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Identification of trans-acting factors regulating SamDC expression in *Oryza sativa*

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ABSTRACT

Abiotic stress affects the growth and productivity of crop plants; to cope with the adverse environmental conditions, plants have developed efficient defense machinery comprising of antioxidants like phenolics and flavonoids, and osmolytes like polyamines. SamDC is a key enzyme in the polyamine biosynthesis pathway in plants. In our present communication we have done *in silico* analysis of the promoter region of SamDC to look for the presence of different cis-regulatory elements contributing to its expression. Based on the presence of different cis-regulatory elements we completed comparative analysis of SamDC gene expression in rice lamina of IR-29 and Nonabokra by qPCR in response to the abiotic stress treatments of salinity, drought, cold and the biotic stress treatments of ABA and light. Additionally, to explore the role of the cis-regulatory elements in regulating the expression of SamDC gene in plants we comparatively analyzed the binding of rice nuclear proteins prepared from IR-29 and Nonabokra undergoing various stress treatments. The intensity of the complex formed was low and inducible in IR-29 in contrast to Nonabokra. Southwestern blot analysis helped in predicting the size of the trans-acting factors binding to these cis-elements. To our knowledge this is the first report on the comprehensive analysis of SamDC gene expression in rice and identification of the trans-acting factors regulating its expression.

1. Introduction

Productivity and growth of rice is highly affected by abiotic stresses like salinity, drought, etc., [1] as well as by biotic stresses like pathogens or hormonal treatments. Plants have developed efficient defense strategies to help in survival against the stress conditions. Accumulation of antioxidants as well as low molecular weight compounds called osmolytes in the cytosol helps in maintaining the cellular osmotic potential via a process called osmotic adjustment. The most prevalent osmolytes includes sucrose, glucose, quaternary ammonium compounds like proline and glycine betaine and higher polyamines like spermine and spermidine [2]. Polyamines have been implicated to play a pivotal role in the growth and development of plants. Polyamine biosynthesis is a very well characterized pathway in plants mediated by arginine decarboxylase (ADC), orninthine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SamDC) [3]. ADC and ODC are involved in the synthesis of putrescine while SamDC is involved in the biosynthesis of spermidine and spermine by providing decarboxylated S-adenosyl methionine as aminopropyl donor [4].

Plants accumulate polyamines in response to salinity and drought stress [1,3] thereby clearly suggesting a protective role for the polyamines. Previously, we have seen that exogenous application of spermine and spermidine helps in alleviating salt stress in three different rice cultivars [5]. In our previous communication we have shown that higher accumulation of polyamines in rice plants is highly correlated with the increased transcript accumulation of SamDC [6,7]. The transcript accumulation of genotypes varied significantly between the salt tolerant and salt susceptible rice genotypes, Nonabokra and IR-29, respectively. This suggests differential regulation of SamDC under salinity or ABA stress treatments. However, there has been no clear information about the regulators of SamDC in rice.

In our present communication we completed differential expression analysis of SamDC by qPCR in response to the different stress treatments of salinity, drought, cold and biotic stress

Abbreviations: SamDC, S-adenosyl methionine decarboxylase; EMSA, electrophoretic mobility shift assay.

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treatments of ABA and light. To get an insight into the mode of SamDC gene regulation, we completed *in silico* analysis of the SamDC promoter region to look for the presence of various regulatory elements. Finally, we have done EMSA using different cis-regulatory elements as a probe using nuclear extracts prepared from lamina of IR-29 and Nonabokra under different stress treatments followed by southwestern blot analysis to identify the trans-acting factors.

2. Material and methods

2.1. cis-Acting regulatory elements and transcription factor-binding sites

The promoter region of SamDC, comprising of 1110 bp promoter and 90 bp downstream sequences obtained from Plant Promoter Db (http://ppdb.gene.nagoya-u.ac.jp/cgi-bin/index.cgi) was used as an input sequence for identifying cis-acting regulatory elements (CAREs). The software programs used were PLACE (http://www.dna.affrc.go.jp/PLACE/) [8] and Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [9].

2.2. Plant material and growth conditions

Seeds were surface sterilized with 0.1% $HgCl_2$ for 20 min, then rinsed thoroughly with distilled water, imbibed in de-ionized water for 6–8 h, kept under anaerobic conditions overnight, then spread over a sterile gauge soaked with sterile water in a Petri dish, and kept in the dark at 37 °C for three days. After three days, the germinated seedlings were grown in a quarter strength of MS medium (Murashige and Skoog complete media, Sigma) at 32 °C for 16 h light and 8 h dark cycle in a plant growth chamber (Nippon, LHP-100-RDS, Tokyo, Japan) for 15 days. Young plants were then treated with either fresh 0.25X MS medium alone as control or in 0.25X MS medium with 200 mM NaCl, or with 20% PEG, or 100 μM ABA, or kept at 4 °C for cold treatment for 6 and 24 h. For the light treatment plants with 0.25X MS medium were kept in continuous light for 24 h or in constant dark for 24 h. After the stress treatments plants were washed thoroughly with de-ionized water and leaves were harvested and fresh weights were taken. The samples were frozen in liquid nitrogen and stored for nuclei and RNA isolation.

2.3. Analysis of gene expression by Real time PCR

To study the expression of SamDC under different treatments quantitative RT-PCR was performed with total RNA prepared from lamina of IR-29 and Nonabokra. The experiments were carried out in three biological replicates with two technical replicates. cDNA synthesis was carried out using 2 μg DNAse treated RNA using GoScript™ Reverse Transcription System (Promega). The qRT-PCR experiments were carried out in triplicate using GoTaq® qPCR Master Mix (Promega) with ubiquitin as standard using CFX-96 Bio-Rad thermocycler (Bio-Rad) (Table 1). Increasing temperatures from 55 to 95 °C (0.5 °C 10 s^{-1}) were used for melt curve analysis. Un-transcribed RNA was also ran as a negative control to check DNA contamination. The relative difference in expression for each sample in individual experiments was determined by normalizing the Ct value for each gene against the Ct value of ubiquitin and was calculated relative to a calibrator using the equation $2^{-\Delta\Delta Ct}$ [10].

2.4. Preparation of radioactive probes, non-radioactive DNA competitors, EMSA and South Western blotting

The oligonucleotides (oligos) were radiolabeled at the 5′end with T4 Polynucleotide Kinase using γ^{32}P-ATP (3700 Ci/mmole from BRIT, JONAKI, Hyderabad) by incubating the reaction mix at 37 °C for 1 h and then stopped by incubating at 70 °C for 15 min, and allowed to cool slowly to room temperature so that the two oligos hybridize to make double stranded DNA probe. The radiolabeled probes were purified using Sephadex G-50 Quick Spin™ Column (Roche, Germany). Incorporation of radioactivity was measured by using Liquid Scintillation Counter (Beckman, USA) after TCA precipitation & counts in CPM/μl were noted. Competitors were prepared by mixing equal concentration of complementary oligonucleotides and heating them by incubating at 80 °C for 10 min to remove secondary structures, followed by slow cooling to room temperature and allowing them to anneal slowly.

20 μg of nuclear extract prepared from equal amount of tissue (20 g) of control and treated lamina of IR-29 and Nonabokra [11] was mixed with 2 μg of Poly(dI). poly(dC) (1 μg/μl, Pharmacia) and the volume was brought up to 35 μl with dialysis buffer. The reaction mixtures were incubated on ice for 15 min and then the radioactive probe (80,000 CPM per reaction) was added followed by incubation at room temperature for 45 min. For competition assays, nuclear extract was pre-incubated with 100-fold molar excess of different non-radioactive competitors for 30 min at room temperature before the addition of the radio labeled probe (Table 1). Southwestern blotting was carried out by using equal amounts of nuclear proteins (20 μg) [12].

3. Results and discussion

3.1. Identification of cis-regulatory elements in the promoter of SamDC

SamDC is a key regulator of polyamine biosynthesis in plants. The function of this enzyme is a key rate limiting step in the polyamine biosynthetic pathway as it aids in the formation of spermidine (Spd) from putrescine (Put) and also the formation of spermine (Spm) from Spd. Previously, it has been shown that SamDC transcript accumulation is highly upregulated by salinity stress and ABA treatment in salt tolerant rice cultivars [6,7], which is consistent with earlier observations where overexpression of SamDC gives tolerance to multiple stresses like salinity, drought, low and high temperature and parquet toxicity in egg plants [13] or tobacco [14]. To study the differential regulation of SamDC in

Table 1
Different primers and oligos used for gene expression analysis by qPCR and EMSA.

Gene name	Forward primer	Reverse primer
Ubiquitin	GTTGAGAGCTCCGACACCAT	AGGGTGGACTCCTTCTGGAT
SamDC	CCAAGGATTCTTGAGCTTGC	GCTTTGCTGGATCTCCAATC
MYB	GAATATAACAAATGTGAATATAACAAATGT	ACATTTGTTATATTCACATTTGTTATATTC
ABREATCAL	GAATACACGCGGTGTGAATACACGCGGTGT	ACACCGCGTGTATTCACACCGCGTGTATTC
W-Box	GAATATGACTGTGAATATGACTGT	ACAGTCATATTCACAGTCATATTC
LTRE	GAATACCGACTGTGAATACCGACCTGT	ACAGGTCGGTATTCACAGTCGGTATTC
GATA	GAATAGATATGTGAATAGATATGT	ACATATCTATTCACATATCTATTC
SORLIP1	GAATAGCCACTGTGAATAGCCACTGT	ACAGTGGCTATTCACAGTGGCTATTC

rice we have performed an *in silico* analysis of the promoter region of SamDC using Place database [8] and Plant Care [9] which revealed the presence of a number of putative cis-elements associated with various environmental signals (Table 2).

3.2. SamDC expression in response to various treatments

To analyze the transcript accumulation of SamDC, qPCR was completed with total RNA isolated from lamina of IR-29 and Nonabokra under different abiotic stress treatments viz. 200 mM NaCl, drought, cold, exogenous ABA and light treatment. SamdC expression was highly induced by all the stress treatments in Nonabokra than IR-29 and the transcript abundance peaked at 24 h after stress treatments (Fig. 1A and B). Our observation thus suggests the existence of multiple regulators that controls expression via ABA dependent and ABA independent pathways.

3.3. Identification of MYB trans-acting factors as regulator of SamDC

A putative MYB (TAACTG), cis-acting regulatory element has been detected by PLACE database analysis in the promoter region of *Oryza sativa* SAMdc gene. Previous studies have demonstrated that MYB motif acts as cis-acting drought responsive elements (DRE) [15]. MYB proteins, a superfamily of transcriptional factors that play regulatory roles in developmental processes and defense responses in plants, are reported to bind on the consensus sequences of MYB-cis-regulatory elements. β-glucoronidase reporter gene driven by the Atmyb2 promoter was reported to be induced by dehydration and salt stress in transgenic Arabidopsis plants [16]. DNA protein interaction studies were further performed to investigate the role of MYB motif in the osmotic stress regulated expression of SamDC gene (Fig. 2A). A very weak interaction was detected in IR-29 control while maximum interaction was detected after 24 h of salinity and drought stress whereas Nonabokra showed strong binding suggesting the inducible nature of the transcription factor. Furthermore, 100 M excess of unlabeled dimeric MYB motif DNA clearly competed out the binding activity. Southwestern blot analysis with the radiolabeled MYB motif identified a ~40 kDa nuclear protein factor in the nuclear extract of Nonabokra treated with 200 mM NaCl for 24 h and 20% PEG-6000 for 24 h (Fig. 4B).

3.4. WRKY transcription factors play a role in regulation of SamDC expression

In plants, W-box cis-elements are known to bind WRKY TFs [17] indicating that these TFs may be important in regulating the expression of the correlated genes. In addition, they have also been documented to mediate abiotic plant responses to freezing [18], wounding [19], oxidative stress [20], drought, salinity, cold, and heat [21,22]. *In sliico* analysis showed the presence of W-box in the SamDC promoter (Table 2). DNA protein interaction performed with W-box showed the presence of two complexes (Fig. 2C) under salinity stress and drought stress treatment in IR-29 and Nonabokra. CII was more predominant in Nonabokra after 24 h of salinity and drought stress, thereby clearly suggesting a key role for this transcription factor in controlling the expression under stress. The complexes were abolished by competition when 100X non-radioactive W-Box was used as competitor against radiolabeled W-box was used as probe. Southwestern blot analysis with the radiolabeled W-Box motif identified a ~25 and 16 kDa nuclear protein factor in the nuclear extract of Nonabokra treated with 200 mM NaCl for 24 h and 20% PEG-6000 for 24 h (Supplementary Fig. 1A).

3.5. ABRE and LTRE binding proteins have a role in SamDC expression

Salinity and drought stress inducible genes are also inducible by ABA indicating that ABA functions as a key player for abiotic stress response [23–25]. The upstream of ABA responsive genes contains a very well characterized cis-element: ABRE (ABA responsive element) which is necessary for gene expression [25]. *In silico* analysis of SamDC promoter showed the presence of ABRE elements (ABREATCAL) (Table 2). Gel mobility shift assays performed with 2X ABREATCAL elements showed the presence of two complexes, the intensity of which increased after salinity and drought stress treatments which was abolished in competition assays suggesting that the trans-acting factors binds specifically to these sequences (Fig. 2A). Southwestern blot analysis identified a ~38 and 28 kDa transcription factor in the nuclear extract of Nonabokra treated with 200 mM NaCl for 24 h and 20% PEG-6000 for 24 h. The intensity of the 38 kDa protein was sharper under drought stress than in salinity stress, thereby clearly suggesting a role for this protein in drought stress response mediated by SamDC (Fig. 4A).

Another key factor affecting the growth and yield of rice is low temperature that causes rolled and withered leaves and reduced plant growth [26]. Moreover, it also causes pollen sterility thereby affecting the reproductive stage of the plant and therefore the yield [27]. The promoter regions of cold stress induced genes contain a core GCCGAC motif called Low Temperature Responsive Element (LTREs) that also forms the core of DRE sequence [28]. *In silico* analysis of SamDC promoter identified LTREs (Table 2) which is consistent with our observation where we have seen that SamDC is induced maximally after cold treatment in Nonabokra in contrast to IR-29 after 24 h. To investigate the role of LTREs in regulating the expression of SamDC we performed EMSA using 2X LTRE as

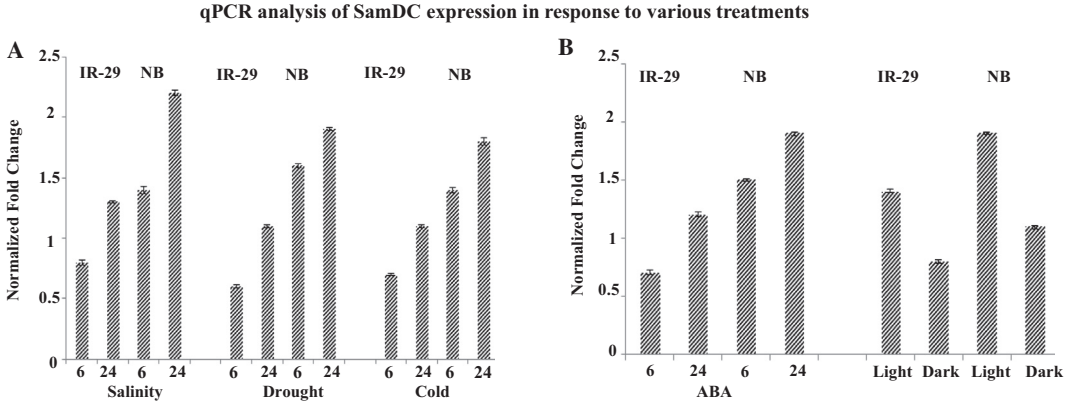

Fig. 1. Analysis of SamDC expression in the lamina of IR-29 and Nonabokra in response to (A) Salinity, Drought and Cold stress (B) ABA and Light treatments.

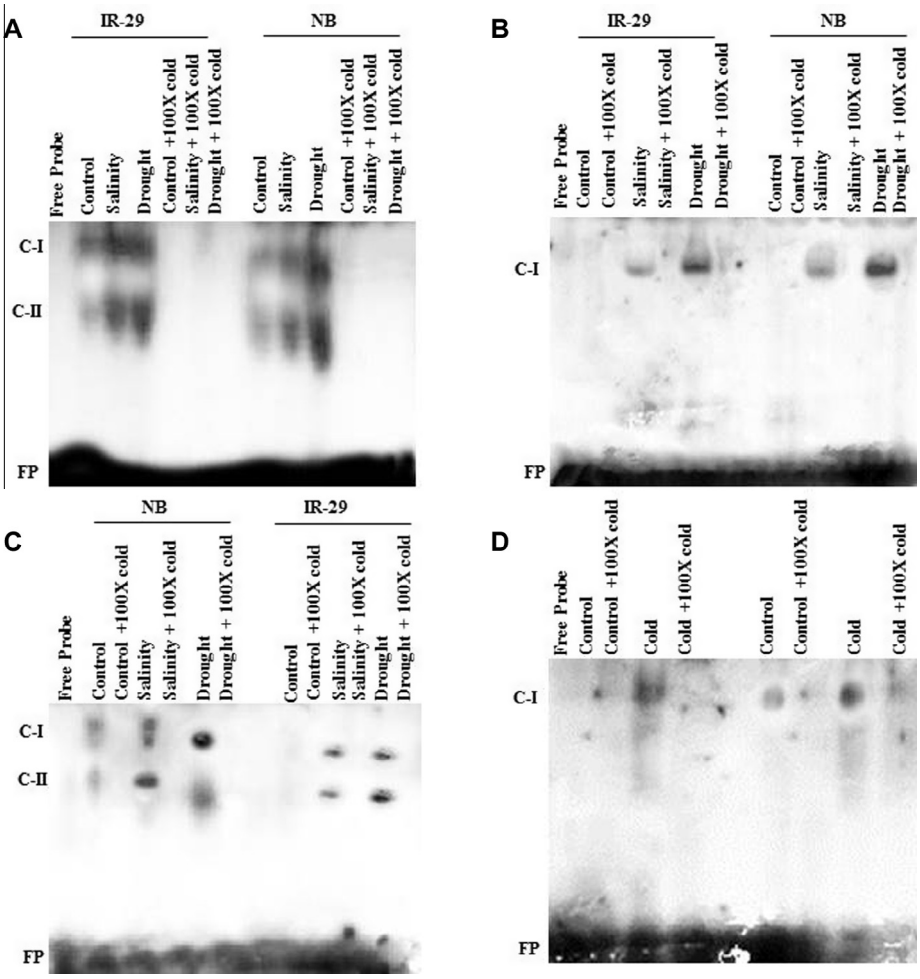

Fig. 2. EMSA and Competition assay with (A) ABREATCAL (B) W-Box (C) MYB and (D) LTRE elements present on the promoter of SamDC gene using nuclear extract prepared from 12-day old lamina of IR-29 and Nonabokra. For competition assays 100 fold molar excess of non-radioactive 2X elements were added in the nuclear extracts from rice plants before the addition of probe and incubated for 30 min at 25 °C.

probe using nuclear extract prepared from lamina of IR-29 and Nonabokra after 24 h. EMSA showed the presence of a complex in the stressed sample after 24 h of treatment in IR-29 and a low intensity complex in the control and maximally induced complex after cold treatment were visible in Nonabokra (Fig. 2D), and southwestern blot identified a ~30 kDa protein in Nonabokra (Fig. 4C).

3.6. Light mediated regulation of SamDC via GATA and SORLIP1

Previous reports in *Pharbatis nil* have shown that SamDC expression is highly induced upon illumination [29], and the photo-response is primarily regulated at the transcriptional level [30]. Analysis of promoter of *phyA* induced genes showed the presence of a well characterized element GATA box or I-box [31] that is highly conserved among *phyA* regulated promoters [32]. Another important cis element found over represented in the promoter of *phyA* regulated genes is SORLIP1 (sequences over-represented in light-induced promoters). SORLIP1 has previously been characterized as an inverted box II like motif in the Arabidopsis GAPB promoter, but later it has been proved that it does not belong to the conventional transcription system as the G-box nor does it bind to HY5 as *b-zip* domain is absent [33], thereby suggesting that it is a separate entity. *In silico* analysis of SamDC promoter revealed the presence of SORLIP1 motif. To investigate the role of GATA and SORLIP1 in regulating the expression of SamDC we performed EMSA using 2X oligos as probe using nuclear extract prepared from lamina of IR-29 and Nonabokra control, plants kept under continuous light or continuous dark for 24 h, respectively. EMSA with GATA showed the presence of two complexes in the control of Nonabokra in and the intensity of the complexes were induced after light treatment but the complexes were abolished in dark while it was absent in IR-29 in all the conditions. EMSA with SORLIP1 showed the presence of a complex in both IR-29 and Nonabokra which was absent in dark treatment in IR-29 while the intensity of this complex was reduced and highly induced by dark and light treatment in Nonabokra respectively (Fig. 3A and B). Southwestern blot analysis with GATA using nuclear extract prepared from Nonabokra identified two proteins of 25 and 16 kDa while with SORLIP1, the protein was 50 kDa. Competition assays proved that the binding of the trans-acting factor was specific to GATA and SORLIP sequences, respectively (Supplementary Fig. 1B and C).

Our study thus clearly shows that SamDC is inducible by both abiotic stresses like salinity, drought and cold stresses as well as by biotic stresses like light and ABA treatment. Moreover our research identified trans-acting factors that play a role in regulating the expression of SamDC in Nonabokra. To conclude, the findings from our research will help to develop the rice plants harboring these genes and thereby help in stress management.

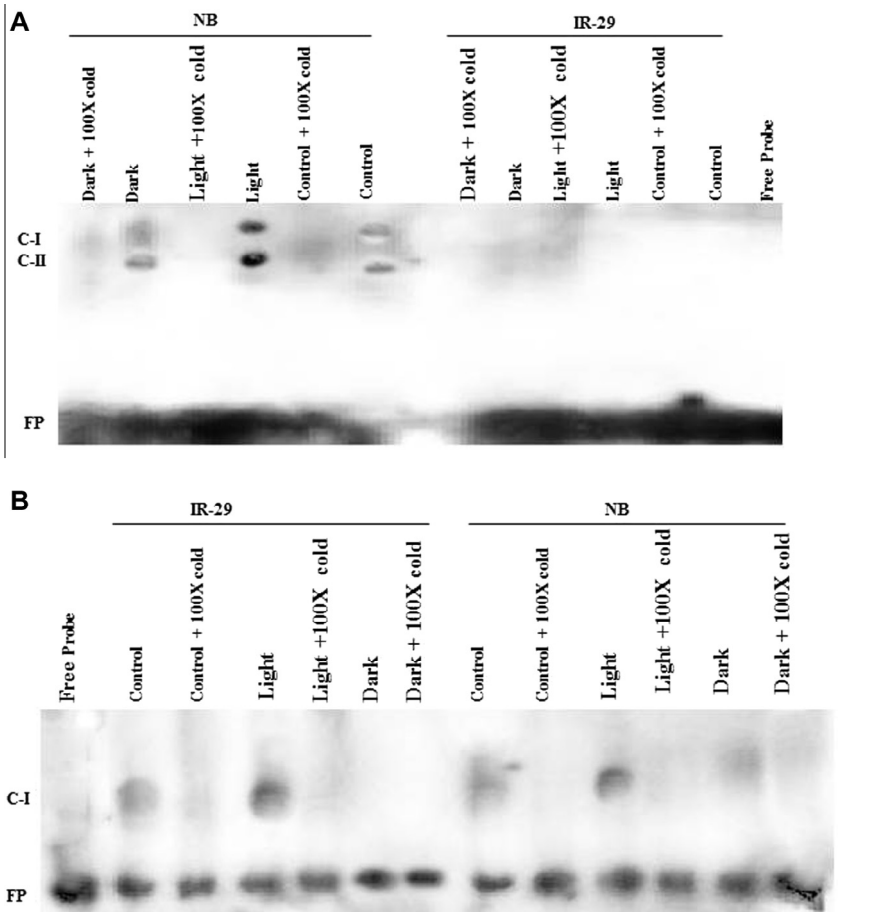

Fig. 3. EMSA and Competition assay with (A) GATA and (B) SORLIP elements present on the promoter of SamDC gene using nuclear extract prepared from 12-day old lamina of IR-29 and Nonabokra. For competition assays 100 fold molar excess of non-radioactive 2X elements were added in the nuclear extracts from rice plants before the addition of probe and incubated for 30 min at 25 °C.

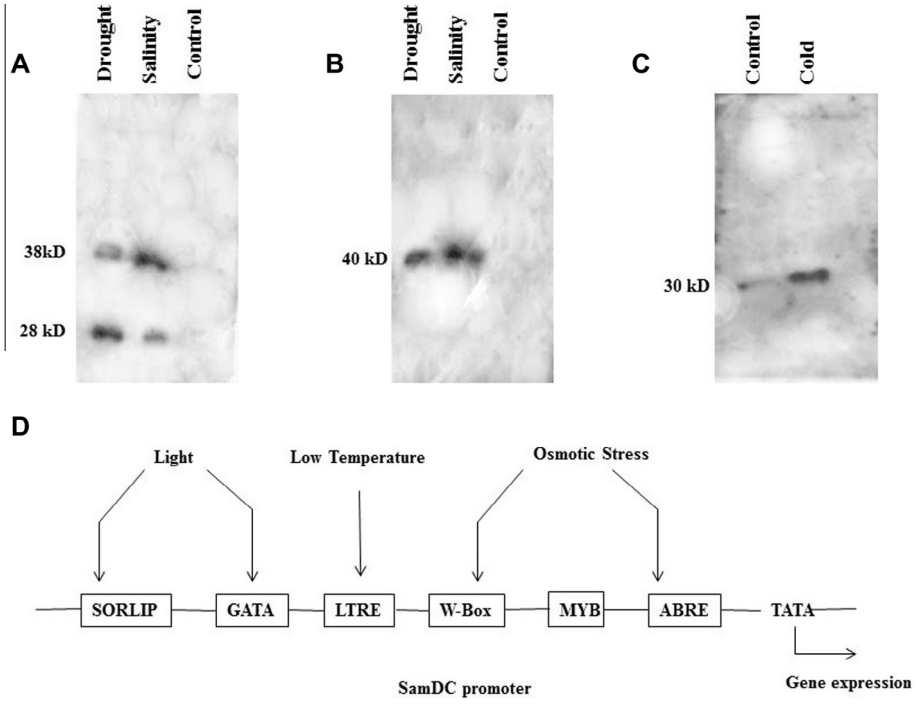

Fig. 4. Southwestern blot analysis with nuclear proteins isolated from Nonabokra using (A) ABREATCAL and (B) MYB and (C) LTRE-Box as probe. Approximately 20 μg of nuclear extract was loaded in each lane. (D) A model for the induction of SamDC gene expression in response to salinity, drought, low temperature and light treatments.

Table 2
Stress related cis-elements in the promoter region of SamDC as revealed by PLACE database.

Factor or site name[a]	Unique number[b]	Signal sequence	Number of elements
TATA and CAAT	S000110 and S000028	TATTAAT and CAAT	1 and 3
ABREATCAL	S000507	MACGYGB	1
MYBGAHV	S000181	TAACAAA	1
W-Box	S000447	TGAC	8
LTRE	S000153	CCGAC	10
GATA	S000039	GATA	8
SORLIP1	S000198	GRWAAW	6
GT1	S000482	GCCAC	1
I-Box	S000124	GATAAG	2

[a] Factors or sites according to their specific cis-acting regulatory elements.
[b] Unique number for each motif in the PLACE database.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.004.

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