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# Salinity and drought response alleviate caffeine content of young leaves of *Coffea canephora* var. Robusta cv. S274

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**ABSTRACT**

With the ever-growing concern of water deficit due to global climatic change, the drought and salinity stress response of plants is a major area of research. However, the effect of these stress on cup-quality of coffee especially, the accumulation of caffeine biosynthetic metabolites, has not been documented. This work studies the methylxanthines (7-methylxanthine, theobromine, caffeine and theophylline) contents in young leaves of coffee in response to PEG-6000 (1.5% and 15% w/v) induced drought and sodium chloride (20mM and 200mM) induced salinity stress. In general, both the stress reduced the caffeine content except for 20mM NaCl. 1.5% PEG reduced caffeine by 0.46 fold and 0.57 fold during first 24hr and 48hr of treatment, respectively; PEG at 15% caused a reduction by 0.36 fold only in the 48hr of treatment compared to untreated plants; and NaCl at 200mM caused a reduction of 0.26 fold and 0.47 fold in the first 24 and 48hrs of treatment, respectively. However 20mM NaCl augmented caffeine by 1.93 and 5.1 fold in the first 24 and 48hrs of treatment, respectively. The levels of caffeine subdued on the withdrawal of the stressor, affirmatively indicating the stress stimuli to be responsible for the observed changes in caffeine levels. The biochemical profile was supported by transcript expression of the caffeine biosynthetic NMT genes and the analysis of regulatory motifs of the promoters. The contents of upstream methylxanthines (7-methylxanthine and theobromine) and the degradation pathway (theophylline) indicate that salinity and drought might have a negative impact on biosynthesis of caffeine but accelerated the rate of caffeine degradation.

## 1. INTRODUCTION

Global climatic changes are projected to be having a high impact on coffee cultivation in the major coffee producing regions of the world [1]. The main agronomic traits of priority consideration for sustainable cultivation of coffee include resistance to biotrophic and necrotrophic pathogens and pests that cause leaf rust [2], berry borer [3] and nematodes [4], high yield, higher cup-quality including low caffeine and tolerance to salinity and drought. Globally, coffee is traded under the regulation of the International Coffee Organization (ICO) with each producing country possessing their respective governmental boards to have a hold on global trade. Recent years observed the 'coffee crisis' period where the trade of coffee was drastically reduced though enough produce was available, leading to heavy losses to the shareholders and finally de-cropping [5]. The coffee crisis years is mainly attributed to sub-standard beans produced during that period (www.ico.org).

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Since the entire cup quality of coffee spoils by the presence of a single damaged bean among hundreds healthy ones, the coffee market slashed to very low during the crisis period. Cup quality of coffee is not only determined by the aroma and flavour of the brew but also by its nutritional value like amount and bio-availability of the antioxidant bio-actives and the content of various anti-nutritional factors like diterpenes and caffeine [6].

Caffeine biosynthesis is catalyzed by the members of the SABATH superfamily of methlytransferases called the *N*-methyltransferase (NMT) [7]. In *Coffea canephora*, 23 NMT genes are present on mainly two chromosomes-Chr1 and Chr9 [8]. Three distinct NMTs are involved in the biosynthesis of caffeine by sequential methylation at the $N_7$, $N_3$ and $N_1$ positions of the committed precursor, xanthosine [9].

These genes are called xanthosine methyltransferases (XMTs), methylxanthine methyltransferases or theobromine synthases (MXMTs) and di-methylxanthine methyltransferases or caffeine synthases (DXMTs) [7]. The regulation of caffeine synthase is unknown; nevertheless the promoter of theobromine synthase like gene has been isolated from *C. canephora* and

indicate the presence of light responsive (GT box and GATA motif), jasmonate responsive (E-box) and salicylate responsive motifs (W-box) [10]. With the coffee genome sequencing completed, information on the sequences of the promoter of all the NMTs is available for a more detailed study [8].

Because of introduction of coffee cultivation to newer locations [11] and due to the global climatic change there may be a need to study the roles of these diverse factors on the final cup quality of the brew. However, research on this stream is mainly focused on the accumulation of metabolites in the bean [12-14]. The area of research focusing on gene expression studies is relatively naive and much effort need to be laid out on the genetic regulatory mechanisms for the cup-quality of the brew. Caffeine is the most studied at the molecular biochemistry level and is known to be regulated by light conditions [15], altitude [16], stress [17] and varietal/species difference [18-19] as well as in the response to salicylic acid/and methyl jasmonate [20]. The effect of drought and salinity on caffeine content has not been thoroughly investigated yet. An earlier report indicated either an increase or a reduction in caffeine content in callus tissue depending on the morphology of the calli [21]. However, there is no study carried out on the effect of salinity and drought on whole plants. With this objective we attempt to study the effect of salinity and drought on caffeine content in young leaves of one year old *C. canephora* seedlings and further discuss the result with transcript profile data of caffeine biosynthetic genes and the *in silico* analysis of the promoter regions obtained from the genome database.

## 2. MATERIALS AND METHODS

### 2.1 Plant materials and treatment

One year old *C. canephora* var. robusta S274 seedlings were selected from nursery raised plants and acclimatized in 400ml of 1X liquid Hoagland's medium [22] in green house conditions under partial doom (temperature 28±3$^{o}$C). Salinity stress was provided by placing the plants in 20mM or 200mM sodium chloride in 1X Liquid Hoagland's medium whereas, drought conditions were mimicked by placing the plants in 1.5% or 15% PEG-6000. The concentration used to induce the salinity and drought response was adopted from earlier work [21]. Sampling was done at 24hrs (NaCl 20-24hrs, NaCl 200-24hrs, PEG 1.5-24hrs, PEG 1.5-24hrs) and (NaCl 20-24hrs, NaCl 200-48hrs, PEG 1.5-48hrs, PEG 1.5-48hrs) interval. In order to affirmatively relate that the changes in caffeine content is caused by the stress induction, two types of control were used: one where the plants were not exposed to the stress (labeled as 'Control') and the other where the plants were exposed to the respective stress till 48hrs and then transferred back to control conditions for the next 48hrs (labeled as 'Control-Retained'). In all cases, the second pair of leaves was sampled for both estimation of methylxanthines and RNA isolation.

### 2.2 Methylxanthine estimation

The leaves were dried overnight at 42$^{o}$C and the dry material was weighed. Approximately 500mg dried tissue was ground in 5ml of 50% (v/v) methanol:water until a fine paste like slurry. The slurry was boiled at 80$^{o}$C for 10 minutes and the supernatant was extracted after centrifugation at 14,000xg for 10 minutes. The pellet was re-extracted similarly with 5ml 50% (v/v) methanol:water four times and the five extracts were pooled. The pooled extracts were freeze-dried and re-dissolved in 50% (v/v) methanol:water at a ratio of 10µl/mg tissue dry weight (dw.) basis. For HPLC analysis, 20µl sample was injected on Shimdazu LC10 HPLC workstation equipped with SPD10A detector and Bondapack $C_{18}$ column. The solvent system used was 0-40% methanol: 50mM sodium acetate gradient and the methodology were adopted from the literature [23]. The standard plots and statistical validation of HPLC equipment and result outputs was carried out using SPSS software similarly as for the earlier published work [15]. The results for 7-methylxanthine (7-MX), theobromine (Tb), Caffeine (Cf) and theophylline (Tp) were indicated in terms of fold change by comparison with the Control samples. Coefficient of variation (CV) of treated and control readings was calculated as the standard deviation by mean ratio and the CV of the quotient for fold change ($CV_{fold}$) of test and control samples was calculated by the formulae as:

$$CV_{fold} = \sqrt{CV^2_{treated} + CV^2_{control}}$$

### 2.3 Transcript profiling

Total RNA was extracted from 100mg fresh tissue using the method of Salmona et al. [24] with slight modification. Briefly, the frozen tissue was ground to fine powder in liquid nitrogen using a mortar and pestle and poured into 5ml extraction buffer (100mM Tris-HCl, pH 8.0, 30mM $Na_2$-EDTA, 2M NaCl, 2% CTAB, 2% PVP). The slurry was incubated for 2hrs in the presence of proteinase-K (100µg/ml slurry) and β-mercaptoethanol (20µl/ml slurry). The sample was extracted twice with equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 8000rpm at 4$^{o}$C for 20 minutes. The total RNA was precipitated overnight at 4$^{o}$C by adding one third volume 8M lithium chloride and centrifuged for 30 minutes at 12000rpm at 4$^{o}$C. The pellet was washed with 1ml 2M lithium chloride and dissolved in 500µl RNase free water. The RNA was precipitated by adding two and a half volumes of absolute ethanol and one tenth volume of 3M sodium acetate, pH 5.2 to the aqueous solution. The pellet was obtained by centrifugation at 12000xg, 10 minutes, 4$^{0}$C, air-dried and finally dissolved in 30µl RNase free water. The RNA was treated with one unit DNaseI (Thermo, Fermentas) using standard protocol and 1µl of it was used in minus reverse transcriptase coupled PCR to check for contaminating DNA. Reverse transcription was carried out on 1µg total RNA primed with 50µg oligo-$dT_{18}$ (Qiagen) using Improm-II reverse transcriptase (Promega) at 42$^{o}$C for one hour. RT was heat inactivated at 65$^{o}$C for 10 minutes in the presence of EDTA and diluted to 1:4 prior to PCR using the primers for XMT, MXMT (including the 378 amino acid variant CaMXMT1-like) and DXMT described in Table 1. The entire PCR product was size fractionated in 1% agarose gel and visualized with ethidium bromide staining. RT

**Table 1:** Details of primers and amplicons.

| Primer name | Gene | Sequence | Amplicon Size | Accession numbers of sequences used for primer designing |
|---|---|---|---|---|
| NMT123-F<br>NMT1-1R | Xanthosine methyltransferase (XMT) | 5' TGT AAA GGA GTT GAA TTA GAC GCC 3'<br>5' CTG CTT TAA TAT GTT CAT CGT CAA T 3' | 250bp | AB048793 (CaXMT1), AB084127 (CaXMT2), DQ422954 (CcXMT1), AB03469 (CmXRS1). |
| TSRT-1F<br>NMT2-1R | Theobromine synthase (MXMT/MXMT1) | 5' ATA GTT TCA ATA TTC CAT TCT TTA C 3'<br>5' GGG TTC GTA AAC TGA TCT AAT TAA T 3' | 209bp | AB048794 (CaMXMT1), 1AB084126 (CaMXMT2), AB034700 (CTS1), AB054841 (CTS2), DQ348077 (PG-1), DQ348078 (PG-4), DQ010011 (PG-5). |
| MXMT1-1F<br>MXMT1-1R | | 5' ACC CAG TAA GAT CCC ATG AAC A 3'<br>5' GAG AGA AAT GAT AAG ATT ATT ATA GC 3' | 201bp | AB048794 (CaMXMT1), AB034700 (CTS1), DQ348077 (PG-1), DQ348078 (PG-4), DQ010011 (PG-5). |
| CcTS3x-3F<br>NMT3-1R | Caffeine synthase (DXMT) | 5' ACG TGG CCG AAT GCT CCT TAC 3'<br>5' GGT TCG AAA ATT GAT CTA ACG ACA 3' | 354bp | AB086414 (CaCCS1), AB084125 (CaDXMT1), DQ422955 (CcDXMT1). |
| GAPDH-F<br>GAPDH-R | Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | 5′ ACG ATA GGT TTG GCA TTG T 3′<br>5′ GTG CTA CTG GGA ATA ATG TT 3′ | 139bp | GQ372995 |
| Ubi-F<br>Ubi-R | Ubiquitin (UBI) | 5′ GGG TGG AGG AGA AAG AAG GAA T 3′<br>5′ CTC CAC CTC TCA GAG CAA GAA C 3′ | 144bp | AF297089 |

reaction was normalized using primers for internal control as glyceraldehyde 3-phosphate dehydrogenase and ubiquitin since these two were indicated to be the best combination for stability of normalization [25].

### 2.4 In silico analysis of the promoter elements

Coffee genome indicates the presence of 23 NMT genes annotated as enzymes involved in caffeine biosynthesis. The genome browser was used to download 3Kb sequence upstream to the start codon as the probable upstream regulatory region. The promoter DNA sequence along with the first exon (75bp) was aligned by MAFFT and the alignment was progressively cured by Muscle or ClustalW2 all on Linux platform. Promoter motifs were identified by Nsite-PL software included in Softberry package by comparing with Plant RegSite database at 0.95, 0.99 and 0.999 significance threshold but only the 0.999 significance level results are represented. *CcNMT16* was omitted from the multiple sequence Nsite-PL analysis due to high sequence variation and improper alignment.

## 3 RESULTS

### 3.1 Methylxanthine contents in salinity and drought treatment

The general trend of methylxanthines indicates a reduction of caffeine content of the young leaves during drought and acute salinity stress (200µM concentration) (Figure 1). Plants treated with 1.5% PEG showed around 50% reduction in caffeine (0.46 fold change in 24 hrs and 0.57 fold change in 48 hrs), plants treated with 15% PEG-6000 exhibited greater than 50% reduction (0.36 fold change) in 48 hrs with no change in first 24 hrs. Plants treated with 200µM NaCl exhibited drastic decrease in caffeine content in 24hrs (0.26 fold change) and 0.47 fold change at 48hrs of treatment. However, plants treated with 20µM NaCl exhibited an increase in caffeine during 24hrs (1.93 fold change compared to control) and 48hrs (5.10 fold change compared to control plants). When the treated plants were shifted back to control (Control-Retained), the plants showed a response of recovering the caffeine levels. Rescued plants had 0.75 fold change caffeine upon rescue from 1.5% PEG-6000 compared to control and 0.78 fold change upon rescue from 15% PEG-6000 treatment compared to control. Caffeine levels were equal to control plant levels upon rescue from 20µM NaCl treatment and exhibited a 0.53 fold change upon rescue from 200µM NaCl treatments when compared to leaf caffeine content of control plants.

Methylxanthines upstream to caffeine in the biosynthetic pathway accumulated to higher levels than control in 1.5% PEG-6000 and 20µM NaCl treatment at least by the 48$^{th}$ hour of stress (1.58 and 2.23 fold change for 7-MX and Tb, respectively during 24hr of treatment with 1.5% PEG-6000; 3.55 and 3.15 fold change for 7-MX and Tb respectively, during 48hr of 20µM NaCl treatment). However, an acute response caused due to high concentration of the stressor lead to a reduction in the 7-MX and Tb contents compared to control (0.72 and 0.80 fold change for 7-MX during 24hr and 48hr treatment, respectively with 15% PEG-6000 and 0.30 fold change for 7-MX during 48hr of 200mM NaCl stress treatment).

Interestingly, theophylline which is the major degradation product of caffeine, accumulated to higher levels than control in most of the stress treatments (3.92 and 3.53 fold change in 24 and 48hrs of 1.5% PEG-6000 treatment, respectively; 1.34 and 3.55 fold change in 24 and 48hrs of 20µM NaCl treatment, respectively; and, 3.54 fold change in 24hrs of 200µM NaCl treatment). However theophylline levels began subduing as the stress treated plants were rescued in control 1x Hoagland's medium (1.92 fold change

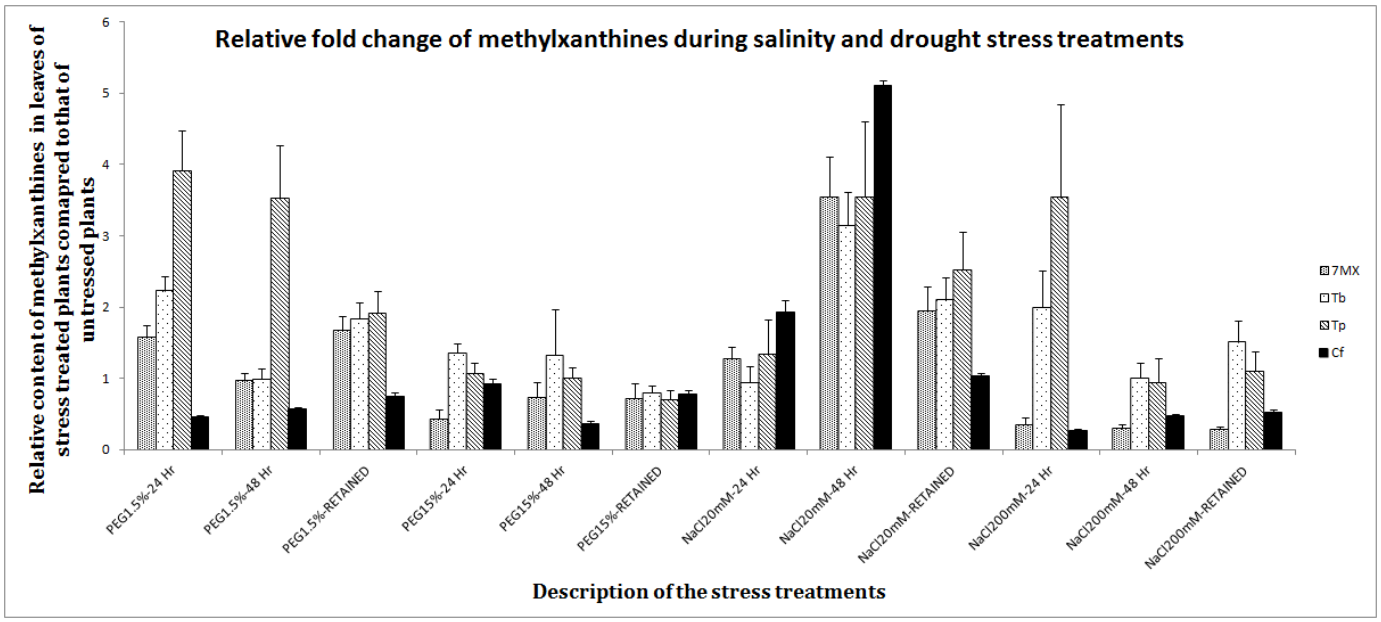


**Figure 1:** Relative fold change of methylxanthines in young leaves during salinity and drought treatments of the plants. The fold increase of 7-methylxanthine (7-MX), Theobromine (Tb), Caffeine (Cf) and Theophylline (Tp) in 24 and 48 hrs of treatment with 20 and 200mM NaCl and 1.5 and 15% PEG-6000 treated seedlings of *C. canephora*. Control-retained refer to respective treated plants subjected to control conditions after 48hrs treatment with the stressor. The fold change was calculated by comparing the values with untreated or control plants.

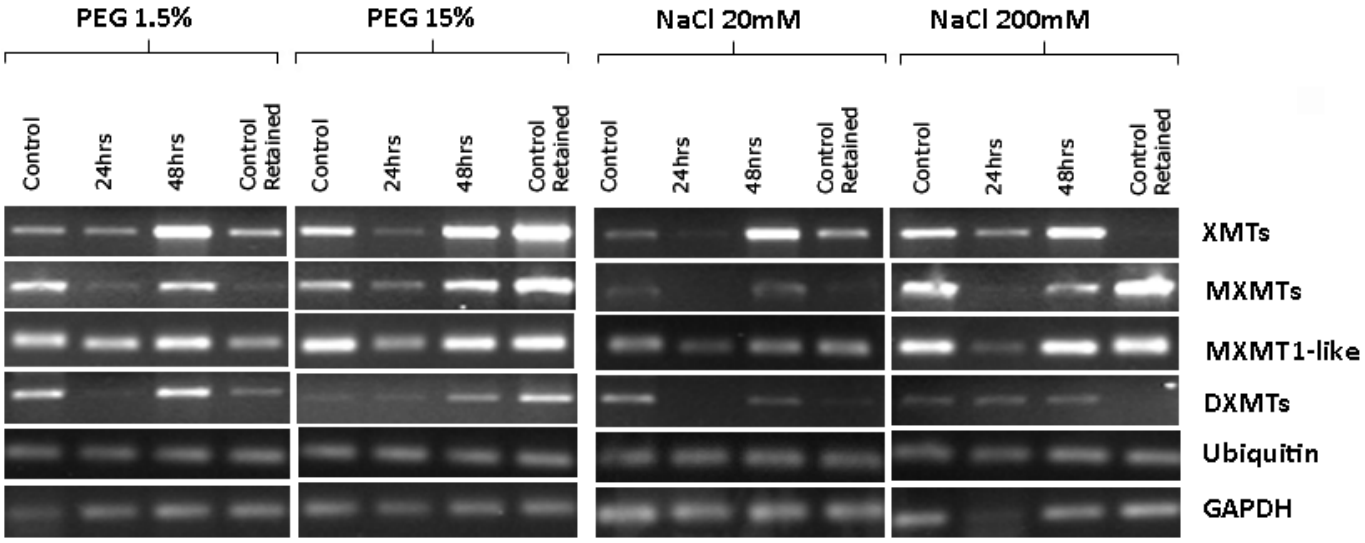


**Figure 2:** Transcript profiling of NMT genes during salinity and drought treatments: semi-quantitative RT-PCR for transcripts for the first (XMTs) second (MXMTs) and third (DXMTs) NMT genes of the caffeine biosynthetic pathway. 'MXMT1-like' refers to the transcript specific to the 378 amino acid variant of *C. arabica* MXMTs. Treatment times and concentrations are same as referred in Figure 1 legend. Glyceraldehyde 3-phosphate dehydrogenase and ubiquitin were used as internal controls.

after rescue from 1.5% PEG-6000 treatment; 2.52 fold change after rescue from 20µM NaCl treatment; and, 1.10 fold change after rescue from 200µM NaCl treatment). Theophylline content remained constant during 15% PEG-6000 treatment. Since theophylline is the major route of caffeine degradation, our results might be indicative of increased caffeine degradation during the salinity and drought stress treatments.

### 3.2 Levels of *N*-methyltransferase transcript during salinity and drought treatment

Since the methylxanthine profile during drought and salinity treatments was indicative of low caffeine content coinciding with accumulation of upstream methlyxanthines, further probe using semi-quantitative gene expression of the three NMTs of the biosynthetic pathway was carried out to study if

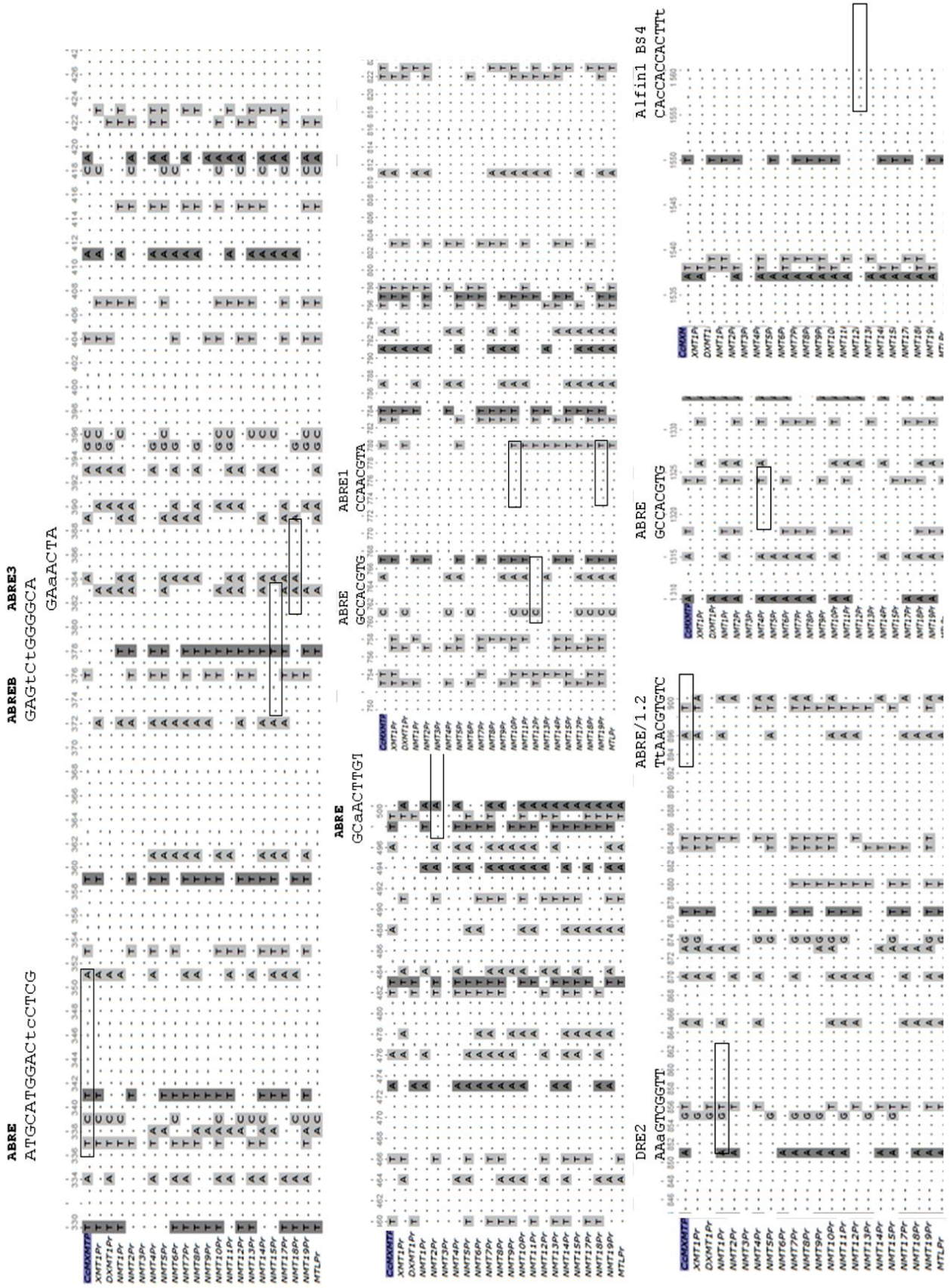


**Fig. 3:** Graphical representation of multiple sequence alignment of 22 NMT promoters and prediction of osmotic responsive elements from position 330-1560 of alignment: *CcNMT*1- *CcNMT*23 except *CcNMT*16 along with *CcXMT*1, *CcMXMT*1, and *CcDXMT*1 promoters downloaded from coffee genome were aligned by MAFFT and used for the graphical representation of regulatory motif prediction software NSITEPL by comparing to Plant RegSite database at 0.999 significance.

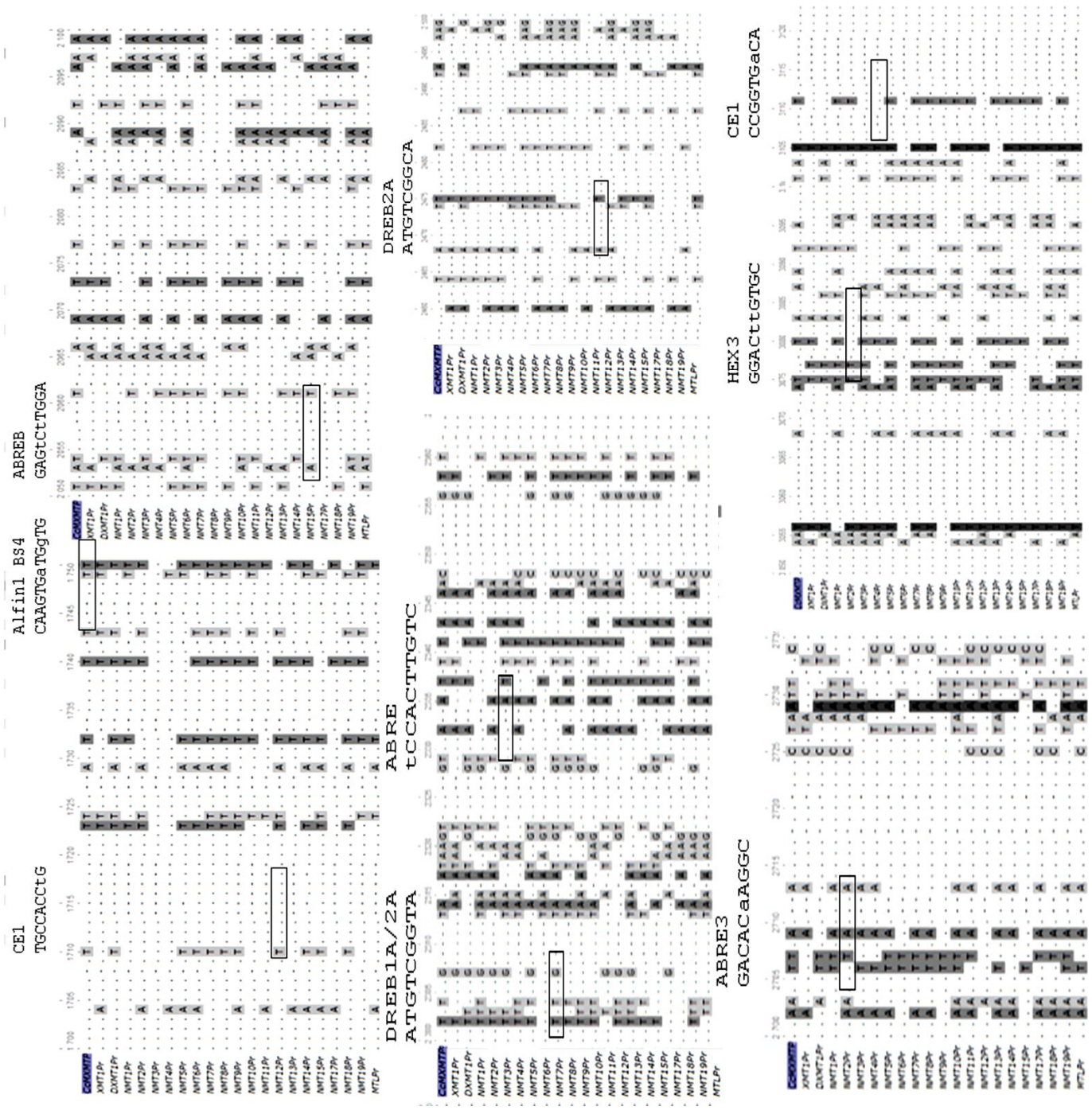


**Fig. 4:** Graphical representation of multiple sequence alignment of 22 NMT promoters and prediction of osmotic responsive elements position from 1700-3124: *CcNMT*1-23 except *CcNMT*16 along with *CcXMT*1, *CcMXMT*1, and *CcDXMT*1 promoters downloaded from coffee genome were aligned by MAFFT and used for the graphical representation of regulatory motif prediction software NSITEPL by comparing to Plant RegSite database at 0.999 significance.

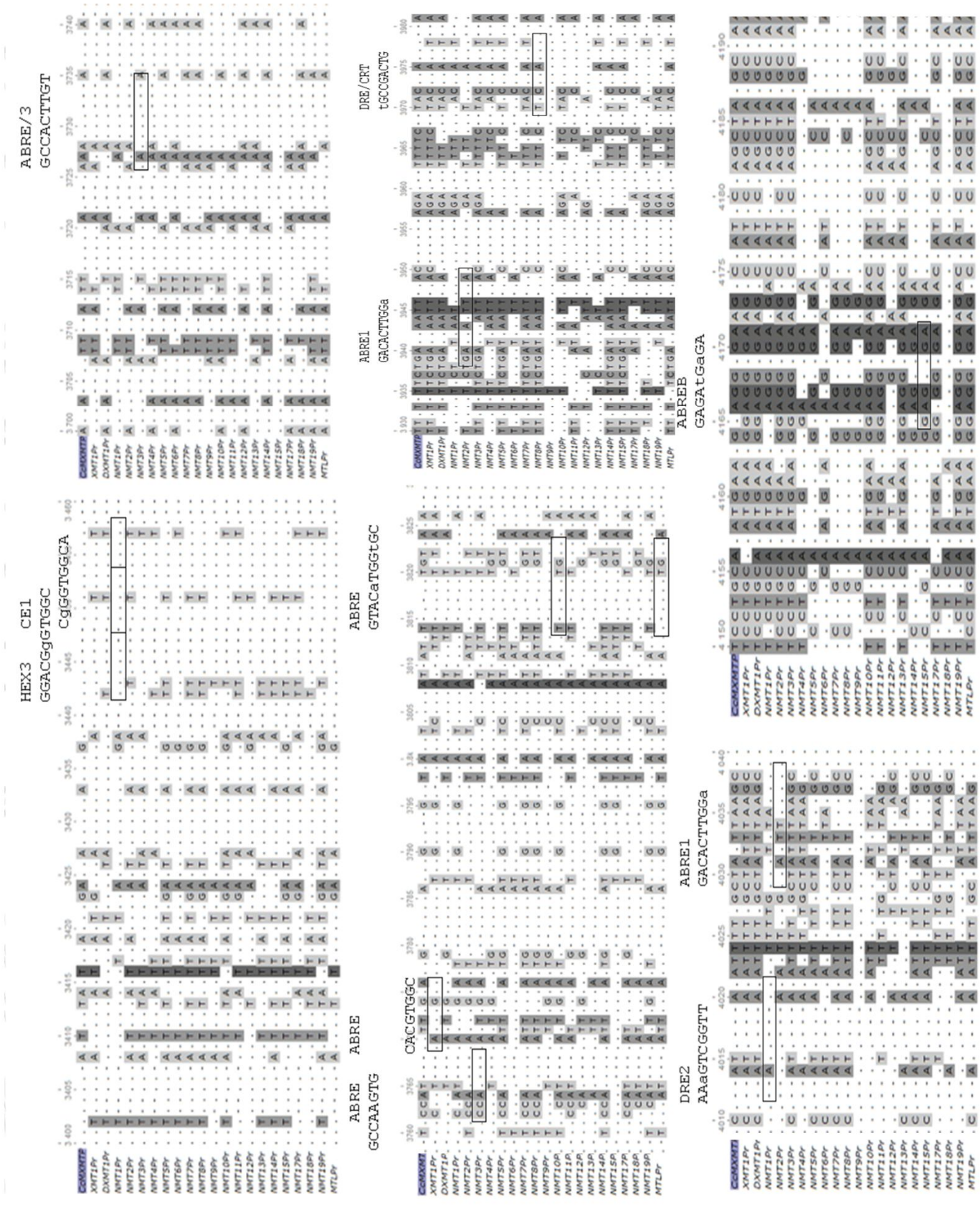


**Fig. 5:** Graphical representation of multiple sequence alignment of 22 NMT promoters and prediction of osmotic responsive elements position from 3402-3980: *CcNMT*1-23 except *CcNMT*16 along with *CcXMT*1, *CcMXMT*1, and *CcDXMT*1 promoters downloaded from coffee genome were aligned by MAFFT and used for the graphical representation of regulatory motif prediction software NSITEPL by comparing to Plant RegSite database at 0.999 significance.

transcriptional changes may be one of the reasons. Lack of characterized gene sequence for theophylline formation hampered the study on gene expression pattern with respect to caffeine degradation. The expression levels of the first NMT of caffeine biosynthetic pathway (XMTs) reduced at 24 hours of all treatments except for 1.5% PEG-6000 treatment where it was similar levels as of control and increased many folds after 48hrs of all treatments (Figure 2). MXMTs exhibited a reduction in 24hr of treatment with further expression similar to control in 48hr under all the treatments. The *CaMXMT-1* like variant of MXMT in *C. canephora* had an expression pattern similar to XMTs but having more prominent increase in 48 hours of 200mM NaCl, 1.5% PEG-6000 and 15% PEG-6000 treatments. Caffeine synthase transcript decreased at 24 hrs of treatment only during exposure to low concentration of stressor (1.5% PEG-6000 and 20µM NaCl). These results may be the reason for 7-MX and Tb accumulation during exposure to low concentration of stressor (1.5% PEG-6000 and 20µM NaCl). In case of high concentration of stressor the DXMTs levels did not change much.

### 3.3 Prediction of NMT gene regulation by *in silico* analysis of the promoter sequences

Analysis of regulatory elements of the 22 NMT-like gene promoters indicated the presence of both ABRE (abscisic acid responsive element) and DRE (drought responsive element) in many of the 22 known gene promoters (Figure 3, 4, 5) indicating that the caffeine biosynthetic NMTs may be under control of both ABA-dependent and ABA-independent response. Among the characterized genes, *CcXMT1* and *CcMXMT1* both carry ABRE elements at 285 bases upstream of start codon. ABRE is also located in the *CcXMT1*-Pr and at 2839 and 2396 base upstream of start codon for *CcMXMT1*-Pr. However, NSite-PL software did not detect any salinity/drought responsive elements in the 3Kb upstream promoter region for the *CcDXMT1* gene. Other elements responsive to salinity/drought for e.g; hex3 in NMT1 and NMT2 promoter, CE1 (Coupling element) in NMT1, NMT4 and NMT12 promoters and Alfin1 BS4 in NMT2 and NMT12 promoters was also detected at 0.999 significance. Different families of transcription factors like Myc/Myb, WRKY, b-Zip, Nac, jasmonate responsive, GT-1 and GATA also have binding elements in the promoters of many of the annotated NMTs implication the role of these in regulating caffeine biosynthesis in coffee. However, further studies are required to validate this observation.

## 4 DISCUSSION

Exposure to low concentration of stressor lead to increase in transcripts of the first two NMTs at 48hrs of treatment but a drop at 24 hours of treatment. Since, the gene expression of DXMTs also dropped in the 24hr of treatment a halt in caffeine synthesis might have caused a small increase in 7-MX and Tb un-utilized in the biochemical pathway at 24hrs of treatment with 1.5% PEG-6000 and 24 and 48hrs of treatment with 20µM NaCl. Furthermore, caffeine content dropped in 1.5% PEG-6000 treatment during both 24 and 48hrs of treatment owing both to reduced DXMT expression and more due to a prominent increase of caffeine degradation as evident from many fold increase of theophylline content in both the time points of 1.5% PEG-6000 treatment. NaCl (20µM) treated plants exhibited very high caffeine accumulation at 48hrs of treatment, although theophylline content also was high. Accumulation of theophylline was not very pronounced in high concentration of both the stressors except for during 24hrs treatment with 200µM NaCl. Theophylline quantities otherwise remained similar to control conditions and might be indicative of normal dynamics of caffeine degradation in high concentration of stressor. Levels of DXMT transcripts also did not change much during the time course of both the high concentration treatments though XMTs and MXMTs increased only during 48hr of 15% PEG-6000 treatment.

As a result, caffeine levels dropped below the levels in leaves of control plants. In conclusion, salinity and drought stress alter caffeine levels in young leaves of *C. canephora* by altering the caffeine degradation route and perhaps to a smaller extent even the regulation of transcripts of the genes involved in the biosynthesis pathway. During mild drought conditions, the caffeine levels reduce due to caffeine degradation and reduced caffeine synthase transcript leading to accumulation 7-MX and Tb during 24hrs of treatment. Our results comply with previous research on caffeine content in developing leaves and berries of *C. arabica* and *C. liberica* var. *dewevrei* that indicate that the total caffeine content is determined by the ratio of rate of biosynthesis to rate of degradation [26].

Salinity and drought stress converge in similar physiological conditions in terms of water deficit [27]. However, salinity stress can additionally induce ionic stress leading to changes in the ionic movement and $Cl^-$ toxicity [28-29]. On the other hand, drought can induce abscisic acid (ABA)-responsive pathway and lead to various responses like stomatal closure [30], increase in antioxidant enzymes (e.g. catalase, peroxidase and dismutase) [31], accumulation of stress metabolites (Glutathione) [32] as well as proteins related to water stress tolerance called as osmolytes (e.g. LEA, dehydrins) [33]. Both water stress or drought and salinity stress lead to reduced water potential thus affecting the plants metabolism, especially the carbon-reduction pathway, light reactions and accumulation of toxic molecules and involve many similar signal transduction pathways [34]. However, in field conditions, the adaptive response to salinity stress is mainly through maintaining ionic homeostasis and the response to drought is through osmotic adjustments by accumulation of ions, solutes or organic compounds [35-36]. Salinity stress proceeds in two phases: the initial intense and rapid phase is the osmotic phase and the later slow and less intense phase is the ionic phase [27]. Osmotic signaling and stress responses in plants follow both an ABA-dependent and ABA-independent pathway [37]. The core regulators of ABA-dependent pathway include transcription factors like, ABRE binding factors (ABF), MYB/MYC whereas, ABA-independent pathways involves the DREB and CBF. The promoter element binding factors may positively or negatively

regulate the gene expression; hence the regulatory element prediction of promoters is the first step to predict a gene regulatory response. Though, the regulatory elements for drought and salinity were predicted at a high significance level (0.999) criterion in NMT promoters using the NSITEPL software, the threshold for identity to consensus was set to relaxed mode. Further studies on the specific factors binding to these elements may provide further clues on the regulatory mechanisms.

Caffeine is a purine alkaloid that might have a function in plant defense against herbivory and pests [38] and is an important component for allelopathic [39] and pollination ecology [40]. There are many reports of studies on drought response in coffee and a few genes expressed in response to osmotic stress are also known [41-42]. Nevertheless, there are previous reports in other plant systems describing salinity treatments leading to increase in levels of alkaloids [43-44]. Various elements like ABRE, DRE, CE1, hex3 and Alfin were observed in the promoter regions of NMTs and many were represented in the characterized caffeine biosynthetic NMTs. ABRE elements (consensus (C/T)ACGTGGC sequence) are a family of sequences known to be involved in ABA dependant osmotic response and bind to ABRE binding factors (ABFs) [45]. The ABA responsive elements lacking the consensus motifs include the coupling element (CE3) (ACGCGTGTCCTC), motif III (GCCGCGTGGC) and hex-3 (GGACGCGTGGC). Recent studies indicate that ABRE alone may not be able to manifest the entire range of effects of ABA but require additional factors called the coupling element (CE-1) (TGCCACCGG) [46]. On the other hand, DRE elements are binding sites for ABA-independent factors like DREBs and CBFs [47]. The elements, hex3 is for binding of ABFs but like CE1 they lack the ACGT core motif of ABRE. Apart from these, Alfin1 proteins may act as co-activators during salinity tolerance [48]. Considering regulation of caffeine biosynthetic genes in response to salicylic acid, methyl jasmonate [20], light [15] and developmental response [49-51] in coffee as well in other plants like cocoa [52-53] it is clear that transcriptional gene regulation of caffeine biosynthetic genes along with the degradation dynamics may have a major role in the accumulation of the methylxanthines in plants. Our results show that caffeine accumulation reduces in response to drought and salinity stress. Since *C. arabica* are more drought tolerant compared to *C. canephora* and even within the *C. canephora* varieties the physiological osmotic stress response in terms of accumulation of proline, nitrogen, phosphorus, potassium, calcium and carbohydrates varies between the tolerant and susceptible varieties, the application of our findings with respect to germplasm selected for quality improvement may not be immediate [54].

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