Structural characterization of a novel full-length transcript promoter from *Horseradish Latent Virus* (HRLV) and its transcriptional regulation by multiple stress responsive transcription factors

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Abstract

Key message **The promoter fragment described in this study can be employed for strong transgene expression under both biotic and abiotic stress conditions.**

Abstract Plant-infecting Caulimoviruses have evolved multiple regulatory mechanisms to address various environmental stimuli during the course of evolution. One such mechanism involves the retention of discrete stress responsive *cis*-elements which are required for their survival and host-specificity. Here we describe the characterization of a novel Caulimoviral promoter isolated from *Horseradish Latent Virus* (HRLV) and its regulation by multiple stress responsive Transcription factors (TFs) namely DREB1, AREB1 and TGA1a. The activity of full length transcript (Flt-) promoter from HRLV (−677 to +283) was investigated in both transient and transgenic assays where we identified H12 (−427 to +73) as the highest expressing fragment having ~2.5-fold stronger activity than the CaMV35S promoter. The H12 promoter was highly active and near-constitutive in the vegetative and reproductive parts of both Tobacco and Arabidopsis transgenic plants. Interestingly, H12 contains a distinct cluster of *cis*-elements like dehydration-responsive element (DRE-core; GCCGAC), an ABA-responsive element (ABRE; ACGTGTC) and *as*-1 element (TGACG) which are known to be induced by cold, drought and pathogen/SA respectively. The specific binding of DREB1, AREB1 and TGA1a to DRE, ABRE and *as*-1 elements respectively were confirmed by the gel-binding assays using H12 promoter-specific probes. Detailed mutational analysis of the H12 promoter suggested that the presence of DRE-core and *as*-1 element was indispensable for its activity which was further confirmed by the transactivation assays. Our studies imply that H12 could be a valuable genetic tool for regulated transgene expression under diverse environmental conditions.

Abbreviations

ABA	Abscisic acid
DRE	Dehydration-responsive element
ABRE	ABA-responsive element
DREBs	DRE-binding proteins
AREBs	ABRE-binding proteins
JRE	Jasmonic response element
ERF	Ethylene-responsive binding factor
AP2	APETALA2
CBF	C-repeat binding factor
LTRE	Low-temperature-responsive element
CRT	C-repeat
bZIP	Basic leucine zipper
CLSM	Confocal Laser Scanning Microscope

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Introduction

Environmental stress conditions arising out of drought, heat, cold, salinity and pathogen attack can adversely affect plant growth and productivity (Akula and Ravishankar 2011). Over the time, plants have developed unique adaptations and mechanisms to counteract such stresses. One of the important mechanisms involved is the expression of specific transcription factors (TFs) that are induced during stress conditions and allow a cascade of signalling events for regulated gene expression. The TFs bind to specific sequences on promoters called 'cis'-acting elements which are the key regulators in determining the functionality of a gene and are widely distributed in nature. While some TFs are recruited upon biotic stress responsive elements such as W-box, JRE and as-1, others bind to the abiotic stress responsive sequences like DRE/CRT, ABRE, G-box, MYB, and MYC (Eulgem et al. 2000; Fujimoto et al. 2000; Nakashima et al. 2007; Park et al. 2001). It is indeed interesting to know how the cis-architecture on a promoter regulates its transcription under various stress-conditions.

In general, plant-infecting Caulimoviruses have long been known to be a rich source of strong constitutive promoters that contain an array of both biotic and abiotic stress responsive elements. Although their involvement in efficient transgene expression has already been established (Assaad and Signer 1990; Gowda et al. 1989; Hirt et al. 1990; Sun et al. 2002); they have not been well studied under stress-inducible conditions. Several efficient promoters have been studied and characterized from members of Caulimoviruses like *Cauliflower Mosaic Virus* (CaMV), *Mirabilis Mosaic Virus* (MMV), *Cassava Vein Mosaic Virus* (CsVMV), *Dahlia Mosaic Virus* (DaMV), *Figwort Mosaic Virus* (FMV), *Peanut Chlorotic Streak Virus* (PClSV), *Strawberry Vein Mosaic Virus* (SVMV) (Dey and Maiti 1999; Kroumova et al. 2013; Maiti and Shepherd 1998; Odell et al. 1981; Pattanaik et al. 2004; Sahoo and Maiti 2014; Sahoo et al. 2014, 2009, 2013). The Caulimovirus genome generally contains two transcriptional promoters, a full-length transcript (Flt) and sub-genomic transcript (Sgt); these are equivalent to the *Cauliflower Mosaic Virus* (CaMV) 35S and 19S transcripts respectively (Odell et al. 1981).

The *Horseradish Latent Virus* (HRLV), first isolated from the horseradish plant in Europe has been assigned to the family Caulimoviridae. It is a double-stranded DNA virus which was first reported by N. Paludan of the State Plant Pathology Institute, Lyngley, Denmark, in April 1973 (Richins and Shepherd 1986). The virus is favourably transmitted by green peach aphids (*Myzus persicae* Sulz.) and known to cause vein-clearing, stunting and mottling

symptoms in Danish cultivars of Horseradish; however it shows no visible symptoms on the American cultivars. The host-range of HRLV majorly includes members of the Brassicaceae family viz. *B. campestris, B. oleraceae* and *B. pekinensis* where it causes chlorotic mottle and yellow-banding on systemically infected leaves (Richins and Shepherd 1986). HRLV contains spherical virions that are nearly 50 nm in diameter and found embedded in inclusion bodies. HRLV is serologically dissimilar to CaMV although the genomic organization remains the same. In addition, significant dissimilarities in coat protein, sequence homology and site-specific discontinuities were also observed between HRLV and CaMV. Another feature that makes HRLV taxonomically distinct is its restriction endonuclease map which does not resemble any other member of the Caulimovirus group. Henceforth, these variations adequately classified HRLV as a new member of Caulimovirus (Richins and Shepherd 1986). Ever since the first report, HRLV remains completely unexplored in context of its regulation by environmental factors (particularly cold) or growth regulators that influence the infection manifested by this virus.

Low temperature is one of the major limiting factors that adversely affect crop production and plant species distribution in a given ecosystem (Thomashow 1999). This could lead to altered function of specific cold-inducible genes. The DRE is a cis-element located on the promoter sequence known to be involved in drought and low-temperature responsive gene expression in higher plants (Shinozaki and Yamaguchi-Shinozaki 2000; Thomashow 1999). It specifically recruits the DREB group of TFs where DREB1 is induced by low-temperatures while DREB2 is induced by dehydration and high salinity (Liu et al. 1998; Stockinger et al. 1997). The DREB is a member of the AP2/EREBP family of TFs and forms an important subfamily consisting of three DREB1 and six DREB2 related genes. It has been reported that DREB1D/CBF4 exhibits both salinity and drought responsiveness (Haake et al. 2002; Sakuma et al. 2002). Since drought and cold are both known to trigger ABA production, the DREB TF is said to be ABA-responsive. The DRE-motif (TACCGACAT) was first identified in the promoter region of *rd-29A* gene in *A. thaliana*, responsible for cold, drought and salinity stress induction (Shinozaki and Yamaguchi-Shinozaki 2000). Later, similar cis-elements such as LTRE and CRT (both having a G/ACC GAC core motif) having low-temperature inducibility were also identified (Baker et al. 1994; Jaglo et al. 2001). One such report is that of a CRT motif (TGGCCGAC) present in the promoter of a cold-inducible gene *cor15a* (Baker et al. 1994). DRE-related elements have also been observed to occur in the promoter regions of *rd17, kin1* and *cor6.6* genes which are cold- and drought-inducible (Iwasaki 1997; Wang et al. 1995). The *BN115* gene of oilseed rape also contains a

core-motif similar to DRE (CCGAC) which is attributed to cold-responsiveness (Jiang et al. 1996) in planta.

The AREB is another class of ABA-responsive TF that selectively binds to the ABRE core-motif (ACGTGG/TC). The AREB family of TFs was first isolated by yeast one-hybrid screening as bait proteins from the *rd29B* promoter (Choi et al. 2000; Uno et al. 2000). They belong to the bZIP group of TFs and comprise of nine homologs in *A. thaliana*. AREB1/ABF2, AREB2/ABF4 and ABF3 are all ABA- and drought- responsive where gain-of function mutants of these TFs exhibited increased drought tolerance (Fujita et al. 2005; Kang et al. 2002; Oh et al. 2005). AREB1 is reported to be regulated by ABA-dependent multi-site phosphorylation that occurs in its conserved domain regions (Furihata et al. 2006). It is hereby well established that both AREB1 and DREB1 function predominantly in drought and cold stress conditions and are interdependent during an ABA-response.

The *as*-1 element is a major player in the Salicylic acid- and auxin-mediated response that exclusively recruits the TGA group of DNA-binding proteins (Fromm et al. 1991; Jupin and Chua 1996). The TGA family of TFs belong to the NPR-1 interacting proteins which get activated during a pathogen infection and mediate defence response in plants via SA-mediated PR-induction (Niggeweg et al. 2000; Zhou et al. 2000). Of the various members of TGA family, TGA1a selectively binds to the 'TGACG' core-motif and is an extensively studied TF. The role of TGA1a in SA- and pathogen-responses in plants has been previously documented in the context of Caulimoviral promoters namely: DaMV and CaMV (Banerjee et al. 2015; Jupin and Chua 1996). The role of TGA1a in enhancing the activity of FMV and DaMV promoters has been demonstrated where both FSgt and DaMVSgt allowed the binding of TGA1a for their positive regulation (Banerjee et al. 2015; Kumar et al. 2012).

In this study, we examined the overlapping regulatory functions of multiple TFs using a novel Caulimoviral promoter isolated from HRLV. We performed in-depth characterization and studied the transcriptional regulation of this promoter under various stress conditions. First, we mapped the TSS of HRLV-Flt using primer extension assay and annotated the position of TATA box (-22 from TSS). We identified the strongest expressing fragment using the promoter leader-deletion approach and found H12 (-427 to $+73$) to have ~2.5 to 3.0-fold stronger activity than the CaMV35S promoter. The promoter activity was evaluated in both transient (protoplast and Agro-infiltration assays) and transgenic (tobacco and Arabidopsis) assays using the *β-glucuronidase* (*GUS*) and *green fluorescent protein (GFP)* reporter genes. The histochemical staining and real time PCR analyses validated the above findings and supported the near-constitutive nature of the H12 promoter. Upon a close inspection of H12, we identified a unique sequence cluster residing both biotic and abiotic stress responsive *cis*-elements. To examine the stress inducibility of the HRLV promoter we provided cold, ABA and SA treatments and recorded the respective alterations in promoter activities. The consolidated effect of the *cis*-elements was determined using the transactivation assays where DREB1, AREB1 and TGA1a were administered in concert with H12 promoter in planta. Gel-binding assays with wild type (WT) and mutant probes confirmed the formation of DNA–protein complexes in vitro. Next, the functional relevance of each *cis*-element was dissected by performing a sequential mutation analysis under various stress conditions. Overall, our studies reveal that the contiguous organization of *cis*-elements on HRLV promoter regulates its activity in a co-ordinated fashion. The Flt- promoter of HRLV (H12) having strongest transcriptional activity and heterogeneous sequence may become a proficient tool for plant genetic engineering and find immensely useful applications for promoting gene expression in plants under multiple stresses.
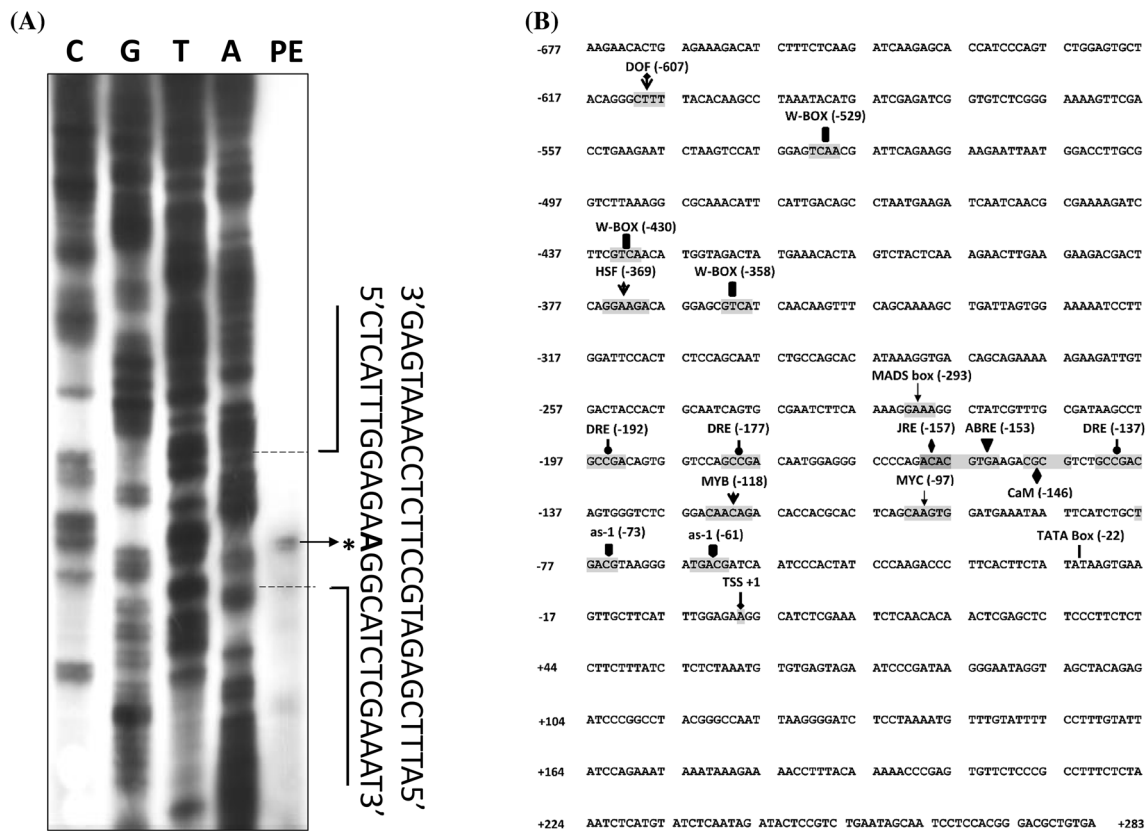
Results

Analysis of HRLV-Flt sequence reveals a 'clustered' organization of both biotic and abiotic stress-responsive *cis*-elements

The HRLV is a member of the Caulimovirus group and has a circular double-stranded DNA of 7954-bp (NCBI Reference Sequence: NC_018858.1; 6641–7590 bp). The Transcription start site (TSS) of HRLV-Flt was determined using the primer extension assay and mapped as an adenine (A) residue present 22 bp downstream of the TATA-Box (Fig. 1a). We subjected the HRLV-Flt (960 bp; -677 to $+283$) promoter sequence to a regulatory database 'PLANTCARE' (Lescot et al. 2002) to extract the putative *cis*-elements present in its 960 bp region. A number of stress-responsive *cis*-elements like *as*-1, MYC, MYB, DRE-core, ABRE, W-box, HSF, and DOF etc. were present on the HRLV-Flt promoter, notable of which were the cold- and drought- responsive DRE-core and ABRE along with an SA-responsive *as*-1 element. A total of three copies of DRE-core (DRE_1; -192, DRE_2; -177, DRE_3; -137), one copy of ABRE (-153) and two of *as*-1 (as-1_1; -73, as-1_2; -61) were closely positioned in a total stretch of 136 bp on HRLV-Flt as depicted in Fig. 1b.

Molecular characterization of HRLV-Flt promoter affirms H12 as the strongest promoter fragment in transient assays

A total of twenty promoter constructs that consisted of 7 (seven) 3′-end deleted (H1–H7) and 13 (thirteen) 5′-end deleted (H8–H20) fragments were generated for

(A)

C G T A PE

3'GAGTAAACCTCTTCCGTAGAGAGCTTTA5'
5'CTCATTTGGAGAGAGGCATCTCGAAAT3'

* (TSS)

(B)

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-677  AAGAACACTG  AGAAAGACAT  CTTTCTCAAG  ATCAAGAGCA  CCATCCCAGT  CTGGAGTGCT
              DOF (-607)
-617  ACAGGGCTTT  TACACAAGCC  TAAATACATG  ATCGAGATCG  GTGTCTCGGG  AAAAGTTCGA
                            W-BOX (-529)
-557  CCTGAAGAAT  CTAAGTCCAT  GGAGTCAACG  ATTCAGAAGG  AAGAATTAAT  GGACCTTGCG
-497  GTCTTAAAGG  CGCAAACATT  CATTGACAGC  CTAATGAAGA  TCAATCAACG  CGAAAAGATC
      W-BOX (-430)
-437  TTCGTCAACA  TGGTAGACTA  TGAAACACTA  GTCTACTCAA  AGAACTTGAA  GAAGACGACT
      HSF (-369)  W-BOX (-358)
-377  CAGGAAGACA  GGAGCGTCAT  CAACAAGTTT  CAGCAAAAGC  TGATTAGTGG  AAAAATCCTT
-317  GGATTCCACT  CTCCAGCAAT  CTGCCAGCAC  ATAAAGGTGA  CAGCAGAAAA  AGAAGATTGT
                                         MADS box (-293)
-257  GACTACCACT  GCAATCAGTG  CGAATCTTCA  AAAGGAAAGG  CTATCGTTTG  CGATAAGCCT
      DRE (-192)  DRE (-177)             JRE (-157)  ABRE (-153) DRE (-137)
-197  GCCGACAGTG  GTCCAGCCGA  CAATGGAGGG  CCCCAGACAC  GTGAAGACGC  GTCTGCCGAC
                  MYB (-118)              MYC (-97)   CaM (-146)
-137  AGTGGGTCTC  GGACAACAGA  CACCACGCAC  TCAGCAAGTG  GATGAAATAA  TTCATCTGCT
      as-1 (-73)  as-1 (-61)                                     TATA Box (-22)
-77   GACGTAAGGG  ATGACGATCA  ATCCCACTAT  CCCAAGACCC  TTCACTTCTA  TATAAGTGAA
                  TSS +1
-17   GTTGCTTCAT  TTGGAGAAGG  CATCTCGAAA  TCTCAACACA  ACTCGAGCTC  TCCCTTCTCT
+44   CTTCTTTATC  TCTCTAAATG  TGTGAGTAGA  ATCCCGATAA  GGGAATAGGT  AGCTACAGAG
+104  ATCCCGGCCT  ACGGGCCAAT  TAAGGGGATC  TCCTAAAATG  TTTGTATTTT  CCTTTGTATT
+164  ATCCAGAAAT  AAATAAAGAA  AACCTTTACA  AAAACCCGAG  TGTTCTCCCG  CCTTTCTCTA
+224  AATCTCATGT  ATCTCAATAG  ATACTCCGTC  TGAATAGCAA  TCCTCCACGG  GACGCTGTGA  +283
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Fig. 1 TSS determination and distribution of putative stress responsive *cis*-elements on HRLV-Flt (−677 to +283) promoter sequence. **a** The TSS of HRLV promoter was mapped using the primer extension assay. The lanes C, G, T and A represent Sanger sequencing reactions. The TSS was positioned at +1 as an adenine residue indicated by a star. **b** A 960 bp fragment of HRLV-Flt promoter showing the relative arrangements of major stress-inducible *cis*-elements. The TATA-box is located at −22 from TSS

leader-deletion analysis. We evaluated the transient activities of each of the twenty constructs coupled to the *GUS* (*uidA*) reporter gene (pUCPMAGUS-H1 to pUCPMAGUS-H20) in tobacco protoplasts (Xanthi Brad) (Fig. 2a). Out of the twenty deletion fragments, the twelfth fragment 'H12' (500 bp; -427 to +73) was found to be the strongest having ~3.0-times greater activity than the CaMV35S promoter. This was followed by H17 (250 bp; −177 to +73) which was ~2.4-times stronger. The fragment H7 (640 bp; −677 to −55) showed negligible GUS activity since it was devoid of the TATA-box (Fig. 2b). Next, the GUS activities of all twenty fragments (pKYLXGUS-H1 to pKYLXGUS-H20) cloned in the plant expression vector pKYLX71 were measured post Agro-infiltration in *Nicotiana tabacum* leaves. A total of three samples were analysed for each construct (in both protoplast and agro-infiltration assays) and the average values were represented with respective SD in Fig. 2b, c. Next, we made a comparative analysis of the activities of all these constructs with the activity obtained from the CaMV35S promoter; subsequently we observed that the H12 and H17 promoter constructs showed ~2.5- and ~1.5-times stronger activity than the CaMV35S promoter respectively.

To check the stable GUS activities of the promoter fragments we employed the transgenic approach, wherein we generated approximately eight to ten independent transgenic tobacco lines for each of the twenty constructs (pKYLX-GUS-H1 to pKYLXGUS-H20). The lines showing single insertion of GUS with appropriate segregation ration (Kan^R:Kan^S::3:1) were selected for further analyses. The GUS activities were measured for three-week old transgenic tobacco seedlings (T$_2$ generation) carrying each of the twenty constructs along with that of CaMV35S promoter and the results were presented in Fig. 2d. We observed that the GUS activity profile was quite similar to that of the transient assays where H12 was the strongest promoter followed by H17.

To further validate the proficiency of the H12 promoter, we went on to perform GFP expression assays in different plant systems. The *GFP* gene was cloned downstream of H5, H12 and H17 and CaMV35S promoters and agro-infiltrated in *N. tabacum* and *Petunia hybrida* leaves. The comparative intensities of H12-GFP and H17-GFP were distinctly observed in both tobacco and petunia leaves. Also, there was strong GFP expression observed in onion epidermal cells
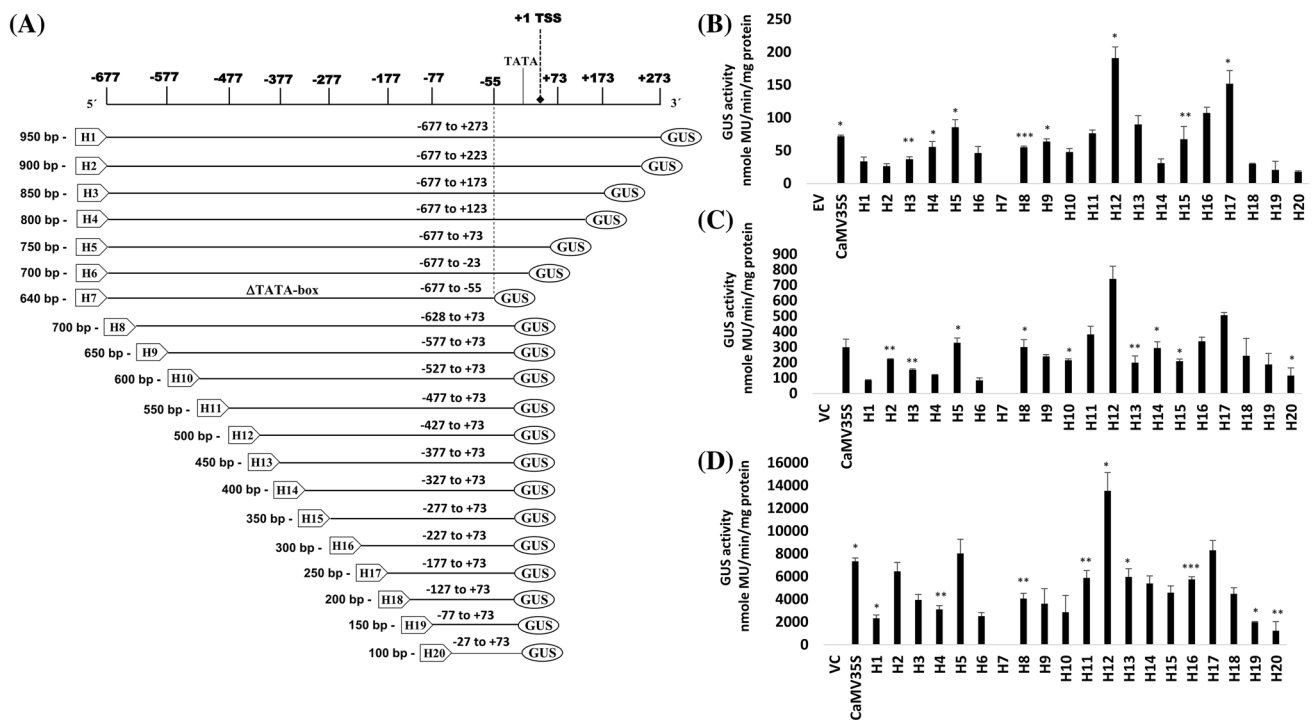
Fig. 2 Transient and transgenic assays of HRLV promoter deletion fragments. **a** A schematic representation of HRLV-Flt promoter leader-deletion constructs (with their respective co-ordinates) fused to the *GUS* reporter gene. The position of TATA box, TSS (+1), and the HRLV-Flt promoter co-ordinates are presented. A total of twenty deletion constructs (H1–H20) were generated of which seven (H1–H7) were 3′-end deleted while thirteen (H8–H20) were 5′-end deleted fragments. The size of each fragment with its respective co-ordinates is also indicated. Transient GUS activities of fragments H1–H20 along with the CaMV35S promoter in **b** tobacco protoplast, **c** Agro-infiltration (in *N. tabacum* leaves) assays represented with their respective Standard deviations (SDs). **d** Stable GUS activities of above fragments in transgenic tobacco plants (T_2 generation progenies) presented along with CaMV35S promoter. The empty vector (EV) and vector control (VC) were used as internal controls. Asterisks indicate level of significance for the data in **b**, **c** and **d** where $p < 0.05$

bombarded with H12-GFP construct (Fig. 3c). The same pattern was followed in Arabidopsis protoplasts electroporated with H12-GFP and H17-GFP where GFP expression was strongly triggered in the cells as shown in Fig. 3d.

The H12 promoter is highly active in both transgenic tobacco and Arabidopsis plants

We performed the histochemical GUS staining to study the expression pattern of HRLV promoter constructs. The leaf petioles and healthy root tips taken from transgenic tobacco plants expressing CaMV35S, H5, H12, H16 and H17 promoter fragments along with a Vector control (VC) line were stained with X-gluc solution (0.3% w/v). A strong blue coloration was observed in H12 and H17 which was preceded by CaMV35S and absolutely no coloration was observed in VC line (Fig. 4a, b). Different floral parts including anther, ovary and pollen grains of H12 transgenic plants were also stained to obtain similar constitutive expression pattern (Fig. 4c). X-gluc staining of 21-days old transgenic tobacco seedlings (T_2 generation)

also showcased maximum GUS accumulation in case of the H12 promoter (Fig. 4d). The q-PCR analysis further confirmed that H12 was ~2.5-fold stronger than the CaMV35S promoter (taken as the baseline = 1.0) followed by H17 and H16 promoters respectively (Fig. 4e).

Since *A. thaliana* is a well-established model for plant genetics, we also raised independent transgenic Arabidopsis lines for the two strongly expressing promoter fragments viz. H12 and H17 along with the CaMV35S promoter and tested for their GUS accumulation. The results of GUS assay are illustrated in Fig. 5a, b which clearly show that H12 activity is ~3.0–3.2 fold greater than the CaMV35S promoter. Analysis of histochemical GUS staining of transgenic *A. thaliana* seedlings (T_2 generation, four weeks old) follows the similar expression profile as tobacco transgenics where the expression of H12 was strongest and constitutive (Fig. 5c). Similar to our previous histochemical studies, we stained different parts of the *A. thaliana* transgenic plants including flower, leaf and roots with X-gluc and observed intense blue colorations (Fig. 5d, e, f). Also, both H12 and H17 promoters
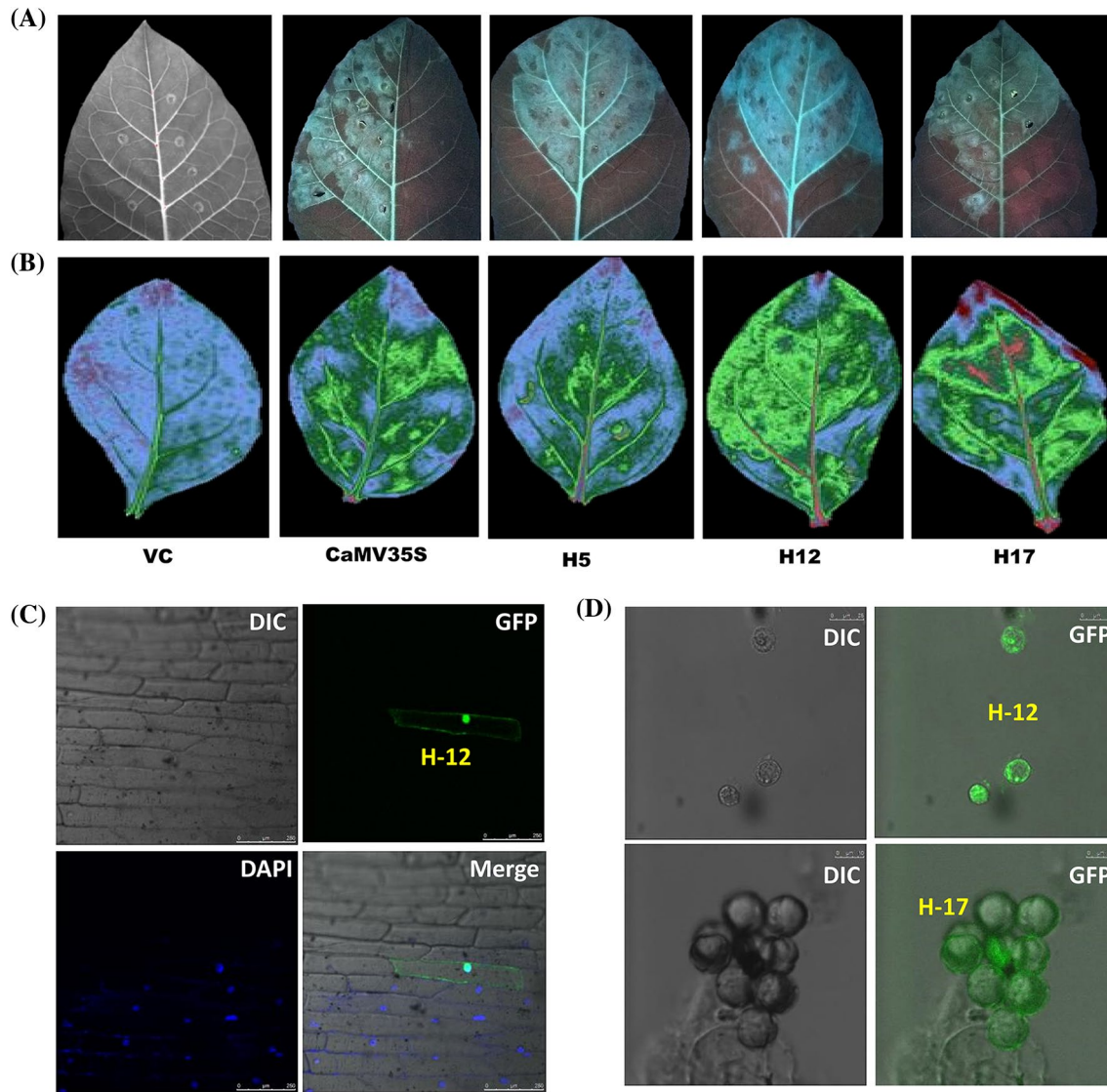
Fig. 3 Transient GFP expression analysis of HRLV promoter in various plant systems. GFP expression of H5, H12, H17, CaMV35S promoters in **a** *N. tabacum* Samsun NN leaves, **b** *Petunia hybrida* leaves following Agro-infiltration assays. **c** CLSM images of GFP expressed under H12 promoter visualized in onion cells after 24 h of bombardment with H12-GFP and **d** CLSM images of Arabidopsis protoplasts electroporated with H12-GFP and H17-GFP constructs

were highly active in most floral parts such as sepal, petal, anther and stigma (Fig. 5g).

The H12 promoter is induced independently by SA, ABA and cold stress conditions

Various *cis*-elements account for stress inducibility of promoters which in turn is followed by a cascade of events primarily involving enhanced gene expression. The *as*-1, DRE-core and ABRE motifs are known to account for SA, cold and ABA responses respectively (Kumar et al. 2012; Shinozaki and Yamaguchi-Shinozaki 2000). The presence of these DNA-binding sites on the H12 promoter prompted

us to investigate its stress inducibility under independent experimental stress conditions. Transgenic *A. thaliana* plants harbouring H12-GUS and H17-GUS constructs were subject to 150 μM each of SA and ABA and 16 h cold stress at 4 °C in three independent experiments following which their respective GUS activities and relative *uidA* transcript levels were measured.

The results presented in Fig. 6a, b showed that the H12 promoter responded exceedingly well to SA-treatment where a ~ 2.4-fold greater *GUS* transcript was recorded as compared to untreated control plants. However, the response was not much notable under ABA stress where only ~ 1.4-fold increased GUS activity was observed (Fig. 6c, d). Upon cold

Fig. 4 Histochemical GUS expression analyses of transgenic tobacco plants harbouring the HRLV promoter fragments H5, H12, H16 and H17. **a** Images of X-gluc stained leaf petiole, **b** Root tip, **c** reproductive parts viz. anther, ovary, ovary base and pollen grains. **d** Images showing GUS expression in transgenic tobacco seedlings (21 days old, T_2 generation). **e** RT-PCR analysis showing the relative abundance of *uidA* transcript (GUS mRNA level) in whole seedlings where the value of CaMV35S promoter as taken as the baseline (value one)
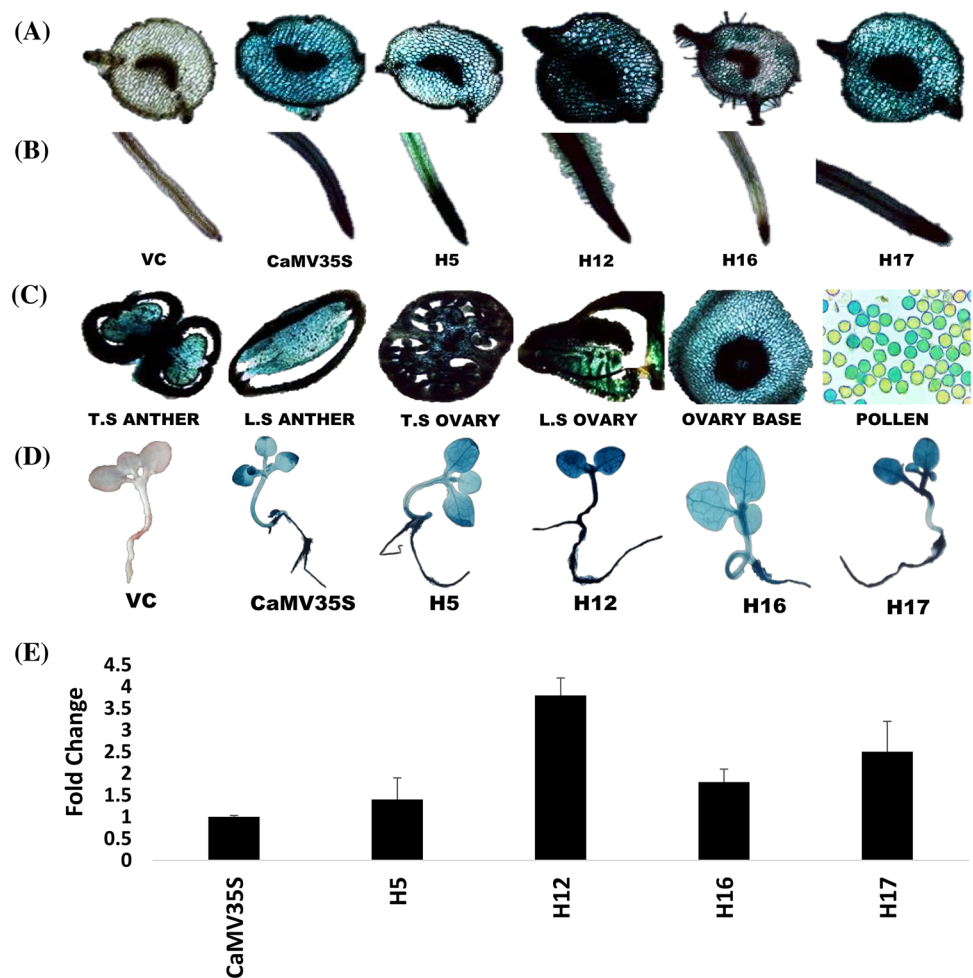
stress, the *GUS* expression under H12 promoter increased by ~2.2-fold (Fig. 6e, f). The above results indicate that H12 is multiple stress-responsive which might be due to the presence of multiple stress-specific *cis*-elements on its sequence.

The H12 promoter recruits TGA1a, AREB1 and DREB1 proteins

The structural analyses of HRLV promoter revealed the presence of several *cis*-elements on its sequence. Based on our previous experiments, we observed that the strongest promoter fragment H12 contains a 136 bp stretch which encompasses the binding sites for TGA1a, AREB1 and DREB1. To experimentally confirm the binding of these TFs, we conducted the electrophoretic mobility shift assays (EMSA) using bacteria-derived recombinant proteins and $\gamma^{32}P$ probes (H12 promoter region specific). Figure 7a represents the scheme of *cis*-element distribution on the H12 promoter. Probe I consisted of two DRE-core elements (GCCGAC) at −192 and −177 while Probe II contained one motif each of ABRE (ACGTGTC) and DRE-core at −153 and −137 respectively. Probe III accommodated two *as*-1 elements

(TGACG) that were positioned at −73 and −61 placed in close proximity.

Gel retardation assays showed strong binding affinity of TGA1a towards the probe which was specific for *as*-1 element, evident from mutational EMSA. Oligonucleotides (Probe III) containing mutations in as-1_1 and as-1_2 showed compromised binding to TGA1a while no binding was observed in case of Δas-1_{1-2} mutant probe (Fig. 7b). For re-affirmation, we performed the competitive EMSA using 10×, 20×, 40× and 100× excess of WT cold probe (Fig. 7c) where binding affinity decreased gradually from 10× to 100×. Recombinant AREB1 protein could bind to WT probe II that was visible as distinct band-shifts in native PAGE gel (Fig. 7d).

Furthermore, recombinant DREB1 displayed strong binding affinity to Probe I (WT1) containing two DRE-core motifs. To check the specificity of this binding, we sequentially mutated each of the DRE-core elements and examined for band-shifts in mutant probes (ΔDRE_1, ΔDRE_2 and ΔDRE_{1-2}). The band-shifts observed in case of both mutant probes (ΔDRE_1 and ΔDRE_2) were slightly greater than that observed for WT1 probe. This was probably because
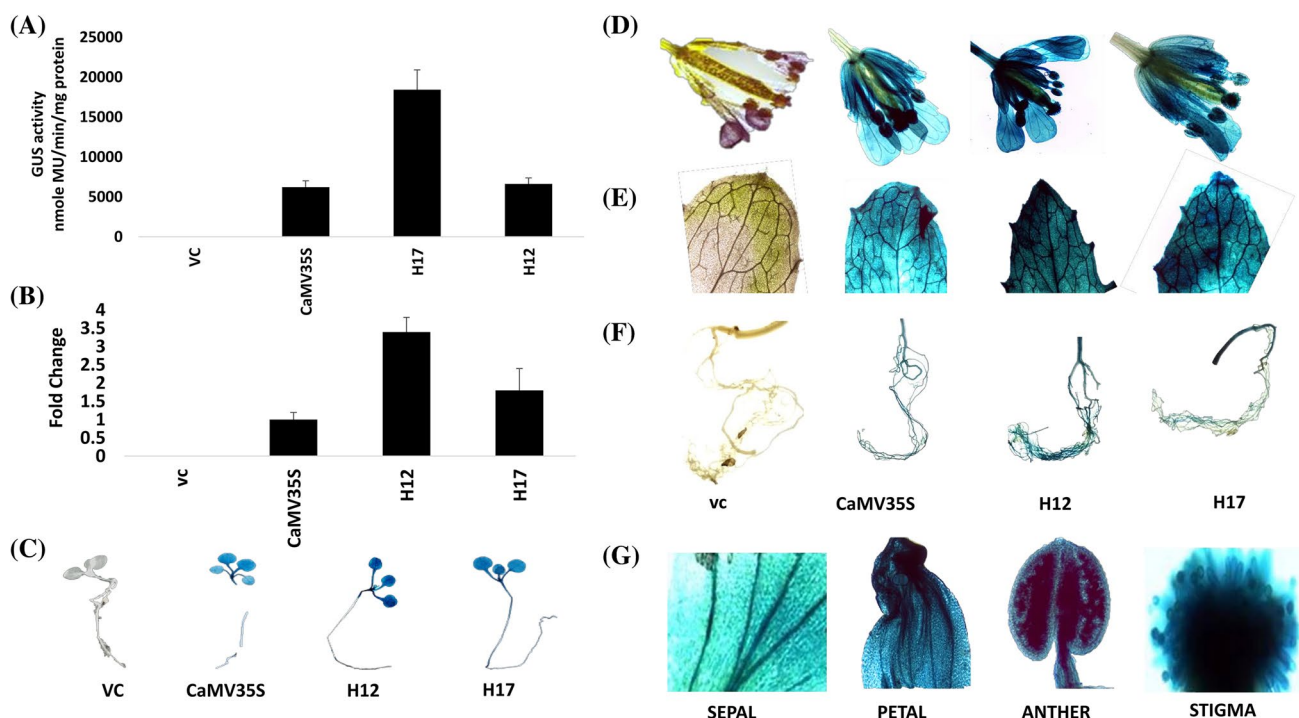
Fig. 5 GUS expression analysis in transgenic *Arabidopsis* plants. **a** Stable GUS activities of H12 and H17 promoter in 28 days old *A. thaliana* T$_2$ generation whole seedlings. **b** Fold change of *uidA* transcript levels of transgenic seedlings under the control of H12, H17 and CaMV35S promoter (where CaMV35S promoter was normalized to 1 unit). **c** Images showing histochemical GUS staining of trans-genic Arabidopsis whole seedlings. **d** Expression of the *GUS* reporter gene in flower, **e** Leaf, **f** Root under the control of H12 and H17 promoters, **g** X-gluc stained images of different floral parts viz. sepal, petal, anther and stigma of H12 transgenic plants showing strong promoter activity. VC represents transformed vector control (pKYLX71) lines in each panel which do not exhibit any colour development

DREB1 was able to bind to either of the two DRE-core binding sites present on Probe (I) The binding affinity completely declined when both DRE-core motifs were mutated using base-substitutions (ΔDRE_{1-2}). Simultaneously, we tested for another DREB1 binding site on probe II (W2) where a strong band shift was obtained; while base-substitutions in the third DREB1 binding site (ΔDRE_3) rendered the protein incompetent for binding (Fig. 7e). The binding of DREB1 was re-confirmed in two separate competition EMSAs using excess of cold probes I and (II) The binding affinity of DREB1 gradually declined from 40× to 100× as shown in Fig. 7f, g. Overall the above results suggest that TGA1a, AREB1 and DREB1 proteins bind to specific sequences on H12 promoter.

The H12 promoter is transactivated by TGA1a and involves coordinated activity of DREB1 with AREB1

As suggested by the gel-binding assays, the TFs TGA1a, DREB1 and AREB1 are recruited on the H12 promoter. To determine whether the binding of these TFs is capable of transactivating the H12 promoter-*GUS* fusion gene in plant cells, we performed the transactivation experiments.

Arabidopsis protoplasts were co-transfected with pUCPMA-H12-GUS (reporter construct) and pUCPMA-DREB1/AREB1/TGA1a effector constructs (Fig. 8a).

Since TGA1a poses a SA-mediated response while DREB1 and AREB1 function in cold/ABA/drought response, we used two separate set of experiments to check the individual as well as consolidated effect of these TFs. First the H12 promoter fused with *GUS* reporter gene was co-electroporated with effectors AREB1 and DREB1 one at a time and then in concert. We observed ~1.3- and ~1.9-fold increase in the H12 promoter activity when AREB1 and DREB1 were administered separately. The activity however shooted up as a result of their combinatorial administration where ~2.5-fold enhanced *GUS* transcript was recorded when compared to the mock (only H12) (Fig. 8b, c). In the next set of experiments, the H12-*GUS* reporter construct was co-administered with TGA1a effector where ~2.3 fold increased activity was observed (Fig. 8d, e). These results do justify the transactivation phenomenon exhibited by all three TFs that bind to the H12 promoter, each being capable of positively regulating the promoter activity by enhancing transcription of *GUS* reporter gene. These results also support our previous findings where H12 was found to be induced by cold, ABA as well as SA treatments. The above
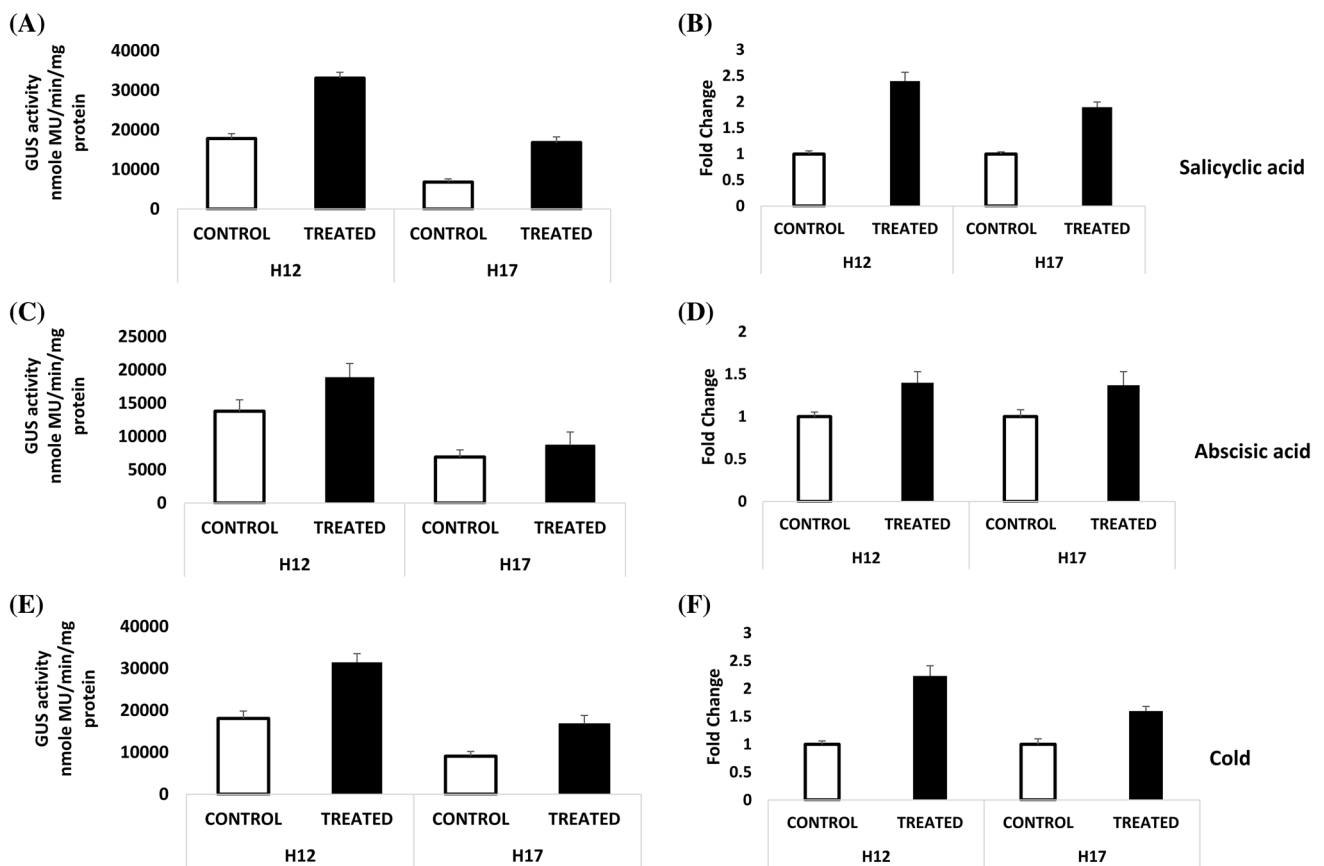
Fig. 6 Multiple Stress-induction assays. GUS activities and corresponding fold changes of *uidA* transcript recorded in *A. thaliana* transgenic seedlings expressing H12 and H17 promoter fragments under **a**, **b** 150 µM exogenous SA administration, **c**, **d** 150 µM exogenous ABA administration and **e**, **f** 16 h cold treatment at 4 °C. Values in **a**, **c** and **e** represent GUS activities of control (untreated) and treated seedlings while those in **b**, **d** and **f** represent relative *uidA* transcript levels of treated seedlings normalized to the untreated controls

experiments authenticate the cumulative effect of AREB1 and DREB1 which function as transcriptional activators involved in cold- and dehydration- responsiveness.

The disposition of *as*-1, DRE-core and ABRE motifs play regulatory roles towards H12 promoter activity

We examined the effect of cold- and SA- treatments on the expression of the H12-*GUS* fusion gene in *Arabidopsis* plants using base-substitution in specific *cis*-elements. The distribution of discrete *cis*-elements on the H12 promoter fragment is depicted in Fig. 9a. Our earlier experiments have shown that H12 is strongly induced by both cold and SA. To further investigate the cumulative regulatory effect of these *cis*-elements, we generated a series of mutants (M1–M10) carrying base-substitutions in each of *as*-1, DRE-core and ABRE motifs employing site-directed mutagenesis and tested their GUS activities. The relative GUS accumulation levels of the cold and SA treated samples were normalized to their respective mocks (untreated samples); after

normalization the values obtained for H12 (WT) was taken as 100 units. Accordingly, we evaluated the normalized GUS activities of mutant constructs (M1–M10) and presented in Fig. 9b, c. The promoter fragments carrying individual mutations in DRE_1 (M1), DRE_2 (M2) and DRE_3 (M4) exhibited little reduction in GUS activity while that for ABRE (M3) was nearly insignificant. Interestingly, we observed that mutating the patch containing ABRE and DRE_3 (M6) was more deleterious for H12 promoter activity compared to mutating both DRE_1 and DRE_2 (M5). Further, the construct comprising of multiple mutations (M7 with mutated $DRE_1 + DRE_2 + ABRE + DRE_3$ motifs) in its sequence displayed the least GUS accumulation levels (51.0% considering the activity of WT H12 promoter as 100 units; Fig. 9b).

Similarly, the fragments carrying mutations in *as*-1 elements were also examined its GUS activity under SA treatment. Here, we observed a significant decline in promoter activity when $as\text{-}1_1$ and $as\text{-}1_2$ were individually mutated (M8 and M9). However, the activity sharply dropped when both $as\text{-}1_1$ and $as\text{-}1_2$ were simultaneously mutated
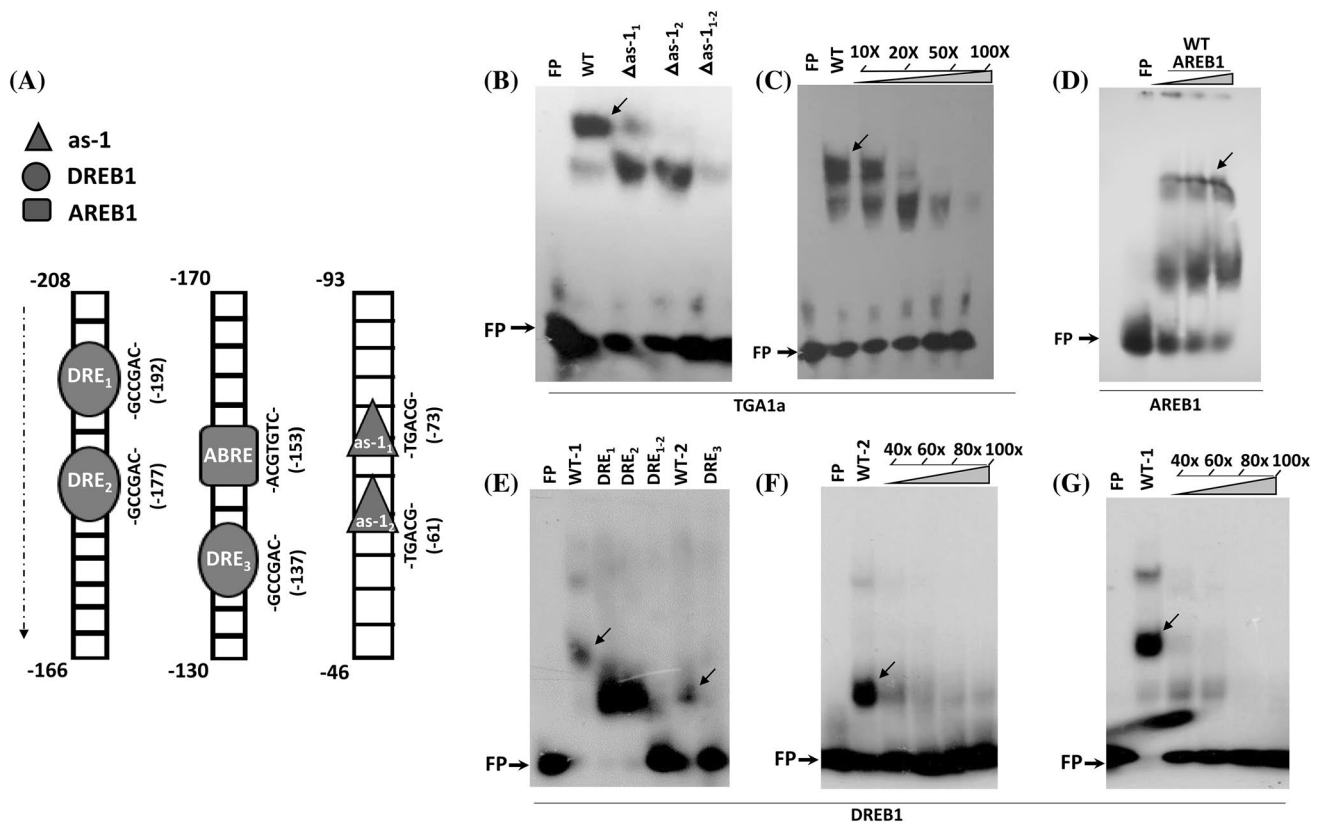
Fig. 7 Gel-Binding assays (EMSA) to depict DNA–protein interactions using γ^{32}P labelled probes. **a** Representation of H12 promoter regions used as Probes I (DRE_1 and DRE_2), II (ABRE and DRE_3) and III (as-1_1 and as-1_2) in this study. Free probe (FP) indicates no added protein. **b** Gel-retardation assays with recombinant TGA1a using WT (Probe III) and mutant-probes (Δas-1_1, Δas-1_2 and Δas-1_{1-2}), **c** Competitive EMSA with TGA1a, **d** Interaction of recombinant AREB1 with Probe II with increasing protein concentrations indicated by the shift of DNA–protein complex in native PAGE. **e** EMSA showing the binding of recombinant DREB1 protein to probe I (WT1) and probe II (WT2) along with mutant-probes (ΔDRE_1, ΔDRE_2, ΔDRE_3 and ΔDRE_{1-2}). **f, g** Competitive EMSA reactions carried out with DREB1 using 40× to 100× molar excess of cold probes I and probe II respectively. Arrows indicate band-shifts created by DNA–protein complexes

(M10) (Fig. 9c). Altogether, the extensive mutation analyses emphasize the importance of as-1 element and also the possible synergy between DRE and ABRE motifs for cold-dependent *GUS* expression under the control of H12 promoter.

Discussion

External environmental conditions have significant impact on all phases of plant growth and development. Extremes in climatic conditions such as cold, heat, disease development, drought etc. pose lethal effects on plant survival, particularly crop productivity (Mittler 2006; Ramegowda and Senthil-Kumar 2015). However, every plant species has its own unique mechanism to cope with such unfavourable conditions imposed on them. This includes the up-regulation of expression of biotic or abiotic stress-responsive gene/s. Here, TFs act as powerful switches for

inducing a wide array of such stress-responsive genes. In several instances, it is suggested that both biotic and abiotic stresses operate together and cannot be directly dealt with a single class of TFs (Atkinson et al. 2013; Jedmowski et al. 2015; Prasch and Sonnewald 2013). Most likely, the incidence of combined stress will drastically increase in the near future and exert significant effects on plant growth and development. The regulatory paths associated with simultaneous exposure of plants to abiotic and biotic stresses have not been explored properly as yet. Recently, major focus has shifted to a 'combined stress-tolerance' based research which aims at developing transgenic plants with built-in multiple stress resistance such that they can thrive well under different environmental insults (Nakashima et al. 2009). For this we require strong and/or inducible plant genetic regulators (promoters) containing specific sets of *cis*-element that can assist in effective gene-expression and build favorable regulatory systems suited to multiple stress conditions.
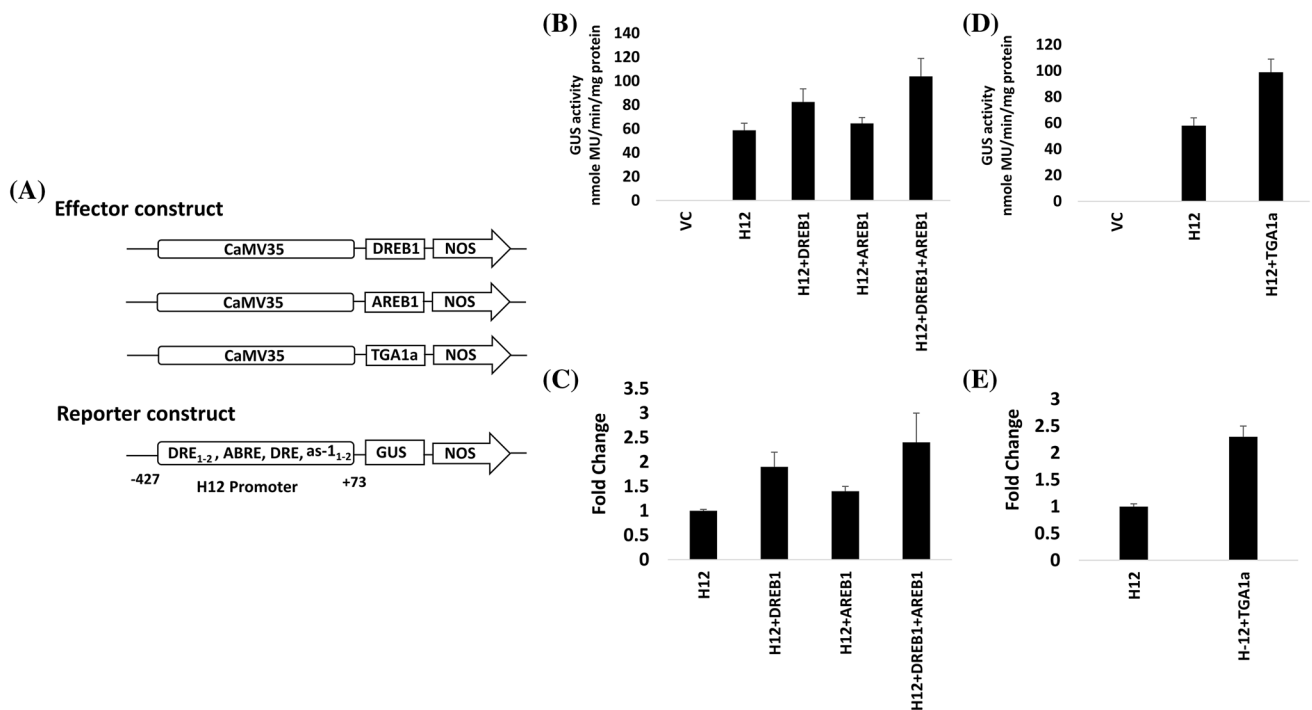
Fig. 8 Transcriptional transactivation assays in tobacco protoplasts. **a** Schematic representation of effector and reporter constructs. A total of three effectors viz. DREB1, AREB1 and TGA1a (each driven by the CaMV35S promoter) and a *GUS* reporter under the control of H12 promoter were constructed. **b**, **c** GUS activities and *uidA* transcript levels measured post-co-electroporation where H12-*GUS* reporter construct was co-administered with DREB1 and AREB1 effectors. **d**, **e** GUS activities and *uidA* transcript levels of H12-*GUS* reporter construct co-administered with TGA1a effector construct. The *uidA* transcript levels in **d** and **e** are normalized to that obtained from H12-*GUS* reporter taken as 1.0 unit

The *cis*-elements are discrete DNA sequences involved in transcriptional regulation; can be both biotic- and abiotic- stress responsive through the cognate binding of stress specific TFs (Fujita et al. 2006; Singh et al. 2002). The recruitment of TFs to *cis*-elements maintains the overall transcriptional pathways and controlled gene-expression in plant cells. The molecular mechanisms responsible for the continuity of such *cis*-regulatory pathways have emerged substantially during the course of evolution not only in higher plants but even in organisms such as Caulimoviruses that have co-evolved with their hosts. The Caulimoviridae is a group of pararetroviruses that cause serious pathological conditions in plants notably foliar-mosaic and banding diseases (Abel et al. 1986; Daubert et al. 1984; Petrzik et al. 1998; Singh et al. 2002). Interestingly, these are host-specific and infect a fairly limited species of plants. The viruses are extremely adaptable and capable of bringing rapid changes in their genome which prepares them to adapt in several environmental niches (Domingo and Holland 1997; Roossinck 1997). Such adaptability in a cell (prokaryotic or eukaryotic) under different environmental changes may be due to altered gene expression governed by interactions between stress-specific *cis*-elements with respective cognate TFs.

It has been well-established that Caulimoviral promoters showed strong constitutive gene-expression patterns in plants (Hull 1978; Speirs et al. 1998) which may be accredited to the positional and combinatorial function of *cis*-elements present on them. The mechanism behind the modulation of gene-expression leading to such rigorous transcription is not well understood and requires in-depth study of transcriptional machinery that includes DNA–protein interactions along with a detailed evaluation of *cis*-elements clustering on the Caulimoviral promoters.

The HRLV is a putative member of family Caulimoviridae which infects horseradish (*Armoracia rusticana*) plants and other members of the Brassicaceae family. It is mostly found in south-eastern parts of Europe and was isolated during a study being conducted on turnip mosaic virus in Denmark (Richins and Shepherd 1986). Since HRLV is prevalent in temperate areas such as Denmark, it is assumed that the virus might inherently consist of certain cold stress responsive *cis*-elements that support its survival when it infects the plant. The physiological response of the plants growing in such lower mean temperatures in turn is guarded by a set of TFs that are particularly cold responsive (Chinnusamy et al. 2007; Kreps et al. 2002).
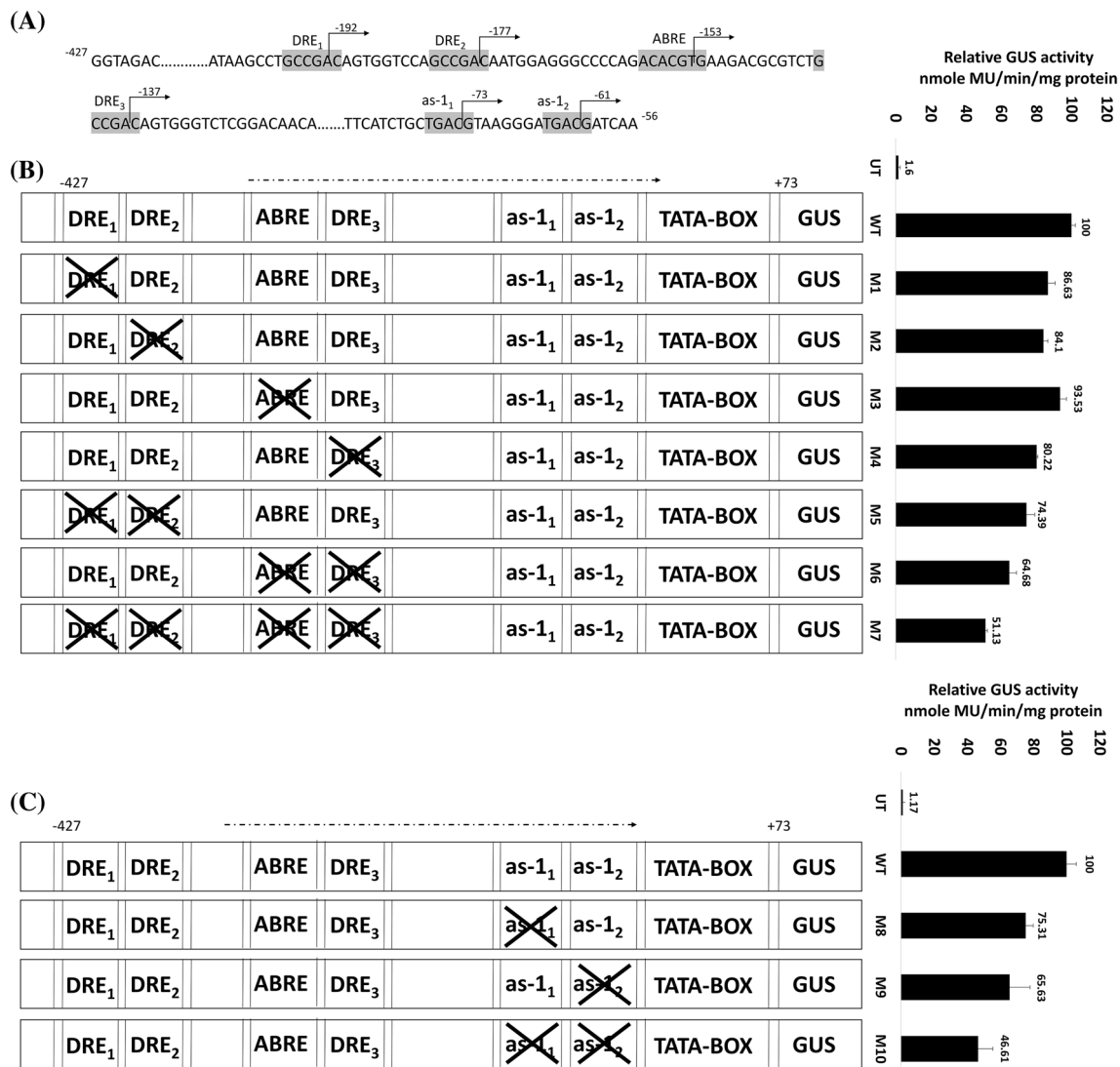
Fig. 9 Base-substitution analysis of H12 promoter in response to cold and SA stress in *Arabidopsis thaliana* leaves. **a** Schematic map of H12 promoter sequence (−427 to −56) indicating the relative positions of three DRE-core motifs (−192, −177, −137), one ABRE motif (−153) and two *as*-1 elements (−73, −61). **b** A series of mutant fragments (M1–M7) showing respective GUS activities under cold stress (16 h at 4 °C). **c** Analyses of GUS expression for fragments M8–M10 carrying mutations in *as*-1 elements under 150 µM exogenous SA administration. Average GUS activity for each fragment (WT and mutant) is presented with its corresponding mock (no stress) values

Since the last two decades, HRLV remains unexplored and no further studies have been carried out on this plant-pathogenic virus. In this backdrop, we performed the structural and functional analyses of HRLV promoter region in lieu to elucidate its regulation under various stress conditions. We identified the TSS of HRLV as an Adenine residue (+1) and the TATA Box at −22 position. We scrutinized the entire HRLV-Flt sequence using in silico analysis and detected several closely spaced *cis*-elements that were particularly stress-responsive in nature. We generated several deletion constructs (H1–H20) using the finer-deletion analysis and assayed their activity in both transient and transgenic

systems. A 500 bp long promoter fragment 'H12' was identified to be the strongest having ∼2.7-fold greater expression than the CaMV35S promoter. Consistent with this, the H12 promoter was also the most efficient in transgenic assays where it exhibited ∼2.5 and ∼3.5 fold stronger activities in tobacco and Arabidopsis transgenic plants respectively.

It was quite interesting to note that the H12 promoter had a unique *cis*-architecture which was mainly composed of three DRE-core, one ABRE and two *as*-1 elements within a stretch of 136 bp which allow the binding of DREB1, AREB1 and TGA1a TFs respectively. We validated this DNA–protein binding by gel-shift assays using suitable

probes along with mutational EMSAs. Co-activation studies demonstrated that DREB1, AREB1 and TGA1a could sufficiently transactivate the H12 promoter both with individual and combined administrations. We also attempted to conduct extensive studies on the regulatory roles of the above three TFs when they are recruited upon the H12 promoter. The stress analyses revealed that H12 promoter was functional in response to cold, ABA and SA treatments which may be accredited to the induction of cognate TFs (Fig. 6). Furthermore, we comprehensively studied the effect of single and combined mutations in each of the above *cis*-elements upon cold and SA treatments.

Interestingly, we observed that the H12 promoter was somewhat similar to that of *A. thaliana rd29 (Responsive to Desiccation)* in context of the spatial distribution of *cis*-elements. A 120 bp region of *rd29* promoter (− 174 to − 55) consists of a DRE (TACCGACAT), a DRE-core (GCCAC), an ABRE motif (TACGTTC) and an *as*-1 sequence (GACGTC). The *rd29* promoter has been reported to be induced by dehydration, high-salinity, cold as well as ABA (Narusaka et al. 2003). While our studies reveal that H12 is also positively regulated by both cold and ABA, it is indeed fascinating that it contains a *cis*-arrangement no different from the *rd29* multiple-stress inducible endogenous plant promoter. Since, the *rd29* promoter is known to respond well to multiple stress signals, it is of immense use for efficient

genetic manipulations through chimeric gene constructions containing reporter genes and TFs (Jia et al. 2012). It hereby appears that this conserved '*cis*-clustering' bestows the virus with a 'selective environmental benefit' for adaptability under stress conditions.

A unique feature of the ABRE-core motifs is that while repeated copies present on a minimal promoter can confer ABA-responsiveness, a single copy alone is unable to do the same. However, the presence of a coupling element can overcome this constrain and render ABA-inducibility (Hobo et al. 1999; Narusaka et al. 2003). Our transactivation assays clearly show that AREB1 could elevate H12 promoter activity while the combinatorial effect of AREB1 and DREB1 was more drastic (Fig. 8). This suggests that DREB1 might be acting as a coupling agent for ABRE1 when the two TFs transactivated the H12 promoter in concert (Fig. 10).

We assume that every change in environmental condition determines the niche of viruses which in turn require being the 'most fit' for natural selection to outcompete the others. The strategy followed by Caulimoviruses predominantly involves withholding the stress-responsive *cis*-elements according to the immediate prevalent surroundings. After a close inspection of the HRLV promoter sequence, we concluded that the presence of stress responsive *cis*-elements notably DRE-core, ABRE motif and *as*-1 element is an indication of the above hypothesis. Also, these elements account
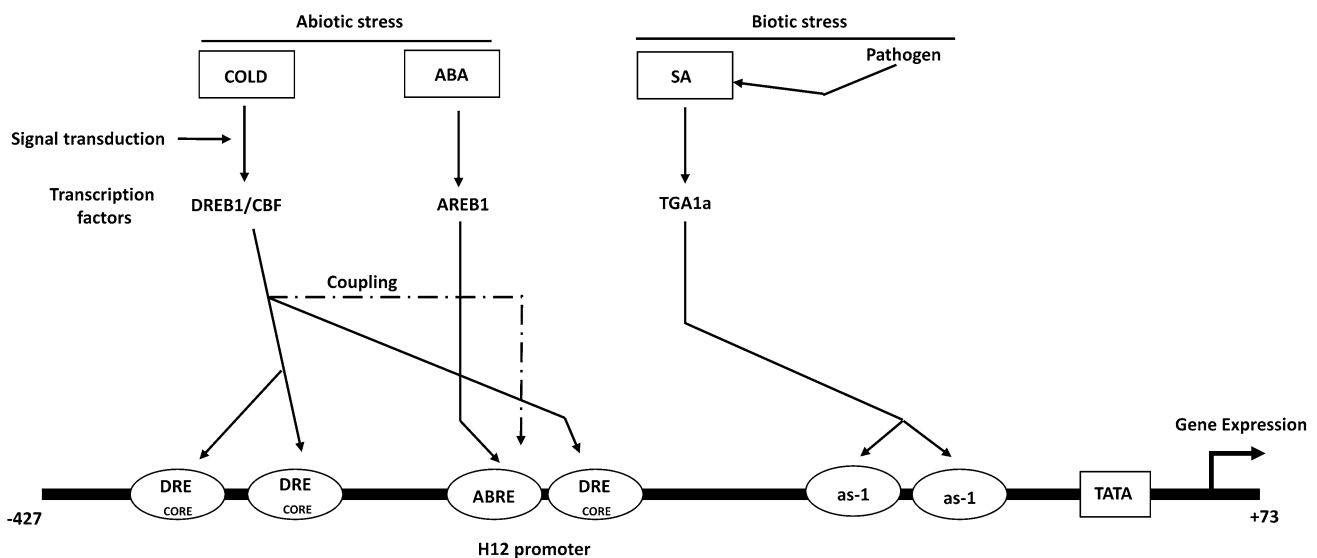
Fig. 10 A proposed model for the transcriptional regulation of H12 promoter under biotic and abiotic stress conditions. Environmental stress signals perceived by plants trigger signal transduction pathways resulting in up-regulation of stress responsive TFs. In case of abiotic stress such as those induced by cold and drought/ABA, the DREB1 and AREB1 TFs are recruited on the H12 promoter, eventually leading to its positive regulation. Here, DREB1 apparently functions as a coupling element that augments AREB1-mediated transcriptional regulation of H12 promoter (indicated by dotted lines). On the other hand, biotic stress imposed due to a pathogen invasion triggers several pathways of which SA is the key intermediate player. Exogenous SA administration which mimics a disease compromised condition, activates the pathogen responsive genes like TGA1a which bind to *as*-1 elements on H12 promoter (positive regulation indicated by solid arrows). Presumably, these events combined, strengthen the activity of H12 promoter and render it as both biotic and abiotic stress responsive

for the strong constitutive expression of H12, identified as the most efficient fragment in this study. Here, we emphasize that the H12 promoter recruits the DREB1 and AREB1 TFs for low temperature and/or ABA tolerance (Fig. 10) coupled to enhanced transcription as supported by the stress and transactivation assays. This may assist their survival in cold temperate areas where they establish a successful host-parasite relationship with the plants they infect. Furthermore, the *as*-1 element involved in SA-signalling pathway during biotic-stress, also imposes a positive regulation on the H12 promoter.

Overall, our findings describe the transcriptional regulation of a novel Caulimoviral promoter H12 which is mediated by stress responsive TFs viz. DREB1, AREB1 and TGA1a. This promoter appears to be an excellent model for studying multiple stress signalling pathways in a plant cell. It also holds excellent prospect for strong gene expression under multiple stress conditions. We propose that H12 could become a well-suited promoter open to gene-pyramiding which in turn implies a wide spectrum of plant biotechnology-based applications.

In conclusion, this novel finding is useful not only for understanding viral adaptation to the environment but for potential biotechnology purposes.

Methods

Plant materials and growth conditions

The wild type tobacco (*N. tabacum* cv. Samsun NN) and Arabidopsis (*A. thaliana* cv. Columbia-0; Col-0) were used for plant transformation. Tobacco plants were grown under the following greenhouse conditions: a photoperiod of 16/8 h (light/dark), light intensity of 220 μmol/m^2/s, temperature of 28 ± 3 °C and humidity of 70–75% while Arabidopsis plants were grown under a photoperiod of 16/8 h (light/dark), light intensity of 220 μmol/m^2/s, temperature of 22 ± 3 °C and humidity of 80–85%.

TSS determination

The TSS was determined using a previously established protocol with slight modifications (Carey et al. 2013). Briefly, a 36 bp long oligonucleotide complementary to a *uidA* mRNA sequence located 43 nucleotides downstream of the start codon of *uidA* was radiolabelled using γ^{32}P ATP. The labelled primer was incubated with total RNA extracted from transgenic tobacco plant harbouring H1 promoter fragment and allowed to anneal with *uidA* specific mRNA. Next, reverse transcription was performed using a first strand c-DNA synthesis protocol following manufacturers' instructions. The reaction was carried out at 37 °C for 1

h to catalyse the elongation of the primer to the 5′- end of the mRNA. The reaction product was analysed on a 6% denaturing polyacrylamide gel electrophoresis next to a sequencing ladder and autoradiographed.

Construction of protoplast and plant expression vectors

A total of twenty fragments generated by leader-deletion analysis were PCR amplified and isolated as *EcoR*I-*Hind*III fragments (Primers listed in Supplementary table). Each promoter construct was cloned into the corresponding sites of protoplast expressing vector pUCPMAGUS (Dey and Maiti 1999) and further sub-cloned into the plant expression vector pKYLX71GUS (Schardl et al. 1987).

Generation of transgenic Tobacco and Arabidopsis plants

For raising transgenic plants, the constructs pKYLXGUS-H1 to pKYLXGUS-H20 were introduced into the *Agrobacterium* strain LBA4404 as described previously (Sahoo et al. 2014). Tobacco leaf discs were transformed with the above constructs employing the *Agrobacterium*-mediated plant transformation method (Cheng et al. 1997) while *A. thaliana* plants were transformed using pKYLXGUS-H12, pKYLXGUS-H17 and pKYLXGUS-35S promoter constructs employing the floral dip method (Zhang et al. 2006). Positive transformants for both tobacco and Arabidopsis were selected on Murashige and Skoog (MS) medium containing 300 and 100 mg/L kanamycin respectively. A total of 10–12 independent transgenic tobacco plant lines were generated for each construct as previously described (Maiti et al. 1993) and maintained under greenhouse conditions (as described in Methods) till setting of seeds. Seeds harvested from T_0 generation transgenic plants were germinated on MS medium containing kanamycin (Kan; 300 mg/L).

The segregation analysis was performed for the T_1 generation seeds followed by a Chi square test (data not shown) where we selected the true transgenic plants displaying a value of < 1.5 ($p \leq 0.05$) (Patro et al. 2015). Transgenic lines showing a segregation ratio of 3:1 (Kan^R:Kan^S::3:1) were then selected and propagated for raising T_2 progenies under proper green-house conditions.

Transient and transgenic assays using fluorometric GUS analysis

For transient assays, the constructs pUCPMAGUS-H1 to pUCPMAGUS-H20 were electroporated into healthy tobacco (*Xanthi* bred) protoplasts following standard protocols (Acharya et al. 2014). Briefly, 5 μg of test plasmids were electroporated for each promoter fragment along with

an empty vector (EV) taken as an internal control. For agro-infiltration assays, healthy leaves of *N. tabacum* were syringe-infiltrated with *Agrobacterium* LBA4404 bacterial suspensions carrying the constructs pKYLXGUS-H1 to pKYLXGUS-H20. Similar protocols were followed for infiltration in *A. thaliana* leaves. Enzymatic GUS activities for each of the construct in protoplast and agro-infiltrated samples (along with CaMV35S promoter) were determined by fluorometric GUS assay using 4-methylumbelliferyl-β-D-glucuronide (MUG) as substrate (Jefferson 1987). In case of transgenic tobacco and Arabidopsis plants, the total protein was isolated from transgenic seedlings (21 days old) carrying the respective constructs (Bradford 1976) and the GUS accumulation values were calculated.

Histochemical GUS staining

For detection of GUS activity, a buffer solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) was prepared. Healthy plant tissues/seedlings were incubated for 48 h at 37 °C in the above solution (Acharya et al. 2014). The samples were washed several times with 70% ethanol post-incubation and then photographed.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from tobacco transgenic seedlings (T$_2$ generation progenies) expressing pKYLX (VC), pKYLXGUS-35S, pKYLXGUS-H5, pKYLXGUS-H12, pKYLXGUS-H16 and pKYLXGUS-H17 using the standard protocol (Kumar et al. 2012). For *A. thaliana*, however, transgenic lines expressing pKYLXGUS-35S, pKYLXGUS-H12 and pKYLXGUS-H17 were used for RNA extraction. The first strand cDNA synthesis was performed using a 1 μg aliquot of mRNA (Thermo Fischer Scientific; Cat No. K1612). The qRT-PCR was performed using *uidA* and *18 s* specific primers in a 7500 Fast-Real Time System (Applied Biosystems). The $2^{-\Delta\Delta CT}$ (CT-comparative threshold cycle) method was used to analyse the obtained results (Livak and Schmittgen 2001) where the transcript level of *uidA* was analysed using 18 s as the normalizer gene.

Stress-treatment assays

The GUS expression of transgenic Arabidopsis plants (3 weeks old, T$_2$ generation) driven by H12 and H17 promoter fragments was analysed under various stress conditions which included a 16 h cold treatment at 4 °C, ABA and SA treatments at 150 μM concentrations (Kumar et al. 2012;

Liu et al. 1998). Each stress was provided in a separate set of experiment which was replicated at least thrice.

Purification of recombinant proteins and gel mobility shift assays

The synthetic TGA1a gene codon-optimized for *Escherichia coli* was cloned into the protein expression vector pET29b with a C-terminal 6× His tag and transformed into the BL21 (DE3) *E. coli* cells. The transformed bacteria were grown until they reached an OD of 0.6 and induced using Isopropyl β-D-1-thiogalactopyranoside. The expressed protein (TGA1a-His) was purified using the Ni–NTA agarose beads according to the standard protocol (Qiagen). On the other hand, full length CDS of DREB1 and AREB1 were cloned into the pGEX4T3 vector which contains a C-terminal GST-tag. The protein expression was carried out as described above and purified using glutathione Sepharose beads according to manufacturers' instructions. The GST-AREB1 and GST-DREB1 fusion proteins were cleaved with Thrombin (T4648 Sigma-Aldrich) and eluted.

For EMSA experiments, 40–50 bp long oligonucleotides were labelled with γ^{32}P ATP and used as probes. Subsequently, each radiolabelled oligonucleotide was annealed to its corresponding complementary pair sequence to make a double-stranded probe. A total of three probes (Probe I, II and III) were designed to contain all three *cis*-elements (DRE-core, ABRE and *as*-1) in sequential arrangement. Both, WT and mutant versions of probes I, II and III carrying base-substitutions in specific positions were synthesized. For, competitive EMSA, 10× to 100× excess cold probe of WT fragment was used. All binding assays were carried out in a reaction mixture (30 μL) containing 1.5 μg of purified recombinant protein, γ^{32}P -labeled probe (1×10^5 cpm), 30 μg Bovine serum albumin and $1 \times$ binding buffer (50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 15% (v/v) glycerol, 5 Mm dTT, 500 ng/μL polydI-dC). The mix was incubated at room temperature for 30 min and the product was resolved on 4% native polyacrylamide gel. The gel was dried at 80 °C for 45 min and autoradiographed.

Transactivation assays

The full length CDS of DREB1, AREB1 and TGA1a were cloned in the pUCPMA vector using gene specific primers (Supplementary Table). These were designated as the respective effector constructs. On the other hand, H12-promoter fused to *GUS* gene was used as the reporter construct along with an empty vector (internal control). The reporter and effector constructs were co-electroporated in

Arabidopsis protoplasts following which the respective GUS activities were recorded (Mizoguchi et al. 1993).

Construction of base-substituted H12 promoter fragments

Mutations in specific *cis*-elements located on H12 promoter were generated using site-directed mutagenesis. A total of ten mutant fragments (M1–M10) were designed employing nested PCR with internal primers containing base-substitutions in specific sequences following an earlier protocol (Reikofski and Tao 1992). All constructs were PCR amplified and cloned into the plant expression vector pKYLX71 and transformed into *Agrobacterium* strain LBA4404. Each construct (including WT H12-GUS) was infiltrated into *A. thaliana* leaves which were then maintained under cold- (M1–M7) and SA- (M8–M10) treatment conditions in two separate set of experiments. The GUS activity of all constructs was measured after a specific time duration viz. 16 h post- cold stress and 24 h post- SA treatments. The absolute GUS activities for each construct (WT and mutants) obtained after treatment were normalized with their respective mock (untreated) values and presented as relative GUS accumulation taking H12 (WT) as 100 units.

Statistical analysis

All experiments were repeated at least thrice and statistical analyses were performed using Graph Pad Prism (version 5.01). The one-way analysis of variance (ANOVA) was performed where a *P* value of <0.05 revealed significance. Supplementary Table: List of oligonucleotides used in this study.

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