene (Genbank accession AY907703) was used as a reference. Primer sequences are given in Table S2. Each 15 μl qRT-PCR contained 7.5 μl Maxima SYBR Green qPCR Master mix buffer (Roche), 0.5 μl 10 μM specific primers, 1.5 μl of a 1:10 dilution of cDNA and 5.5 μl ddH2O. The cycling regime consisted of a denaturation step (95°C/3 min) followed by 18–35 cycles of 95°C/30 s, 60°C/15 s, 72°C/15 s, and a fragment of the soybean TUBULIN gene (GenBank accession AY907703) was used as an internal control. Primer sequences are given in Table S2. The relative expression level of the target sequence was determined using the 2^ΔΔCt method [38]. Each estimate was derived from the mean of three independent biological replicates.

Bisulfite DNA sequencing

A 2 μg DNA aliquot was dissolved in 50 μl ddH2O and denatured by adding 3.5 μl 3 M NaOH and incubating for 30 min at 42°C. Thereafter, 510 μl 2.3 M sodium bisulfite (pH 5.0), 30 μl 10 mM hydroquinone and 65 μl ddH2O were added, and the solution overlayed with mineral oil and held for 16h at 55°C. The DNA was recovered using a Wizard® DNA Clean-Up System kit (Promega A7280), and a 90 μl aliquot treated with 10 μl 3 M NaOH for 15 min at 37°C, then neutralized by adding 70 μl 10 M ammonium acetate. Finally, the DNA was precipitated by adding 400 μl ethanol and 10 μl glycogen, and re-suspended in 50 μl ddH2O to provide the template for a series of PCRs based on the gene-specific primers listed in Table S2. A fragment of Glyma20g32730 featuring many CG, CNG and CNN sites was amplified from genomic DNA, then inserted into pMD18-T vector and transferred into Dm- E. coli strain JM110. The plasmid was released from the bacterial cells by the plasmid extraction kit (TianGen. Cat. DP103-03) and treated with bisulfite in parallel with the soybean genomic DNA as a control to monitor the transformation efficiency of unmethylated cytosine to thymine. The subsequent PCR consisted of 34–37 cycles of 94°C/30 s, 55°C/30 s, and 72°C/40 s. The resulting amplicons were purified with a Wizard® DNA Clean-Up System kit, ligated into the pMD18-T vector (TaKaRa) and transferred into E. coli for sequencing. Ten clones from each amplicon were sequenced.
Figure 3. Methylation status of the promoter region of four salinity-responsive TFs in untreated (S0) and salinity-stressed (S1–S24) seedlings (S1: 1h, S3: 3h, S6: 6h, S12: 12h, S24: 24h). (a) The black and white boxes indicate, respectively, exon and untranslated regions. The short bars annotated with "I, II, III", "a" or "b" indicate, respectively, the sequences subjected to ChIP analysis, genomic bisulfite sequencing and those used as probes for Southern blotting. The long vertical bars marked "c" display the distribution of CG dinucleotides (marked with red vertical lines), and CNG (blue vertical lines) and CNN (black vertical lines) trinucleotides. The red vertical lines marked with a rectangular indicate CCGG sites analyzed by Southern blotting. The thick black vertical lines represent the proportion of methylated cytosine. Ten positive clones from each gene’s amplicon were sequenced. The data reflect the outcome of three independent experiments, and error bars represent standard error (SD). (b) The efficiency of the bisulfite treatment to transform unmethylated cytosine to thymine. A fragment of Glyma20g32730 with numerous cytosines was cloned into Dm-E. coli cells and the plasmid was treated with bisulfite in parallel with the soybean genomic DNA. All clones processed showed a transformation rate >99.7%. (c) Methylation-sensitive DNA gel blot analysis of non-stressed (S0) and salinity-stressed seedlings (S1–S24). Genomic DNA was digested to generate large fragments, then with one or other of the restriction enzymes HpaII or MspI. Hybridization probes indicated. A DNA fragment amplified from the probe sequence was used as a positive control (+), and ddH2O was used as a negative control (−). doi:10.1371/journal.pone.0041274.g003
The process was repeated three times using biologically independent samples.

Chromatin immunoprecipitation (ChIP) assay

The ChIP protocol was modified from that of Johnson et al. (2002). Briefly, 1 g of plant tissue was fixed by immersion in 1% v/v formaldehyde under vacuum for 10 min. The extracted DNA/protein complex was then sheared by sonication to a size range of ~100–1000 bp. After centrifugation, the complex was immunoprecipitated by challenging with H3K9ac, H3K4me3 and H3K9me2 antibodies (Millipore cat. 07–392, 07–473 and 05–768R) at a titer of 1:100. The residual protein was degraded by the addition of 10 µl (20 mg/ml) proteinase K, followed by a phenol/chloroform extraction. A 2 µl aliquot of the final solution was used as a template for qRT-PCR analysis as described above. A 1000x diluted input DNA (Input) obtained from 500 µl of extract was purified in parallel with the immunoprecipitated samples as a control, and ChIP reactions were also performed in the absence of antibody (No AB) to detect the occurrence of any non-specific binding. Relative levels of H3K9 acetylation, H3K9 dimethylation and H3K4 trimethylation were normalized to an internal control (GenBank accession AY907703). The sequences of all PCR primers used are given in Table S2. The mean and standard deviation are shown for three independent ChIP experiments and the significance of differences between means assessed with a t test.

Methylation-sensitive Southern blot analysis

Genomic DNA (100 µg) extracted from both non-stressed and salinity-stressed seedlings was treated for at least 6 h with 100 U of the appropriate restriction enzymes (EcoRI and NdeI for Glyma11g02400; SacI for Glyma16g27950; BglII for Glyma20g230840; SacI and BglII for Glyma08g41450) (TaKaRa) to generate large fragments containing the target sequences. The digested DNA was extracted by phenol/chloroform and divided into two equal aliquots, one of which was treated with HpaII and the other with its schizomer MspI [39]. The digested DNA was re-extracted, electrophoretically separated through an 0.8% agarose gel and transferred onto a Hybond N+ membrane (Amersham). Probes for each gene were designed to detect the methylation status within the target sequence that analysed by genomic bisulfite sequencing.

About 3 µg of probe DNA was labeled using a DIG-High Prime kit (Roche), and the subsequent hybridization and detection procedure was performed using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche), according to the manufacturer’s instructions. The positive control consisted of a 100x diluted DNA fragment that amplified from the genomic DNA in the same regions that prepared for probes, while the negative control was ddH2O.

Results

The identification of salinity stress responsive TFs in soybean

A set of differentially expressed genes were identified by comparing the soybean Affymetrix microarray profiles generated by probing with RNA extracted from salinity-stressed and non-stressed plants. We mainly focused on the four groups of AP2/EREB, bZIP, NAC and MIB transcription factors that have been verified for salt stress in Arabidopsis or other plants. Of the 1,335 MIB, NAC, AP2/DREB and b-ZIP TFs represented on the microarray, 49 appeared to be up-regulated (fold change of hybridization signal >2, p<0.01) by salinity stress. These consisted of 15 (of 448) GmMIBs, 9 (of 226) GmNACs, 16 (of 426) GmAP2/DREBs and 9 (of 235) Gmb-ZIPs (Table S1). When the expression of these 49 TFs was assayed by sqRT-PCR in both mock-stressed (M0–M24) and salinity-stressed soybean seedlings (S1–S24) with gene-specific primers (Table S1), 14 of the GmMIBs, 8 of the GmNACs, 15 of the GmAP2/DREBs and 8 of the Gm-ZIPs were confirmed to be markedly induced by salinity stress (Figure 1). Expression pattern analysis indicated that 19 of them were strongly induced at a relatively early stage of exposure to salinity (1–3 h), while the others were induced somewhat later (6–24 h).

Expression of the salinity induced TFs in the presence of 5-ADC

The expression of the 45 salinity-induced TFs was then monitored in the mock treated (M0–M72) and the seedlings that exposed to 5-ADC for various periods (A0–A72). As a result, ten of the them showed higher levels of expression in treated (M0–M72) than in mock-treated (A0–A72) seedlings; these ten TFs consisted...
of four GmMYB (Glyma11g02400, Glyma20g30840, Glyma15g07230, Glyma12g34650), one GmNAC (Glyma15g08480), four GmAP2/ DREB (Glyma20g32730, Glyma20g30840, Glyma16g27950, Glyma10g00980) and one Gmb-ZIP genes (Glyma08g41450) (Figure 2).

The expression level of nine of these TFs was very low for the first 12h of exposure, but thereafter rose substantially; the exception was the Gmb-ZIP Glyma08g41450, the expression of which was induced somewhat earlier.

Figure 5. Promoter methylation status in four salinity-responsive TFs in non-treated (A0) and 5-ADC treated seedlings (A12–A72).

For Figure legend please refer to Figure 3 legend.

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Figure 6. Expression, DNA methylation and histone modification status of *Glyma11g02400, Glyma16g27950, Glyma08g41450* and *Glyma20g30840* in none treated (S0) and salinity-stressed (S1–S24) seedlings. (a) Relative H3K9 demethylation, acetylation and H3K4
Methylation status as affected by salinity stress

To investigate the DNA methylation status of above candidate genes under salinity stress, the sequence corresponding to the translation start codon and the promoter region of the ten TFs was subjected to bisulfite sequencing. First, the efficiency of the sodium bisulfite treatment to convert cytosine to thymine was estimated. The efficiency of the sodium bisulfite treatment to convert cytosine to thymine in the cytosine rich segment of Glyma20g32730 was estimated to be 99.7%. Bisulfite sequencing result indicated that the Glyma11g02400, Glyma08g41450, Glyma16g27950 and Glyma20g30840 promoters all appeared to be differentially methylated by the imposition of salinity stress (Figure 3a, b, Figure S1), but those of the other six genes were largely non-methylated (Figure S2). In the Glyma11g02400 promoter from position -518 to -274, most of the cytosines were demethylated following exposure to salinity stress for 1–24h (Figure 3a). In the immediate downstream region of the Glyma16g27950 transcription start codon (+24 to +233), about 35% of the cytosines were methylated before the salinity stress was imposed and for the first three hours of stress, but thereafter only few methylated cytosines remained (Figure 3a). In the Glyma20g30840 promoter region 1 (~87 to +163), 51% of the cytosines were methylated prior to exposure to salinity stress, but this proportion fell to 27% after 1h, 12% after 3h, and to even lower levels as the stress was prolonged further (Figure 3a); meanwhile in region 2 of the same promoter (~163 to -405), 42% of the cytosines were methylated at the start of the stress period, and this proportion hardly altered thereafter (Figure 3a). In the Glyma08g41450 region immediately downstream of the transcription start codon (+24 to +233), 35% of the cytosines were methylated prior to the imposition of stress, and the same proportion was maintained throughout (Figure 3a).

DNA methylation-sensitive Southern blotting was applied to verify these observations. The restriction fragments including Glyma11g02400, Glyma16g27950 and Glyma20g30840 were more readily digested by HpaII after 6h of salinity stress than the same samples obtained from non-stressed seedlings, which consistent with a reduction in global cytosine methylation caused by salinity stress analyzed by bisulfite sequencing (Figure 3a, c). The sequence surrounding Glyma08g41450 was not digestible by HpaII, but several small restriction fragments were generated byMspI digestion, suggesting that CCGG sites in the region of this TF were hypermethylated in both non-stressed and stressed seedlings (Figure 3c). Thus the Southern bloting outcomes were in general consistent with the bisulfite sequence data.

The analysis of DNA methylation pattern of them indicated that methylation was affecting either CG dinucleotides or CNN/CNN trimucleotides under salinity stressed process (Figure 4). In the Glyma11g02400 promoter, 98% of the CG’s, 60% of the CNN’s and 6% of the CNN’s were methylated in the non-stressed seedlings, but almost all the CG’s, CNN’s and CNN’s were demethylated in plants exposed to salinity stress for more than 6h (Figure 4). For Glyma16g27950, some 80% of the CG’s, 72% of the CNN’s and 4% of the CNN’s were methylated in non-stressed seedlings and those during the early phase (1–3h) of the salinity treatment, but by 6h a significant fall in CG and CNN methylation was observed (Figure 4). Within region 1 of the Glyma20g30840 promoter, 95% of the CG’s, along with 40% of the CNN’s and 4% of the CNN’s, were methylated at 3h after the imposition of stress, but by 6h, a marked reduction in CG and CNN methylation had occurred. CG, CNN and CNN methylation in the Glyma08g41450 promoter was unaffected by salinity stress (Figure 4). Clearly, DNA methylation in Glyma11g02400, Glyma16g27950 and Glyma20g30840 (region 1) varied over the period of the salinity stress episode.

Methylation status of Glyma11g02400, Glyma08g41450, Glyma16g27950 and Glyma20g30840 as affected by the presence of 5-ADC

To identify whether the up-regulation of these four genes were related with cytosine demethylation under 5-ADC treatment, the effect on the DNA methylation status of the four responsive TFs in plants treated with 5-ADC was analyzed using genomic bisulfite sequencing. The relevant test for the transformation efficiency of unmethylated cytosine to thymine using Glyma20g32730 is illustrated. As a result, all four TFs were hypermethylated in non-treated seedlings; after a 24h exposure to 5-ADC, some evidence of demethylation was obtained, but from 48h onwards it was clear that a substantial level of demethylation had occurred (Figure 5a, b). This observation was supported by a DNA methylation-sensitive DNA gel blot (Figure 5c). All of the four TFs showed an increased digestion with HpaII after salinity stress for more than 48h, suggesting a reduction of cytosine methylation in 5-ADC stressed seedlings (Figure 5c).

Histone modification of hypermethylated genes induced by salinity stress

The histone content (H3K4me3, H3K9ace and the inactive H3K9me2) of the four TFs (Glyma11g02400, Glyma08g41450, Glyma16g27950 and Glyma20g30840) which responded to salinity stress by altering their methylation status was then examined, using a combination of ChIP and qRT-PCR (Figure 6a). An unmethylation gene Glyma20g32730 was also analysed in parallel with them as a control (Figure S3). When Glyma11g02400 was induced by salinity stress, a significant increase in H3K4me3 (regions I, II and III) and a decrease in H3K9me2 (regions I and II) was observed, but the H3K9ace sites remained unmodified (Figure 6a). Within Glyma20g30840 (regions II and III) and Glyma08g41450 (region III), a high level of H3K9me2 and a low level of H3K4me3 and H3K9ace was present in both non-stressed seedlings and those sampled during the early phase (1–3h) of stress; at later time points (6–24h), a significant decrease in H3K9me2 and increase of H3K4me3 and H3K9ace content was observed (Figure 6a). A similar H3K4me3, H3K9ace or H3K9me2 signal was detected in all three regions of the Glyma16g27950 promoter (Figure 6a). Thus, like the DNA methylation, histone modification was also subject to dynamic change during the course of the salinity stress episode.

Changes of epigenetic modification in regulating the TFs expression during salinity stress

The expression of Glyma11g02400 was low in non-stressed seedlings, while its promoter was hypermethylated and was highly enriched for H3K9me2 and depleted for H3K4me3 (Figure 6a, b, c).
c). When the seedlings were exposed to salinity stress, its expression rose rapidly, while its promoter became gradually demethylated, the level of H3K9me2 fell and that of H3K4me3 increased (Figure 6a, b, c). Glyma20g30840 behaved similarly, but the establishment of H3K4me3, DNA demethylation and gene expression were not contemporaneous. Demethylation was noticeable within 1h of the imposition of salinity stress, but the establishment of H3K4me3 did not occur until 3h and the up-regulation of expression only at 6h (Figure 6a, b, c). Similarly, Glyma08g41450 was up-regulated by 12h after the start of the stress episode, but its promoter was hypermethylated throughout. Between 6h and 24h after the stress had begun, the level of H3K9me2 fell and that of H3K4me3 and H3K9ac rose (Figure 6a, b, c), Glyma16g27950 expression was repressed in non-stressed seedlings and during the early phase of the stress episode, when its promoter was hypermethylated. Later the TF was gradually induced and its promoter demethylated, while the level of enrichment of H3K9me2, H3K4me3 and H3K9ac did not change. A correlation analysis of their expression, methylation levels and histone modifications indicated that up-regulation of Glyma11g24000 was associated with a decreased level of DNA methylation \( (r = -0.89) \), H3K9me2 \( (r = -0.965) \), on average of regions II and III and an increased level of H3K4me3 \( (r = 0.7) \), on average of regions I and II (Figure 6; Table S3). The expression of Glyma20g30840 correlated negatively with DNA methylation \( (r = -0.78) \), H3K9me2 \( (r = -0.93) \), on average of regions II and III and positively with H3K9ac \( (r = 0.96) \) and H3K4me3 \( (r = 0.93) \) on average (Figure 6; Table S3). Up-regulation of Glyma16g27950 just correlated negatively with DNA methylation \( (r = -0.97) \), did not with histone modifications, while the up-regulation of Glyma08g41450 correlated negatively with H3K9me2 \( (r = -0.67) \) and positively with H3K9ac \( (r = 0.84) \) and H3K4me3 \( (r = -0.78) \), did not with DNA methylation during salinity stress (Figure 6; Table S3). Therefore, the histone accumulation/depletion and/or cytosine methylation appears to underlie the activation by salinity stress of these four TFs.

**Discussion**

TF transcription can be influenced by both DNA methylation and/or histone modification in a region-specific manner

The regulation of genes via cytosine methylation and histone modification is a well recognized component of the plant stress response [14,34,40,41]. Both these epigenetic modifications are region-specific, and can be dynamic over time [42,43,44,45]. Here we have identified a set of ten salinity-induced, cytosine methylation-dependent TFs, three of which displayed the expected relationship between promoter cytosine methylation and gene expression during the salinity stress process, but one of which (Glyma08g41450) remained up-regulated even though it was in a highly methylated state (Figure 1, Figure 3a). A similar unexpected relationship has been noted for an embryogenesis-related gene in carrot [42]. Many genes are expressed despite their promoter region being highly methylated, so it seems probable that for Glyma08g41450, its up-regulation in plants exposed to salinity stress is independent of DNA methylation. One region of the Glyma20g30840 promoter was hypermethylated in both stressed and non-stressed plants, while its neighbouring region responded to the stress by a reduction in methylation (Figure 3a). This behaviour provides an example of region-specific regulation of methylation. The gradual up-regulation of Glyma11g24000 in salinity-stressed seedlings was accompanied by a decrease in CG, CNG and CNN methylation (Figure 4), while the rapid up-regulation of Glyma20g30840 and Glyma16g27950 was accompanied by a decrease in only CG and CNN methylation (Figure 4), suggesting heterogeneity in the genome for gene expression regulation via DNA methylation.

Histone modification provides a second major mechanism of epigenetic control over gene expression [46]. In a range of *A. thaliana* stress-responsive genes, salinity stress has been shown to increase trimethylation at H3K4 and decrease H3K9 demethylation [29]. The up-regulation of Glyma20g30840 and Glyma08g41450 during the course of the salinity stress episode may have been achieved by the depletion of H3K9me2 and the enrichment of H3K4me3 and H3K9ac in regions II and III of Glyma20g30840 and in region III of Glyma08g41450 (Figure 6a, b). The up-regulation of Glyma11g24000 may have been brought about by the depletion of H3K9me2 and the enrichment of H3K4me3 in regions I, II and III (Figure 6a, b), thus, as for DNA methylation, the effect of salinity stress on histone modification appears to be heterogeneous across the genome.

**Transcriptional activation, DNA methylation and histone modification are not simultaneous events**

There was a distinct time lag between transcriptional activation, DNA methylation and/or histone modification of Glyma11g24000, Glyma20g30840 and Glyma08g41450. Thus, most of the cytosine content of the Glyma11g24000 promoter was demethylated very soon (within 1h) of the imposition of stress, but there was no evidence for the TF’s activation before 3h (Figure 6b, c). Similarly, Glyma20g30840 was progressively demethylated over the full 24h of the stress episode, but its up-regulation was complete within 6h (Figure 6b, c). In Glyma11g24000, Glyma20g30840 and Glyma08g41450, the H3K4me3 content was already increasing by 1h, but the up-regulation of TF expression occurred substantially later (Figure 6a, b). Similar time lags have been noted for the build-up of H3K4me3 within the coding regions of the *A. thaliana* drought-related genes *RD29A* and *RAP2.4* during drought stress [36]. Furthermore, the DNA demethylation of Glyma20g30840 was prior to the erasure or establishment of H3K9me2, H3K4me3 and H3K9ac (Figure 6a, c) and all the three genes (Glyma11g24000, Glyma20g30840 and Glyma08g41450) show an earlier establishment of H3K4me3 than H3K9me2 and H3K9ac (Figure 6a). Suggesting that there were also a time lags between the DNA demethylation and establishment of histone modifications.

The interplay between DNA methylation and histone modification in the context of patterns of gene expression

In *A. thaliana*, it has been demonstrated that the loss of CG methylation in *met1* plants has a large effect on H3K9me2 content, leading to the idea that cytosine methylation can influence H3K9 modification [17,47,48,49]. H3K9me2, mediated by KYP/SUVH4 and SUVH2, is also known to direct non-CG methylation [39,50]. In the ‘two-step’ hypothesis for the regulation of transcription, CG methylation directs H3K9 methylation and H3K9 methylation recruits non-CG methylation [51]. Moreover, hypermethylation of the stress inducible genes in Arabidopsis correlated with the enrichment of H3K9me2 and depletion of H3K9ac histones under salt stress conditions [16]. In this study, the behaviour of Glyma11g24000 and Glyma20g30840 was consistent with this model; as CG was progressively demethylated in these TFs, the content of H3K9me2, H3K4me3 and/or H3K9ac rose and meanwhile a lower level of non-CG methylation was observed (Figure 6a, Figure 4). Note, however, that for
Glyma16g27950, while salinity stress led to a noticeable demethylation of the DNA, it had little effect on the level of histone modification (Figure 6a, c). The Glyma16g27950 promoter was hypermethylated throughout the salinity stress period, while H3K9me2 was depleted and H3K4me3 and H3K9ac accumulated (Figure 6a, c). For these two TFs, therefore, the evidence is that DNA methylation had no influence on histone modification, consistent with the behaviour of the A. thaliana genes TOUSED and RPA2 [52,53].

Supporting Information

Figure S1 Monitoring the non-methylated cytosine to thymine transformation efficiency of the bisulphite sequencing procedure. A fragment of Glyma20g32730 with numerous cytosines was cloned into Dm- E. coli cells and the plasmid was treated with bisulphite. Nine positive clones of this gene from the bisulphite treated DNA were sequenced and compared with the untreated DNA sequence.

Figure S2 Genomic bisulphite sequencing of six unmethylated genes in plants not exposed to salinity (S0) and those stressed for 1–24 h (S1–S24). Ten clones of each gene were sequenced. The short thick bars indicate the regions subjected to sequencing, and the long vertical ones show the distribution of CG dinucleotides (black circles), and CNG (triangles) and CNN (unmarked) trinucleotides. The black vertical line represents the proportion of methylated sites. Data represent the mean of three biological replicates.

Figure S3 ChIP analysis to assess the unmethylated gene Glyma20g32730’s H3K9me2, H3K9ac and H3K4me3 content in plants challenged with salinity. The short bars marked “a” indicate regions subjected to genomic bisulphite sequencing (+1 to +393); “I, II and III” indicate the regions subjected to ChIP analysis.

Table S1 The set of TFs identified by microarray analysis to be inducible by salinity stress.

Table S2 Sequences of primers employed in this research.

Table S3 Correlation analysis among methylation levels, gene expression and histone modifications of the four TFs during salinity stress. NC: no correlation; *P<0.05, **P<0.01.

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Author Contributions

Conceived and designed the experiments: FX. Performed the experiments: YS DJ SL PW QL. Analyzed the data: YS. Contributed reagents/materials/analysis tools: FX. Wrote the paper: YS.

References

1. Phang TH, Shao G, Lam HM (2008) Salt tolerance in Soybean. J Integr Plant Biol 50: 1196–1212.
2. Cheong YH, Moon BC, Kim JK, Kim CY, Kim MC, et al. (2003) BWMK1, a rice tigon-activated protein kinase, locates in the nucleus and mediates pathogenesis-related gene expression by activation of a transcription factor. Plant Physiol 132: 1961–1972.
3. Choi HI, Hong JH, Ha JO, Kang JY, Kim SY (2000) ABF1, a family of ABA-responsive element binding factors. J Biol Chem 275: 1723–1730.
4. Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, et al. (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci U S A 97: 11632–11637.
5. Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, et al. (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBS, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem Biophys Res Commun 290: 998–1009.
6. Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AMY2C (bHLH) and AMY2B (MYB) function as transcriptional activators in abscisic acid signalling. The Plant Cell 15: 63–78.
7. Chen M, Xu Z, Xia I, Li L, Cheng X, et al. (2009) Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, GmDREB3, in soybean (Glycine max L.). J Exp Bot 60: 121–35.
8. R Qu, Jiang HH, Li CR, Zhao QZ, Zhang J, et al. (2008) Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signalized defense responses. Cell Res 18: 756–767.
9. Liao Y, Zou HF, Wei W, Hao YJ, Tian AG, et al. (2008b) Soybean GmZIP4, GmZIP2 and GmZIP7 genes function as negative regulator of ABA signaling and confer salinity and freezing tolerance in transgenic Arabidopsis. Planta 229: 225–240.
10. Liao Y, Zou HF, Wang HW, Zhang WK, Ma R, Z, et al. (2008b) Soybean GlymMYB76, GlymMYB92, and GlymMYB177 genes confer stress tolerance in transgenic Arabidopsis plants. Cell Research 18: 1047–1060.
11. Chen M, Wang QY, Cheng XG., Xu ZS, Li LC, et al. (2007) GmDREB2, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants. Biochim Biophys Acta 1769: 295–307.
12. Hao YJ, Wei W, Song QX, Chen HW, Zhang YQ, et al. (2011) Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. Plant J 68: 302–313.
13. Zhu JK (2006) Epigenetic sequencing comes of age. Cell 133: 393–397.
14. Chinnusamy, Gong Z, Zhu JK (2008) ABA-mediated epigenetic processes in plant development and stress responses. J Integr Plant Biol 50: 1167–1195.
15. Boyko A, Kovachlik I (2008) Epigenetic control of plant stress response. Environ Mol Mutagen 49: 61–72.
16. Bilichak A, Isaynkyi Y, Hollunder J, Kovachlik I (2012) The progeny of Arabidopsis thaliana plants exposed to salt exhibit changes in DNA methylation, histone modifications and gene expression. PLoS One 7(1): e30513.
17. Tariq M, Paszkowski J (2004) DNA and histone methylation in plants. Trends Genet 20: 244–252.
18. Peters AH, Schubeler D (2005) Methylation of histones: playing memory with DNA. Curr Opin Cell Biol 17: 230–238.
19. Tariq M, Demidov D, Houben A, Schubert I (2006) Chromosomal histone modification patterns – from conservation to diversity. Trends Plant Sci 11: 206–209.
20. Paszkowski J, Whitham SA (2001) Gene silencing and DNA methylation processes. Curr Opin Plant Biol 4: 125–129.
21. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes & Development 16: 6–21.
22. Finnegan EJ, Genger RK, Peacock WJ, Dennis ES (1998) DNA methylation in plants. Annu Rev Physiol Plant Biol 58: 261–284.
23. Zhang NY, Yacik J, Sundenasan A, Cokus S, Chan SW, et al. (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in Arabidopsis. Cell 126: 1189–1201.
24. Vaillant I, Paszkowski J (2007) Role of histone and DNA methylation in gene regulation. Curr Opin Plant Biol 10: 526–533.
25. Lachner M, O’Sullivan RJ, Jennewein T (2003) An epigenetic road map for histone lysine methylation. J Cell Sci 116: 2117–2124.
26. Earley K, Lawrence RJ, Pontes O, Renther R, Enciso AJ, et al. (2006) Erasure of histone acetylation by Arabidopsis HDA6 mediates large-scale gene silencing in nuclear dominance. Genetics 172: 1283–1293.
27. Chen ZJ, Tian L (2007) Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochem Biophys Acta 1769: 295–307.
28. Chen LT, Lao M, Wang YY, Wu K (2010) Involvement of Arabidopsis histone deacetylase HDA6 in ABA and salt stress response. J Exp Bot 61: 3345–3353.
29. Tamaru H, Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature 414: 277–283.
30. Jackson JP, Johnson L, Jasencakova Z, Zhang X, Perez-Burgos L, et al. (2004) Stabilization of histone H3 lysine 9 methyltransferase 2 in Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in Arabidopsis. Cell 126: 1189–1201.
31. Earley K, Lawrence RJ, Pontes O, Renther R, Enciso AJ, et al. (2006) Erasure of histone acetylation by Arabidopsis HDA6 mediates large-scale gene silencing in nuclear dominance. Genetics 172: 1283–1293.
32. Chen LT, Lao M, Wang YY, Wu K (2010) Involvement of Arabidopsis histone deacetylase HDA6 in ABA and salt stress response. J Exp Bot 61: 3345–3353.
33. Tamaru H, Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature 414: 277–283.
34. Jackson JP, Johnson L, Jasencakova Z, Zhang X, Perez-Burgos L, et al. (2004) Stabilization of histone H3 lysine 9 methyltransferase 2 in Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in Arabidopsis. Cell 126: 1189–1201.
32. Fahrner JA, Eguchi S, Herman JG, Baylin SB (2002) Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. Cancer Res 62: 7213–7218.

33. Steward N, Ito M, Yamaguchi Y, Koizumi N, Sano H (2002) Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. J Biol Chem 277: 37741–37746.

34. Choi H, Sano H (2007) Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. Mol Genet Genomics 277: 389–600.

35. Tsuji H, Saika H, Tsutsumi N, Hirai A, Nakazono M (2006) Dynamic and reversible changes in histone H3-Lys4 methylation and H3 acetylation occurring at submergence-inducible genes in rice. Plant Cell Physiol 47: 995–1003.

36. Kim JM, To TK, Ishida J, Morosawa T, Kawaihara M, et al. (2006) Alterations of lysine modifications on histone H3 N-tail under drought stress conditions in Arabidopsis thaliana. Plant Cell Physiol 49: 1580–1588.

37. Smyth GK, Yang YH, Speed TP (2003) Statistical issues in microarray data analysis. In: Brownstein MJ, Khodursky AB, eds. Functional Genomics: Methods and Protocols. Volume 224, 111–136 Humana Press, Totowa.

38. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

39. Jackson JP, Lindroth AM, Cao X, Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature 416: 556–560.

40. Zhu J, Jeong JC, Zhu Y, Sokolchik I, Miyazaki S, et al. (2007) Involvement of Arabidopsis HIS15 in histone deacetylation and cold tolerance. Proc Natl Acad Sci U S A 104: 4945–4950.

41. Agino F, Kappos A, Zhu JK (2006) Role of the Arabidopsis DNA glycosylase/lvase ROS1 in active DNA demethylation. Proc Natl Acad Sci U S A 103: 11796–11801.

42. Shibukawa T, Yazawa K, Kikuchi A, Kamada H (2009) Possible involvement of DNA methylation on expression regulation of carrot LEC1 gene in its 5' upstream region. Gene 437: 22–31.

43. Pokholok DK, Harbison CT, Levine S, Cole M, Hannett NM, et al. (2005) Genome-wide map of nucleosome acetylation and methylation in yeast. Cell 122: 517–527.

44. Millar CR, Grunstein M (2006) Genome-wide patterns of histone modifications in yeast. Nat Rev Mol Cell Biol 7: 657–666.

45. Li X, Wang X, He K, Ma Y, Su N, et al. (2008) High-resolution mapping of epigenetic modifications of the rice genome uncovers interplay between DNA methylation, histone methylation, and gene expression. Plant Cell 20: 259–276.

46. Egger G, Liang GN, Sarker A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429: 457–465.

47. Mathieu O, Probst AV, Paszkowski J (2005) Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. EMBO J 24: 2783–2791.

48. Tariq M, Saze H, Probst AV, Lichota J, Habu Y, et al. (2003) Eruation of CpG methylation in Arabidopsis alters patterns of histone H3 methylation in heterochromatin. Proc Natl Acad Sci U S A 100: 8823–8827.

49. Soppe WJ, Jasencakova Z, Houben A, Kakutani T, Meister A, et al. (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in Arabidopsis. EMBO J 21: 6549–6559.

50. Malagnac F, Bartee L, Bender J (2002) An Arabidopsis SET domain protein required for maintenance but not establishment of DNA methylation. EMBO J 21: 6842–6852.

51. Naumann K, Fischer A, Hofmann I, Krauss V, Phalke S, et al. (2005) Pivotal role of AtSUVH2 in heterochromatic histone methylation and gene silencing in Arabidopsis. EMBO J 24: 1418–1429.

52. Xia R, Wang J, Liu C, Wang Y, Zha J, et al. (2006) ROR1/RPA2A, a putative replication protein A2, functions in epigenetic gene silencing and in regulation of meristem development in Arabidopsis. Plant Cell 18: 85–103.

53. Kapoor A, Agarwal M, Andreucci A, Zheng X, Gong Z, et al. (2005) Mutations in a conserved replication protein suppress transcriptional gene silencing in a DNA-methylation-independent manner in Arabidopsis. Curr Biol 15: 1912–1918.