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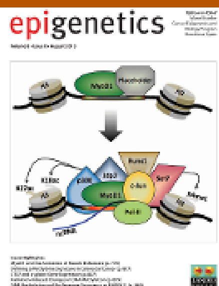

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Epigenetic marks in an adaptive water stress-responsive gene in tomato roots under normal and drought conditions

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Keywords: epigenetics, methylation, *Asr2*, water stress, tomato, roots

Abbreviations: Asr1, abscisic acid stress and ripening 1; Asr2, abscisic acid stress and ripening 2; bp, base pairs; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; MET1, methyltransferase 1; DNMT1, DNA methyltransferase 1; CMT3, chromomethylase 3; H3K4me3, tri-methylated lysine 4 of histone 3; H3K9me2, di-methylated lysine 9 of histone 3; H3K27me3, tri-methylated lysine 27 of histone 3; KYP, kryptonite: histone 3 lysine 9 methyltransferase; DRM2, domains rearranged methyltransferase 2; LEA, late embryogenesis abundant; MYA, million years ago; PCR, polymerase chain reaction; qRT-PCR, quantitative real time – polymerase chain reaction; FWA, flowering wageningen; VIM1, variant in methylation 1; SUVH9, SU (Var) 3-9 homolog 9; CTAB, cetyl trimethyl ammonium bromide; SDS, sodium dodecyl sulfate; ToRTL, tomato copia-type retrotransposon; EF-1, elongation factor 1; nt, nucleotide(s); mC, methylcytosine

Tolerance to water deficits was evolutionarily relevant to the conquest of land by primitive plants. In this context, epigenetic events may have played important roles in the establishment of drought stress responses. We decided to inspect epigenetic marks in the plant organ that is crucial in the sensing of drought stress: the root. Using tomato as a crop model plant, we detected the methylated epialleles of *Asr2*, a protein-coding gene widespread in the plant kingdom and thought to alleviate restricted water availability. We found 3 contexts (CG, CNG, and CNN) of methylated cytosines in the regulatory region of *Solanum lycopersicum Asr2* but only one context (CG) in the gene body. To test the hypothesis of a link between epigenetics marks and the adaptation of plants to drought, we explored the cytosine methylation status of *Asr2* in the root resulting from water-deficit stress conditions. We found that a brief exposure to simulated drought conditions caused the removal of methyl marks in the regulatory region at 77 of the 142 CNN sites. In addition, the study of histone modifications around this model gene in the roots revealed that the distal regulatory region was rich in H3K27me3 but that its abundance did not change as a consequence of stress. Additionally, under normal conditions, both the regulatory and coding regions contained the typically repressive H3K9me2 mark, which was lost after 30 min of water deprivation. As analogously conjectured for the paralogous gene *Asr1*, rapidly acquired new *Asr2* epialleles in somatic cells due to desiccation might be stable enough and heritable through the germ line across generations, thereby efficiently contributing to constitutive, adaptive gene expression during the evolution of desiccation-tolerant populations or species.

Introduction

Epigenetic research has generated a fair amount of information on certain plant models, such as *Arabidopsis*,[1,2] rice,[3] and maize.[4] Eukaryotic DNA methylation is one of the most studied epigenetic processes as it results in a direct and heritable covalent modification triggered by external stimuli. While methylation in animal genomes occurs mostly in regulatory regions, methylation in *Arabidopsis* is also found in transcribed sequences at not only canonical CG sites but also CNG (N denotes A, C, or T) and CNN (asymmetric) sites. The latter sites are preferentially methylated in repetitive elements and transposons.[5,6]

Mutant analyses in *Arabidopsis* ascertained that methylation in each context arises from specialized enzymatic machineries, such as the following examples: (1) the enzyme MET1, orthologous to mammalian DNMT1, which maintains CG methylation;[7] (2) the enzyme CMT3,[6] the structure of which was recently elucidated by X-ray diffraction crystallography,[8] which, together with the histone methyltransferase KYP, is responsible for transferring the methyl group in the CNG context;[9] and

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(3) the methyltransferase DRM2,[10] guided by short sequence-specific RNAs, which appears to catalyze de novo methylation in all contexts, including CNN.[11] In any case, intragenic DNA methylation mechanisms are essential as they regulate gene expression and plant development,[12] but there are still additional molecular players to be explored in more depth, such as those that prevent genes from undergoing ectopic deposition of methylation.[13]

We are particularly interested in investigating the epigenetics of plant species with larger and more complex genomes than *Arabidopsis*, specifically with respect to the alterations elicited by abiotic stress. Among the different types of environmental stresses amenable to study, we selected water shortage because primitive plants have coped with this stress since they colonized land habitats approximately 400 MYA.[14] Our studies centered on the tomato plant (*Solanum lycopersicum*), an edible plant crop (http://mips.helmholtz-muenchen.de/plant/tomato/index.jsp) of great economic importance with a genome[15] that is almost 10 times larger than that of *Arabidopsis* and with very few epigenetics surveys.[16-18] Using this model system, we previously investigated the cytosine methylation status in the leaf of *Asr1*,[17] a non-transposon, protein-coding, desiccation stress-inducible gene of the LEA superfamily[19] that is conserved in the plant kingdom but lacks an orthologous counterpart in *Arabidopsis*. The ancestral (300 MYA) ASR gene family[20] has been extensively studied by us and other groups at the DNA,[20] RNA,[21] and protein[22-24] levels and in terms of physiological function.[25]

We now report a methylation study on the paralogous gene *Asr2*, which has been a target of positive selection during the evolution of the *Solanum* genus in arid environments.[20] Moreover, this model gene displays intraspecific nucleotide variation, evidence of non-neutral evolution in different populations ascribed to different rainfall regimes.[26] In addition to the two latter relevant reasons related to the adaptation to threatening environmental demands, we selected this gene because it has a very simple organization, consisting of exon 1 and exon 2 with 159 and 186 nt, respectively, separated by a short intron. In addition, its sequence upstream of the transcription start site, also analyzed in this work, has been functionally tested to efficiently drive the transcription of a reporter gene in transgenic plants.[27] We also chose the root as the source of genomic DNA for these epigenetic studies for two reasons: (1) it is the organ, through its hairs, involved in the primary sensing of drought stress;[28] and (2) it is the organ in which *Asr2* expression is the highest upon water stress.[21]

In summary, our group's main interest was the link between epigenetics and stress in plants,[29-31] particularly water-deficit stress[32] in species with large genomes. For the above-mentioned reasons, *Asr2* is a suitable and attractive model gene for this purpose. As stress-induced physiological responses in *Arabidopsis* are thought to depend on altered DNA methylation[33] and histone modifications,[34] we tested this hypothesis experimentally in a crop species by examining the gains and losses of cytosine methylation and histone marks in the roots as a consequence of imposing water stress conditions on tomato plants.

Results

Indicator of drought conditions. We first assessed that drought conditions really occurred. To this end, we applied a simple procedure consisting of weighing thoroughly wiped roots before and after the stress treatments (**Fig. S1**). The results indicated that the weight decreased, strongly suggesting the loss of internal water as a consequence of the stress.

Overall non-CG methylation in the *Asr2* promoter/enhancer region and gene body. We wanted to gain insight into the methylation events in cytosine contexts other than CG. To this end, we used the bisulfite conversion procedure,[35] taking care to address methodological concerns, namely parallel bisulfite control reactions on non-methylated or in vitro-methylated plasmid DNA (see Materials and Methods). In addition, to understand the molecular mechanisms underlying the adaptation of plants to abiotic stress, we analyzed root DNA methylation after imposing water-shortage stress on whole tomato plants through root drying.

For that purpose, we performed an inspection of the methylated sites at the functionally studied regulatory region of tomato *Asr2*,[27] spanning 968 nt upstream of the transcription start site (**Fig. 1A and C**), in the root. As all the observed epialleles showed different methylation patterns at CNN sites, we did not need to address the concerns of Henderson et al.[36] or the model of Peng and Ecker[37] to eliminate sibling clones. After grouping the results according to each methylation type, we concluded that there were substantial levels of the three types of methylation (CG, CNG, and CNN) in that region under non-water stress conditions (**Fig. 1A**). After stress, the overall CNN methylation showed a slight but significant ($P < 0.05$) decrease (**Fig. 1A**; **Fig. S2**).

In parallel, we analyzed the cytosine methylation in the gene body of *Asr2*, consisting of 2 exons and one intron and spanning 603 nt, in the root (**Fig. 1B and C**). The raw data (**Fig. S3**) were grouped by methylation type, leading to the conclusion that a considerable proportion (near 80%) of the clones showed methylation at the few existing CG sites (**Fig. 1B**) under both environmental conditions. In contrast, very few of the numerous CNG and CNN sites were methylated under either normal or non-water stress conditions (**Fig. 1B**). It is worth mentioning that after the water stress, 2 distinct clones appeared to be strongly methylated in all contexts (**Fig. S3B**), leading to an overall increase in CNG and CNN methylation when analyzed comprehensively.

Detailed picture of CNN demethylation in the *Asr2* promoter/enhancer upon stress. We next decided to carry out a close-up inspection of the upstream regulatory region by performing calculations of the mean (methylation level values) in a recursive fashion, that is, depending on a previously calculated value. This computational tool is known as a "moving average" (http://lorien.ncl.ac.uk/ming/filter/filmav.htm). In our case, this procedure consisted of a moving window of 10 contiguous cytosines (see Materials and Methods for an explanation). This approach allowed the observation of a slight increase due to stress in cytosine methylation distant (–1227/–1174) to the

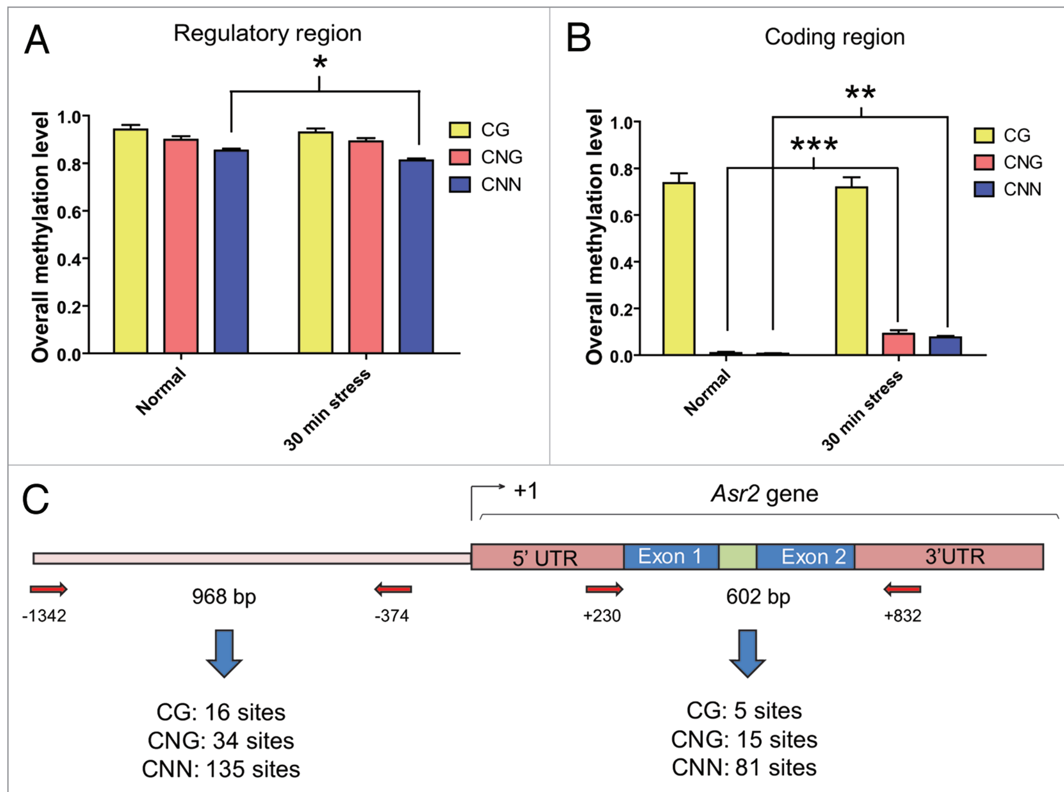

Figure 1. Overall methylation levels in the *Asr2* regulatory region and gene body. Data were grouped by methylation context in the regulatory region, which is located upstream of the transcription start site (**A**), and coding region (**B**) under both normal conditions and after 30 min of water stress. (**C**) Schematic representation of the entire gene, aligned to **A** and **B**, showing the annealing positions of the primers relative to the +1 site designed for the post-bisulfite PCR analysis. The amplicon sizes and number of cytosine residues in each context existing in the amplicons analyzed for **A** and **B** are also shown. The error bars indicate the standard error (SEM). The slight decrease in the overall CNN methylation in the regulatory region was significant (*$P < 0.05$). The increased values in the CNG (***$P < 0.001$) and CNN (**$P < 0.01$) methylation in the coding region were also significant. The bisulfite treatments were performed as indicated in *Materials and Methods*. The primers for post-bisulfite PCR are listed **Table S1**. GenBank accession numbers for *Asr2*: L20756, CU468249, and X74907.

gene body and more evidently in the –840 to –596 interval. The sequence between these 2 regions and the proximal regulatory region showed a marked trend toward demethylation (**Fig. 2**). In particular, we found that the simulated drought conditions caused the removal of methyl marks at 77 of the 142 asymmetric (CNN) sites present in the upstream regulatory region. When focusing on other regions of *Asr2*, no striking tendencies were revealed.

Histone marks in *Asr2*. As gene expression is also influenced by post-translational histone modifications,[34] we decided to explore H3K27me3, H3K9me2, and H3K4me3, abundant signatures of gene repression (H3K27me3, H3K9me2), and activation (H3K4me3) in *Arabidopsis*.[38] H3K27me3 and H3K4me3 marks in the upstream region closest to the gene (–316/–207 bp) were barely detected (**Fig. 3A, C, and G**). However, in the upstream distal region (–987/–820 bp), the H3K27me3 repressive mark was detected ($P < 0.05$, $P < 0.01$), but it did not change as a result of stress (**Fig. 3C**). Surprisingly, these histone methylation marks within the *Asr2* coding region were not appreciably perceived (**Fig. 3B and D**). In contrast, the usually repressive H3K9me2 mark was clearly evident in all regions analyzed (**Fig. 3E and F**), decreasing after the water shortage.

Given that some negative results were obtained, we assessed the performance of the antibodies on the constitutively expressed *actin* gene and the transposable element *ToRTL*, which were selected as control loci. Although we detected only H3K9me2 mark on the body of *actin*, which was reduced after stress ($P < 0.001$, **Fig. S4C**), immunoprecipitation with the same antibodies effectively captured all the studied methylation marks in the chromatin wrapping the transposon; however, these marks were lost as a result of water stress ($P < 0.05$, $P < 0.01$) (**Fig. S4**). These results confirmed the good quality of the antibodies specific to the inspected histone marks.

***Asr2* expression in roots upon water stress.** To identify an eventual correlation between any type of methylation (CG, CNG, and CNN) and expression of our model gene, we performed qRT-PCR for both the normal and stress conditions (**Fig. 4**). After normalization against the housekeeping *EF-1* mRNA, the results showed a slight increase in the *Asr2* mRNA levels at as early as 10 min of water stress ($P < 0.05$, data not shown) and an even higher expression at 30 min of stress, the time point at which we performed the methylation analysis ($P < 0.001$, **Fig. 4B**). Longer times resulted in the occurrence of massive cell death (**Fig. S5**), which would have made an epigenetic evaluation impossible.

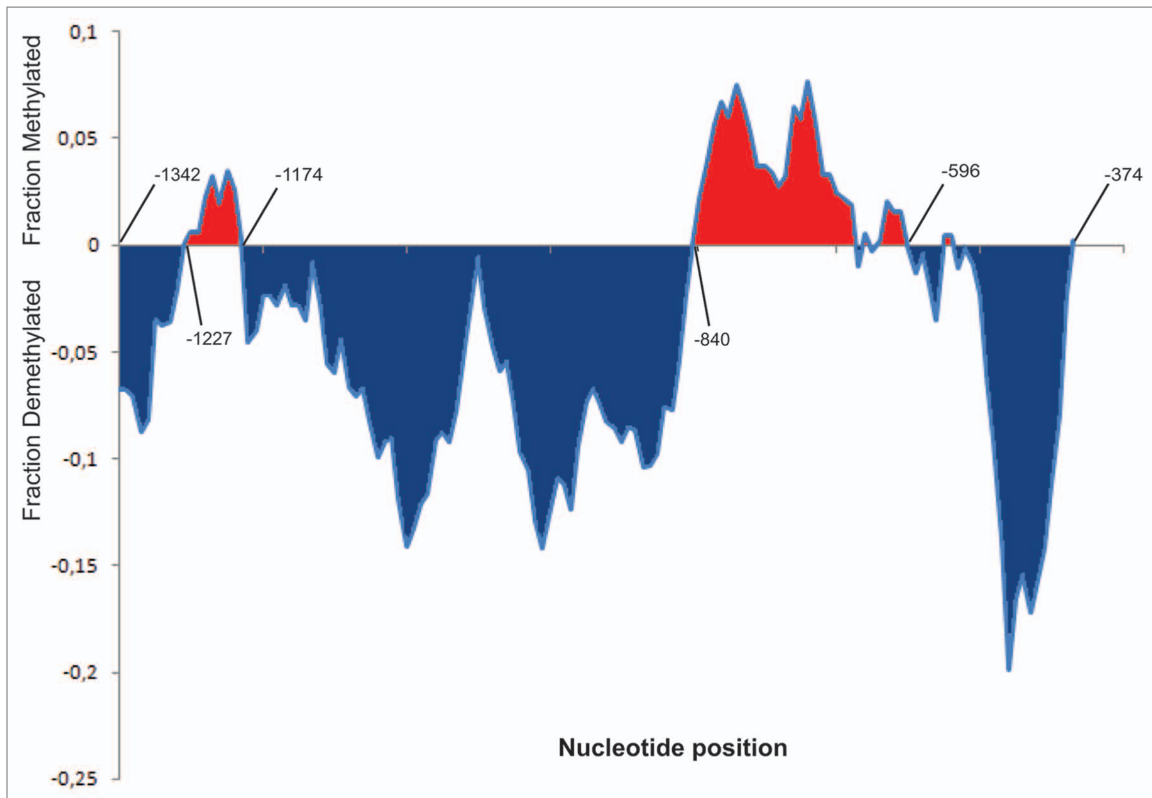

Figure 2. Detailed inspection of the stress-induced changes in asymmetric methylation (CNN) within the *Asr2* regulatory region. The moving average statistical tool (http://lorien.ncl.ac.uk/ming/filter/filmav.htm) was employed. The X-axis represents 10-cytosine (5 left + 5 right) windows for each position corresponding to the 142 cytosine residues (CNN) existing in this region. Y-axis units represent the moving average of the differences between the methylation level for each cytosine under stress and the methylation level for the same cytosine under no stress. Some position numbers relative to the +1 transcription start site are indicated.

Discussion

In plants, the classical CG methylation context not only is linked to the regulatory region of the gene, as in mammals, but also is found in the coding region of the gene.[39,40] Another particularity of plant species is that this methylation context is not necessarily associated with gene silencing.[31] Moreover, these marks are often present even in the body of housekeeping genes, such as *actin*,[41] and appear to provide a conserved pattern in different organs of the same species. For example, the few methylated CG sites in the *Asr2* gene body we found in the roots strikingly matched those found in the same gene in fruit by the recent genome-wide methylome project performed by Giovannoni's group.[18] However, we observed no stress-provoked higher CG methylation, in contrast to our previous report on another model gene of the same family in tomato leaves.[17]

Regarding non-CG methylation, its existence in tandem repeats has been demonstrated by Jacobsen's group[42] along with its conservation across duplicated regions of the genome.[43] In this work on the drought-responsive gene *Asr2* in roots, in contrast to our previous report on leaf *Asr1*, no asymmetric CNN methylation in the transcribed sequence was found in the absence of stress. However, we uncovered extensive methylation at these nonconventional acceptor sites in the upstream regulatory region. Moreover, we noticed that this epigenetic mark in combination with CG and CNG methylation correlated with poor expression, consistent with previous evidence.[44] We also showed that upon stress, many of these CNN sites were demethylated, similar to what occurs with those in the gene body of leaf *Asr1*.[17] Analogously, a null DRM2 mutant was reported to block non-CG methylation, which allowed for full desilencing of the FWA gene, resulting in a late-flowering phenotype.[45] Along the same lines, evidence from Matzke's lab showed that the combination of CG and non-CG methylation in at least some coding regions was associated with gene silencing in synergid cells.[46]

It is worth noting at this point that the various clones with dissimilar patterns displayed in the Kismeth schemes may have arisen from different cell types present together in the root samples under examination, with each having a distinct epigenetic behavior, as conjectured by Peng and Ecker (2012).[37]

With respect to the chromatin structure, a link between cytosine methylation and histone methylation is currently known to exist.[47,48] A clear example of this relationship is given by KYP and VIM1, which bind to methylated cytosines and act as histone methyltransferases.[49] Other protein complexes, however, lack methyltransferase activity but bind to methylated cytosines and cooperate with the DNA methylation machinery, such as in the case of SUVH9 and DRM2, the methyltransferase devoted, but not exclusively, to the asymmetric context.[45] It is also worth

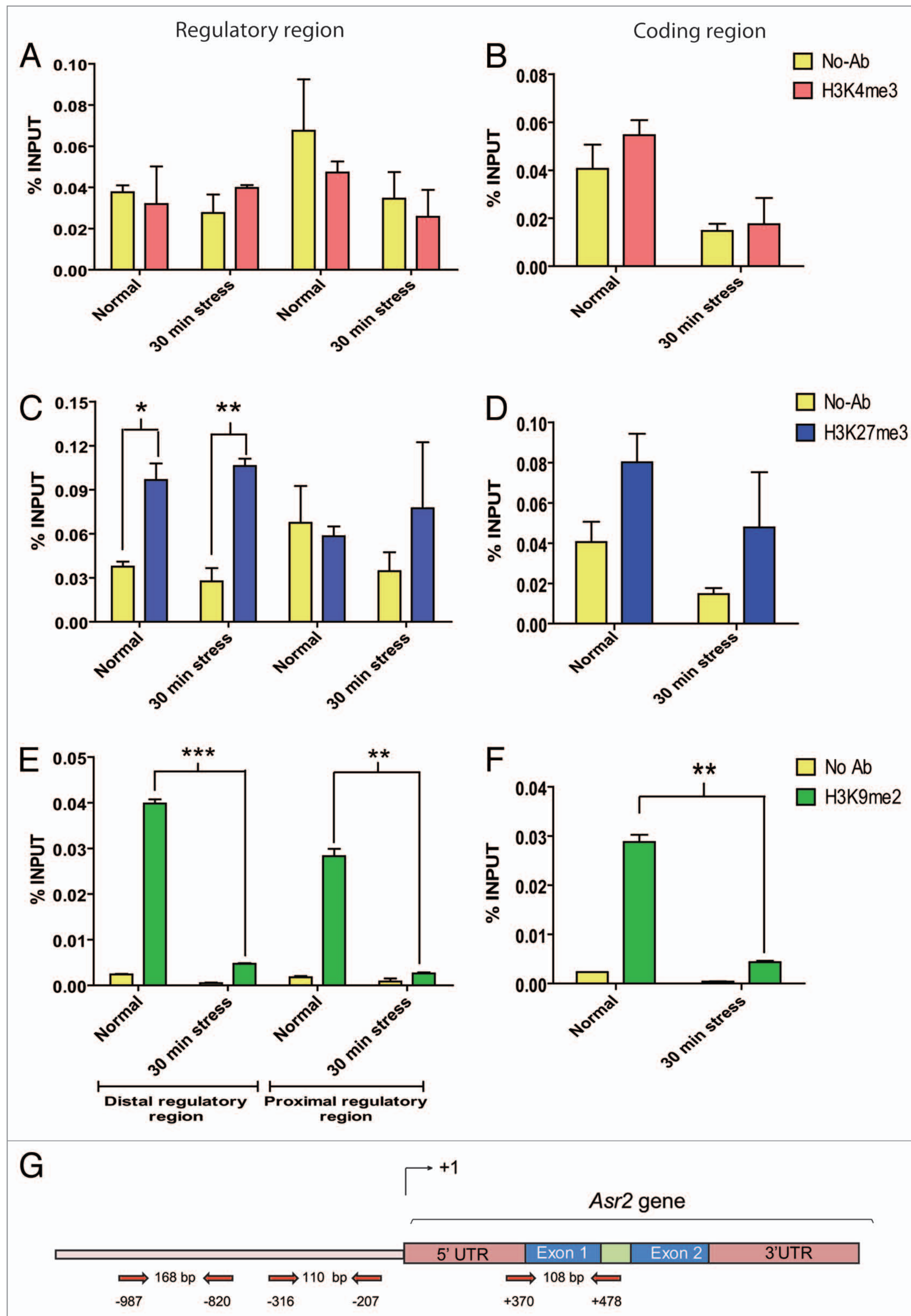

Figure 3. Histone marks on the *Asr2* regulatory and coding regions. The H3K4me3 (**A and B**), H3K27me3 (**C and D**), and H3K9me2 (**E and F**) levels in the regulatory (**A, C, and E**) and coding (**B, D, and F**) regions under both normal conditions and after 30 min of water stress are expressed as % input. ChIP was performed as described in Materials and Methods. (**G**) Schematic representation of the entire gene, aligned to **A–F**, showing the annealing positions of the primers designed for qPCR and the sizes of the analyzed amplicons. The error bars indicate the standard error (SEM). Statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) histone marks are highlighted. Statistical significance between no-antibody (noise signal) and real ChiP signal is shown except for the evident H3K9me2 mark (**E and F**) under normal conditions ($P < 0.001$) for the sake of simplicity, not to mask the pronounced effect of stress on demethylation in both gene regions. The qPCR primers are listed in **Table S1**.

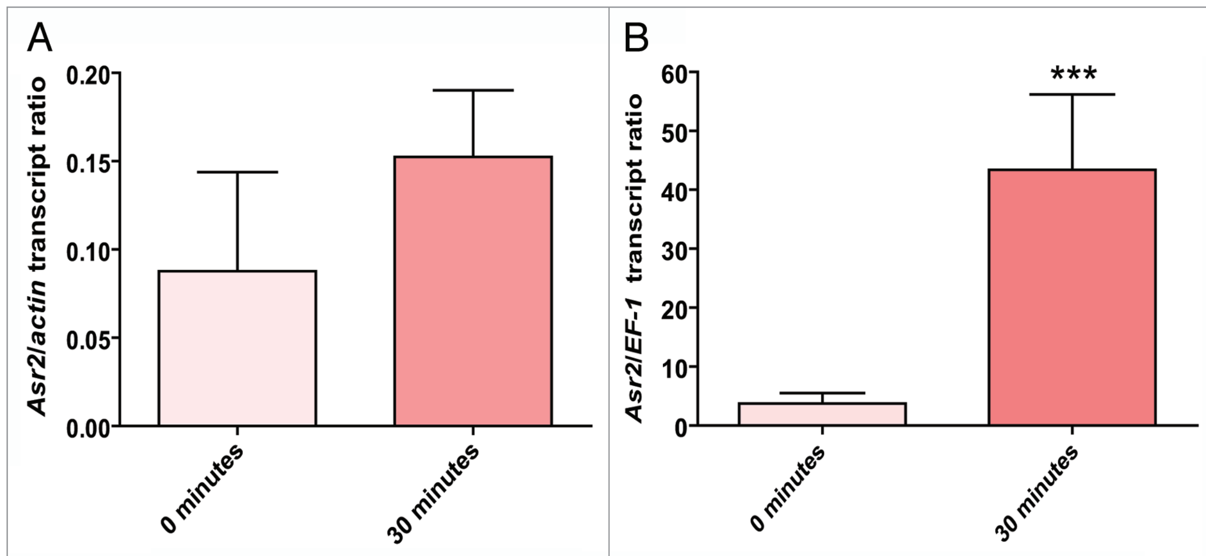

Figure 4. *Asr2* expression as a consequence of water stress. The root mRNA steady-state levels were measured by qRT-PCR as described in Materials and Methods. The data were normalized to *actin* (**A**) or *EF-1* (**B**) mRNA at each stress time before comparing the effects of the different stress treatments. Normalized against *EF-1*, the difference in the expression levels of *Asr2* was statistically significant at 30 min (****P* < 0.001). The qRT-PCR primers are listed in **Table S1**. The error bars indicate the standard error (SEM).

mentioning that any conclusions regarding the assignment of different plant cytosine methyltransferases to particular substrate sites have been derived solely through reverse genetics by analyzing mutants[6,10,50] and not from in vitro experiments with purified enzymes. However, there are recent advances on the structure of the maize crystallized CMT3 protein, highlighting its domains involved in binding to nucleosomes and catalysis on CNG sites.[8] In this context of chromatin modifications, we found no significant stress-induced variations in the levels of either H3K4me3 or H3K27me3 marks (associated with the opposite effects on transcription in *Arabidopsis*[38]) in our model gene. Because methylated H3K27 is independent of and potentially mutually exclusive with DNA methylation,[51] this mark is not very informative for understanding the relationship among chromatin modifications, DNA methylation and gene regulation at this locus. Therefore, we also explored the repressive mark H3K9me2, which turned out to correlate with CNN methylation, both pointing in the same direction and, in turn, inversely associated with *Asr2* gene expression.

The data presented herein provide a new example of a not very frequent genomic location for CNN methylation in plants, namely, a non-repetitive, non-transposon gene. However, there are certainly other examples, e.g., Baek et al. (2010) in *Arabidopsis*,[52] Zhang et al. (2011) in sorghum,[53] and Li et al. (2012) in rice young panicles.[54] Certainly, more biochemical studies will help elaborate new models to understand the in vivo maintenance of CNN methylation during DNA replication, which is difficult to envisage, as there are no local cytosine residues to be methylated in the nascent complementary strand.

As high throughput technologies for uncovering specific methylomes are becoming more and more accessible, comparisons of genome-wide cytosine methylation in all contexts or of average methylation levels can be easily made among different plant sources. For example, in rice, the average methylation levels in the 3 contexts are approximately 44%, 24%, and 5%, respectively,[55] revealing that the rice genome has a much higher level of DNA methylation than *Arabidopsis*.[56] Strikingly, in tomato, the percentages of mC in the CG, CNG, and CNN contexts are even higher: approximately 85%, 56%, and 8%, respectively, for leaves and oscillating from 74–79%, 52–54%, and 13–14%, respectively, for fruits at different maturation stages.[18] A noteworthy bonus of the present work was that it was conducted on the root, a plant organ almost neglected at the epigenetic level, particularly with respect to methylation, except for a few works in *Arabidopsis*[37,52] and sorghum.[53]

With respect to the appealing connection between plant epigenetics and stress, our findings in the tomato plant were consistent with the hypothesis highlighted by the Kovalchuk group[30] in *Arabidopsis* and experimentally supported in rice,[55] in which at least some stress-induced phenotypes depend on altered DNA methylation. In this regard, the change in asymmetric epigenetic marks we observed upon environmental drought conditions may represent an alternative mechanism for adaptation in plants, not only in *Arabidopsis* but also in species with larger and more complex genomes. The rapid emergence of these newly acquired epialleles in roots, coupled with the unique ability of plants to produce germ line cells late during development, may allow for the inheritance of these marks across (not necessarily stressed) generations,[57-60] thus efficiently contributing to the constitutive, adaptive gene expression during evolution of drought tolerance in crop species. We have no evidence of such a conjecture, and this issue might be resolved by future research.

Materials and Methods

Plant material. Commercial tomato (*Solanum lycopersicum*) seeds were bleached by sinking in a 20 g/l sodium hypochlorite

solution for 30 min. After the treatment, the seeds were placed on dampened blotting paper and left in the dark for 72 h. Plantlets were placed in a growth chamber at 23°C with a photoperiod of 12 h light/12 h dark for 5 d followed by transplantation to pots. The plants were then returned to the growth chamber and watered twice weekly until the experiments were performed.

Water stress conditions. Three-week-old plants were taken from the pots, and their roots were carefully cleaned. The roots were cut off, wiped, weighed, and frozen in liquid nitrogen (non-stressed plants) or placed onto blotting paper for different times up to 30 min, weighed, and immediately frozen (stressed plants). The decrease in weight (**Fig. S1**) was indicative of water loss. For each condition, the roots of 8 plants were pooled together to achieve the amount of DNA necessary for the subsequent bisulfite treatment.

DNA extraction. Peralta and Spooner's protocol[61] was followed with some modifications. This procedure included the use of CTAB as a detergent instead of SDS, which is appropriate for tomato due to its high content of sugars and polyphenols. The DNA quality was assessed by spectrophotometry using the A_{260}/A_{280} ratio. Only samples with an A_{260}/A_{280} ratio between 1.7 and 2.0 were used.

Bisulfite conversion procedure. We used the protocol described by Clark et al.[35] with some modifications. The DNA was digested with Dra I (5'-TTTAAA-3') at 37 °C overnight to obtain DNA fragments of approximately 2000 bp in average length, which were purified by extraction with phenol:chloroform (1:1). Total genomic DNA (1 μg) was then treated with bisulfite. The conversion step was performed for 16 h at 55 °C. The treated DNA was then purified using the commercial Wizard DNA Clean-Up System kit (Promega).

Post-bisulfite PCR. Both the regulatory region of *Asr2*, which was defined as comprising 1500 bp upstream of the +1 transcription start site[23] on chromosome 4, and the gene body of *Asr2* were separately amplified. The primers were designed using the Beacons Designer software (http://www.premierbiosoft.com/molecular_beacons/index.html) to have the highest possible content of T residues, especially at the 3' ends, to favor the selective amplification of bisulfite-converted molecules.[62]

For the amplification reaction of the regulatory region (968-bp amplicon), 5 μl of bisulfite-treated product was amplified by Taq DNA Polymerase (Invitrogen) in an MJ Research PTC-100 (MJ Research Inc.) according to the following program: 40 cycles of denaturation (94 °C, 30 s), annealing (61 °C, 30 s), and elongation (72 °C, 1.30 min). This PCR was performed using the primers listed in **Table S1**.

For the body region (602-bp amplicon), we used the same conditions, but the program was the following: 40 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 45 s), and elongation (72 °C, 45 s). For this purpose, we used the primers listed in **Table S1**.

In all PCR reactions, we used 0.625 U of Taq DNA polymerase, 6 μM $MgCl_2$, 0.2 μM dNTPs, and 0.2 μM of each primer in a final volume of 50 μl.

Validation of bisulfite conversion efficiency. Full conversion was assessed as described in Gonzalez et al.;[17] essentially, p-Bluescript SK (+) plasmid DNA (Stratagene) was in vitro methylated with mHaeIII methyltransferase (NEB) and incubated with bisulfite in parallel with the genomic DNA samples, followed by PCR with specific primers. The bisulfite treatment of the plasmid destroyed two pre-existing Bfa I restriction sites and created a new BfaI site, as expected. In addition, we ruled out a possible unintentional overestimation of cytosine methylation due to an eventual inefficient bisulfite conversion by using primers specifically designed to amplify the converted template but incapable of annealing to the natural sequence.[62]

Subcloning and sequencing. Subcloning was performed with the pGEM-T "easy vector" (Promega). Plasmid minipreps were processed from randomly picked insert-positive colonies using the GeneJET Plasmid Miniprep Kit (Fermentas). Sanger sequencing was performed with SP6 and T7 universal primers. For the regulatory region, 13 clones were sequenced in the case of plants under normal conditions and 16 for plants after 30 min of drought. For the coding region, 22 clones each were sequenced in both cases.

Methylation data analysis. To analyze the methylation data, we used the Kismeth software[63] (http://katahdin.mssm.edu/kismeth/revpage.pl), which allows visualization of the proportion of clones (epialleles) showing methylation at every particular site. Once the methylation level data for each site were gathered, GraphPad software (www.GraphPad.com) was used for the statistical analysis. The data were grouped according to methylation type (CpG, CpNpG, and CpNpN). Statistical significance was determined using the Mann–Whitney test at the 95% significance level. For a detailed analysis of the CNN methylation level on the regulatory region (**Fig. 2**), we employed the moving average statistical tool (http://lorien.ncl.ac.uk/ming/filter/filmav.htm), which calculates the mean in a recursive fashion, that is, depending on a previously calculated value. In our case, this method involved taking a window of a fixed size of 10 contiguous cytosines, calculating the average methylation value for those 10 cytosines, and then displacing the window one position (one cytosine) at a time for the successive calculations of the mean.

Chromatin immunoprecipitation (ChIP) specific for histone modifications. We followed the protocol of Ricardi et al.[64] but used Dynabeads protein A (Invitrogen) instead of agarose beads. We used 2 μg of DNA for the input and 8 μg for all the incubations with the antibody. To ensure that those amounts remained constant, the volumes were variable according to the DNA initial concentrations. Anti-H3K4me3, H3K9me2, and H3K27me3 antibodies were purchased from Abcam.

For the qPCR, we used 0.625 U of Maxima Hot Start Taq (Fermentas), 3 mM magnesium chloride, 2 mM dNTP mixture (Fermentas), 0.2 μM of each primer (IDT Inc.), and 2 μl of the template in a final volume of 25 μl. As the fluorophore, we used Sybr Green® (Roche). The reactions were conducted in a Stratagene Mx3000P equipment under the following cycling conditions: 2 min of denaturation at 94 °C followed by 40 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 67 °C, and 30 s of elongation at 72 °C. The melting curve was performed between 65 °C and 95 °C, with readings every 0.5 °C. The primers are listed in **Table S1**.

Expression analysis (RNA extraction, reverse transcription, and qRT-PCR). Total RNA was extracted with Trizol (Invitrogen) from 100-mg mortar-ground roots in liquid nitrogen followed by incubation with 12.5 U of DNAseI (Invitrogen). The reverse transcription was achieved using 2 μl RNA, 50 U MMLV-RT (Promega), and oligo-dT (50 pmoles) in a 25-μl final volume for 1 h at 42 °C. To prevent RNA degradation, 10 U RNaseOUT (Invitrogen) were added. The qRT-PCR was performed under the same conditions indicated for the ChIP experiments, using the primers listed in **Table S1**.

The data obtained for *Asr2* mRNA were normalized to *actin* or *EF-1* mRNA at each stress time before comparing the effects of the different stress treatments.

Determination of cell viability under water stress conditions by staining. We followed the protocol described by Tamás,[65] with some modifications, based on the use of Evans Blue, a dye capable of penetrating only dead cells that is subsequently extracted by dimethylformamide. In detail, the plants were stressed for different lengths of time. The roots were then cut into fragments of approximately 5 mm with a scalpel and weighed before the test. Subsequently, a solution of 0.25% Evans Blue was added with gently shaking for 15 min, followed by three washes with mQ water for 10 min each with stirring. The root pieces were then incubated for 1 h with dimethylformamide with continuous stirring and then centrifuged at 21 500 × g for 5 min. Finally, the supernatant was removed. The absorbance at 595 nm was measured using a microplate reader (Biorad) and normalized to the starting mass of the roots.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing interests.

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RMG performed all the experimental work, generated the data, and extensively revised the manuscript together with MMR and NDI. MMR set up the experimental conditions for ChIP. NDI introduced the theoretical frame, coordinated the project, and drafted the manuscript. All 3 authors read and approved the final manuscript.

MMR and RMG hold post-doctorate fellowships from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. NDI is an Independent Researcher at CONICET.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/25524

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