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1 Original paper submitted to "Plant Cell Reports"

2 Characterization of a novel vascular bundle localizing *Gossypium hirsutum* NAC4 3 transcription factor promoter for its role in environmental stress responses

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11 Key message:

12 GhNAC4 transcription factor from cotton, localizes to vascular bundles and is highly upregulated by phytohormones 13 and environmental stresses.

14 Abstract:

15 We have studied the expression of *GhNAC4*, a NAC domain-containing transcription factor from *Gossypium* 16 *hirsutum*. The expression of *GhNAC4* was upregulated by ABA, cytokinin, JA, GA, auxin, and ethylene. Its 17 expression was also highly induced by drought, osmotic stress, oxidative stress, salinity, high and low-temperature 18 stress, and wounding. To corroborate these observations, we have conducted a spatio-temporal analysis of the 19 promoter of the *GhNAC4* gene, by using *uidA* (GUS) gene in a transcriptional fusion in transgenic tobacco. The 20 GUS expression was mostly localized to the vascular bundles along with shoot apical meristem and guard cells. We 21 also observed intense staining in other cells upon wounding. Sequence analysis of the promoter revealed the 22 presence of several motifs pertaining to phytohormone responsiveness, stress-inducibility, light responsive, sugar- 23 responsive and tissue-specificity. These data suggests that GhNAC4 is a vascular tissue localizing NAC 24 transcription factor, which may act as a node integrating environmental stress signals for modulating plant growth 25 and development with the aid of phytohormonal stimuli.

26 Keywords:

27 NAC transcription factor, promoter characterization, vascular bundle localization, phytohormones, environmental 28 stress

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1 **Abbreviations:**

2 4-MU 4-Methylumbelliferone
3 ABA Abscisic acid
4 BAP 6-Benzyl aminopurine
5 GA Gibberellic acid
6 GUS β-Glucuronidase
7 IAA Indole-3-acetic acid
8 MeJA Methyl Jasmonic acid
9 MUG 4-Methylumbelliferyl-β-Dglucuronide
10 MV Methyl viologen
11 NAC NAM ATAF CUC
12 PEG Polyethylene glycol
13 SA Salicyclic acid
14 TF Transcription factor

15 **Introduction:**

16 Plants have developed intricate mechanisms for sensing various environmental stresses and suitably responding to
17 them. These stresses can induce a wide range of local and long-distance signals, which have to be synchronized and
18 integrated into the signal transduction pathway at the whole plant level (Tran et al. 2007). The processes like growth
19 and development have to be regulated in the plant for it to tolerate and survive under stress (Chaves et al. 2002). One
20 of the most common ways for a plant to connect local stimuli to systemic responses is via the regulation of
21 phytohormone levels, which in turn regulate gene expression (Verma et al. 2016). Understanding hormonal cross
22 talk is thus vital to deciphering how plants activate the required set of responses to a particular stress condition. The
23 first step to achieve this goal is to identify the regulatory components of signaling pathways (Tian & Lu 2006).
24 Transcription factors (TFs) function as regulatory trans-acting proteins that bind to specific *cis*-acting elements in
25 the promoters of target genes and lead to the activation or repression of gene expression. Hence, the expression
26 pattern of a given gene is a direct consequence of the promoter function, and *cis*-acting elements are important
27 molecular switches that play a crucial role in transcriptional regulation (Yamaguchi-Shinozaki & Shinozaki 2005).
28 They control a complex and dynamic network of gene expression, which affects biological processes. Accurate
29 functional analysis of *cis*-acting elements would help to understand the complex gene regulatory networks
30 underlying plant adaptation to stress (Yamaguchi-Shinozaki & Shinozaki 2005). The identification of stress-
31 inducible and tissue-specific promoters is of significant theoretical and practical importance, as they would eliminate
32 the need for deployment of constitutive promoters that cause unnecessary metabolic penalties in transgenic plants
33 (Venter 2007).

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1 Vascular tissues consist of xylem and phloem, and are derived from the meristematic vascular procambium and
2 cambium. The proliferation of cambium, development, differentiation, and patterning of vascular tissue requires a
3 hormonal response, transcriptional regulators, and peptide signaling components. Various transcription factors (TFs)
4 like NAC, MYB, and HD-ZIP have been implicated in vascular bundle development (Yang & Wang 2016).

5 NAC (NAM ATAF CUC) TFs constitute one of the largest plant-specific TF super-family with 171 members in
6 Arabidopsis and 151 members in rice (Nuruzzaman et al. 2010). So far 283 NAC genes in *Gossypium hirsutum,* 147
7 in *G. arboreum*, 267 in *G. barbadense, and* 149 in *G. raimondii* have been identified (Sun et al. 2018). A typical
8 NAC TF consists of two domains, a conserved N-terminal domain and a highly divergent C-terminal domain (Olsen
9 et al. 2005). Previous studies have shown that NAC TFs play an important role in regulating a wide variety of
10 biological processes such as shoot apical meristem development (Souer et al. 1996), seed development (Sperotto et
11 al. 2009), leaf senescence (Guo & Gan 2006), flower development (Sablowski & Meyerowitz 1998), fibre
12 development (Ko et al. 2007), abiotic stress response (Hu et al. 2006) and biotic stress response (Nakashima et al.
13 2007).

14 The biological functions of NAC TFs in vascular development have been emerging recently. NAC TFs act as master
15 switches regulating the vascular tissue differentiation and secondary cell wall formation (Wang & Dixon 2012).
16 VASCULAR-RELATED NAC-DOMAIN1 (VND1) to VND7 are specifically expressed in vascular tissues. VND6
17 and VND7 regulate the vessel differentiation at inner metaxylem and protoxylem in the roots respectively (Kubo et
18 al. 2005). NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, and
19 SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 1 (SND1)/NST3/ANAC012 act as
20 transcriptional switches and regulate secondary cell wall thickening in various tissues and are localized to xylary and
21 extraxylary fibers in the inflorescence stem (Ko et al. 2007; Mitsuda et al. 2005; Mitsuda et al. 2007). XYLEM NAC
22 DOMAIN 1 (XND1) is specifically expressed in xylem and negatively regulates tracheary element growth (Zhao et
23 al. 2008). So far, very little information is available about the role of NAC TFs in vascular development in cotton.
24 GhXND1, a cotton NAC TF is involved in negative regulation of xylem development. Ectopic expression of
25 GhXND1 resulted in a decrease in the number of xylem vessels and reduction of interfascicular fibers thickness (Li
26 et al. 2014). *GhFSN1* (fiber secondary cell wall-related NAC1) is expressed in cotton fibers and is important for
27 secondary cell wall synthesis (Zhang et al. 2018).

28 Comprehensive expression analysis of NAC TFs would be useful in understanding their biological function.
29 However, very few studies have investigated the spatio-temporal expression of NAC TFs during stress responses
30 and development.

31 Cotton is one of the most widely cultivated and economically important crops and is a primary source of natural
32 fiber. However, cotton-growing areas are prone to high and low temperatures, extreme drought, salinity, and pest
33 infestation that can hinder cotton productivity. Very little information is available about the molecular mechanisms

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1 regulating the responses of cotton plant to environmental stresses. Hence, advancements to accelerate stress
2 tolerance are very important for cotton production.

3 GhNAC4 is one of the first reported NAC TF expressing in vascular bundles in cotton. To understand the molecular
4 mechanisms regulating NAC TFs and accelerate the use of NAC TFs in engineering transgenic cotton, we have
5 characterized the differential regulation of *GhNAC4*, isolated earlier by Meng et al. (2009). In this direction, we
6 performed an expression analysis of *GhNAC4* in response to various environmental stress conditions and
7 phytohormones treatments, identified putative *cis*-acting elements in the promoter region, and observed the spatio-
8 temporal localization of *GhNAC4* in various plant developmental stages. Our findings show that GhNAC4 is a novel
9 vascular bundle localizing NAC TF, which is responsive to environmental stress and phytohormone signals and
10 might be involved in plant growth and development.

11 **Materials and methods:**

12 **Plant material and stress treatments:**

13 Cotton (*Gossypium hirsutum* var. JK Durga) seeds were surface sterilized with 70% ethanol for 2 min followed by
14 4% sodium hypochlorite for 15 min. The seeds were then rinsed four times with sterilized water and soaked for 5-6
15 h. The seeds were germinated and grown for two weeks on blotting water placed on top of 0.5X MS media without
16 any growth regulators.

17 For hormonal treatments, the filter paper on which the seedlings were grown, was moistened with either
18 100 µM ABA, 100 µM MeJA, 100 µM SA, 20 µM 6-BAP, 20 µM GA_3 or 20 µM IAA and incubated for 24 h prior
19 to sampling. Untreated seedlings and filter paper moistened with water, having the same quantity of sodium
20 hydroxide or alcohol used for dissolving the hormones were also used as controls. Ethylene treatment was carried
21 out for 24 h by placing the seedlings on filter paper in a sealed container, and Ethephon was added to the box and
22 diluted to a final concentration of 10 ppm in distilled water. Seedlings in a similar container having air were used as
23 a control.

24 High salt and osmotic stress were induced by moistening the filter paper with 0.3 M NaCl and 0.3 M
25 mannitol respectively, and the seedlings were allowed to grow for 24 h. Oxidative stress was induced by moistening
26 the filter paper with 10 µM methyl viologen and incubated for 24 h. For inducing drought stress, the seedlings were
27 air dried for 30 min. Flooding stress was achieved by immersing the seedlings in distilled water for 24 h. For the
28 wounding treatment, the leaves of seedlings were squeezed with a forceps, and the wounded seedlings were
29 harvested after 30 min. For the combination of dark and cold treatment, the seedlings were wrapped in aluminum
30 foil and incubated at 4 ^{o}C for 24 h, while the dark treatment was carried out at 25 ^{o}C for 24 h. In the high-
31 temperature stress, the seedlings were subjected to 42 ^{o}C for 12 h. Following all the treatments, the leaves were
32 immediately frozen in liquid nitrogen to analyze the expression levels of *GhNAC4* gene.

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1 To determine the degree of promoter activation, homozygous T_2 tobacco seedlings were subjected to different
2 hormonal treatments and environmental stresses following the same methodology as applied to cotton seedlings, for
3 GUS activity measurement.

4 **RNA isolation and real-time quantitative PCR:**

5 Total RNA was extracted from the leaves of control and treated cotton seedlings by a CTAB (cetyltriethyl
6 ammonium bromide) extraction procedure as described by Chang et al. (1993). To avoid DNA contamination, the
7 total RNA was treated with RNase free DNase1 (Epicentre biotechnologies, USA) by incubating at 37 ^{o}C for 15
8 min. One µg of total RNA was used for synthesizing the first-strand cDNA using RevertAid 1st strand cDNA
9 synthesis kit (Thermo Fischer Scientific, USA) following the manufacturer's instructions. An oligo(dT)$_{-18}$ was used
10 as the primer in the reverse transcription reaction. For the real-time quantitative PCR, cDNA was diluted to 100
11 ng/µl and was mixed with the SYBR master mix (Kapa Biosystems, USA), and amplification was carried out
12 according to the manufacturer's protocol in the StepOne Plus machine (Applied Biosystems, USA). The
13 constitutively expressing Ubiquitin gene (*GhUBQ7*, GenBank accession no. DQ116441) from cotton, was used as an
14 internal reference gene. The primer sequences used to amplify the internal regions of *GhNAC4* (NAC4-RTF and
15 NAC4-RTR), and *GhUBQ7* (UBQ7-RTF and UBQ7-RTR, Kuppu et al. 2013) are mentioned in Table 1. To ensure
16 the gene specificity, the amplicons obtained by these primers were sequenced and confirmed. The fold change in the
17 *GhNAC4* gene levels relative to *GhUBQ7* gene was determined using the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001).
18 The experiment was performed in triplicates, and two independent biological replicates were used.
19

20 **Isolation and cloning of *GhNAC4* promoter from cotton:**

21 The full-length CDS of *GhNAC4* (GenBank accession number EU706342.1) was used as a query to retrieve the 5'
22 upstream sequence from Phytozome (https://phytozome.jgi.doe.gov/). Genomic DNA was isolated from the leaves
23 of cotton by the CTAB method(Murray & Thompson 1980). It was used as a template for PCR amplification of a
24 DNA fragment, in the range of-1492 bp to +119 bp (relative to the transcription start site). The primer sequences
25 (GhNACPRO-F, GhNACPRO-R) used for the amplification are mentioned in the Table 1. The amplicon was cloned
26 into pTZ57R/T (ThermoFisher Scientific, USA) and the accuracy was verified by sequencing. The fragment was
27 excised and sub-cloned into BamHI and PstI restriction sites of the promoter-less vector, pCAMBIA 1381Z
28 (Cambia, Australia) to generate a fusion gene having 5' upstream region of *GhNAC4* and *uidA* gene
29 (p*GhNAC4*Pro:GUS). This construct was used for *Agrobacterium tumefaciens* (strain EHA105) transformation by
30 using the freeze-thaw method.

31 **Generation of tobacco transgenics:**

32 *In vitro* grown three weeks old *Nicotiana tobacum* cv. Samsun leaves were used as explants, for transformation with
33 Agrobacterium harboring either the empty pCAMBIA 1381Z (vector control) or the $pPro_{GhNAC4}$:GUS vector,
34 according to Horsch et al. (1985). Explants were co-cultivated with Agrobacterium on a co-cultivation medium (MS

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1 salts with 2 mg/l BAP, 0.1 mg/l NAA and 3% sucrose pH 5.8) for 48 h in dark and later were transferred to the shoot
2 induction medium (MS salts with 2 mg/l BAP, 0.1 mg/l NAA, 3% sucrose, 10 mg/l hygromycin and 250 mg/l
3 Cefotaxime pH 5.8) with 16/8 h light/dark photoperiod at 24±2 ^{o}C. The regenerated shoots were sub-cultured on
4 shoot elongation medium (MS salts with 1 mg/l BAP, 0.05 mg/l NAA, 3% sucrose, 15 mg/l hygromycin and 250
5 mg/l Cefotaxime pH 5.8). The hygromycin resistant shoots were further sub-cultured on rooting medium (0.5xMS
6 salts with 0.1 mg/l NAA and 20 mg/l hygromycin pH 5.8). Plants with well-developed roots were transferred to
7 sterile soil in small plastic cups for acclimatization.

8 Further, well-established plants were shifted to pots and allowed to grow to maturity in green house and set seeds.
9 The putative primary transformants (T_0) were screened by genomic PCR for the presence of *GhNAC4* promoter
10 sequence and hygromycin gene (*HptII*) (with primers, HptII-F and HptII-R) and allowed to self-pollinate and set
11 seeds. The T_1 seeds from selected plants were germinated on 0.5X MS medium supplemented with 25 mg/l
12 hygromycin. Copy number of the DNA integration was determined by Hyg^R segregation test. Segregation analysis
13 was carried out by counting the number of green and bleached seedlings. The hygromycin-resistant (green)
14 seedlings were later transferred to soil in the green house after 2-3 weeks and allowed to set seeds. The T_2 progeny
15 were germinated on 0.5X MS medium supplemented with 25 mg/l hygromycin to identify the homozygous T_2 lines.

16 **Histochemical localization and fluorometric measurement of GUS activity:**

17 β-glucuronidase (GUS) activity was assayed as described by Jefferson et al. (1989) with minor modifications. For
18 histochemical staining, the tissues were vacuum infiltrated with the solution containing 1 mM X-Gluc (5-bromo-4-
19 chloro-3-indolyl-b-D-glucuronide), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 1 mM EDTA and
20 0.1% Triton X-100 in 50 mM phosphate buffer (pH 7.0) and were incubated at 37 ^{o}C for 12-14 h in the dark. After
21 staining, the tissues were fixed in a solution containing 4% formaldehyde in 50 mM phosphate buffer (pH 7.0) for
22 12 h at 4 ^{o}C and subsequently cleared in 70% ethanol at room temperature. Photographs were taken using M165 FC
23 and DM6B microscopes (Leica Microsystems, Germany).
24 For the fluorometric assay, the tissue was homogenized in 400 µl GUS extraction buffer containing 10 mM
25 EDTA and 0.1% Triton X-100 in 50 mM phosphate buffer (pH 7.0). After centrifugation at 12,000 rpm (4 ^{o}C) for 15
26 min, 5 µl of homogenate was diluted with 95 µl of extraction buffer and mixed with 100µl of extraction buffer
27 having 2 mM 4-methyl-umbelliferyl-β-D-glucuronide (4-MUG, Duchefa, Netherlands) and incubated at 37 ^{o}C for 1
28 h. The reaction was terminated by the addition of 1.8 ml of 200 mM sodium carbonate. Total protein concentration
29 in the homogenate was assessed by Bradford method (Bradford, 1976) with BSA as standard. Fluorescence
30 (excitation 363 nm, emission 447 nm) was determined by Infinite 200 plate reader (Tecan, Switzerland) and GUS
31 activity was expressed as pmol 4-methyl-umbelliferone (4-MU, Sigma, USA) per µg protein per min. 4-MU in the
32 range of 20 nM-100 µM was used to generate a standard curve. Each MUG assay was performed in triplicate and
33 repeated three times. The data shown represent mean (n = 3) ± standard error of the mean.

34 **Bioinformatics analysis of *GhNAC4* promoter sequence:**

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1 A search for the putative *cis*-acting regulatory elements in the promoter sequence was conducted using the
2 PlantPAN 2.0 (Chang et al. 2008, http://plantpan2.itps.ncku.edu.tw/), PLACE (Higo et al.
3 1999, http://www.dna.affrc.go.jp/htdocs/PLACE/) and PlantCARE (Lescot et al. 2002,
4 http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases.

5 **Statistical analysis:**

6 All experiments were repeated at least three times and the data were expressed as the mean ± SD. Error bars shown
7 are the standard deviation of the experimental data. Data was analyzed by one-way analysis of variance (ANOVA)
8 using SigmaPlot 11.0 software. *** P<0.001, **P□<□0.01 and *P□<□0.05 represents significant differences at
9 0.1, 1 and 5% level respectively. 'ns' represents no significant difference.

10 **Results:**

11 **Expression analysis revealed the responsiveness of *GhNAC4* gene to various phytohormones and**
12 **environmental stresses:**

13 Plant stress responses are thought to be regulated by various hormones and multiple signaling pathways. They show
14 significant overlap with the gene expression patterns (Singh et al. 2002). To gain an insight into the impact of
15 environmental stresses and phytohormones on gene expression of *GhNAC4*, qRT-PCR analysis was carried out by
16 subjecting cotton seedlings to various treatments (see materials and methods). Fig. 1 shows that *GhNAC4* responded
17 differentially to several phytohormones. Phytohormones like BAP, ABA, GA3, and MeJa significantly upregulated
18 the expression of *GhNAC4*. BAP caused induction of expression by 10.4 fold, GA3 by 11.5 fold, ABA by 6.9 fold
19 and MeJA by 7.8 fold at 24 h. However, other hormones such as IAA, Ethylene and SA caused weak upregulation
20 of its expression (4-5 fold) at 24 h.

21 PEG-induced drought treatment resulted in very high upregulation of *GhNAC4* expression (~184 fold) at 24 h. Other
22 abiotic stress treatments like high salinity and osmotic stress (caused by mannitol) also lead to high upregulation of
23 expression (43.6 and 58.7 fold respectively). Air-drying caused 10.2 fold, cold by 19.4 and MV by 14.5 fold
24 upregulation in the *GhNAC4* transcripts. However, *GhNAC4* was weakly upregulated by high temperatures,
25 flooding, wounding and dark treatments (2-6 fold) compared to other treatments as shown in Fig. 2.

26 **Sequence analysis of *GhNAC4* promoter:**

27 A total of 1612 bp (-1492 bp to +119 bp) was amplified from *G. hirsutum* (Var. JK Durga) and was sequenced.
28 Sequence similarity of 91.6% (LALIGN, https://www.ebi.ac.uk/Tools/psa/lalign/) was observed between the
29 promoter region of *G. raimondii* and *G. hirsutum* of *NAC4* gene. No significant difference was observed between
30 the predicted motifs in the two sequences. The composition of GC content of *GhNAC4* promoter was 29.9%
31 (BioEdit, Hall 1999) which is in accordance with the observed range (Joshi 1987) for a plant promoter. The putative

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1 transcription start site (TSS) predicted by Softberry database (www.softberry.com) using the default settings, was
2 located 119 bp upstream of the ATG translation start codon, which was consistent with the features of a eukaryotic
3 promoter as shown in Fig. 3. The predicted TATA box was located 16 bp upstream of TSS, and a CAAT box was
4 located 179 bp upstream of TATA box. Several other CAAT boxes were also predicted in the entire length of the
5 sequence.

6 **Multiple hormone-related, environmental stress-related and tissue-specific *cis*-elements were**
7 **predicted on the *GhNAC4* promoter:**

8 Putative *cis*-acting regulatory elements and their location were searched using the PlantPAN 2.0, PlantCare and
9 PLACE software tools. Only statistically significant motifs (P value >0.9) were selected. The resulting putative *cis*-
10 acting regulatory elements were grouped into six classes, as shown in Table 2, including phytohormone-responsive
11 motifs, stress-responsive motifs, light responsive, basic transcription elements, tissue-specific elements, and other
12 TF binding sites. We observed a relatively high proportion of tissue-specific motifs and transcription factors binding
13 sites followed by light specific, hormone responsive and stress responsive elements (Fig. 4).

14 Several motifs responding to phytohormones were revealed in the *GhNAC4* promoter. Various ABA-responsive
15 elements such as ABRELATERD1, ACGTABREMOTIFA2OSEM, and ABRERATCAL (found upstream of Ca^{+2}
16 responsive genes) were observed in the promoter sequence. Auxin-responsive motifs such as one AUXREPSIAA4,
17 one TGA element, one GGTCCCATGMSAUR, and two CATATGGMSAUR motifs, were predicted. Salicylic acid
18 responsive motifs like TCA element and WBOXATNPR1 (recognition site of SA-induced WRKY TFs) were also
19 identified. TGACG and CGTCA motifs, required for jasmonic acid induction were found in the sequence.
20 Gibberellins responsive elements such as GARE1OSREP1, GAREAT, and GADOWNAT were found. ARR1AT
21 TF, a cytokinin response regulator, and CPBCSPOR motif, critical for cytokinin enhanced expression were also
22 observed. The *cis*-elements known to play a role in ethylene induction such as ERELEE4 and binding sites of
23 EIN/EIL TF, a positive regulator in the ethylene response pathway were also identified in the promoter sequence.
24 Binding sites of BES1 TF, key TFs in the Brassinosteroids signaling pathway were also observed in the putative
25 promoter sequence. Furthermore, several recognition sites of AP2/ERF TFs were also found in the sequence, which
26 are key regulators in integrating various hormone signals and play a role in stress response.

27 A scan of the motifs showed that some potential elements, such as the MYB recognition sites like MYB1AT and
28 MYB2CONSENSUSAT, that are stress-induced regulatory motifs found in the promoters of the dehydration-
29 responsive gene were identified. *Cis*-elements such as DRECRTCORE, HSE, CBFHV, and a binding site for CSD
30 and HSF TFs, known to regulate responses to cold shock and heat stress were predicted. The binding site for bZIP
31 (ACGTTBOX), WRKY (WBBOXPCWRKY1) and MYB (MYB1LEPR) TFs that were known to play important
32 roles in stress response were found. *GhNAC4* promoter sequence also contained a *cis*-element for nutrient deficiency
33 response like the GMHDLGMVSPB, which is a binding site of homeodomain-leucine zipper protein, which is found
34 in the phosphate response domain. Various motifs and target sites of TFs, vital for biotic stress response were also
35 predicted in the promoter sequence. Many fungal elicitor response elements like BOXLCOREDCPAL, TC- RICH

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REPEATS, and GT1GMSCAM4 were observed. Numerous binding sites of BELL homeodomain TF, BIHD1OS, known to play a vital role in disease response were also observed in the sequence. Hypersensitive response element like HSRENTHSR203J was also observed. Motifs involved in wounding response such as T/GBOXATPIN2 and WBOXNTERF3 were also found. ANAERO1CONSENSUS, a motif found upstream of anaerobic induced genes was observed.

cis-Elements involved in sugar repression like PYRIMIDINEBOXOSRAMY1A, UP2ATMSD and WBOXHVISO1 (SUSIBA2 binds to W-box element) TFs were identified; they are also known to plays a role in auxiliary bud outgrowth after stem decapitation.

Several motifs essential for light-regulated transcriptional activation like SORLIP2AT, IBOXCORE, GT1CONSENSUS (that activate and stabilize the transcription machinery), GATA (that also plays a role in vascular-specific expression), and CGACGOSAMY3 (G Box/BOXII) were identified in the putative promoter sequence suggesting that *GhNAC4* might be highly regulated by light. Motifs important for plastid-specific expression such as BOXIINTPATPB, -10PEHVPSBD, and PRECONSCRHSP70A were also present in the *GhNAC4* promoter.

The specific expression is crucial for genes functioning at particular stages and in a particular tissue(s). A plethora of motifs essential for tissue-specific expression was predicted in the *GhNAC4* promoter sequence. Motifs important for embryo maturation and seed development such as SEF4MOTIFGM7S, SEF1MOTIF, and CANBNNAPA were found. Binding sites for TALE TFs, that are known to play to a role in meristem function and involved in maintaining cells in an undifferentiated state, were also observed. Motifs involved in root specific expression like OSE2ROOTNODULE, ROOTMOTIFTAPOX and recognition sites for MYBST1 were identified. Several copies of CACTFTPPCA1, which is an important motif for mesophyll expression, were found. Numerous binding sites of DOFCOREZM involved in regulation of carbon metabolism and for shoot and leaf-specific expression were identified. Binding sites of WOX TF, known to be involved in several key developmental processes was also observed. TAAAGSTKST1, a motif known to play a role in controlling guard cell-specific gene expression, was also found. Numerous binding sites for TFs significant for vascular-specific expression like DOF, GATA, and AT hook TFs were observed. Many target sites for TCR, SBP and MYBPZMTFs and motifs like CARGCW8GAT and TGTCACACMCUCUMISIN significant for flower and fruit development were present. Motifs for pollen specific expression such as POLLEN1LELAT52 and GTGANTG10 were also identified.

cis-Elements involved in gene expression induction in actively dividing cells such as MYBCOREATCYCB1, E2FCONSENSUS and CELLCYCLESC were predicted in the sequence. Motifs such as MARARS, MARABOX1, and MARTBOX are known to play a role in scaffold attachment region were also found.

Several potential binding sites for transcription factors, such as NAC, MYB/SANT, ZF-HD, bHLH, Storekeeper, bZIP, GRAS, and TCP were also identified.

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1 Basal motifs, playing a critical role in the core transcription initiation, were found in the promoter of *GhNAC4*.
2 Several TATA box motifs, essential for accurate initiation of transcription and synergistic enhancement of
3 transcription were found. CAAT-box motifs that are universal enhancer elements in promoters were also predicted.
4 In addition to basal regulatory elements, a number of transcriptional regulatory elements were found. Several
5 enhancer elements, for example, CTRMCAMV35S, QARBNEXTA, QELEMENTZMZM13, and 5' UTR -
6 PYRIMIDINE STRETCH and binding sites of NF-Y TF were identified. Interestingly, a repressor element like
7 RE1ASPHYA3 was also found in the promoter. These elements suggest that *GhNAC4* promoter is functional *in situ*.
8 The presence of these motifs hints that regulation of *GhNAC4* gene expression is complex and it may play a possible
9 role during plant growth and is responsive to phytohormones, environmental stresses, and light.

10 **Generation and analysis of tobacco transgenics of PRO$_{GhNAC4}$:GUS:**

11 To evaluate the promoter activity of *GhNAC4* gene, 1612bp fragment was amplified by PCR and was
12 cloned into pTZ57R/T vector for confirmation. The promoter sequence was then cloned into a binary
13 vector, pCAMBIA1381Z for fusing it to *GUS* reporter gene in a transcriptional fusion. Transgenic
14 tobacco plants carrying the PRO$_{GhNAC4}$:GUS were generated using the Agrobacterium-mediated leaf disc
15 transformation method. A total of 12 hygromycin resistant T_0 plants were generated and were confirmed
16 by genomic PCR. Out of these, 11 plants exhibited the expected band sizes of 1612 bp and 1026 bp
17 corresponding to the size of *GhNAC4* promoter sequence and *HptII* gene, respectively. To eliminate the
18 effect of gene copy number on GUS activity, only single copy T_1 progenies, P7, P9, and P17 were used
19 for further generation of T_2 seeds. The homozygosity of T_2 lines was confirmed by uniform 100%
20 germination on a medium supplemented with hygromycin. The T_2 progenies of three lines, P7.1, P9.5,
21 and P17.3,were used for tissue specific localization and MUG assay.

22 ***GhNAC4* localizes to various tissues during growth and development:**

23 As several motifs pertaining to tissue localization were observed in the promoter sequence, we used GUS
24 histochemical assay to precisely define the spatial-temporal expression patterns of *GhNAC4* promoter throughout
25 plant development in T_2 generation transgenics tobacco plants. Fig. 5 shows the localization of *GhNAC4* gene from
26 vegetative to reproductive tissues in tobacco.

27 In the early stages of Tobacco growth (1 d old seedlings), GUS activity was first observed in the emerging radical
28 (Fig. 5a) and later, it was detected in emerging cotyledons, root tip and shoot apex of 3 d old seedlings, but the GUS
29 activity was relatively weaker in hypocotyl tissues (Fig. 5b). In the 7, and 15 d old PRO$_{GhNAC4}$:GUS transformed
30 tobacco seedling, GUS expression was detected in the leaf veins, petioles, stem and root (Fig. 5c-d). Similar GUS
31 activities were maintained in the one month old seedling (Fig. 5e). The main and the lateral roots showed GUS
32 expression, which was absent in the root cap region and the root hairs (Fig. 5f-g). Intense GUS activity was also
33 observed in the mid rib and the lateral veins, but the leaf lamina showed little staining (Fig. 5h). We also observed

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1 the GUS activity in the guard cells (Fig. 5i) and the developing mid rib regions of a young leaf (Fig. 5j). Fig. 5k-m
2 shows the staining of the floral structures, which revealed that the GUS activity was present in the sepals, anthers,
3 pollen grains, and stigma and to a lesser extent in the petal edges. However, it was absent in the ovary and pedicel.
4 No expression was detected in the seedlings harboring a promoter-less *GUS* gene regardless of the developmental
5 stage (Fig. 5n). This strongly indicates that *GhNAC4* expression is under developmental and temporal control and its
6 promoter modulates a precise transcriptional regulation throughout the plant growth and development process
7 consistent with the bioinformatics data.

8 ***GhNAC4* localizes to vascular bundles and is wound-inducible:**

9 To obtain a better understanding of tissue specificity of GUS activity, thin cross-sections of various tissues using a
10 razor blade were made. GUS staining was only detected in the vascular bundles, especially in the phloem of leaf
11 veins, petiole, stem, and root (Fig. 6a-e). Other cell types, like the pith, cortex, and epidermis remained unstained.
12 However, upon wounding, cortex, and epidermis also showed intense GUS expression (Fig. 5k and 6f).

13 ***GhNAC4* promoter is induced by various phytohormones in transgenic tobacco seedlings:**

14 To explore the possible regulation of *GhNAC4* gene expression by phytohormones, the activity of GUS was
15 examined by fluorometric MUG assay, and treating the PRO_{GhNAC4}:GUS T_2 transgenic tobacco seedlings with
16 various hormones. The corresponding results have been depicted in Fig. 7. The specific activity of GUS enzyme
17 without any hormone treatments in GhNAC4 seedlings was measured to be 2.42±0.42pmol μg^{-1} min^{-1}. This is in
18 agreement with the histochemical staining results, where GUS staining was observed even in untreated seedlings.
19 When 2-week old tobacco seedlings were treated with different hormones for 24 h, the highest level of induction
20 was seen upon BAP treatment (~3.1 fold) and followed by ABA (~2.7 fold). GUS activities were also moderately
21 enhanced by IAA (~2.4 fold) and MeJA (~2.2 fold).The promoter weakly responded to GA3 (~2.1 fold) and
22 Ethylene (~1.8 fold) but not to SA as compared with the untreated control samples. This suggests that *GhNAC4* gene
23 expression is regulated largely by multiple phytohormones. This data is consistent with the pattern and presence of
24 *cis*-acting elements observed in the promoter sequence.

25 ***GhNAC4* promoter is responsive to various environmental stress treatments:**

26 Since we have observed that *GhNAC4* gene expression was regulated by various stresses, we studied the promoter
27 activity in tobacco transgenics plants harboring the PRO_{GhNAC4}:GUS construct by treating them with various
28 environmental stresses for varying time points. This study was encouraged by the presence of several stress-
29 responsive motifs in the promoter sequence, and the GUS activity was measured by the fluorometric MUG assay.
30 An approximately three-fold increase in GUS activity was observed when the seedlings were subjected to salt,
31 mannitol, PEG, MV, and air-drying as compared to untreated seedlings. A combination of cold and dark treatment
32 also activated the promoter (~2.4 fold) as compared to dark alone (~1.7 fold). Flooding stress caused by submerging
33 the seedlings in water moderately induced the promoter (~1.9 fold). High temperatures and wounding weakly
34 activated the promoter (~1.7 fold) as shown in Fig. 8.

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1 There are some differences in the expression levels among experimental systems such as cotton seedlings and 2 tobacco transgenics, suggesting the presence of complicated factors in experiments in terms of treatments. The 3 differences among the experimental systems remained to be determined.

4 **Discussion:**

5 Transcriptional control is essential for mediating the responses of an organism to environmental cues. The promoter 6 of a gene is critical in determining its spatial and temporal expression in the plant and during its development (Singh 7 1998). The promoter sequence specifies the recruitment of TFs, which regulate its gene expression. The short *cis*- 8 acting elements that specify the binding for the TFs are the vital functional components of promoter function. 9 Therefore, investigations into the *cis*-acting elements and their interactions would aid in the understanding of the 10 mechanism of transcriptional regulation (Hudson & Quail 2003).

11 To identify the putative *cis*-regulatory elements, a motif search was carried out for the promoter sequence of 12 *GhNAC4*. We found that *GhNAC4* promoter region contained several *cis*-acting elements linked to various stress 13 responses, phytohormone induction, tissue-specific localization, sugar response, light response, and transcriptional 14 activation.

15 To precisely evaluate the function of *GhNAC4*, we generated transgenic tobacco plants having the PRO_{GhNAC4}:GUS 16 construct and analyzed the spatial, temporal, and developmental expression of GUS. We also studied the expression 17 under various external phytohormones and environmental stress treatments.

18 Understanding how hormones and genes interact in a changing environment to synchronize plant growth and 19 development is an important breakthrough in plant developmental biology. In a stressed condition, phytohormones 20 can exhibit either synergistic or antagonistic interactions to modulate plant growth (Robert-Seilanian et al. 2011). 21 Several phytohormones highly induced the expression of *GhNAC4* such as GA3, BAP, ABA, and MeJA by 7-11 22 folds. Other hormones such as ethylene, SA and IAA lead to moderate increase in expression levels (4-5 fold). 23 GhNAC4 promoter region contains several key motifs linked to be involved in hormone responses.

24 ABA is an important stress hormone that plays crucial roles in plant adaptation to different stress conditions like 25 osmotic imbalance, salinity, and drought. ABA regulates the expression of many genes that have functions in abiotic 26 stress tolerance (Fujita et al. 2011). *GhNAC4* shows several folds induction by ABA (Fig. 1 & 7) and its promoter 27 carried several motifs known for ABA responsiveness such as six ACGTABREMOTIFA2OSEM, eight 28 ABRELATERD1, and one ABREOSRAB21 motif. ACGTABREMOTIFA2OSEM is an ABA-responsive motif 29 found in the promoter of rice *OsEm* gene, which is, regulated by the seed-specific VP1 TF (Hattori et al. 2002). The 30 ABRELATERD1 motif is related to the ABRE motif, and has been found upstream of Early Response to 31 Dehydration1 (*ERD1*) gene, and is responsive to ABA and showing significant upregulation under water stress 32 (Simpson et al. 2003). A single copy of ABRE is insufficient for ABA-responsive transcription. Either multiple 33 copies of ABRE motif or ABRE along with a coupling element, forming the active ABA Response Complex 34 (ABRC) are essential for ABA-induced gene expression (Skriver et al. 1991). *GhNAC4* promoter was predicted to

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1 have one *CE3* coupling element. The minimal ABRC of the *HVA1* promoter from barley contained one coupling
2 element and along with one ABRE motif, which were enough to confer high levels of ABA induction when linked
3 to a minimal promoter (Shen et al. 1996).

4 Regulation of gene expression by cellular calcium plays a crucial role in plant responses to environmental stresses.
5 The stress stimuli trigger a burst of cytosolic calcium ions which confers changes in gene expression, thereby
6 allowing plants to adapt to the stress conditions (Knight 2000). The ABRERATCAL motif is a sequence related to
7 ABRE, found upstream of calcium ion responsive genes. Quite a few genes upregulated by calcium ion having
8 ABRERATCAL motifs were early stress-induced genes (Kaplan et al. 2006). The *GhNAC4* promoter region was
9 predicted to have seven ABRERATCAL motifs suggesting that it may also be responsive to calcium and ABA-
10 mediated stress adaptation.

11 Gibberellins (GA) are essential for many plant developmental processes such as seed germination, plant growth, and
12 flower development (Richards et al. 2001). GA and ABA are known to mediate several plant developmental
13 processes antagonistically, but recent data suggests the involvement of GA in plant adaptation to stress (Colebrook
14 et al. 2014). *GhNAC4* was highly upregulated by GA3 (Fig. 1 & 7) and its promoter has four copies GAREAT and
15 two copies of GARE1OSREP1 motifs, which are gibberellins responsive elements (Skriver et al. 1991). An R2R3-
16 type MYB TF called the GAMYB directly interacts with the GARE motif in the promoter of barley amylase gene
17 and seemed to be essential for the GA induced gene expression (Gubler et al. 1995). Recent evidence indicates that
18 GAMYB is also a target of the antagonistic effects of ABA signaling (Gomez-Cadenas et al. 2001; Zentella et al.
19 2002). Multiple copies of GARE alone or in association with other motifs such as PYRIMIDINE box or TATCCAC
20 box (forming the Gibberellins Response Complex), are essential for GA induced gene expression at higher levels
21 (Lanahan et al. 1992; Rogers et al. 1994). *GhNAC4* promoter region also exhibits four copies of
22 PYRIMIDINEBOXOSRAMY1A motif. A DOF TF (OsDOF3) binds to the pyrimidine box in the promoter region
23 of rice *RAMY1a* gene that is one of the most predominant GA-responsive genes (Washio 2001). GAMYB and
24 OsDOF3 are shown to interact with each other and synergistically cause the GA induction of gene expression
25 (Washio 2003). GAs are main targets for stress-induced growth modulation, and recent evidence indicates the
26 involvement of GA in either promotion or suppression of growth depending on the type of stress (Colebrook et al.
27 2014). GADOWNAT motif is a common sequence found in the genes down-regulated by GA. Interestingly, this
28 motif is identical to ABRE (Ogawa et al. 2003). Five copies of this motif are present in the *GhNAC4* promoter
29 suggesting coordinated regulation of *GhNAC4* by GA and ABA.

30 Ethylene is essential for plant growth and development and plays a crucial role in responses to environmental stress
31 such as pathogen attack, wounding, flooding, high temperatures, and drought (Abiri et al. 2017). *GhNAC4* promoter
32 contained four binding sites for ethylene insensitive (EIN) TF, and the ethylene treatment upregulated the transcript
33 levels of *GhNAC4* (Fig. 1 & 7). AtNAC2 TF upregulation under salinity treatment was induced in ethylene-
34 overproducing mutant *eto1-1* but was repressed in ethylene-insensitive mutants *etr1-1* and *ein2-1* (He et al. 2005)
35 suggesting that ethylene plays a positive role in the salt response of AtNAC2 TF. Most of the ethylene responses
36 appear to be mediated by EIN3 TF along with ethylene insensitive-like (EIL) protein (Solano et al. 1998). EIN3 TF

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1 was shown to enhance salt tolerance in Arabidopsis (Peng et al. 2014) and also act synergistically with SOS2 to
2 modulate salt stress response (Quan et al. 2017). Furthermore, EIN3/EIL1 act as a node integrating ethylene and JA
3 signaling for regulating plant growth and stress responses (Zhu et al. 2011).

4 Cytokinin regulates many important aspects of plant growth and development such as development of vasculature,
5 photomorphogenesis, and stress responses (Werner & Schmülling 2009). We have observed high upregulation of
6 *GhNAC4* transcripts in cytokinin (BAP) treatment (Fig. 1 & 7), and the promoter region of this gene was predicted
7 to have one CPBCSPOR motif and 16 binding sites for Authentic Response Regulators1 (ARR1). ARR1 is an
8 important signaling component, acting at the head of transcriptional cascade to regulate cytokinin response and is
9 known to be involved in cytokinin-mediated differentiation of protoxylem (Yokoyama et al. 2007). They are also
10 known to regulate plant responses to abiotic stresses (Nguyen et al. 2016; Jeon & Kim 2013).

11 Auxin is a phytohormone that plays a crucial role in growth and development. It regulates the development of
12 primary and lateral roots and has a most profound role in vascular differentiation and venation pattern formation
13 (Zhao 2010). Auxin (IAA) was shown to induce the expression of *GhNAC4* (Fig. 1 & 7), and the promoter region
14 shows an AUXREPSIAA4, one TGA element, one GGTCCCATGMSAUR, and two CATATGGMSAUR motifs.
15 AUXREPSIAA4 is an auxin-responsive element (AUXRE) found upstream of pea *IAA4* gene, which is an early
16 auxin-inducible gene (Ballas et al. 1993). GGTCCCATGMSAUR and CATATGGMSAUR are AUXREs found in
17 the promoter region of maize *SAUR* (Small Auxin-Up RNA) gene (Li et al. 1994; Xu et al. 1997). Each of these
18 promoters is rapidly (within few minutes) and specifically induced by auxin. Auxin Response Factors (ARFs) bind
19 to AUXRE and mediate hormone response. ARF5 is required for the formation of vascular strands at all stages and
20 critically required for embryonic root formation (Hardtke & Berleth 1998). Auxin role in stress response is emerging
21 and Bouzroud et al. (2018) suggested that ARFs are potential mediators of auxin action to environmental stresses.
22 Vascular bundle localization of GUS in PRO_{GhNAC4}:GUS transgenic plants (Fig. 6) could be attributed to the
23 regulation of *GhNAC4* promoter by auxin and cytokinin.

24 Jasmonates are important regulators of plant defense responses to pathogen and insect attack, herbivory and
25 wounding (Wasternack 2007). MeJA and wounding induced the expression of *GhNAC4* by several folds (Fig. 1 & 2)
26 and its promoter contains motifs such as T/GBOXATPIN2, TGACG, and CGTCA. JAMYC/AtMYC2 TF binds to
27 the T/GBOXATPIN2 motif, found in the promoter of JA responsive and wound-inducible Protease Inhibitor II
28 (*PIN2*) gene. JAMYC act as a conserved master switches regulating the expression of several JA-regulated defense
29 genes especially during wounding response (Lorenzo et al. 2004; Boter et al. 2004). A bZIP TF, TGA1, is a positive
30 regulator of disease resistance, and binds to the TGACG motif and its palindrome CGTCA motif which is known for
31 JA responsiveness (Schindler et al. 1992; Shearer et al. 2012). TGACG motif is found in promoters of various JA
32 responsive genes like *OsOPR1* known to play important roles in defense responses in rice (Sobajima et al. 2007).

33 Phytohormones act synergistically and antagonistically with each other to regulate plant growth and development in
34 association with a changing environment by forming a complex cross-talks network (Robert-Seilaniantz et al. 2011).
35 One of the TF families modulating these multiple regulatory responses is Apetala2/ Ethylene responsive factor

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1 (AP2/ERF) TFs (Licausi et al. 2013). Apart from ethylene, JA, ABA, auxin, and cytokinin also regulate many
2 members of the AP2/ERF TF family. AP2/ERF TFs also modulate the content of these phytohormones by regulating
3 their biosynthesis pathways. The stimulated TFs would further regulate the downstream target genes resulting in
4 changes in plant growth and development, environmental stress responses (Gu et al. 2017). They mediate
5 downstream responses by binding to GCC box (ethylene response element) and/or DREB element (Fujimoto et al.
6 2000). *GhNAC4* promoter has four copies of GCC box motif. Taken together, this suggests that GhNAC4 may act
7 downstream of AP2/ERF TF.

8 As *GhNAC4* expression is regulated by many phytohormones essential for stress response and plant development
9 and the promoter contains several motifs required for regulation by phytohormones, our data suggest that GhNAC4
10 TF can act as a node modulating hormonal response in a changing environment to regulate plant development.

11 Plants being sessile have developed capabilities to grow and propagate even under extreme environmental
12 conditions such as high salt, severe drought, heavy metal stress, low or high temperatures. Plants have become
13 specialized in rapidly sensing and responding to adverse environmental conditions by having a complex network of
14 cellular processes for stress adaptation (Wang et al. 2003). Various genes induced during stress response not only
15 play a role in stress tolerance but also play a role in sensing and transcriptional regulation (Zhu 2016). *GhNAC4* was
16 highly upregulated by drought (~184 fold), salinity (~43 fold), osmotic stress (~58 fold) caused by mannitol,
17 oxidative stress (~14 fold) induced by methyl viologen, cold (~19 fold), high temperature stresses (~6 fold), and
18 wounding (~5 fold) (Fig. 2). Binding sites for various TF such as MYB, CSD, HSF, WRKY, BELL, and C2H2,
19 known to be involved in environmental stress responses were observed in the *GhNAC4* promoter region.

20 Low-temperature stress and drought are two major limiting physiological factors affecting plant growth,
21 productivity, and geographical distribution (Shinozaki & Yamaguchi-Shinozaki 2000). *GhNAC4* is significantly
22 upregulated by cold stress and drought (Fig. 2 & 8), and its promoter region carries many motifs required for
23 drought and cold responsiveness like MYB2CONSENSUSAT, C-REPEAT/DRE, DRECRTCOREAT, CSD, and
24 CBFHV TF binding sites. Two copies of MYB2CONSENSUSAT motifs are found in the *GhNAC4* promoter, which
25 is the binding site of AtMYB2 TF, that is required for drought inducibility of *rd22* gene by binding to its promoter
26 region (Abe 2003). Cold responsive motif, C-repeat (CRT) motif is responsible for the regulation of many cold-
27 inducible genes in an ABA-independent manner. It is also involved in dehydration responsiveness (Stockinger et al.
28 1997). An AP2 domain-containing TF, CBF/DREB binds to this motif. Three copies of the DRECRTCORE motif
29 are found in the *GhNAC4* promoter. CBFHV motif is a binding site of an AP2 domain containing cold-inducible TF,
30 HvCBF1 charaterized in barley (Xue 2002). Three copies of CBFHV motif are found in the *GhNAC4* promoter.
31 Eight copies of binding sites for cold shock domain (CSD) proteins are also found in the *GhNAC4* promoter and are
32 highly upregulated during low-temperature stress. Apart from being important during cold adaptation, they are also
33 known to play a role in plant development (Chaikam & Karlson 2008).

34 Heat stress damages components of the photosynthetic apparatus and leads to oxidative stress and thereby affecting
35 plant growth and productivity (Kotak et al. 2007). High temperture and oxidative stress highly induced *GhNAC4*

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transcripts (Fig. 2 & 8). Heat stress proteins (HSP) are molecular chaperones essential for restoration of homeostasis during heat stress, and the transcriptional regulation of HSPs are controlled by heat stress transcription factors (HSF) (Al-Whaibi 2011). They bind to the heat stress responsive element (HSE) and modulate transcription (Baniwal et al. 2004). Four copies of HSF binding sites were present in the *GhNAC4* promoter. Binding sites for C2H2 TF were also identified in the *GhNAC4* promoter region. C2H2 TFs such as ZAT7, ZAT10, and ZPT2 are known to be important in regulating responses to abiotic and biotic stress tolerance (Kiełbowicz-Matuk 2012).

Plants defend themselves against pathogen attacks by developing strong physical barriers in their cell walls and a complex signaling network that involves inducible defense mechanisms. *GhNAC4* promoter contains several binding sites of TFs like WRKY, MYB, BELL and also many copies of motifs such as WBOXNTCHN48, BOXLCROREDCPAL, HSRENTHSR203J, and GT1GMSCAM4 known to be essential for elicitor-induced activation of defense genes.

Elicitor inducible WRKY TF is involved in transcription of defense genes like chalcone synthase (*CHN48*) in tobacco by binding to the WBOXNTCHN48 motif (Yamamoto et al. 2004). DcMYB1 TF binds to the BOXLCROREDCPAL motif in the promoter of carrot phenylalanine ammonia-lyase (*PAL*) gene and is essential for induction by elicitor treatment or UV-B irradiation (Maeda et al. 2005). *HSR203J* gene is rapidly and specifically upregulated during a hypersensitive response and HSRENTHSR203J (HSR203 responsive element) motif is responsible for the induction of HSR203J during incompatible plant-pathogen interactions (Pontier et al. 2001). Soybean calmodulin (SCaM-4) is rapidly induced by pathogen attack and salt stress. This is mediated by the binding of GT-1 TF to the promoter at the GT1GMSCAM4 motif (Park et al. 2004). Six such motifs are also present in the *GhNAC4* promoter. OsBIHD1 TF is a BELL homeodomain TF known to induce resistance in rice in response to *Magnaporthe grisea* (Luo et al. 2005). Six copies of OsBIHD1 binding sites were found in the *GhNAC4* promoter. Mechanical wounding induced the expression of *GhNAC4* by several folds (Fig. 2 & 8) and we observed intense GUS staining in the wounded area (Fig. 6). *GhNAC4* promoter consists of four copies of WBOXNTERF3 motifs. WRKY TF binds to the WBOXNTERF3 motif in the promoter of *ERF3* gene of tobacco and upon wounding causes rapid activation (Nishiuchi et al., 2004). Thus, these motifs may modulate the expression of *GhNAC4* gene in response to various abioitc and bioitc stress.

Presence of ABA-dependent and independent abiotic stress-related motifs and also elicitor-induced motifs in the *GhNAC4* promoter, suggests that there is a cross-talk between various stress signaling pathways and *GhNAC4* may act as a node for the integration of these pathways. This maybe modulated by the interactions of different motifs present in the *GhNAC4* promoter.

Stress responses occur primarily at the level of transcription, leading to the regulation of spatial and temporal expression of stress-induced genes (Geng et al. 2013). Spatial and temporal patterns under the control of *GhNAC4* promoter were monitored in various tissues by the detection of GUS activity in transgenic tobacco plants. In the present investigation, we have observed the expression of *GhNAC4* at the seedling stage, in the emerging radicle, plumule, and cotyledons. *GhNAC4* expression was found in the the vascular bundles of the stem, leaf, main and

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1 lateral roots, in the later stages of plant growth. It was also observed in the guard cells and meristems. During
2 flowering, sepals, petal edges, and pollens showed *GhNAC4* expression (Fig. 5 & 6). Since *GhNAC4* promoter has
3 very strong vasculature specific activities, the *GhNAC4* promoter carried several motifs responsible for this tissue-
4 specific expression. Several copies of SEF1MOTIF, SEF3MOTIFGM, and SEF4MOTIFGM7S motifs were
5 observed in the *GhNAC4* promoter. These are developing embryo, and seed-specific motifs found upstream of
6 soybean seed storage β-conglycinin gene (Lessard et al. 1991). Binding sites for three-amino-acid-loop-extension
7 (TALE) TF, that are known to control the formation and maintenance of meristem, were observed in the promoter of
8 *GhNAC4* gene. These homeo-proteins provide a gene regulatory link between hormonal stimuli and development of
9 shoot apical meristem (Hamant & Pautot 2010). OSE1ROOTNODULE and OSE2ROOTNODULE motifs are root
10 specific elements found in the promoter of *Vicia faba* leghaemoglobin gene and are important for nodule formation
11 by arbuscular mycorrhiza (Fehlberg et al. 2005). The ROOTMOTIFTAPOX1 element is found in the promoter of
12 the *rolD* gene of *Agrobacterium rhizogenes,* which has a distinctive pattern in the root elongation zone and vascular
13 bundle (Elmayan & Tepfer 1995). The CACTFTPPCA1 motif is a key component of mesophyll expression module
14 1 (MEM1) in the promoter of *Flaveria trinervia* C4 phosphoenolpyruvate carboxylase gene and is sufficient for high
15 mesophyll-specific expression (Gowik et al. 2004). The TAAAGSTKST1 is a guard cell-specific motif found
16 upstream of K^+ influx channel gene (*KST1*) in potato and is bound by StDof1 TF (Plesch et al. 2001).

17 Binding sites for quite a few other TF known to play roles in organogenesis and tissue-specific expression were
18 predicted such as MYBST1, DOFCOREZM, SBP TF, TCR TF, AT HOOK TF, and WOX TF. *GhNAC4* has 26
19 copies of DOFCOREZM motif, which is a binding site of DOF TFs that are plant specific and have a unique single
20 zinc finger DNA binding domain. Key role of DOF transcription factors in the formation and functioning of plant
21 vascular bundles is emerging (Le Hir & Bellini 2013). They directly or indirectly regulate the processes associated
22 with the establishment and maintenance of the vascular system. HMG-I/Y TFs have an AT-hook region, which is a
23 short conserved peptide that binds to AT-rich tracts of DNA in the minor groove and cause DNA bending. These
24 TFs play an important role in chromatin structure and regulate gene expression by acting as TF co-factors. HMG-I/Y
25 TFs are expressed in high levels in tissues showing rapid cell division. And, they are also known to play an
26 important role in development and defense responses (Klosterman & Hadwiger 2002). Squamosa promoter binding
27 protein (SBP) TFs are plant-specific TFs that are known to play a role both in vegetative phase change and flower
28 development pathway, and their role in stress response is also emerging (Hou et al. 2013; Klein et al. 1996).
29 Wuschel-related homeobox (WOX) TFs plays an important role in cell fate determination during all stages of plant
30 development and shows very specific spatial and temporal expression (Hedman et al. 2013).Various motifs such as
31 CARCGW8GAT, TGTCACACMCUCUMISIN and binding sites for MYBPZM, and TCR TFs known to be
32 important for flower and fruit development were found. CARCGW8GAT is the binding site of MADS-box TF,
33 AGAMOUS-like 15 (AGL15). AGl15 accumulates during embryo development and regulates somatic
34 embryogenesis (Tang & Perry 2003). TGTCACACMCUCUMISIN is a fruit-specific motif found upstream of a
35 subtilisin-like serine protease, cucumisin gene in melon (Yamagata et al. 2002). MYBPZM is an MYB homolog, P,
36 transcription factor from maize that binds to CCT/AACC sequence and it controls red pigmentation in the floral
37 organs by regulating the flavonoid biosynthetic pathway (Grotewold et al. 1994). Two pollen-specific motifs are

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1 also found in the *GhNAC4* promoter, POLLEN1LELAT52, and GTGANTG10. POLLEN1LELAT52 is one of the
2 two co-dependent motifs, found upstream of tomato *LAT52* gene that is essential for pollen development (Bate &
3 Twell 1998). The GTGANTG10 motif is important for the pollen-specific expression of tobacco pectate lyases, *g10*
4 gene (Rogers et al. 2001). GhNAC4 has a unique tissue-specific expression pattern suggesting that this gene may be
5 essential for plant growth and development.

6 In plants, soluble sugars not only function as a nutrient source but also act as signals regulating various growth and
7 development pathways (Rolland et al. 2006). There is a cross-talk between the environmental stress response and the
8 sugar signaling pathways to modulate plant metabolic responses by differential regulation of many genes (Ho et al.
9 2001). Nutritional stress and sugar starvation responses may have an overlap with the response of a plant to
10 environmental stresses (Couée et al. 2006). Exogenous application of glucose helped in the alleviation of the
11 negative effects of salt stress in wheat seedlings by maintaining the ion homeostasis and activation of the antioxidant
12 system (Hu et al. 2012). *GhNAC4* promoter carried 16 copies of CGACGOSAMY, two copies of WBOXHVISO1
13 and one copy of SREATMSD motif. CGACGOSAMY3 motif acts as a coupling element for the G-box motif in the
14 promoter of sugar starvation regulated rice amylase 3D (*OsAMY3D*) gene and plays a role in sugar responsiveness
15 coupled to environmental stress regulation (Hwang et al. 1998). SUGAR SIGNALING IN BARLEY 2 (SUSIBA2)
16 is a sugar-inducible WRKY TF which binds to both WBOX (WBOXHVISO1) and SURE (Sugar responsive
17 element) in the barley isoamylase1 (*iso1*) promoter and is involved in the regulation of starch synthesis (Sun et al.
18 2003). SREATMSD is a sugar-repressive element, found upstream of genes down-regulated during auxiliary bud
19 growth after main stem decapitation (Tatematsu et al. 2005). SREATMSD is also involved in feed-back sugar
20 repression of gene expression. Sugar-dependent repression of gene expression is an important regulatory mechanism
21 for adjusting to the changes in the availability of carbon source and maintaining energy homeostasis between the
22 source and sink tissues (Yu et al. 2015).

23 Plants responses to light are complex, and most of these responses such as development and stress signaling require
24 the regulation in the expression of various nuclear and plastid-encoded genes and are mediated by photoreceptors.
25 More than one photoreceptors may act on light-regulated genes thus allowing for tight control of their expression
26 upon light stimulation (Kami et al. 2010). It has been hypothesized that light-responsive elements (LRE) have
27 complex structures and consist of aggregates of connected binding sites for different transcription factors (Terzaghi
28 & Cashmore 1995). Combinatorial interaction of different LREs in the promoter sequence such as GATA motif,
29 ASL box, BOXII, IBOX, GT1 element, SORLIPs, TBOX, AE-BOX, and ATC motif, are required to confer light
30 inducibility (Argüello-Astorga & Herrera-Estrella 1998). The combinatorial pairing of tetrameric repeats of GATA
31 and GT1 motif to a minimal promoter responded to a broad spectrum of light as compared to multimeric repeats of
32 these motifs alone (Chattopadhyay et al. 1998). GATA motif plays a role in light responsiveness and tissue
33 specificity and has been shown to be involved in the light-dependent development of phloem tissue (Yin et al.
34 1997). Vascular localization of *GhNAC4* (Fig. 6) could be partially attributed to the presence of 42 copies of the
35 GATA motif in its promoter region. Several copies of GT1CONSENSUS motif were found in the *GhNAC4*
36 promoter sequence. GT1CONSENSUS sequence (GRWAAW) was initially identified as Box II motif and is the

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binding site of GT-1 TF. It is involved in light activation or dark repression and tissue-specificity (Hiratsuka et al. 1994). SORLIPs (sequence over-represented in light-induced promoters) are light-responsive elements found in the promoters of genes that respond to the phytochrome A receptor pathway (Hudson & Quail 2003). SORLIP1AT and SORLIP2AT were found in the *GhNAC4* promoter sequence. TBOXATGAPB motif is found upstream of Arabidopsis *GAPDH* subunit B gene and serves as a positive modulator of light inducibility (Chan et al. 2001). This suggests that the tissue specificity of GhNAC4 to a certain extent is dependent on light inducibility.

Abiotic stress impedes plant growth and development which would compel the regulation of photosynthesis and carbohydrate partitioning. *GhNAC4* showed the presence of sugar and light regulatory motifs and is also up-regulated by abiotic stresses. This indicates that GhNAC4 may act to integrate stress responses with plant growth regulation.

Presence of key motifs related to various stresses in the promoter of *GhNAC4* indicates their putative function in the response of a cotton plant to environmental stresses. The transcript abundance of *GhNAC4* was altered by salinity, osmotic stress, oxidative stress, wounding, low and high temperatures and drought, as well as, by exogenous application of various phytohormones like ABA, BAP, MeJA, GA and ethylene implying that *GhNAC4* may participate in cotton plant's response to environmental stress. This work would provide a foundation for a comprehensive functional investigation of GhNAC4 TF in the future.

Conclusion:

In conclusion, expression, histochemical, and bioinformatics analyses showed that GhNAC4 not only expressed in vascular bundle and guard cells and is responsive to phytohormones like ABA, JA, CK, and auxin. But it is also up-regulated under external stimuli like drought, oxidative stress, osmotic stress, salinity and cold suggesting that GhNAC4 TF may be a common regulator of the molecular mechanism controlling plant development and stress responses. *GhNAC4* promoter may be employed in biotechnological approaches as a vascular-specific and stress-inducible promoter. In the future studies, characterization of the GhNAC4 promoter by the 5' deletion analysis would be carried out. Further characterization of GhNAC4 would provide new insights into the mechanisms integrating plant development with environmental stress conditions aided by hormonal stimuli. We are currently investigating the effects of GhNAC4 expression in transgenic plants and how GHNAC4 integrates stress response with plant developmental processes.

Author contribution statement:

TSV, PBK and PG conceived and designed the experiments. TSV, SM and PB performed the experiments. TSV analyzed the data. TSV and PBK wrote the manuscript. All authors read and approved the manuscript.

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3 **Conflct of interest:**

4 The authors declare they have no conflict of interest.

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13 **Tables:**

14 **Table 1**

Primer	Sequence (5'-3')	Description
GhNACPRO-F	TATCTGGATCCGGCCTCATAATTGCATCAC	Cloning primers for *GhNAC4* promoter
GhNACPRO-R	TACATACTGCAGGATTCTAAAGTTTTCTTGCCG	
HptII-F	TATTTCTTTGCCCTCGGACGA	*HptII* transcript screening primers
HptII-R	ATGAAAAAGCCTGAACTCACC	
NAC4-RTF	TTCTCGTAAAAGTGGTAGCTCC	*GhNAC4* real-time PCR primers
NAC4-RTR	TCCAGTTGTGAAGAAGACGATG	
UBQ7-RTF	AGAGGTCGAGTCTTCGGACA	*UBQ7* real-time PCR primers
UBQ7-RTR	GCTTGATCTTCTTGGGCTTG	

15

16 **Table 2**

MOTIFS/TFs RELATED TO HORMONE RESPONSE					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	TGA-ELEMENT	1	PC	AACGAC	Cis acting regulatory element involved in auxin responsiveness
2	GGTCCCATGM SAUR	1	S000360	GGTCCCAT	
3	CATATGGMSA UR	2	S000370	CATATG	
4	AUXREPSIAA4	1	S000026	KGTCCCAT	Auxin responsive element
2	ABRERATCAL	7	S000507	MACGYGB	ABARE related sequence, found upstream of Calcium ion responsive genes

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3	CE3	1	PC	GACGCGTG TC	Involved in ABA and VP1 response
4	ABREOSRAB21	1	S000012	ACGTSSSC	Motifs involved in ABA responsiveness
5	ABRELATERD1	8	S000414	ACGTG	
6	CGTGT SPHZMC1	1	S000294	CGTGTCGTC CATGCAT	
7	ACGTABREMO TIFA2OSEM	6	S000394	ACGTGKC	ACGT-core of motif A in ABRE
8	WBOXATNPR1	4	S000390	TTGAC	Recognition site of salicylic acid (SA)-induced WRKY TFs
9	TCA ELEMENT	1	PC	GAGAAGAA TA	Involved in salicylic acid response
10	CPBCSPOR	1	S000491	TATTAG	Critical for Cytokinin-enhanced expression
11	ARR1AT	16	S000454	NGATT	Binding site of ARR1, a cytokine response regulator
12	TGACG-MOTIF	1	PC	TGACG	Involved in jasmonate (JA) induction, plays a role in wounding response
13	T/GBOXAT PIN2	1	S000458	AACGTG	
14	PYRIMIDINEBO XOSRAMY1A	4	S000259	CCTTTT	
15	CGTCA-MOTIF	1	PC	CGTCA	
16	GADOWNAT	5	S000438	ACGTGTC	Motif for down-regulation of expression by GA
17	GARE1OSREP1	2	S000419	TAACAGA	Gibberellins-responsive element (GARE)
18	GAREAT	4	S000439	TAACAAR	
19	EIN-EIL TF	4	-	ATGCA	Positive regulator in the ethylene response pathway
20	BES1 TF	7	-	CACGTG	Key transcription factors in Brassinosteroids signaling pathway
21	AP2/ERF TF	3	-	GCCGAC	key regulator integrating various hormone signals, also plays a role in stress response
MOTIFS/TFs RELATED TO STRESS RESPONSE					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	ANAERO1 CONSENSUS	2	S000477	AAACAAA	Motifs found upstream of anaerobic induced genes
2	UP2ATMSD	2	S000472	AAACCCTA	Plays a role in auxiliary bud outgrowth after stem decapitation
3	SREATMSD	1	S000470	TTATCC	Cis elements involved in sugar repression
4	CGACGCOSAM Y3	16	S000205	CGACG	May function as a coupling element for the G-box motif
5	WBOXHVISO1	2	S000442	TGACT	SUSIBA2 bind to W-box element, plays a role in sugar

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					signaling
6	GMHDL GMVSPB	1	S000372	CATTAATTA G	Binding site of the soybean homeodomain leucine zipper proteins (GmHdl56, GmHdl57); Found in the phosphate response domain of the soybean *VspB* promoter
7	MYB2 CONSENSUSAT	2	S000409	YAACKG	MYB recognition site found in the promoters of the dehydration-responsive gene
8	MYB1AT	4	S000408	WAACCA	
9	HSE	1	PC	AGAAAATT CG	Involved in heat stress responsiveness
10	C-REPEAT/DRE	1	PC	TGGCCGAC	Essential for cold and dehydration stress response
11	CBFHV	3	S000497	RYCGAC	
12	DRECRT COREAT	3	S000418	RCCGAC	
13	CSD TF	8	-	AATAAA	Plays a role in cold shock response
14	HSF TF	4	-	CTNGAANN TTCNAG	Heat shock responsive transcription factor
15	C2H2 TF	11	-	ACACT	Play diverse roles in the plant stress response and the hormone signal transduction
16	BOXLCORE DCPAL	1	S000492	ACCWWCC	Core sequences of box-L-like sequences, involved in elicitor induction
17	WBOX NTCHN48	1	S000508	CTGACY	Involved in elicitor-respsonsive transcription of defense genes
18	HSRE NTHSR203J	1	S000466	CAAAATTTT GTA	"HSRE (HSR203 responsive element)" responsible for the marked induction during the HR (hypersensitive response); HSR203J is specifically activated during the early steps of incompatible plant/pathogen interactions;
19	GT1GMSCAM4	6	S000453	GAAAAA	Plays a role in pathogen- and salt-induced gene expression
20	TC- RICH REPEATS	1	PC	GTTTTCTTA C	Involved in defense and stress response
21	BIHD1OS	6	S000498	TGTCA	Binding site of BELL homeodomain transcription factor, known to play a role in disease response
22	WBOXNTERF3	4	S000457	TGACY	W-box, May be involved in activation by wounding
23	WBBOX PCWRKY1	2	S000310	TTTGACY	W-box, binding site of WRKY TFs

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24	MYB1LEPR	1	S000443	GTTAGTT	MYB TF binding site
25	ACGTTBOX	2	S000132	AACGTT	Binding site of bZIP TFs

MOTIFS/TFs RELATED TO TISSUE SPECIFIC LOCALIZATION					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	SEF1MOTIF	3	S00006	ATATTTAW W	Motifs required for seed specific expression
2	SEF3MOTIFGM	1	S000115	AACCCA	
3	SEF4MOTIFGM 7S	12	S000103	RTTTTTR	
4	CANBNNAPA	1	S000148	CNAACAC	Required for seed specificity
5	TALE TF	12	-	TGAC	Plays a role in meristem function; Involved in maintaining cells in an undifferentiated, meristematic state
6	ROOTMOTIF TAPOX1	11	S000098	ATATT	Motif found both in promoters of *rolD*
7	OSE1ROOT NODULE	1	S000467	AAAGAT	Consensus sequence motifs of organ-specific elements (OSE) characteristic of infected cells of root nodules
8	OSE2ROOT NODULE	10	S000468	CTCTT	
9	TELOBOX ATEEF1AA1	1	S000309	AAACCCTA A	Required for expression in the root
10	RHERPATEXPA 7	3	S000512	KCACGW	Right part of Root Hair-specific cis-Element
11	MYBST1	2	S000180	GGATA	Core motif of MybSt1 (a potato MYB homolog) binding site.
12	CACTFTPPCA1	22	S000449	YACT	Tetranucleotide (CACT) is a key component of Mem1 (mesophyll expression module 1)
13	DOFCOREZM	26	S000265	AAAG	Core site required for binding of Dof proteins; involved in regulation of carbon metabolism
14	MYBPZM	1	S000179	CCWACC	Core of consensus maize P (myb homolog) binding site and specifies red pigmentation of kernel pericarp, cob, and other floral organ
15	SBP TF	8	-	CGTAC	Plays a role in flower and fruit development as well as other physiological processes
16	TCR TF	12	-	-	Plays a role in development of both male and female reproductive tissues
17	CARGCW8GAT	6	S000431	CWWWWW	Binding site for AGL15

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				WWWG	(AGAMOUS-like 15)
18	TGTCACACMC UCUMISIN	4	S000422	TGTCACA	Enhancer element necessary for fruit-specific expression
19	AT hook TF	50	-	AAAAT	Known to play a role in vascular specific expression
20	POLLEN1 LELAT52	9	S000245	AGAAA	One of two co-dependent regulatory elements responsible for pollen specific activation
21	GTGANTG10	13	S000378	GTGA	"GTGA motif"; plays a role in pollen specific expression
22	TAAAGSTKST1	5	S000387	TAAAG	Target site for Dof1 TF controlling guard cell-specific gene expression
23	WOX TF	5	-	TTAAT	Involved in several key developmental processes
MOTIFS/TFs RELATED TO LIGHT RESPONSE					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	GATA MOTIF	1	PC	GATAGGA	Part of a module for light response, Also plays a role in vascular specific expression
2	GATA TF	42	-	GATA	Part of a module for light response,
3	IBOXCORENT	3	S000424	GATAAGR	Conserved sequence upstream of light regulated genes
4	IBOX	3	S000124	GATAAG	
5	IBOXCORE	11	S000199	GATAA	
6	GT1 CONSENSUS	23	S000198	GRWAAW	Plays a role in light regulation; Consensus GT-1 binding site, can activate and stabilize the transcription machinery
7	SORLIP1AT	1	S000483	GGGCC	Cis acting elements involved in the phytochrome A regulated gene expression
8	SORLIP2AT	2	S000482	GCCAC	
9	TBOX ATGAPB	1	S000383	ACTTTG	Involved in light activated transcription
10	3-AF1	1	PC	AAGAGATA TTT	Light responsive element
11	ACE	1	PC	AAAACGTT TA	Part of a module for light response
12	AE-BOX	1	PC	AGAAACAA	
13	AT-1	1	PC	AATTATTTT TTATT	
14	ATC MOTIF	1	PC	AGTAATCT	
15	AT-C	1	PC	AATTATTTT TTATT	
16	RBCS CONSENSUS	1	S000127	AATCCAA	rbcS general consensus sequence
17	-10PEHVPSBD	2	S000392	TATTCT	-10 promoter element, involved

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					in chloroplast expression
18	BOXIINTPATPB		S000296	ATAGAA	Conserved sequence found in the promoters of plastid genes
19	PRECONSCRHSP 70A		S000506	SCGAYNRN NNNNNNNN NNNNNNHD	Consensus sequence of plastid response element (PRE) in the promoters of HSP70A

MOTIFS/TFs RELATED TO CELL CYCLE

S.No	MOTIF/TF NAME	No.	DB ID	SEQUENCE	EXPECTED FUCTION
1	E2F CONSENSUS	6	S000476	WTTSSCSS	Motifs involved in gene expression induction at the G1/S transition of the cell cycle in actively dividing cells
2	CELLCYCLESC	1	S000031	CACGAAAA	
3	MYBCORE ATCYCB1	1	S000502	AACGG	Myb core" in the 18 bp sequence which is able to activate expression without leading to M-phase-specific expression
4	MARARS	4	S000064	WTTTATRTT TW	Motifs found in SAR (scaffold attachment region); or matrix attachment region, (MAR)
5	MARTBOX	6	S000067	TTWTWTTW TT	
6	MARABOX1	4	S000063	AATAAAYA AA	

MOTIFS/TFs RELATED TO BASIC TRANSCRIPTION AND EXPRESSION LEVELS

S.No	MOTIF/TF NAME	No.	DB ID	SEQUENCE	EXPECTED FUCTION
1	CAATBOX1	19	S000028	CAAT	Common cis element in promoter and enhancer regions
2	TATABOX OSPAL	4	S000400	TATTTAA	Binding site of TATA binding protein (TBP); for essential for accurate initiation of transcription and synergistic enhancement of transcription
3	TATABOX3	2	S000110	TATTAAT	
4	TATABOX4	1	S000111	TATATAA	
5	TATABOX2	2	S000109	TATAAAT	
6	TATA BOX	5	PC	TATA	
7	POLASIG3	3	S000088	AATAAT	Consensus sequence for plant polyadenylation signal
8	MRNA STA1CRPSBD	1	S000274	CUCUUTGU TTUU	mRNA stability determinant; Required for the stable accumulation
9	NF-Y TF	5	-	CCAAT	Component of the NF-Y/HAP tf complex. The NF-Y complex stimulates the transcription of various genes by recognizing and binding to a CCAAT motif in promoters
10	CTRM CAMV35S	1	S000405	TCTCTCTCT	Cis acting element conferring high transcription levels
11	5'UTR-	1	PC	TTTCTTCTC	

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	PYRIMIDINE STRETCH			T	
12	QARBNEXTA	1	S000244	AACGTGT	
13	QELEMENT ZMZM13	2	S000254	AGGTCA	
14	EECCRCAH1	1	S000494	GANTTNC	
15	RE1ASPHYA3	2	S000195	CATGGGCG CGG	RE1 (putative repressor element)
OTHER TFs BINDING SITES					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	NAC/NAM TF	2	-	CGTR	Plays a role in developmental process and stress responses
2	TCP TF	6	-	GGCCCAW W	Plays a pivotal role in shaping plant morphology and also plays a role in synthesis of bioactive compounds
3	STOREKEEPER TF	3	-	GGTCG	Plays a role in sucrose inducible expression of patatin gene
4	GRAS TF	1	-	GTACGG	Play a role in various processes like GA signaling, root radial patterning and floral development
5	DOF TF	19	-	AAAG	Known to play a role in seed development, photoperiodic flowering, photosynthesis, stress tolerance and cell-specific gene expression
6	MYB/SANT TF	43	-	AGATATTT	Play diverse role in developmental and physiological processes, hormone response and stress tolerance
7	bHLH TF	32	-	CANNTG	Known to play many different functions in essential physiological and developmental process
8	ZF-HD TF	36	-	ATTAT	Plays an important role in developmental processes and stress responses
9	WRKY TF	4	-	GTCAA	Forms an integral parts of signaling webs that modulate many plant processes
10	bZIP TF	2	-	ACGT CORE	Play diverse role in developmental and physiological processes

1

2 **Legends to figures:**

3 **Fig. 1**

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1 Expression patterns of *GhNAC4* transcript in response to various phytohormones. qRT-PCR expression analysis of
2 the *GhNAC4* gene in *G. hirsutum* leaves after ABA, MeJA, SA, 6-BAP, GA_3, IAA, or Ethephon treatment. Two
3 weeks-old cotton seedlings incubated for 24 h under the treatment, were used for analysis. mRNA levels of
4 GhNAC4 gene were normalized to that of Ubiquitin gene, *GhUBQ7*. The data are shown as the means ± SE (n=3).
5 A statistical analysis with one-way ANOVA indicates significant differences (** $P<0.01$, *** $P<0.001$, ns - not
6 significant)

7 **Fig. 2**

8 Expression patterns of *GhNAC4* transcript in response to various environmental stresses. qRT-PCR expression
9 analysis of the *GhNAC4* gene in *G. hirsutum* leaves after NaCl, mannitol, PEG, methyl viologen, high temperature,
10 air drying, submergence, wounding, dark or combination of dark and cold treatment. Two weeks-old cotton
11 seedlings were used for analysis. mRNA levels of *GhNAC4* gene were normalized to that of Ubiquitin gene,
12 *GhUBQ7*. The data are shown as the means ± SE (n=3). A statistical analysis with one-way ANOVA indicates
13 significant differences (*** $P<0.001$, ns - not significant)

14 **Fig. 3**

15 Physical map of *GhNAC4* promoter. Nucleotide sequence of the 1492 bp upstream region of *GhNAC4* gene from *G.*
16 *hirsutum* var JK Durga. The putative transcription start site is in bold and is designated as +1. The translation start
17 site is bolded and underlined. The numbers on the left side indicate the distance from the transcription start site. The
18 sequence was analyzed by PLACE, PlantCARE and PlantPAN2.0 programs. All the predicted motifs are indicted by
19 arrow and their names are mentioned above. '→' and '←' indicates that the predicted motif is on positive (5'-3')
20 and negative (3'-5') strand respectively. Stress inducible motifs are represented in red, phytohormone responsive
21 motifs are in green, light inducible motifs are in orange, tissue specificity motifs are in blue, transcriptional related
22 motifs in purple and transcription factors binding sites are in teal color

23 **Fig. 4**

24 Functional classification of *cis*-elements present in the *GhNAC4* promoter. The motifs in the GhNAC4 promoter
25 were classified into five functional categories. There are a relatively high proportion of tissue-specific motifs and
26 transcription factors binding sites.

27 **Fig. 5**

28 Histochemical localization of GUS activity in tobacco transgenic plants containing PRO_{GhNAC4}:GUS construct. **a**-**e**
29 seedlings grown on MS media with hygromycin at - **a** Day1; **b** Day 3; **c** Day 7; **d** Day 15; **e** Day 30. **f**-**j** various
30 tissues of a 30-day old transgenic tobacco plant - **f** Main root; **g** Lateral root; **h** True leaf; **i** Guard cells; **j** Developing
31 leaf. **k**-**m** floral structures – **k** Mature flower; **l** dissected flower showing various tissues; **m** cross-section of an
32 anther. **n** 15 days old tobacco seedling carrying empty pCAMBIA 1381Z vector. AW, anther wall; CO, cotyledon;
33 GC, guard cell; HY, hypocotyl; LR, lateral root; LRJ, lateral root junction; LV, lateral vein; OV, ovary; PD, pedicel;
34 PE, petal; PG, pollen grains; PL, plumule; RAM, root apical meristem; RD, radicle; RH, root hairs; SAM, shoot
35 apical meristem; SE, sepal; TL, true leaf; VB, vascular bundle; WA, wounded area. All arrows show strong GUS
36 activity or no activity. Bars of each panel are as shown

37 **Fig. 6**

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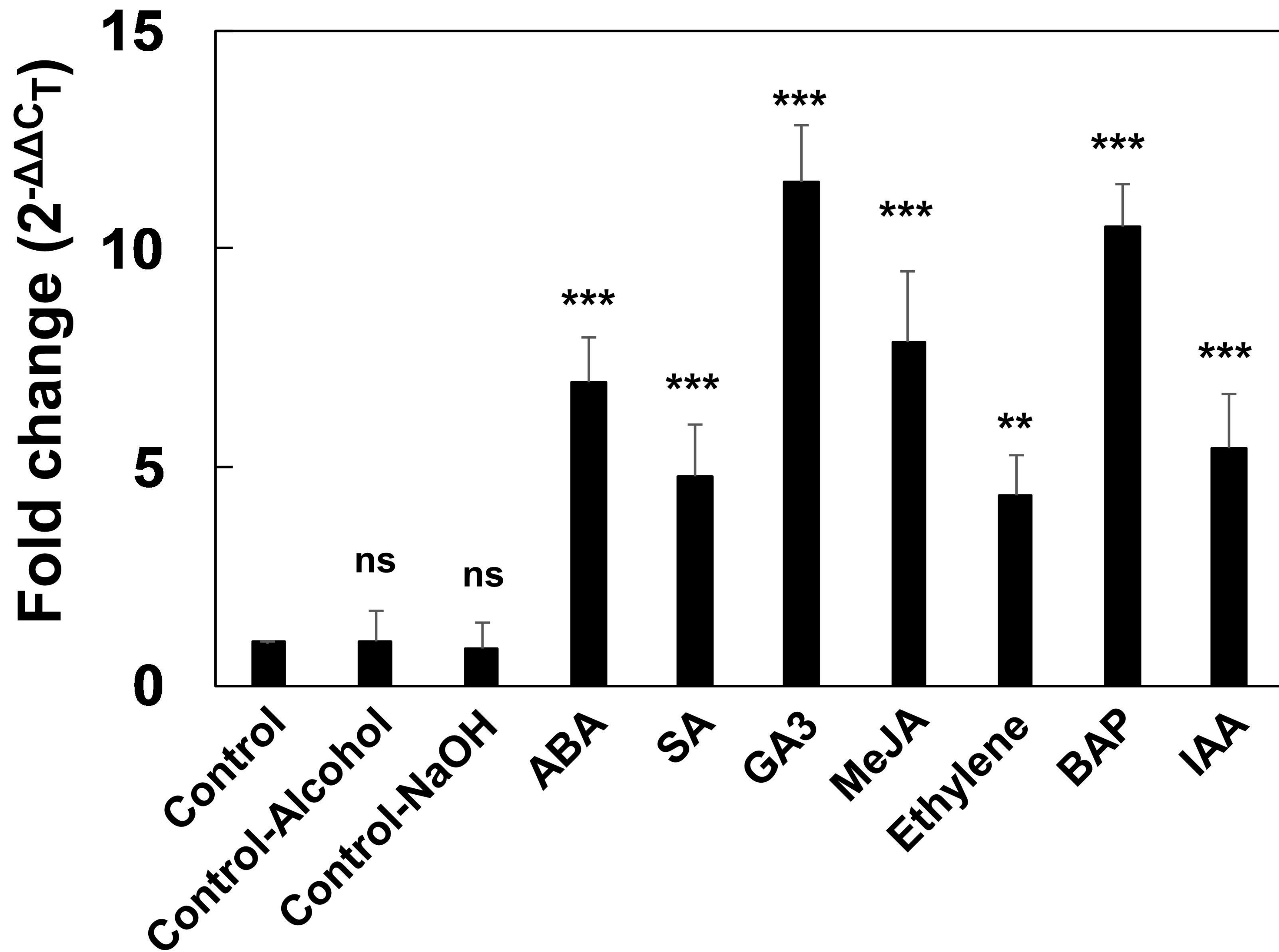
1 GUS activity in free hand cross-sections tobacco transgenic plants containing PRO_{GhNAC4}:GUS construct. **a** leaf; **b**
2 petiole; **c** stem **d** magnified view of the stem; **e** root; **f** wounded stem. abP, abaxial phloem; adP, adaxial phloem;
3 CO, cortex; P, phloem; VB, vascular bundle; XY, xylem. All arrows show strong GUS activity or no activity. Bars
4 of each panel are as shown
5 **Fig. 7**
6 Effect of various phytohormones on the GUS activity of PRO_{GhNAC4}:GUS tobacco transgenics. Fluorometric analysis
7 of the *GUS* gene in tobacco seedlings after ABA, MeJA, SA, 6-BAP, GA_3, IAA, or Ethephon treatment. Two
8 weeks-old PRO_{GhNAC4}:GUS tobacco transgenic seedlings incubated for 24 h under the treatment, were used for
9 analysis. pCAMBIA 1381Z empty vector harboring tobacco seedlings were used as a negative control. The data are
10 shown as the means ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (***
11 $P<0.001$, ns - not significant)
12 **Fig. 8**
13 Effect of various stresses on the GUS activity of PRO_{GhNAC4}:GUS tobacco transgenics. Fluorometric analysis of the
14 *GUS* gene in tobacco seedlings after NaCl, mannitol, PEG, methyl viologen, high temperature, air drying,
15 submergence, wounding, dark or combination of dark and cold treatment. Two weeks-old PRO_{GhNAC4}:GUS tobacco
16 transgenic seedlings, were used for analysis. The data are shown as the means ± SE (n=3). A statistical analysis with
17 one-way ANOVA indicates significant differences (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)
18 **TABLE 1**
19 Primer sequences used in the experiments. Underlined sequences are restriction enzyme recognition site
20 **TABLE 2**
21 Putative motifs in the *GhNAC4* promoter identified by in silico analysis. DB ID represents the PLACE database
22 accession number. PC represents the motifs identified by PlantCare database


Fold change ($2^{-\Delta\Delta C_T}$)
15
10
5
0
ns
ns
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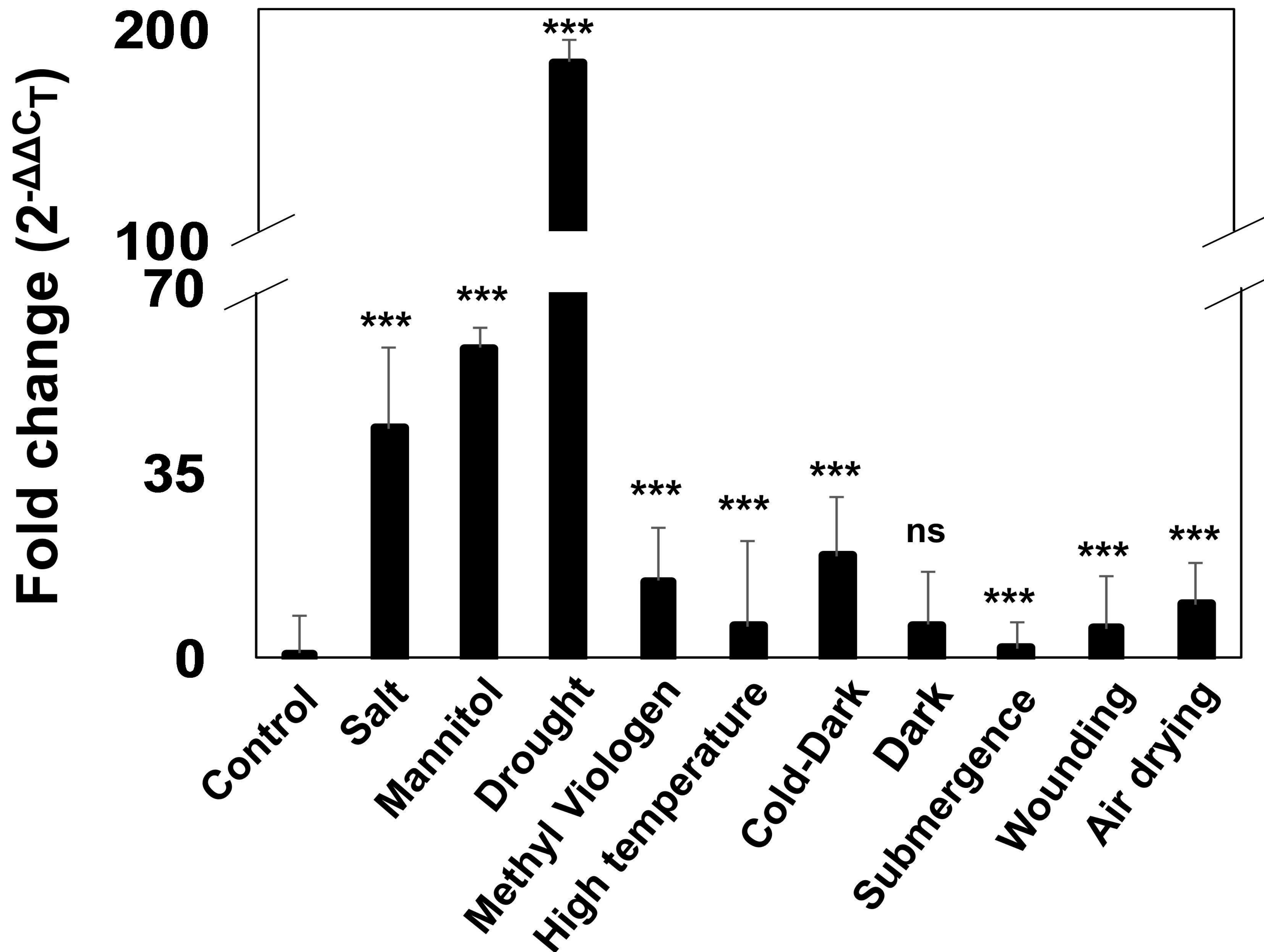
***
Control
Control-Alcohol
Control-NaOH
ABA
SA
GA3
MeJA
Ethylene
BAP
IAA


Fold change ($2^{-\Delta\Delta C_T}$)
200
100
70
35
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ns
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Control
Salt
Mannitol
Drought
Methyl Viologen
High temperature
Cold-Dark
Dark
Submergence
Wounding
Air drying

MYB2CONSENSUSAT GTGANTG10 IBOX CORE SEF4MOTIF GM7S DOFCOREZM SEF1MOTIF

-1492 GGCCTCATAATTGCATCACCGTTGAAATCACAAGATAAGTTTTTGTTCTTTTTGTATTATCTATATTTAA

GT1 CONSENSUS POLLEN1 LEALAT52

-1422 AATTGAAAATAAAAATTGAGTTTAAAATTTTTTAGTCTTGATTTGATAATAAGAAAATTATATGTAGATT

OSE2ROOT NODULE ARR1AT

-1352 CTCTTCGAATTTTATTTTGATTTAACCTTCACATTAGTAGGTTATTGAATTGTTATAATAGATGCCGATA

BOXII NTPATPB GT1GM SCAM4 MARTBOX TCA ELEMENT

-1282 ATGGCAATAGAATGAACGAAAAATAAGAAAAATAAAAACACACAAAAACCACGGAAGAGGAGAAGAAAA

ANAERO1 CONSENSUS TAAAG STKST1

-1212 CA[illegible]ATTAAAAAAACCCTA

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ROOTMOTIF TAPOX1 C2H2 TFBS SORLIP2AT

-1142 TTCTAATTAATATCAAATAAAATAAGTGTAGTTTTATACAGATTCTACTTATGTGGCCCTCAACCTCTTG

E2F CONSENSUS GAREAT TALE TFBS

-1072 TCCCCACATATCTCTTATTTTGCCACTGTATTTATTTATTTTTAACAAGTGATAGGATTTAGGTCACACA

CPBCSPOR

-1002 AACTCTAACATGAGCAATTAAAAAAAATCATTCTAATTTTAATTCTAAAACCATTCGAATAAAAGAAGCTA

PYRIMIDINEBOX OSRAMY1A ZF-HD TFBS

-932 ATAAAAGGGGAAAATATTTGATATATTGCTAATGTAGTTTTTAGACTTCACAAGTATTAATGAATTTATA

GATA TFBS

-862 AGTGTCCAATATAAATATAGTAAGATATTAAAAAATAAATAAATATTTTTAAAAATGTGATAATTTGTTA

CACTF PPCA1 POLASIG3

-792 AGATTACTTATGAAATAAATAAAAATACACTACCAAAATACATGATACTAACCCATAAAAAAATAATAGAA

WBOXATNPR1 DOF TFBS WBOXNTERF3 NAC TFBS CARGCW8GAT

-722 TATAATTCCTTTGACAAAAAAAAGCAAAAACTTTGACCTATATAATAAATAATCAATCAAATTTCATTATT

TGTCACAM CUCUMISIN EIN TFBS

-653 TTGAATGTGTGACACATCAATTGAATAAATGGTCCAAAACTCCATGAATGCATTAAAACTTGTAAGCTAG

TCR TFBS

-582 TTCGTAGAAGTTATTGAGTGAAAGAAACCCTAATCCCAAACATTTAAATCAACGAAAATGAAAATCCAAC

WBOX HVISO1 GADOWNAT ABRERATCAL

-512 AAATAAAAAAAAAAGTCAATTAAAACGTGTCACGAGTCACAACCAGCCAATTGTTTTTTATTTCCCTTTTT

HSE ABARE LATERD1 CGACG OSAMY3

-442 ACCCTCATCATTCCCCTTTACTTTAATTTCCACAAAATTCTAGCCTAACTCTTCCACACGTGTTGGCCGA

ACGTTBOX QARBNEXTA AT-HOOK TFBS MARAS

-372 CGTGTCCTCCCTTCTTTCAAGGCATGATAAACGTTAAACTCTCCAATAAAAATTAAAAATAAAATAACAA

MYB/SANT TFBS

-302 AAGGACAAAAGCTCCAAACTCCATAATTTTTTTTATTGCCTTACAGAATTTGGGGGAGATTCTAGAAACA

CAATBOX ACGTABRE MOTIFA2OSEM TGACG MOTIF

-232 AAGCAAGAAAAATTGACACGTGTCCTAAAACTTTTATGACACTCTTTTTGTAAATTTTCGTGACGGTTCA

MYBST1 DRECRT COREAT BIHD1OS TCA ELEMENT MYBCORE ATCYCB1

-162 TGTACCCTGTTTGGTAGGATAAGGTCGGCCGTAGATGTCAGGCACGTGTCGTTAGTTAACGGTGATAAAG

-92 TGGGTCCCACTTAGACAAAAGTTAGACAAGGACGACCGTACTTATCTCTTTTTAGACACTATCGATCTAT

TATABOX OSPAL +1

-22 CCTCCCTTCTATTTAAATACCTTCCCTTTCCCCTAACCCTGAAAAAAAAAGAGAGAGAAAAACCTTTTTT

+49 TACTGTGTTTTATTTTCTCTTGTACTAAAACTGTTTGGTCGGCAAGAAAACTTTAGAATCATGGGAGTGC

+119 CGATGGGAGTGCCGGAAACT

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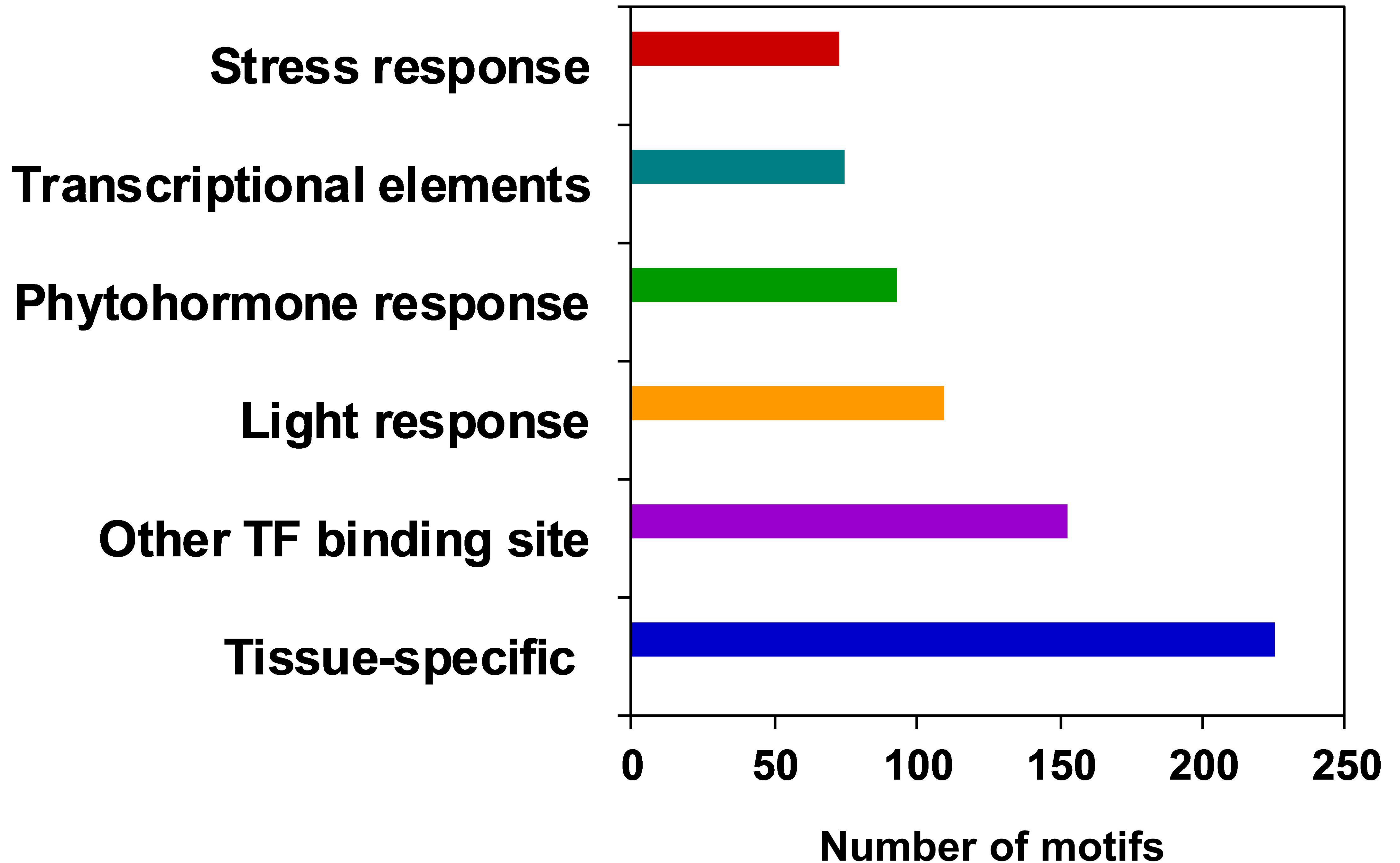

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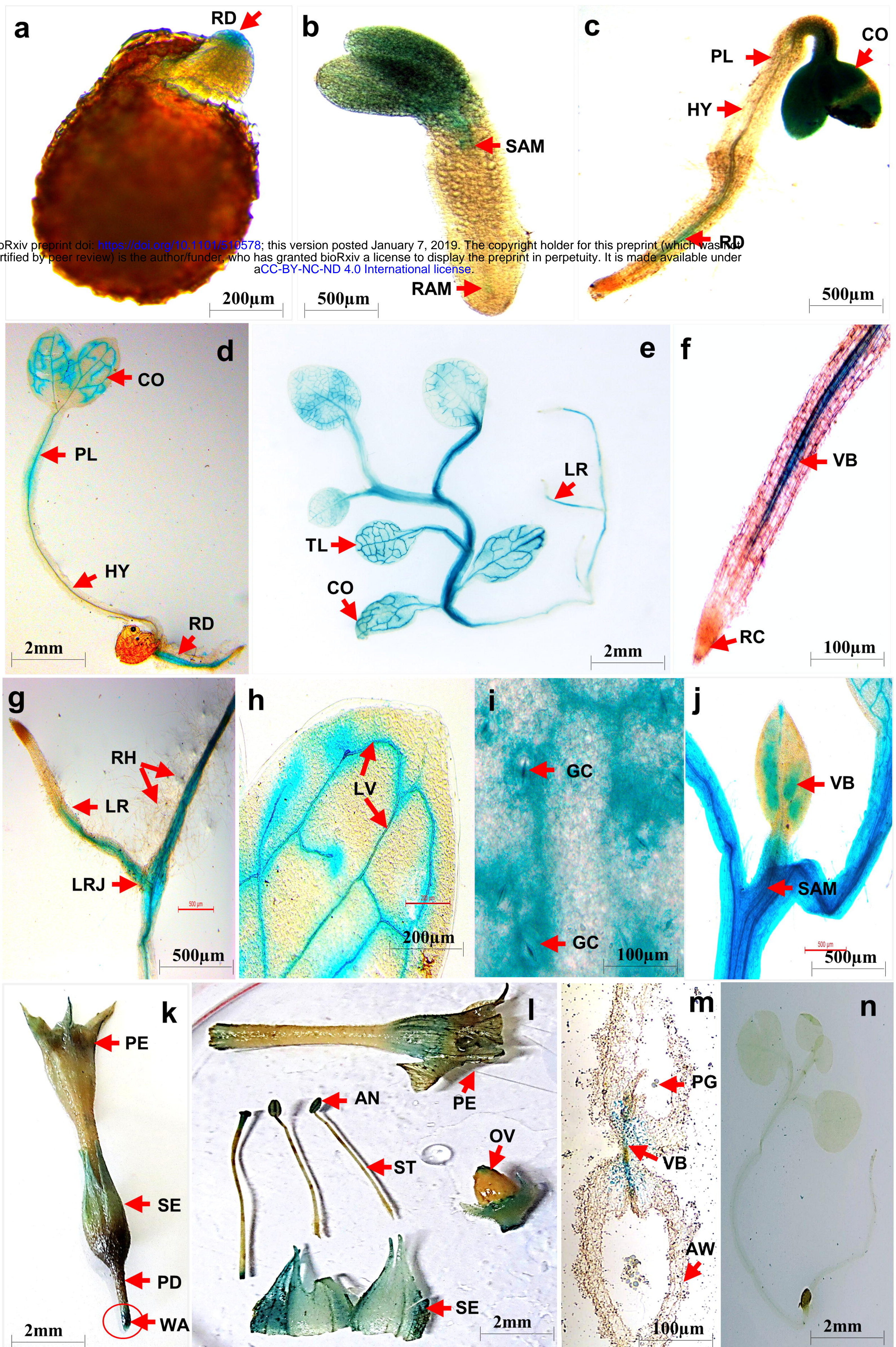

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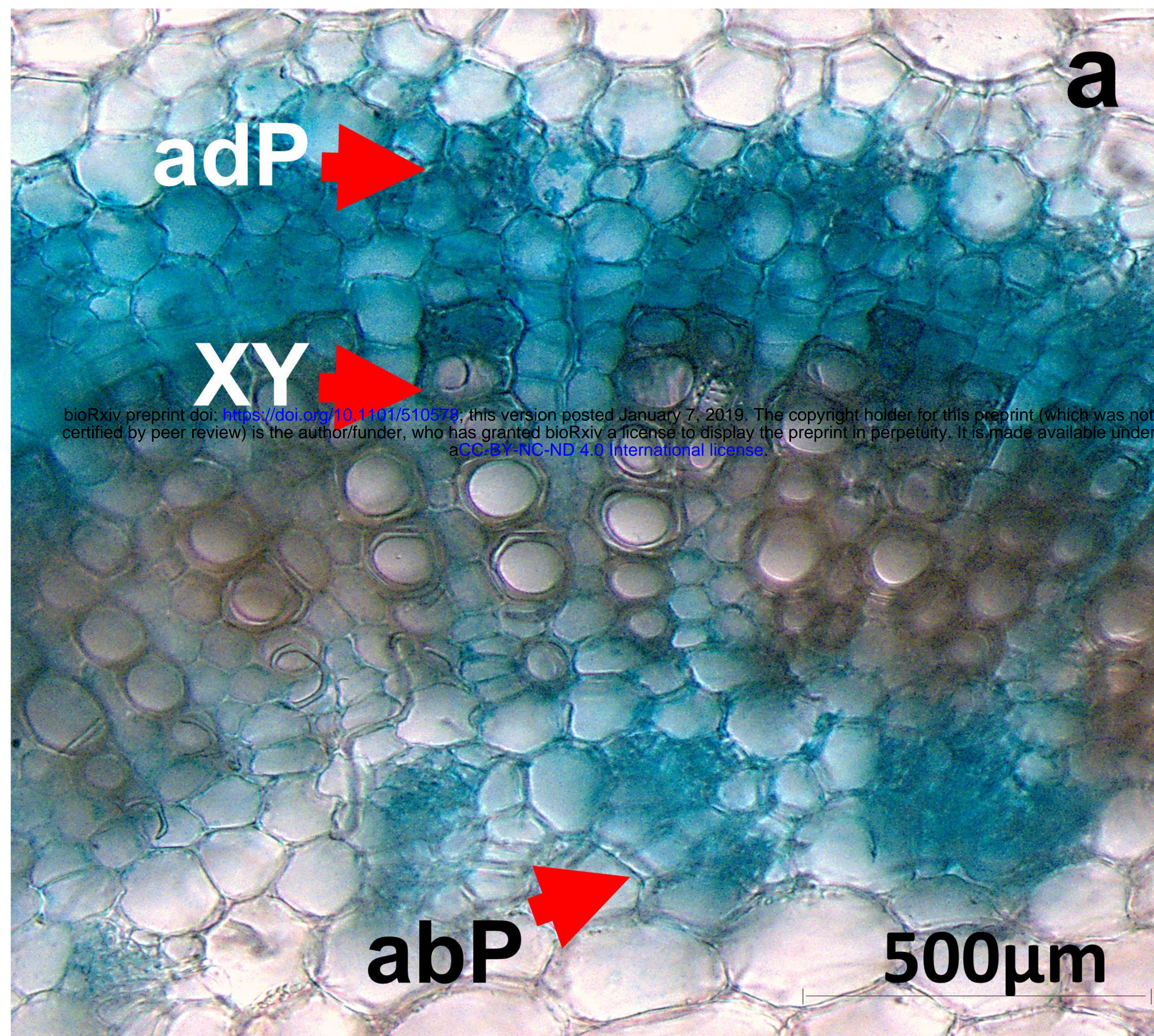

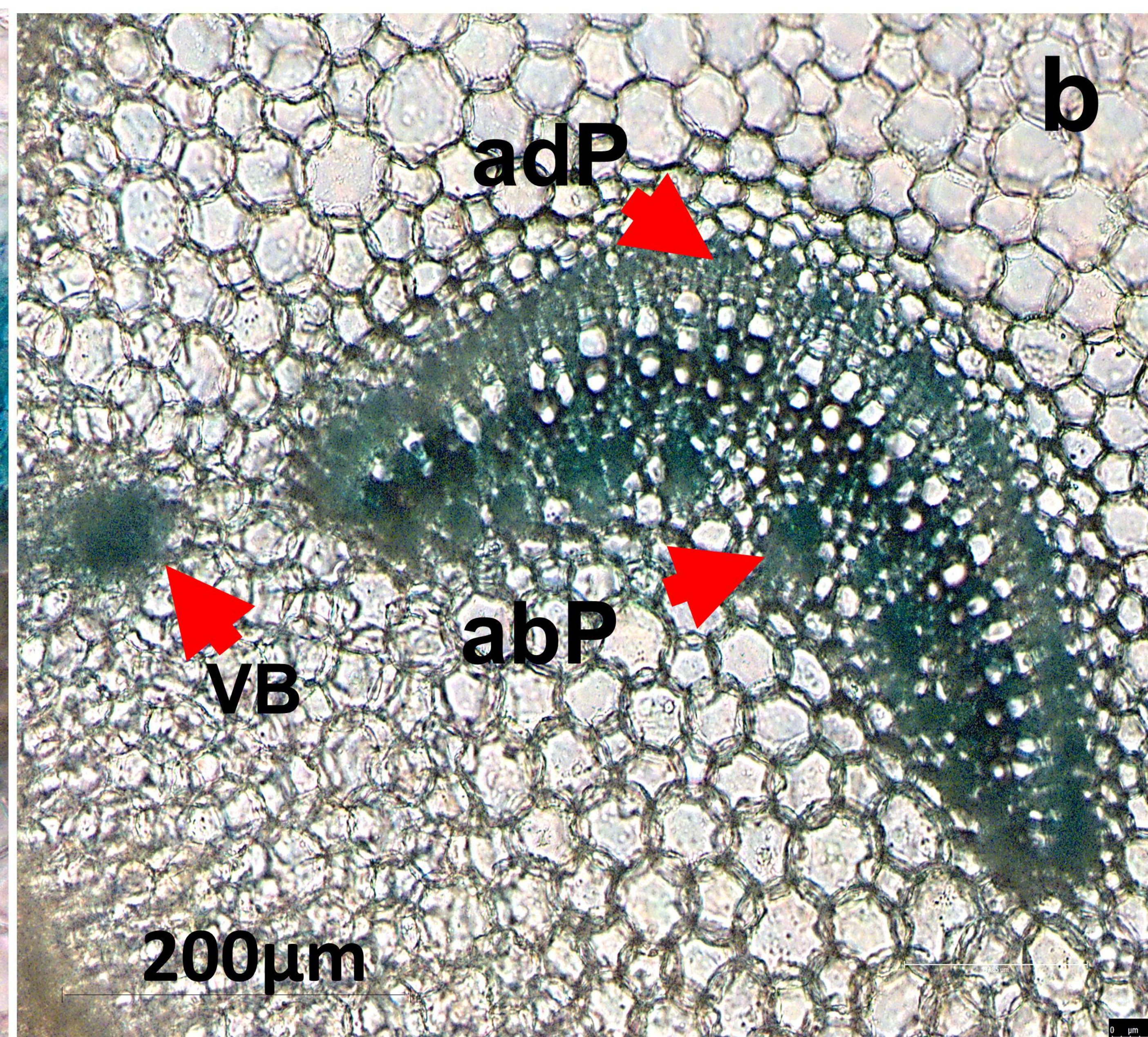

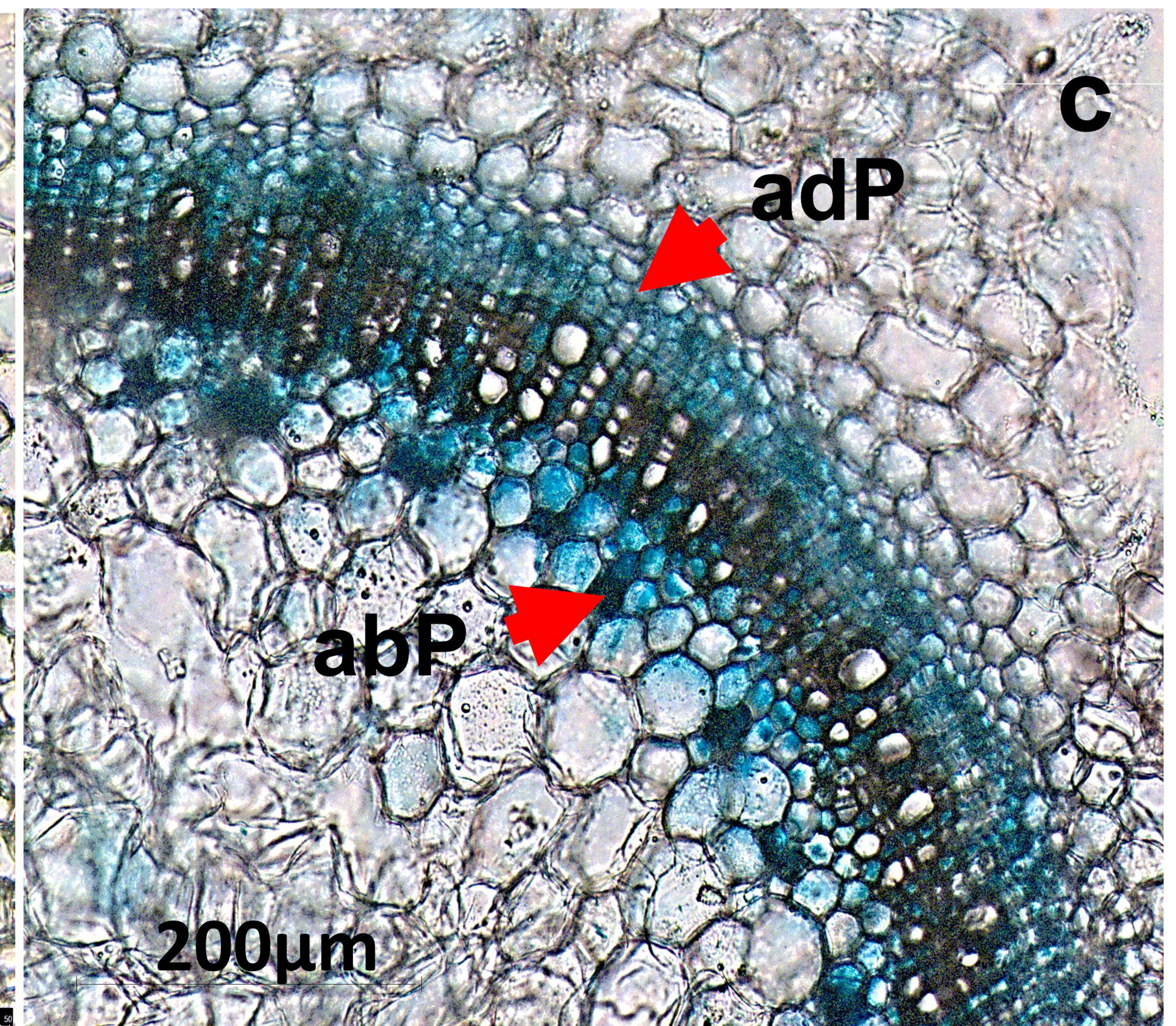

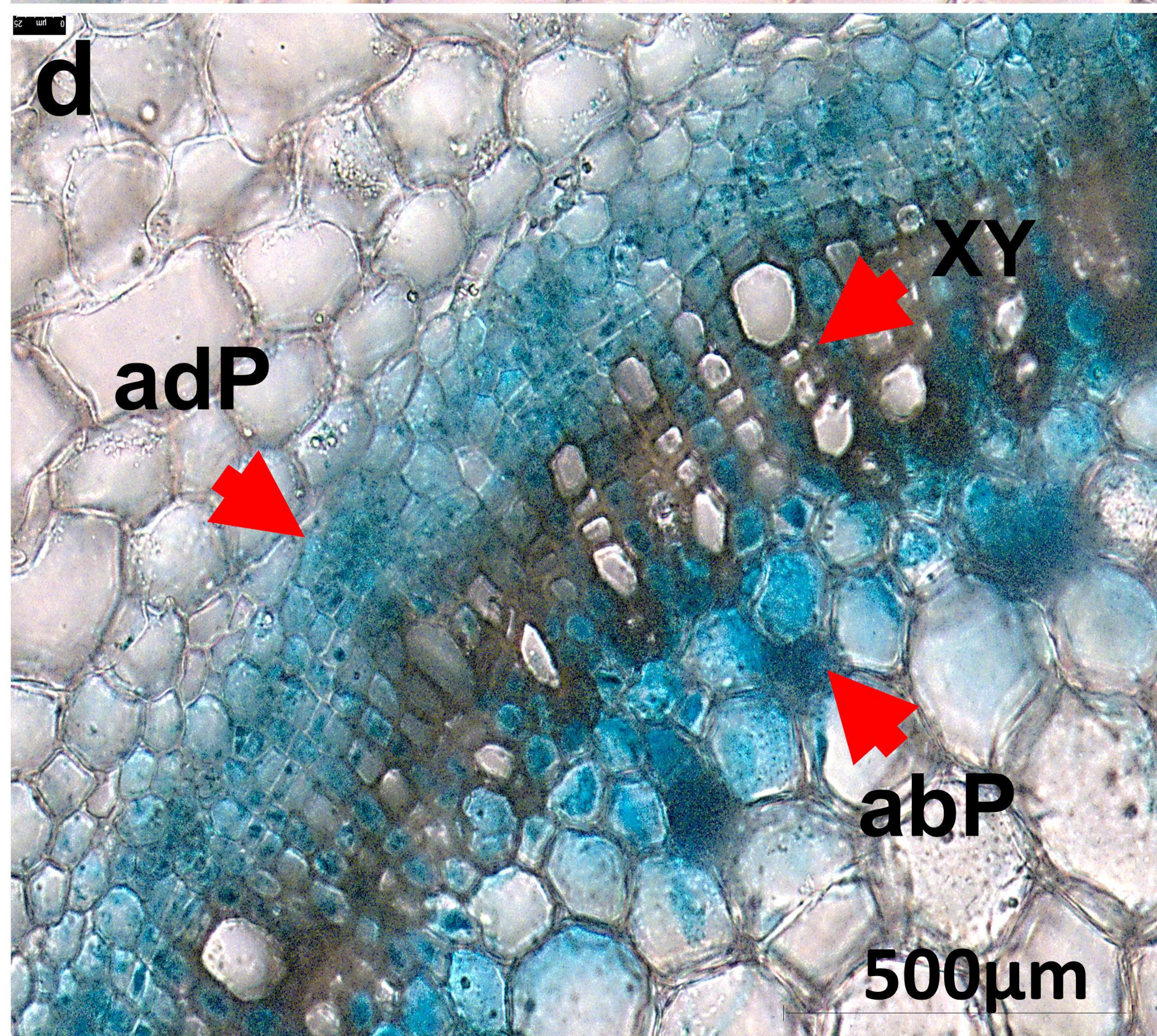

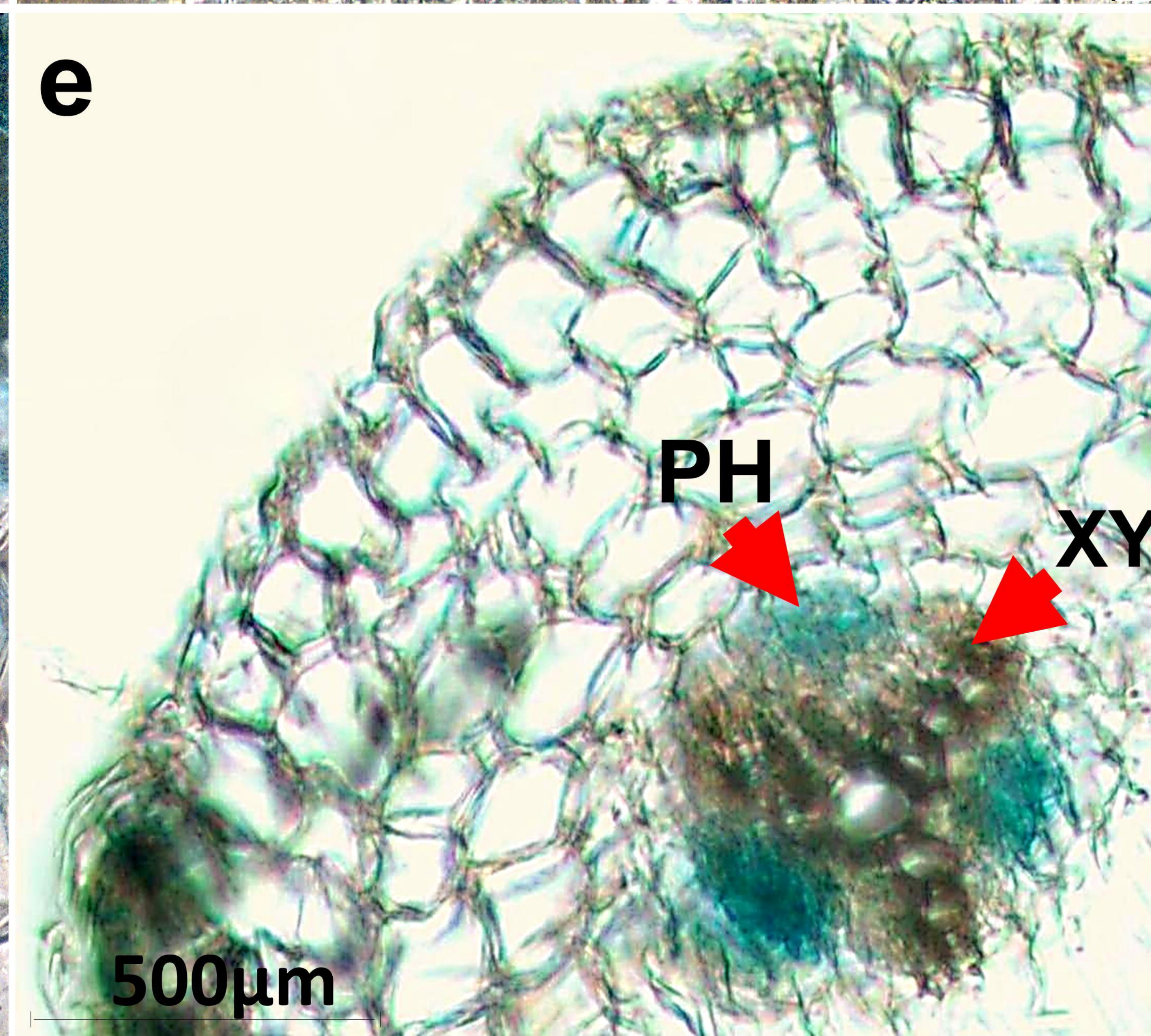

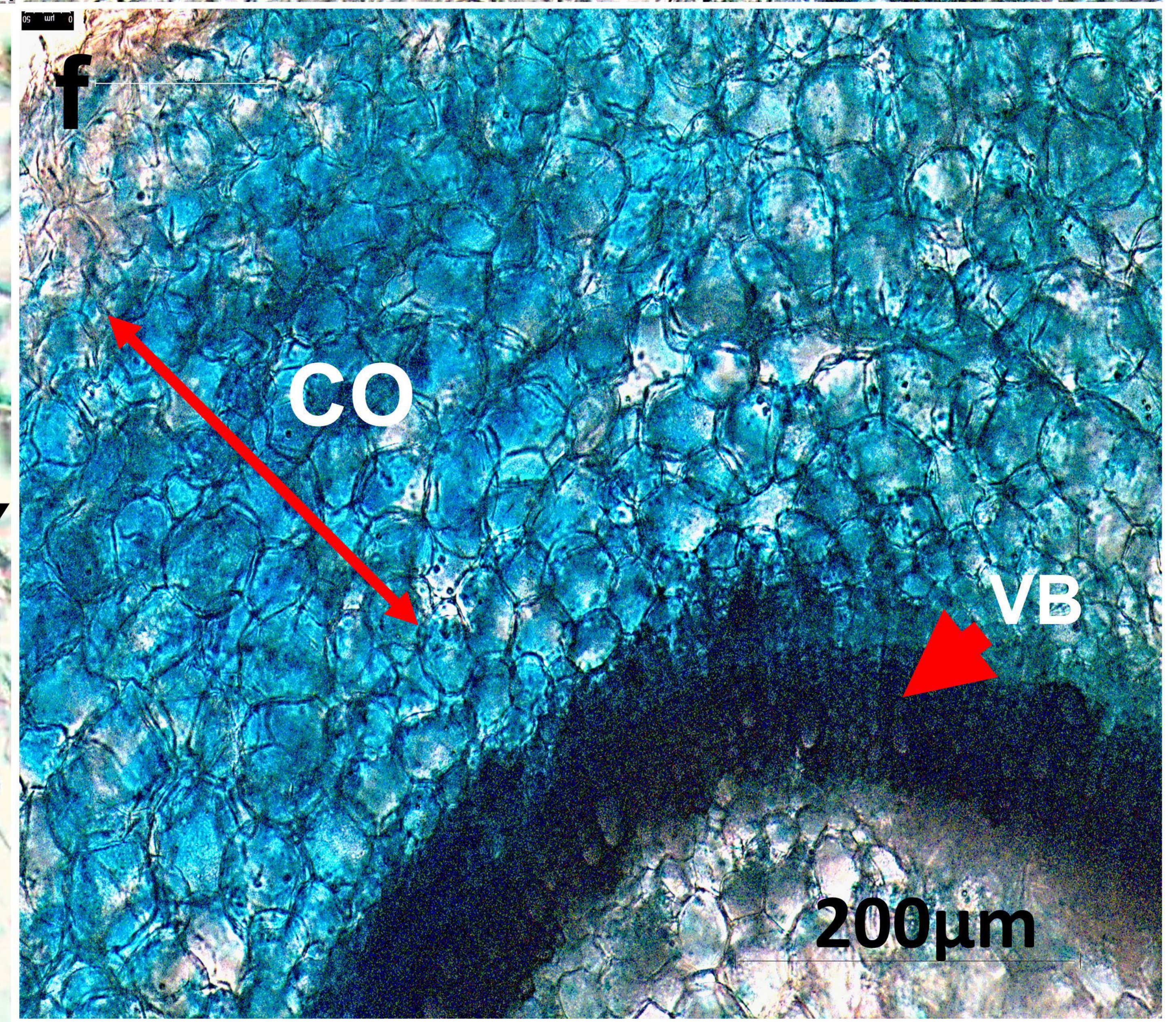

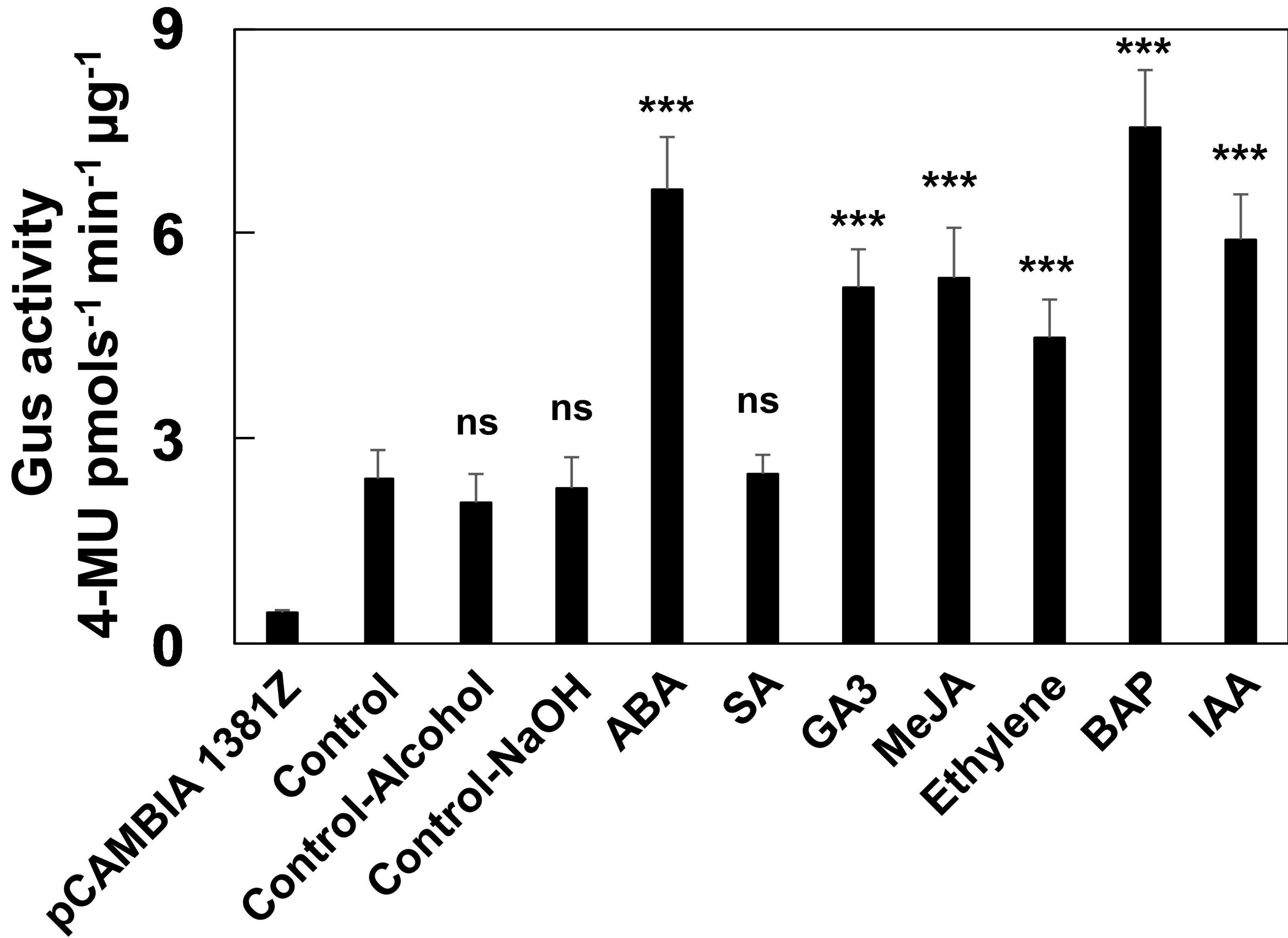
Gus activity
4-MU pmols-1 min-1 µg-1
9
6
3
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ns
ns
ns
pCAMBIA 1381Z
Control
Control-Alcohol
Control-NaOH
ABA
SA
GA3
MeJA
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BAP
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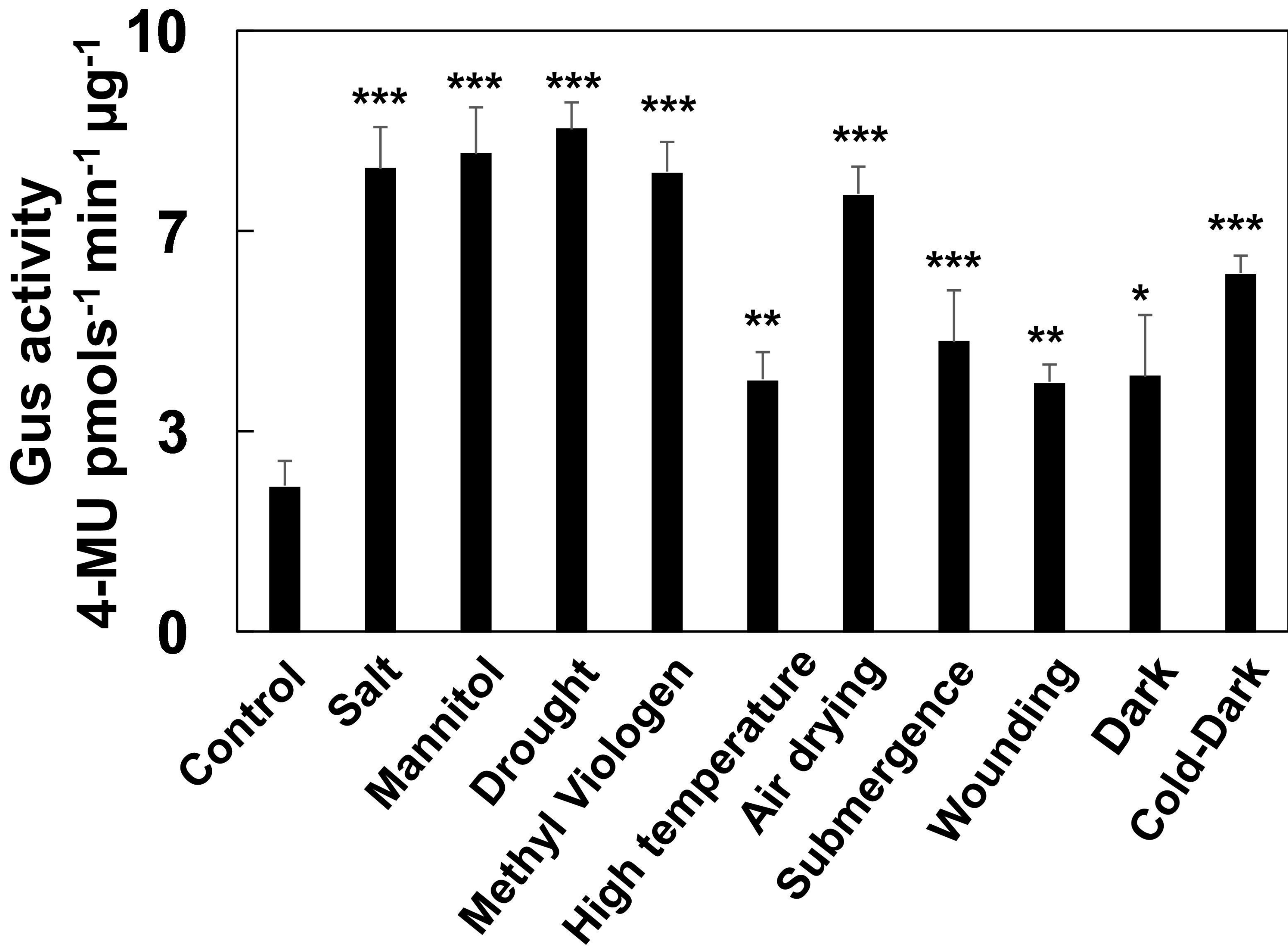
Gus activity
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Control
Salt
Mannitol
Drought
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Air drying
Submergence
Wounding
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