Biotechnol Lett (2020) 42:2013–2033
https://doi.org/10.1007/s10529-020-02961-7

ORIGINAL RESEARCH PAPER

Characterization of *STP4* promoter in Indian mustard *Brassica juncea* for use as an aphid responsive promoter

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Received: 23 November 2019 / Accepted: 3 July 2020 / Published online: 16 July 2020

Abstract
Objective *Brassica juncea*, a major oilseed crop, suffers substantial yield losses due to infestation by mustard aphids (*Lipaphis erysimi*). Unavailability of resistance genes within the accessible gene pool underpins significance of the transgenic strategy in developing aphid resistance. In this study, we aimed for the identification of an aphid-responsive promoter from *B. juncea*, based on the available genomic resources.
Results A monosaccharide transporter gene, *STP4* in *B. juncea* was activated by aphids and sustained increased expression as the aphids colonized the plants. We cloned the upstream intergenic region of *STP4* and validated its stand-alone aphid-responsive promoter activity. Further, deletion analysis identified the putative *cis*-elements important for the aphid responsive promoter activity.
Conclusion The identified STP4 promoter can potentially be used for driving high level aphid-inducible expression of transgenes in plants. Use of aphid-responsive promoter instead of constitutive promoters can potentially reduce the metabolic burden of transgene-expression on the host plant.

Keywords Aphid-responsive genes · Plant inducible promoter · Promoter analysis · *Brassica juncea* · Agroinfiltration · Transgenics

Introduction

Among the rapeseed-mustard group of crops, Indian mustard [*Brassica juncea* (L.) Czern.] is the predominant oil yielding crop in India. It alone occupies 85% of the total rapeseed-mustard growing area in India (DRMR 2015). Because of intrinsic tolerance to salinity and moisture deficit, mustard cultivation fits well across the diverse agroclimatic regimes, including marginal lands of resource poor farmers. However, the productivity of this crop is severely affected due to infestation by mustard aphid [*Lipaphis erysimi* (Kalt)] (Bakhetia 1987; Rohilla et al. 1987; Shekhawat et al. 2012). The severity of infestation may cause 35.4–91.3% yield loss which is equivalent to

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10529-020-02961-7) contains supplementary material, which is available to authorized users.

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11–32% loss in oil (Singh and Sachan 1994). In financial terms, the average loss imposed by aphids may extend to Rs. 1575 crores annually (derived). Aphids are hemipteran, phloem-feeding insects. While feeding, the nymphs and the adults divert excessive photo-assimilates from the host and exudate sugar-rich honeydews. Production of honeydews hinders photosynthetic activities and alters defense response of the host (Schwartzberg and Tumlinson 2014). The salivary components of aphids potentially attenuate host-defense response and establish uninterrupted feeding (Miles 1999; Ilarduya et al. 2003; Zhu-Salzman et al. 2004; Park et al. 2006). Aphids also serve as potential vectors of plant luteo viruses (Hogenhout et al. 2008; Lu et al. 2016).

The gene(s) for genetic resistance is either obscure among the *Brassica* germplasms or even if reported in a few wild accessions, largely remains uncharacterized (Kumar et al. 2011; Atri et al. 2012; Sarkar et al. 2016). Thus, the scope for developing resistance through conventional breeding is limited (Bhadoria et al. 1995; Dutta et al. 2005). For overcoming such bottleneck, transgenic expression of insecticidal genes from distant sources has been considered as a potential avenue for developing the aphid resistant plant types (reviewed in Bhatia et al. 2011; Das et al. 2018). For example, several plant lectin genes have been expressed in *B. juncea* for developing transgenic-mediated aphid resistance (Kanrar et al. 2002; Sharma et al. 2004; Dutta et al. 2005; Hossain et al. 2006; Saha et al. 2006; Sadeghi 2007). However, none of these transgenics could be advanced to field trials or released as cultivar. This clearly advocated the lack of field-applicable resistance in these transgenics. In these studies, either the constitutive promoter CaMV 35S or, only in few cases, a phloem-specific promoter had been used for expressing the transgene (Kanrar et al. 2002; Dutta et al. 2005; Hossain et al. 2006). Constitutive expression of the transgene leads to various disadvantages such as metabolic payoffs (Cipollini et al. 2003; Walters and Heil 2007; Garrido et al. 2017) and pleiotropic effects on the plants (Li et al. 2002; Liu et al. 2008; Brini et al. 2011). Thus, use of specific promoters for tissue and stress specific expression of the transgene will be more desirable.

Interestingly, aphids have evolved to bypass the host-defense while feeding on the host plants. It secretes effector proteins such as COO2, MP1, VPS52 etc. into the host cells which inactivate defense signaling in the surrounding tissues (Pitino and Hogenhout 2012; Jaouannet et al. 2014; Rodriguez et al. 2017). Consequently, several independent studies have led to hypothesize countering of host-defense suppression by aphid-inducible expression of endogenous defense genes (Ellis et al. 2002; Boughton et al. 2006; Koramutla et al. 2014). However, validation of such possibility as well as precise temporal expression of aphid deterring genes, will require aphid responsive promoters. Transcriptome data on several plant–aphid interaction studies are available which can be analysed for identifying the host-genes activated due to colonization and feeding by aphids (Voelckel et al. 2004; Smith and Boyko 2007; Kusnierczyk et al. 2008). Mining on these transcripts and their upstream intergenic regions will be the most relevant assignment in identification of the aphid-responsive promoters.

Recently, white fly- and aphid-specific promoters have been described in *Arabidopsis* (Dubey et al. 2018). However, an aphid responsive promoter is not known in rapeseed-mustard group of crops including *B. juncea*. In the present study, we have isolated and characterized a potential aphid-responsive promoter in *B. juncea* by screening the host genes which are transcriptionally activated by aphid infestation. We have also attempted to delineate the important regions of the promoter for the aphid-responsive promoter-activity through deletion analysis.

Materials and methods

Planting materials and growth conditions

Seeds of *B. juncea* cv. Varuna and *Nicotiana benthamiana* were available at ICAR-National Institute for Plant Biotechnology, New Delhi. The plants were grown in 8-inch plastic pots filled with a mixture of field soil, soilrite and leaf compost. The plants were maintained in glass house at 24 ± 2 °C with 65–70% relative humidity and with 16/8 h light/dark cycle. The plants were watered twice a week with Hoagland solution (Hi-media, India).

Aphid infestation, sample collection and cDNA preparation

Maintenance of aphid population, aphid inoculation on the experimental plants, collection of leaves for the

gene expression studies, isolation of RNA and cDNA synthesis were performed according to the methods as described in Ram et al. (2017).

Mining of aphid-inducible genes from plant-aphid interaction studies

Transcriptome and microarray data of plant-aphid interaction studies (Table 1) were retrieved from GEO database (https://www.ncbi.nlm.nih.gov/gds) and analyzed with GEO2R online tool of NCBI (https://www.ncbi.nlm.nih.gov/geo/geo2r). The top 250 genes from each data set were selected and further filtered for > 2.0-fold change in expression. Additionally, the genes validated for the up-regulation during aphid infestation in plant-aphid interaction studies were also included. Annotation clustering and categorization for the biological functions of the identified genes were carried out using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 bioinformatic resources (https://david.abcc.ncifcrf.gov) with ease threshold at p-value < 0.05 and Gene Ontology enrichment anaLysis and visuaLizAtion (GORILLA; https://geneontology.org/page/go-enrichment-analysis) tool, respectively. The Gene Ontology (GO) analysis of selected genes was performed using Blast2GO software (https://www.blast2go.com).

PCR and qRT-PCR assay

For designing of gene-specific primers, homologs of the selected genes were identified in *Arabidopsis* database (https://www.arabidopsis.org). The gene-specific primers (Supplementary Table 1) were designed from intron spanning regions of the genes, using PrimerQuest tool of Integrated DNA Technology (https://eu.idtdna.com/PrimerQuest/Home/Index). PCR was performed in 20 µl reaction volume containing 2.0 µl of 10X Taq DNA polymerase buffer, 0.5 µl of dNTP mix (10 mM), 1.0 µl each of the forward and reverse primer (10 µM), 1 U of Taq DNA polymerase (TAKARA Bio Inc., Japan) and 1.0 µl of template DNA (50 ng/µl). The final volume was made up by nuclease-free water. PCR reaction was performed in a thermal cycler (Applied Biosystems, USA) with the following PCR programme: initial denaturation at 95 °C for 4 min followed by 30 repeated cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 30 s and primer extension at 72 °C for 1 min followed by final extension at 72 °C for 7 min. The amplicons were analysed on 1.5% agarose gel. The desired amplicons were validated by sequencing (SciGenom Labs Pvt. Ltd., India).

The qRT-PCR analysis was performed using the SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (TAKARA Bio Inc., Japan) in a StepOnePlus Real-Time PCR system (Applied Biosystems, USA). *CAC* gene has been identified as the best reference gene for *B. juncea* samples treated with aphid stress (Ram et al. 2017). Thus, in case of *B. juncea*, the gene expression data was normalized using *CAC*. For analysing *GUS* gene expression in tobacco samples, *GAPDH* was taken as the internal control. A 10 µl reaction cocktail contained the following components: 5 µl of 2X SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.2 µl of 50X ROX dye, 0.4 mM of each forward and reverse primers, 1 µl of diluted cDNA and 3.3 µl of nuclease-

Table 1 Plant-aphid interaction studies used in analysis by GEO2R

S. no	Plant-aphid interaction	Type of study	References
1	*Arabidopsis-Myzus persicae*	Transcriptome	Moran et al. (2002), De Vos et al. (2005)
2	Sorghum-*Schizaphis graminum*	Transcriptome	Zhu-Salzman et al. (2004)
3	Tobacco-*M. nicotianae*	Transcriptome	Voelckel et al. (2004)
4	Tobacco-*M. nicotianae*	Transcriptome	Heidel and Baldwin (2004)
5	Celery-*M. persicae*	Microarray	Divol et al. (2005)
6	*Arabidopsis-M. persicae*	Microarray	Couldridge et al. (2007)
7	*Arabidopsis-M. persicae*	Microarray	De Vos and Jander (2009)
8	*Arabidopsis-M. persicae*	Microarray	Barah et al. (2013)
9	*Arabidopsis-M. persicae*	Microarray	Appel et al. (2014)

free water. PCR was carried out with one cycle of initial denaturation at 95 °C for 2 min followed by 40 cycles at 95 °C for 10 s, 58–60 °C for 30 s and 72 °C for 30 s. Amplification specificity was confirmed by dissociation curve analysis with gradual increase in melting temperature from 60 to 95 °C. All the qRT-PCR experiments were performed independently in three biological replicates with minimum three technical replicates each time. Fold-change in gene-expression was calculated using $2^{-\Delta\Delta Ct}$ method (Pfaffl et al. 2004).

Identification and cloning of STP4 promoter

For identifying upstream region of the *STP4* gene, the homologous sequence of *STP4* CDS was identified in *B. rapa* database (https://Brassicadb.org) through BLAST analysis. The full length *STP4* gene was identified in BAC clone KBrH046K16 using FGENESH software (https://www.softberry.com/fgenesh). The transcription start site (TSS) of the *STP4* gene was predicted using TSSP online tool (https://www.softberry.com/berry.phtml? topic = promoter). The corresponding locus in *B. rapa*, was searched in Genome Browse of *B. rapa* database (https://Brassicadb.org/cgi-bin/gbrowse/Brassica/). Based on the translation start site (ATG) of the locus Bra017776, an upstream region (2 kb approx.) was retrieved as the putative promoter of the *STP4* gene.

The putative promoter was validated in silico by various promoter prediction tools and amplified from *B. juncea* DNA using sequence-specific primers (proBjSTP4F and proBjSTP4R). Restriction sites for *Bam*HI and *Nco*I were incorporated at the 5′ end of the forward and reverse primers, respectively. Thus, the forward primer was designed 2.0 kb upstream from the translation initiation codon (ATG) and the reverse primer was designed at the 5′UTR region immediately upstream to the translation initiation codon. PCR amplification was performed in 20 µl of reaction volume containing 18.0 µl of Platinum PCR Super Mix High Fidelity (Invitrogen, USA), 0.4 µM each of the forward and reverse primer and 1.0 µl of DNA (50 ng/µl). The PCR reaction was performed in a thermocycler (Applied Biosystems, USA) with following conditions: one cycle of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 54 °C for 30 s and primer extension at 68 °C for 4 min and one cycle of final extension at 68 °C for 15 min. The PCR amplified fragment (proBjSTP4) was cloned in pGEM-T Easy cloning vector (Promega, USA) and validated through sequencing. In silico analysis of the putative promoter for identifying *cis*-regulatory elements was carried out using Plant CARE (https://bioinformatics.psb.ugent.be/webtools/plantcare).

Development of proBjSTP4-GUS fusion construct and assay of the promoter activity

The proBjSTP4 fragment was sub-cloned in *Bam*HI–*Nco*I sites of the binary vector pCAMBIA1305.1 by substituting the CaMV 35S promoter of the parent vector. Subsequently, the binary construct was mobilized into a C58 type *Agrobacterium* strain GV3101. For verifying any bacterial expression of GUS, the *Agrobacterium* cells harbouring the pCAMBIA-proBjSTP4 construct was incubated with all the components of histochemical assay at 37 °C for 1 h. *Agrobacterium* strains harbouring pBI121 and pORER2, as they show bacterial expression of GUS, was used as positive controls in the experiment.

Development of deletion constructs

Four deletions, starting from 5′ end of the STP4 upstream sequence, named as proSTP4DC1, proSTP4DC2, proSTP4DC3 and proSTP4DC4 were created by PCR amplifications using 4 set of forward primers and a common reverse primer proBjSTP4R (Supplementary Table 2). All the deletion fragments were cloned in pGEM-T easy vector and validated by sequencing. All these deletion fragments were further cloned in pCAMBIA1305.1, substituting the CaMV 35S promoter upstream to GUS.

Agroinfiltration of *B. juncea* and *N. benthamiana* leaves

Agroinfiltration of leaves were carried out according to Xu et al. (2008) with some modifications. The detached leaves of *B. juncea* were completely dipped into the infiltration culture and subjected to vacuum at 30 psi for 30 min. Density of the *Agrobacterium* cells were kept uniform every time based on OD600 reading. The vacuum infiltrated leaves were stabilized for 24 h with their petioles immersed in Hoagland solution before further treatment and histochemical

assay. The Agroinfiltration in *N. benthamiana* leaves were performed according to Sparkes et al. (2006) with minor modifications. The *N. benthamiana* plants at 4–6 leaf stage were Agroinfiltrated at 3–4 spots using 2 ml syringe with 100 µl infiltration suspension containing the *Agrobacterium* cells. The infiltrated plants were kept for 24 h at normal growth conditions before wound or hormonal treatment and histochemical assay.

Wound and hormonal treatment of Agroinfiltrated *N. benthamiana*

Wound was inflicted towards petiole of the infiltrated tobacco leaf across the mid vein of at least 40% leaf area and samples were collected in a time course manner at 2 and 6 h. Infiltrated leaves without wounding were collected as control for each time point. In hormonal treatments, 2 mM methyl jasmonate (MJ) and 5 mM salicylic acid (SA), prepared in water with Triton X-100, were sprayed on infiltrated tobacco plants kept in desiccators in independent experiments. Leaf samples were collected at 2 and 6 h time points following the treatments. Mock treated (water with Triton X-100) infiltrated leaves were taken as control for each time point. The collected samples were immediately frozen in liquid nitrogen and stored at − 80 °C until further use. The experiments were repeated three times. Significant difference in mean values was evaluated by Student's t-test at p-value < 0.05.

Aphid bioassay on detached leaves

Forty-to-fifty wingless aphids of assorted life stages were released on each Agroinfiltrated leaf and kept in a desiccator in moist condition. Leaf samples were collected at 0, 24 and 48 h post infestation along with parallel controls sans infestation. The samples were collected in duplicates for histochemical as well as qRT-PCR analysis. The experiments were repeated three times as biological replicates with three technical replicates at each time. Significant difference in means was evaluated by Student's t-test at p-value < 0.05.

Histochemical and gene expression analysis of GUS

Histochemical staining of GUS activity was conducted according to the method described by Jefferson et al. (1987) with minor modifications. The samples were incubated in GUS staining solution containing 10 mM sodium phosphate buffer at pH 7.0, 10 mM Na_2-EDTA, 0.1% Triton X-100, 1 mg/mL X-Gluc, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide at 37 °C for 10–12 h after 30 min of vacuum infiltration at 30 psi pressure. After GUS staining, the samples were incubated in 70% ethanol for removing the chlorophylls. Relative abundance of *GUS* transcripts was assayed by qRT-PCR of RNA from the leaves, as described earlier.

Results

Mining of aphid-inducible genes

Studies on gene-expression in case of *B. juncea*-aphid interaction are limited (Bandopadhyay et al. 2013; Koramutla et al. 2014). Thus, for identifying the genes transcriptionally activated by aphids we mined the transcriptome and microarray data from other plant-aphid interaction studies (Table 1). The genes up-regulated by > 2.0-fold due to aphid infestation were identified and thus, 261 up-regulated genes were selected. A flow diagram representing the pipeline used for the identification of the aphid-inducible genes is given in Fig. 1.

The selected 261 transcripts were subjected to functional clustering which revealed 14 annotation clusters (named A to N), each representing 1–22% of the transcripts at p-value < 0.05 (Fig. 2a). The cluster A represented 57 (22%) defence responsive genes followed by cluster C representing 47 (18%) metabolic genes. Many of the earlier reports suggested that, the defence genes transiently activated by aphid probing were eventually suppressed in susceptible hosts (De Vos et al. 2005; Koramutla et al. 2014; Schwartzberg and Tumlinson 2014). Therefore, expression of such host-genes may not be persistent under aphid colonization (Gao et al. 2007). Thus, the defence pathway related genes were excluded. The remaining 204 genes were screened for identifying their involvement in various biological processes such

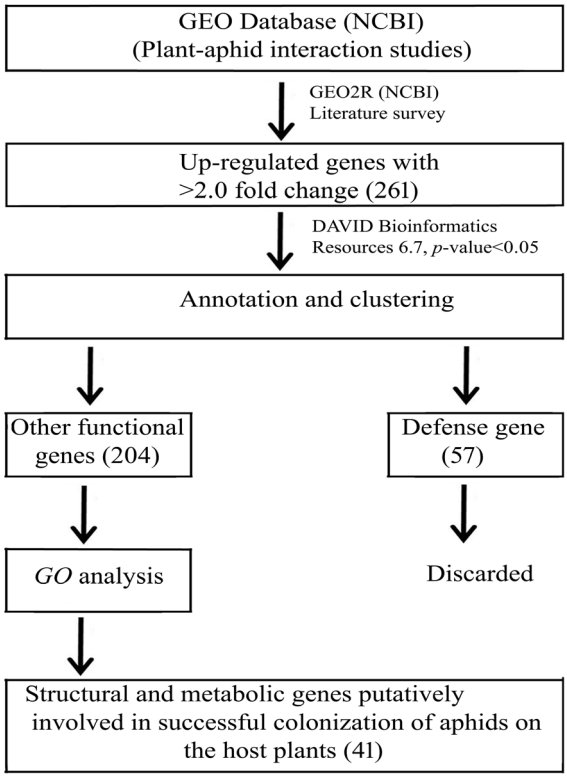


Fig. 1 A flow diagram of the pipeline used for the identification of aphid inducible genes. The aphid inducible genes were identified from different plant-aphid interaction studies on the basis of > 2.0 fold-change in expression due to treatment. The selected genes were clustered based on their functions using DAVID v6.7 software at threshold p value < 0.05

as cell wall modification, water transport, vitamin biosynthesis, carbon and nitrogen metabolism and mobilization. We hypothesized that the genes involved in such metabolic processes may be modulated by aphids for generating a favourable metabolic pool supportive to rapid colonization of the host plants. Based on this hypothesis, 41 genes (Table 2) were identified. Gene ontology (*GO*) analysis of the selected 41 genes revealed majority of them (60.53%) being associated with transporter activity followed by a few (20%) being DNA binding proteins (Fig. 2b). Similarly, when categorized based on involvement in biological processes, majority of the genes were involved in cellular processes (74%) followed by transport (70%, Fig. 2c). Further, based on cell components, majority of them (75%) were found residing on the cell membrane followed by cytoplasmic components (Fig. 2d).

Gene-expression study of the aphid-inducible genes in *B. juncea*

Heterologous primers based on the *Arabidopsis* sequences could amplify 39 of the selected genes in *B. juncea* DNA and cDNA. Amplicons with desired specificity in length were sequenced for validation and designing of the gene-specific qPCR primers (Supplementary Table 1). In qRT-PCR based gene expression study, 18 genes revealed differential expression in response to aphid infestation over the time course of 6–96 h (Fig. 3). Based on the expression pattern, these genes were grouped into three categories: (a) significantly up-regulated genes with sustained expression which included *CAT2*, *CAT9*, *STP4*, *β-fruct*, *NRT2*, *MSAM3*, *ERD6* and *MIPS,* (b) genes showing early response, but down regulated within 96 h or later time points viz. *PINV*, *PME*, *EXPA1*, *EXPA2*, *LHT7*, *THI1* and *MT* and (c) genes showing insignificant activation viz. *SDH, GS* and *MS.* Eight significantly up-regulated genes were further compared based on three criteria namely, minimum basal level of expression, significant level of transcriptional activation in response to aphids and sustained level of activated expression at least more than 96 h of treatment. The candidate genes *STP4*, *IPS* and *β-fruct* were found to be the most appropriate in terms of the above criteria. Among the three, *STP4* gene was selected for isolation of its promoter. Aphid mediated transcriptional activation of the *STP4* gene was also analysed across the major tissues such as leaf, stem, flower and siliques, which are highly infested by aphids. The study validated transcriptional activation of the STP4 promoter, albeit at variable level, across the tissues upon aphid infestation. The highest transcriptional activation of more than fivefold was recorded in the flowers whereas, other tissues such as leaf, stem and siliques showed an activation ranging from 1.6 to 2.5-fold compared to their counterparts from non-infested plants (Fig. 4).

Isolation and in silico analysis of STP4 promoter in *B. juncea*

The *STP4* coding sequence of *B. juncea* shared 89% and 95% homology to *AtSTP4* (At3g19930) gene and the BAC clone of *B. rapa* subsp. *Pekinensis* (KbrH046K16), respectively. Since *B. rapa* is one of the progenitors of *B. juncea,* the BAC clone

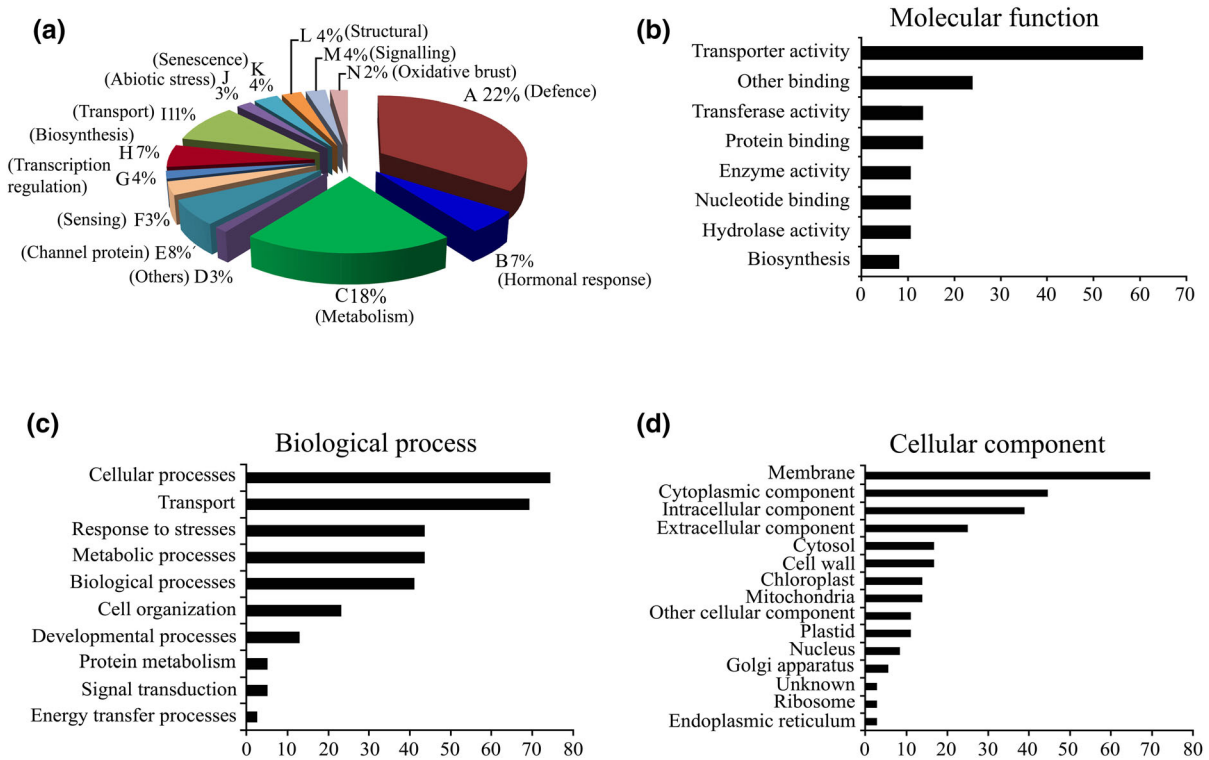


Fig. 2 Clustering and GO categorization of aphid-responsive genes. **a** Functional clustering of 261 aphid responsive genes. **b–d** GO analysis of the selected 41 aphid-responsive genes for molecular function, biological processes and cellular components. The analysis was performed using DAVID v6.7 at p value of ≤ 0.05 and Blast 2 GO programme. Value at X-axis represents percentage of gene ontology

KbrH046K16 was used as genomic resource for the identification of upstream sequences of *STP4*. The *STP4* gene in the BAC clone spanned over 1.9 kb consisting of 4 exons and was mapped on chromosome 3 (A03) of *B. rapa*. Based on the defined TSS and ATG of the *STP4* gene of *B. rapa* (Bra001766), around 2.0 kb upstream region was retrieved from *B. rapa* BAC clone. The identified upstream sequence was PCR amplified in *B. juncea* using a specific set of primers. The PCR amplified 2.0 kb fragment was cloned in pGEMT Easy vector and upon sequencing showed 99% homology with the upstream region of Bra001766. The sequence cloned as the putative promoter and named as proBjSTP4, was analysed by Plant CARE software for identification of the *cis*-regulatory elements (Fig. 5). The basal promoter elements like TATA-box (TATAAATT) and CAAT-box (GACCAA) was found at − 36 and − 94 bp upstream to the TSS, respectively. In silico analysis also revealed the presence of other important *cis*-regulatory elements, which have been listed in Supplementary Table 3.

Functional assay of proBjSTP4

Prior to Agroinfiltration in *B. juncea* leaves, it was imperative to ensure absence of any bacterial expression of the reporter cassette proBjSTP4::GUS. In histochemical assay, the *Agrobacterium* cells harbouring proBjSTP4::GUS cassette did not show any GUS activity ruling out any bacterial expression, whereas pBI121 and pORER2 generated blue colour due to bacterial expression of the *GUS* gene (Supplementary Fig. 1). This is due to the presence of an intron in the *GUS* gene of pCAMBIA1305.1. The proBjSTP4::-GUS construct was Agroinfiltrated into the *N. benthamiana* leaves and in histochemical assay after 24 h the treated leaves produced blue colour which demonstrated stand-alone promoter activity of the proBjSTP4.

Table 2 Aphid-responsive genes identified for gene-expression analysis

S. No	Gene	Locus ID	Biological function(s)	References
1	*CAT9*	At1g05940	Amino acid transmembrane transport	Su et al. (2004), Hanada et al. (2011)
2	*AAP8*	At1g10010	Amino acid transmembrane transport	Tegeder and Ward (2012), Santiago and Tegeder (2016)
3	*LHT7*	At4g35180	Amino acid transport, ER unfolded protein response, negative regulation of defense response	Lee and Tegeder (2004), Tegeder and Ward (2012)
4	*LHT2*	At1g24400	Amino acid transport	Lee and Tegeder (2004), Tegeder and Ward (2012)
5	*Nod*	At1g44800	Amino acid homeostasis and transport	Hu et al. (2003), Ladwig et al. (2012)
6	*CAT2*	At1g58030	Amino acid transmembrane transport Biochemical and physiological response	Su et al. (2004), Hanada et al. (2011)
7	*AAP1*	At1g58360	Amino acid transmembrane transport	Tegeder and Ward (2012), Santiago and Tegeder (2016)
8	*ATLP2*	At1g25530	Amino acid transport	Lee and Tegeder (2004), Tegeder and Ward (2012)
9	*BAT1*	At2g01170	Amino acid transmembrane transport	Dündar and Bush (2009)
10	*AQA*	At1g52180	Water channel	Johanson et al. (2001)
11	*STP4*	At3g19930	Monosaccharide, nitrate transport, sucrose:H^+ symport	Truernit et al. (1996), Fotopoulos et al. (2003)
12	*B-fruct*	At3g13790	Carbohydrate metabolic process, wound response	Sherson et al. (2003)
13	*CAM1*	At5g37780	Signalling transduction	Reddy et al. (1993)
14	*PINV*	At5g62360	Cellular component	Ascencio-Ibáñez et al. (2008)
15	*PME*	At2g45220	Cell wall modification	Louvet et al. (2006)
16	*NS*	At1g09240	Nicotianamine biosynthetic process, phloem transport	Schuler et al. (2012)
17	*SugT*	At1g08930	Sugar transport	Schneider et al. (2006)
18	*ERD6*	At1g08930	Carbohydrate transmembrane transport	Kiyosue et al. (1998)
19	*CAT8*	At1g17120	Amino acid transmembrane transport	Su et al. (2004), Hanada et al. (2011)
20	*CAT5*	At2g34960	Amino acid transmembrane transport	Su et al. (2004), Hanada et al. (2011)
21	*CAT4*	At3g03720	Amino acid transmembrane transport	Su et al. (2004), Hanada et al. (2011)
22	*CAT7*	At3g10600	Amino acid transmembrane transport	Su et al. (2004), Hanada et al. (2011)
23	*CAT3*	At5g36940	Amino acid transmembrane transport	Su et al. (2004), Hanada et al. (2011)
24	*EXPA1*	At1g69530	Cell wall modification, stress response	Wei et al. (2011)
25	*EXPA2*	At5g05290	Cell wall modification, stress response	Wei et al. (2011)
26	*MT*	At1g07600	Metal response	Ruta et al. (2017)
27	*XET3*	At3g44990	Cell wall biogenesis	Zhu et al. (2012)
28	*MS*	At5g17920	Methionine biosynthetic process	Gallardo et al. (2002)
29	*MIP*	At4g00430	Water transport, iron ion transport	Tian et al. (2016)
30	*MSAM3*	At4g01850	S-adenosylmethionine biosynthetic process	TAIR 10 www.Arabidopsis.org
31	*THI1*	At5g54770	Thiamine biosynthetic process	Garcia et al. (2014)
32	*NRT2*	At1g08090	Nitrate transport	Krapp et al. (2014), Lezhneva et al. (2014)

Table 2 continued

S. No	Gene	Locus ID	Biological function(s)	References
33	*SDH*	At5g51970	Oxidation–reduction process	Aguayo et al. (2013)
34	*IPS*	At2g22240	Respond to phosphate starvation, Metabolic activities	Donahue et al. (2010)
35	*PHT1*	At2g38940	Phosphate ion transport	Liu et al. (2011), Jost et al. (2015)
36	*NR1*	At1g77760	Nitrate assimilation	Krapp et al. (2014)
37	*CES*	At5g09870	Cell wall biosynthesis	Sullivan et al. (2011)
38	*GS*	At1g66200	Nitrate assimilation, Metabolism	Lothier et al. (2011)
39	*GLT1*	At5g16150	Glucose transmembrane transport	Cho et al. (2011)
40	*SWEET9*	At2g39060	Sugar transmembrane transport	Chen et al. (2012)
41	*VGLT1*	At3g03090	Monosaccharide transport	Aluri and Büttner (2007)

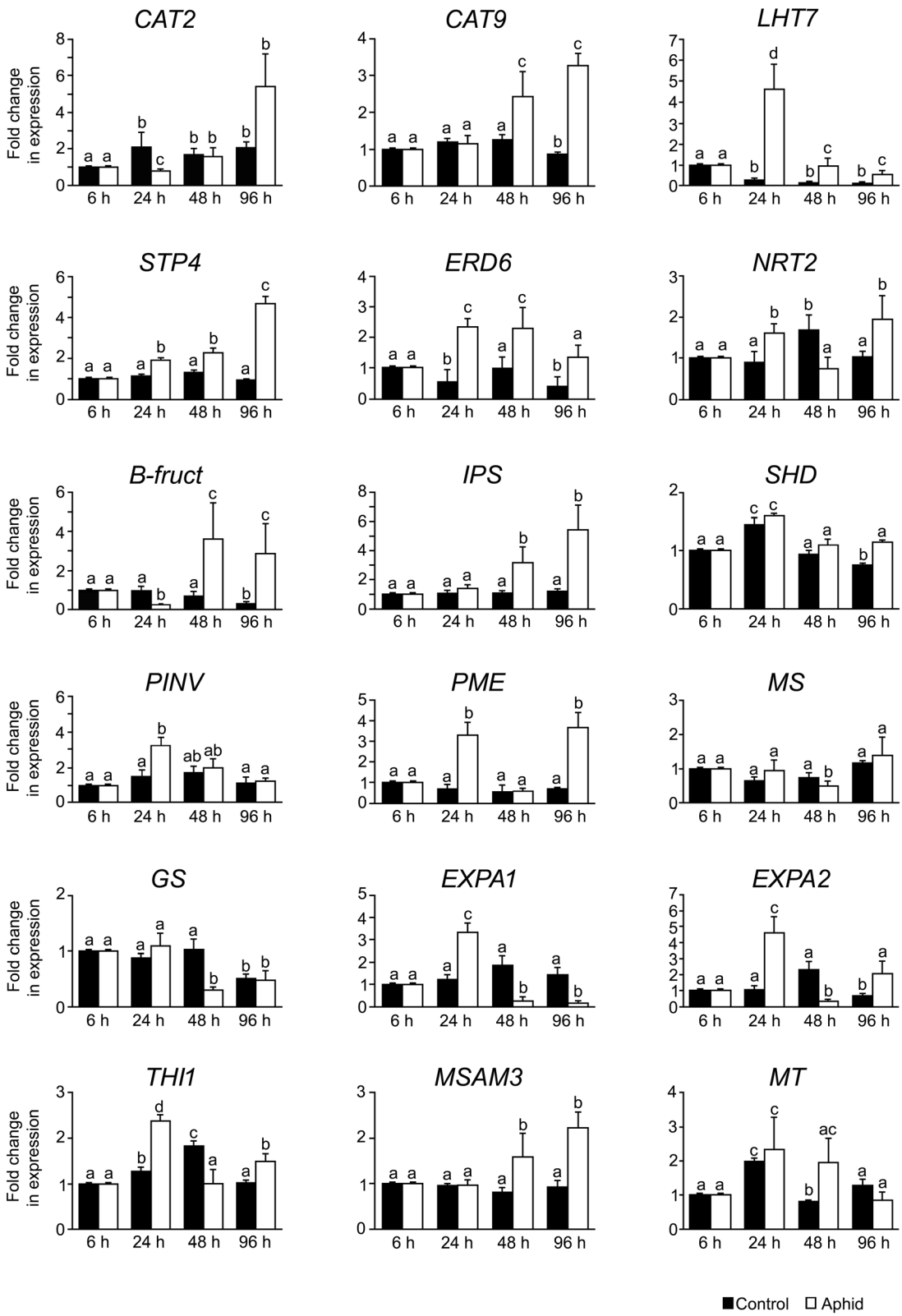
Wound and hormone responsive activity of proBjSTP4 in *N. benthamiana*

In order to assay the activation of proBjSTP4 promoter in response to wounding, *N. benthamiana* leaves Agroinfiltrated with pCAMBIA (proBjSTP4::GUS), were wounded across the midrib after 24 h of Agroinfiltration. Histochemical assay of GUS expression in the wounded leaves showed higher intensity of GUS activity in the wounded leaves compared to the unwounded leaves. The results suggested activation of proBjSTP4 promoter activity by wound response (Fig. 6a). The intensity of blue colour also varied between samples collected at 2 and 6 h post wounding. In parallel, qRT-PCR based assessment of GUS transcript level across the samples reaffirmed the wound mediated activation of the proBjSTP4 promoter activity (Fig. 6b). The quantitative analysis revealed a twofold and sixfold increase of GUS transcript level in wounded samples at 2 h and 6 h time points, respectively, compared to the samples from unwounded plants.

For assessing effect of the defense related hormones viz. methyl jasmonate (MJ) and salicylic acid (SA) on proBjSTP4 promoter activity, the leaves of *N. benthamiana* prior Agroinfiltrated with pCAMBIA (proBjSTP4::GUS) were treated with either MJ or SA in independent experiments. In each experiment, the treated and mock-treated leaf samples were collected at 2 and 6 h after the treatment. Histochemical analysis of GUS expression demonstrated significant increase in GUS expression due to MJ (Fig. 6c, d) and SA treatments (Fig. 6e, f). Transcript analysis by qRT-PCR indicated significant and continuous increase in GUS transcript levels from 2 to 6 h due to treatment with MJ and SA. Without any treatment, basal transcript level of GUS driven by the constitutive promoter CaMV 35S was significantly higher compared to the basal transcript level of GUS driven by proBjSTP4 in mock-treated samples. As expected, there was no GUS activity observed in case of plants infiltrated with empty *Agrobacterium* cells.

Aphid-induced activity of proBjSTP4 in *B. juncea*

The promoter activity of proBjSTP4 in response to infestation by mustard aphids was assayed in Agroinfiltrated *B. juncea* leaves. Forty-to-fifty wingless aphids were released on detached leaves of *B. juncea* after 24 h of Agroinfiltration with the pCAMBIA (proBjSTP4::GUS) construct. Histochemical analysis for GUS was performed in a time course manner on the infested samples. The GUS assay revealed higher accumulation of blue colour in the infested leaves compared to the uninfested leaves (Fig. 7a). A time course experiment indicated increasing level of GUS activity with increased duration of aphid-feeding. qRT-PCR based quantification of GUS transcripts across the samples corroborated the results obtained in histochemical assay (Fig. 7b). Gradual activation in transcript level of *GUS* at 24 and 48 h following the aphid infestation unambiguously demonstrated aphid


CAT2
CAT9
LHT7
STP4
ERD6
NRT2
B-fruct
IPS
SHD
PINV
PME
MS
GS
EXPA1
EXPA2
THI1
MSAM3
MT
Fold change in expression
6 h
24 h
48 h
96 h
Control
Aphid

◀**Fig. 3** Gene expression studies of the aphid-inducible genes in *B. juncea*. The fold-change in expression of the genes across the samples was determined by qRT-PCR and calculated using Pfaffl equation (Pfaffl et al. 2004). Different lower-case alphabets indicate statistically significant difference between the samples. The samples were collected in three biological replicates and each sample assayed in three technical replicates. Significant difference in mean was evaluated by Student's t test at $P < 0.05$ and represented as mean ± SE ($n = 3$)

mediated activation of the proBjSTP4 promoter activity. Also, aphid-induced expression of *GUS* gene under proBjSTP4 promoter at 48 h of feeding was on par with the constitutive expression of *GUS* gene under the CaMV 35S promoter.

Deletion analysis of proBjSTP4 for aphid-responsive promoter activity in *B. juncea*

Based on distribution of the putative *cis*-regulatory elements in proBjSTP4 region, four deletion constructs were designed and named as proSTP4DC1-4. For determining promoter activity, the deletion constructs were Agroinfiltrated in detached leaves of *B. juncea*, followed by the release of aphids. Histochemical analysis revealed that any decrease in the promoter activity in deletion construct proSTP4DC1 (with deletion of − 2017 to − 1618) was insignificant and the promoter activity remained on par with proBjSTP4 and CaMV35S::GUS. However, in deletion construct proSTP4DC2 (with deletion of − 2017 to − 1377) and deletion construct proSTP4DC3 (with deletion of − 2017 to − 918), reduced promoter activity due to the deletions were evident (Fig. 7c). In case of deletion construct proSTP4DC4 (with deletion of − 2017 to − 308), the promoter activity was further reduced significantly compared to the promoter activities in other deletion constructs and proBjSTP4. Transcript analysis by qRT-PCR empirically supported the reduced pattern of expression in the deletion constructs (Fig. 7d). No significant difference in *GUS* transcript level was observed between deletion construct 2 and deletion construct 3. Thus, the deletion analysis revealed indispensable association of the sequence elements up to -1617 upstream region in aphid mediated activation of proBjSTP4 promoter.

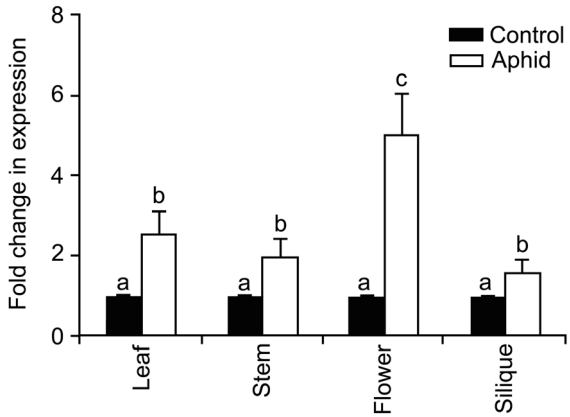
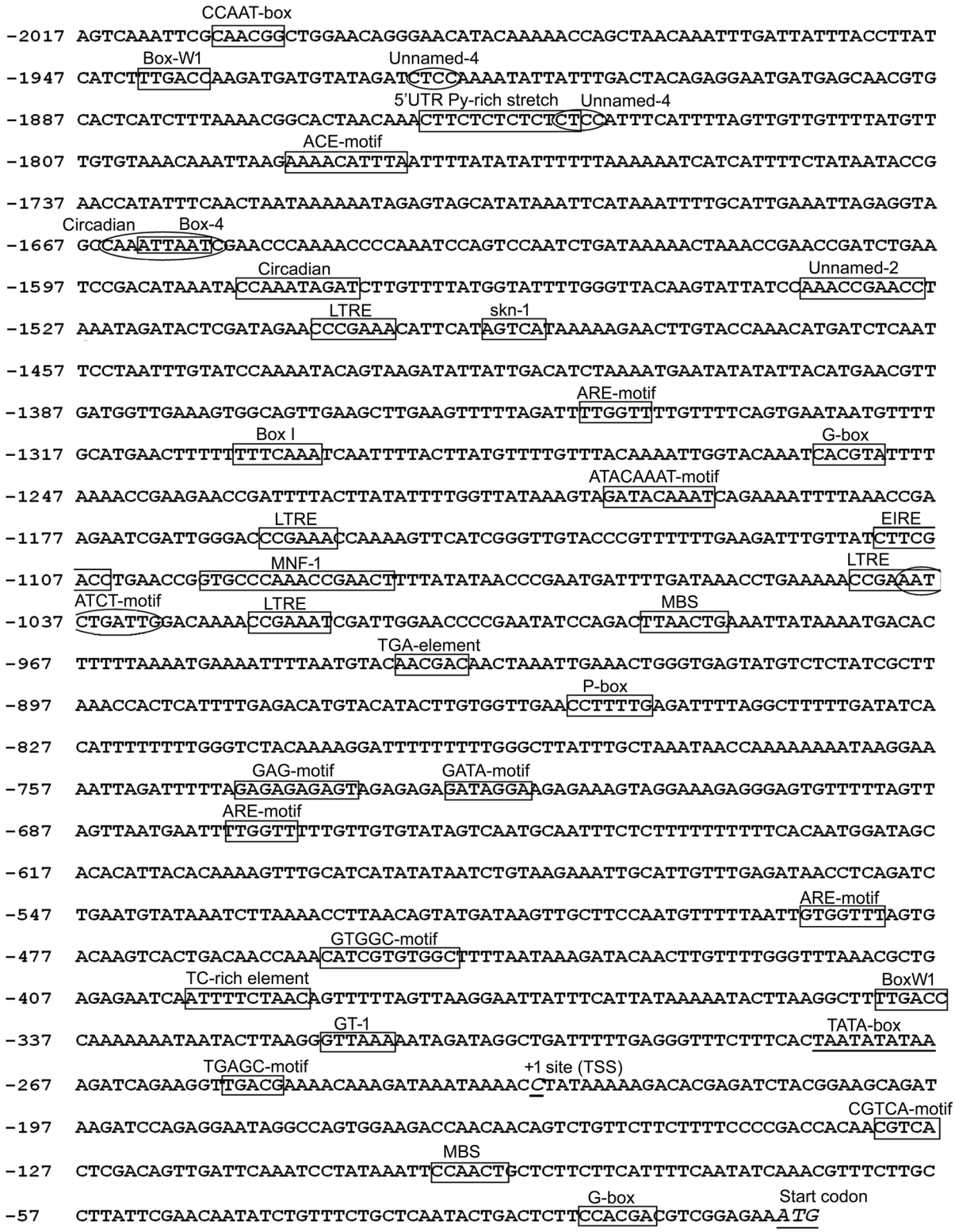


Fig. 4 Gene-expression of *STP4* in different tissues of *B. juncea* under aphid infestation. The fold-change in gene-expression was determined by qRT-PCR and calculated using Pfaffl equation (Pfaffl et al. 2004). The different lower-case alphabets indicate significant difference in mean derived from three biological replicates with three technical replicates each. Significant difference in mean was evaluated by Student's t test at $P < 0.05$ and represented as mean ± SE ($n = 3$)

Discussion

Aphids create strong sink around the host-feeding sites and modulate the source-to-sink relationship of the host towards its own favour (Will et al. 2007). Thus, the majority of genes up-regulated during the early stages of aphid infestation were in the category of multi facilitator super family. These genes are essentially involved in facilitating the transport of carbon and nitrogen assimilates such as sugars, amino acids, etc. along with metabolites to the growing tissues of the plants (Lemoine et al. 2013; Tegeder 2014). Many of the up-regulated transcripts also represent early activated genes as a part of induced defense response against insects. In a susceptible plant-aphid interaction, expressions of defense response genes are short-lived and subjected to host-defense suppression by aphids (De Vos and Jander 2009; War et al. 2012). Thus, up-regulated transcripts which were related to early defense response were not taken into account in this study. Aphids secrete honeydews which attract moulds and bacterial growth on the leaf surface. Therefore, the genes known for early induction due to pathogen attack were also discarded in further narrowing down to the possible candidate genes (Zust and Agrawal 2016). Thus, the screening pipeline primarily considered host metabolic genes related to resource allocation phenomena as these genes have been


CCAAT-box
-2017 AGTCAAATTCGCAACGGCTGGAACAGGGAACATACAAAAACCAGCTAACAAATTTGATTATTTACCTTAT
Box-W1
Unnamed-4
-1947 CATCTTTGACCAAGATGATGTATAGATCTCCAAAATATTATTTGACTACAGAGGAATGATGAGCAACGTG
5'UTR Py-rich stretch
Unnamed-4
-1887 CACTCATCTTTAAAACGGCACTAACAAACTTCTCTCTCTCTCCATTTCATTTTAGTTGTTGTTTTATGTT
ACE-motif
-1807 TGTGTAAACAAATTAAGAAAACATTTAATTTTATATATTTTTTAAAAAATCATCATTTTCTATAATACCG
-1737 AACCATATTTCAACTAATAAAAAATAGAGTAGCATATAAATTCATAAATTTTGCATTGAAATTAGAGGTA
Circadian
Box-4
-1667 GCCAAATTAATCGAACCCAAAACCCCAAATCCAGTCCAATCTGATAAAAACTAAACCGAACCGATCTGAA
Circadian
Unnamed-2
-1597 TCCGACATAAATACCAAATAGATCTTGTTTTATGGTATTTTGGGTTACAAGTATTATCCAAACCGAACCT
LTRE
skn-1
-1527 AAATAGATACTCGATAGAACCCGAAACATTCATAGTCATAAAAAGAACTTGTACCAAACATGATCTCAAT
-1457 TCCTAATTTGTATCCAAAATACAGTAAGATATTATTGACATCTAAAATGAATATATATTACATGAACGTT
ARE-motif
-1387 GATGGTTGAAAGTGGCAGTTGAAGCTTGAAGTTTTTAGATTTTGGTTTTGTTTTCAGTGAATAATGTTTT
Box I
G-box
-1317 GCATGAACTTTTTTTTTCAAATCAATTTTACTTATGTTTTGTTTACAAAATTGGTACAAATCACGTATTTT
ATACAAAT-motif
-1247 AAAACCGAAGAACCGATTTTACTTATATTTTGGTTATAAAGTAGATACAAATCAGAAAATTTTAAACCGA
LTRE
EIRE
-1177 AGAATCGATTGGGACCCGAAACCAAAAGTTCATCGGGTTGTACCCGTTTTTTGAAGATTTGTTATCTTCG
MNF-1
LTRE
-1107 ACCTGAACCGGTGCCCAAACCGAACTTTTATATAACCCGAATGATTTTGATAAACCTGAAAAACCGAAAT
ATCT-motif
LTRE
MBS
-1037 CTGATTGGACAAAACCGAAATCGATTGGAACCCCGAATATCCAGACTTAACTGAAATTATAAAATGACAC
TGA-element
-967 TTTTTAAAATGAAAATTTTAATGTACAACGACAACTAAATTGAAACTGGGTGAGTATGTCTCTATCGCTT
P-box
-897 AAACCACTCATTTTGAGACATGTACATACTTGTGGTTGAACCTTTTGAGATTTTAGGCTTTTTGATATCA
-827 CATTTTTTTTGGGTCTACAAAAGGATTTTTTTTTGGGCTTATTTGCTAAATAACCAAAAAAAATAAGGAA
GAG-motif
GATA-motif
-757 AATTAGATTTTTAGAGAGAGAGTAGAGAGAGATAGGAAGAGAAAGTAGGAAAGAGGGAGTGTTTTTAGTT
ARE-motif
-687 AGTTAATGAATTTTGGTTTTTGTTGTGTATAGTCAATGCAATTTCTCTTTTTTTTTTTCACAATGGATAGC
-617 ACACATTACACAAAAGTTTGCATCATATATAATCTGTAAGAAATTGCATTGTTTGAGATAACCTCAGATC
ARE-motif
-547 TGAATGTATAAATCTTAAAACCTTAACAGTATGATAAGTTGCTTCCAATGTTTTTAATTGTGGTTTAGTG
GTGGC-motif
-477 ACAAGTCACTGACAACCAAACATCGTGTGGCTTTTAATAAAGATACAACTTGTTTTGGGTTTAAACGCTG
TC-rich element
BoxW1
-407 AGAGAATCAATTTTCTAACAGTTTTTAGTTAAGGAATTATTTCATTATAAAAATACTTAAGGCTTTTGACC
GT-1
TATA-box
-337 CAAAAAAATAATACTTAAGGGTTAAAAATAGATAGGCTGATTTTTGAGGGTTTCTTTCACTAATATATAA
TGAGC-motif
+1 site (TSS)
-267 AGATCAGAAGGTTGACGAAAACAAAGATAAATAAAACCTATAAAAAGACACGAGATCTACGGAAGCAGAT
CGTCA-motif
-197 AAGATCCAGAGGAATAGGCCAGTGGAAGACCAACAACAGTCTGTTCTTCTTTTCCCCGACCACAACGTCA
MBS
-127 CTCGACAGTTGATTCAAATCCTATAAATTCCAACTGCTCTTCTTCATTTTCAATATCAAACGTTTCTTGC
G-box
Start codon
-57 CTTATTCGAACAATATCTGTTTCTGCTCAATACTGACTCTTCCACGACGTCGGAGAAAATG

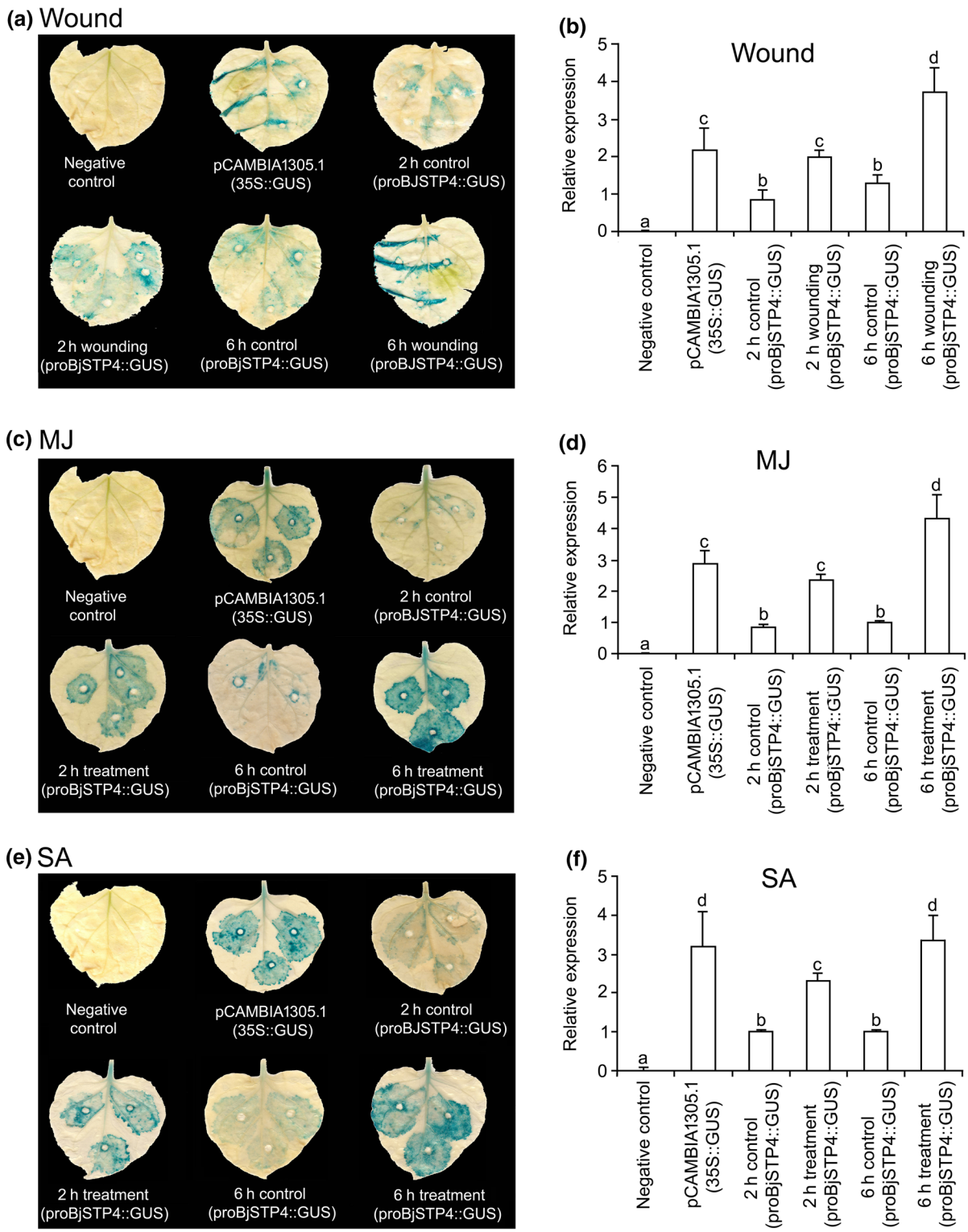
◀**Fig. 5** Mapping of *cis*-regulatory elements in proBjSTP4. The putative *cis*-regulatory elements in proBjSTP4 upstream sequence was predicted by using PlantCare software and indicated in boxes and circles. The + 1 site and italicized bold letters indicate the TSS and translation initiation codon of the *STP4* gene, respectively

hypothesized to be activated by aphids with sustained expression (Dorschner et al. 1987; Sandström et al. 2000; Rehill and Schultz 2003; Girousse et al. 2005; Goggin 2007). However, their tissue and temporal specificity in gene-expression might differ from one species to another (Sunilkumar et al. 2002; Zhu-Salzman et al. 2004; Heidel and Baldwin, 2004; Divol et al. 2005; Appel et al. 2014). Therefore, gene-expression of the in silico identified genes were validated for their temporal expression pattern upon aphid infestation in *B. juncea* (Fig. 3). In further comparison among the identified genes, three criteria: minimal basal level of expression, rapid induction in response to aphids and sustained expression have been adhered to identify the most appropriate candidate gene for the promoter isolation. Minimal basal level of expression in case of transgene expression by such promoter is likely to minimize metabolic burden on the host plant in absence of the stress.

The monosaccharide transporter gene *STP4* showed gradual increase in gene-expression as the aphids rapidly multiplied on the *B. juncea* plants. The uptake of carbon assimilates from the source tissues (reservoir) and loading it towards the growing tissues (sink) is the key role of sugar transporter proteins (Truernit et al. 1996; Fotopoulos et al. 2003). Earlier, the *STP4* gene was characterized during wounding and bacterial infection in *Arabidopsis* (Truernit et al. 1996). Subsequently, its expression was also analysed during aphid infestation in *Arabidopsis* which showed tenfold increase in transcript levels due to aphid feeding (Moran and Thompson 2001). Thus, multi-fold increase in *STP4* expression during aphid colonization in *B. juncea* as shown in this study was consistent with the previous results. Additionally, transcriptional activation of *STP4* gene along with the gene encoding invertase enzyme during growth and development in *Arabidopsis* strongly supported the involvement of STP4 in maintaining the source-to-sink relationship in plants (Sherson et al. 2003). In the present investigation, induced expression of *STP4* gene was observed in leaves, stem, flowers and siliques of aphid infested *B. juncea* plants. Since leaves, flowers and siliques constitute the major feeding sites for the aphids, regulation of *STP4* gene-expression was expected to be more evident in these tissues.

With the availability of genome sequences of various plant species, it has become advantageous to predict upstream promoter sequence of a gene (Kim et al. 2005). During the course of this investigation, genome sequence of *B. juncea* was not available. *B. rapa* is one of the progenitors in amphidiploid genome of *B. juncea* (Kaur et al. 2014). Therefore, the available genomic resources of *B. rapa* was utilized for identification of the upstream sequence of *STP4*. Based on the genomic organization of *STP4* in *B. rapa*, a pair of primers were designed and the homologous upstream counterpart in *B. juncea* was cloned. Though, the genome of *B. juncea* is amphidiploid the pair of primers led to the amplification of a single amplicon possibly targeting the *STP4* paralogue which was descended from *B. rapa* and conserved the sequence homology.

The *cis*-regulatory elements in the promoter sequence play pivotal role in interactions with the transcriptional factors for tissue and stress specific expression of a gene (Davuluri et al. 2003; Kaur et al. 2017). The putative *cis*-regulatory elements identified on the promoter proBjSTP4 were found to be mostly associated with gene-expression during growth and development, biotic stresses including pathogen infection, elicitor treatment, treatments by defense hormones, wounding and insect-infestation. For functional analysis of the putative promoter, Agroinfiltration mediated transient assay was used. Transient assay has been preferred because of its technical simplicity which led to rapid assay of a large number of constructs. Also, as it did not involve any plant transformation or gene integration event, confounding effect of the site of integration or positional effect on the promoter activity could be eliminated. In functional assays, proBjSTP4 promoter activity was found to be activated by wound, MJ and SA treatment in a


(a) Wound
Negative control
pCAMBIA1305.1 (35S::GUS)
2 h control (proBJSTP4::GUS)
2 h wounding (proBjSTP4::GUS)
6 h control (proBjSTP4::GUS)
6 h wounding (proBJSTP4::GUS)
(b)
Wound
Relative expression
0
1
2
3
4
5
a
c
b
c
b
d
Negative control
pCAMBIA1305.1 (35S::GUS)
2 h control (proBjSTP4::GUS)
2 h wounding (proBjSTP4::GUS)
6 h control (proBjSTP4::GUS)
6 h wounding (proBjSTP4::GUS)
(c) MJ
Negative control
pCAMBIA1305.1 (35S::GUS)
2 h control (proBJSTP4::GUS)
2 h treatment (proBjSTP4::GUS)
6 h control (proBjSTP4::GUS)
6 h treatment (proBjSTP4::GUS)
(d)
MJ
Relative expression
0
1
2
3
4
5
6
a
c
b
c
b
d
Negative control
pCAMBIA1305.1 (35S::GUS)
2 h control (proBjSTP4::GUS)
2 h treatment (proBjSTP4::GUS)
6 h control (proBjSTP4::GUS)
6 h treatment (proBjSTP4::GUS)
(e) SA
Negative control
pCAMBIA1305.1 (35S::GUS)
2 h control (proBJSTP4::GUS)
2 h treatment (proBjSTP4::GUS)
6 h control (proBjSTP4::GUS)
6 h treatment (proBjSTP4::GUS)
(f)
SA
Relative expression
0
1
2
3
4
5
a
d
b
c
b
d
Negative control
pCAMBIA1305.1 (35S::GUS)
2 h control (proBjSTP4::GUS)
2 h treatment (proBjSTP4::GUS)
6 h control (proBjSTP4::GUS)
6 h treatment (proBjSTP4::GUS)

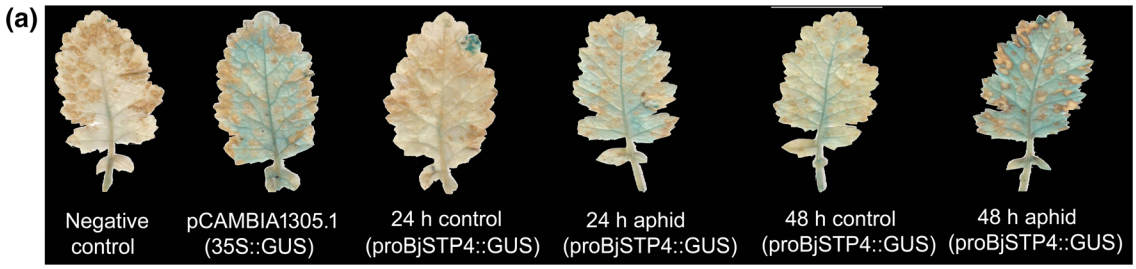
◀**Fig. 6** Activation of proBjSTP4 promoter activity. Histochemical analysis of GUS-activity and qRT-PCR based quantification of GUS-transcripts following the treatment of wounding (**a**, **b**), MJ (**c**, **d**) and SA (**e**, **f**) in Agroinfiltrated leaves of *N. benthaminana*. A positive (pCAMBIA1305.1) and negative (Agrobacterium without vector) control were included for validation of the experimental procedure. Different lower-case alphabets indicate significant difference in mean, derived from three biological replicates with three technical replicates each. Significant difference in mean was evaluated by student's t-test at $P < 0.05$ and represented as mean ± SE ($n = 3$)

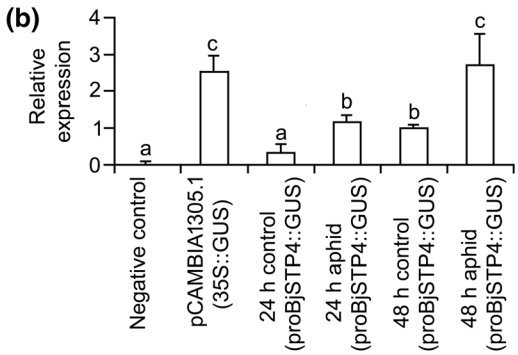
gradual manner from 2 to 6 h of the treatments and peaked higher compared to the CaMV 35S. However, the basal promoter activity of proBjSTP4 in control plants was significantly lower compared to the CaMV 35S promoter. It empirically demonstrated the potential applicability of proBjSTP4 in reducing metabolic burden of constitutive transgene-expression and at the same time high inducibility of proBjSTP4 under aphid-stress in the host plant.

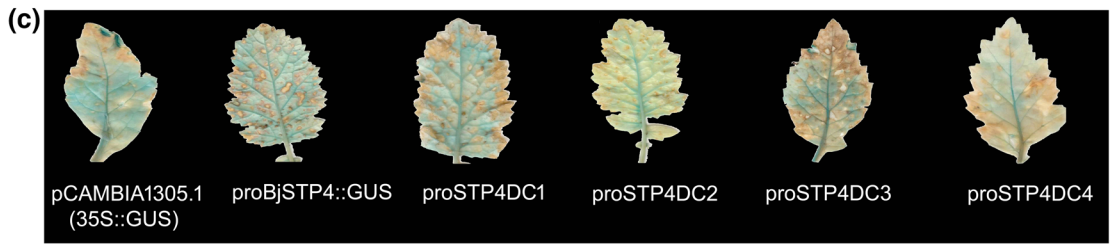
Conventionally, for elucidating the minimal functional region of a promoter, deletion analysis is carried out in promoter analysis (Sugaya and Uchimiya 1992; Ijaz et al. 2020). It is carried out to shorten the promoter length required for stand-alone function of the promoter and also for explicating specific role of the *cis*-regulatory elements of the promoter (Mahoney et al. 2016). In the present study, four deletion constructs of proBjSTP4 were generated in order to identify the indispensable regions of the promoter for aphid-responsive promoter-activity. Histochemical analysis of GUS activity in conjunction with qRT-PCR based transcript analysis suggested that, deletion of 5′ end (− 2017) up to − 1618 (deletion 1) did not result into any significant decrease in aphid-responsive promoter activity. Thus, the identified elements over this region viz. CCAAT box, Box-W1, 5′UTR Py-rich repeat, ACE motif, Box-4, circadian-regulated elements, LTRE and skn-1 presumably were not very significant to proBjSTP4 activity; though these elements were found to be crucial for the inducible activity of many promoters (Sun et al. 2003; Xu et al. 2006; Mikkelsen and Thomashow 2009; Gao et al. 2016; Kaur et al. 2017). However, in case of deletion construct proSTP4DC2, with further deletion of − 1616 to − 1377, the aphid-responsive promoter activity was significantly diminished indicating indispensability of this region (Fig. 5). A further deletion to − 916 bp in deletion construct proSTP4DC3 led to similar reduced promoter activity with no further significant decrease compared to proSTP4DC2. Thus, the deletion analysis suggested that, the putative *cis*-elements viz CAAT-box, Box-W1, 5′UTR Py-rich repeat, ACE motif, Box-4, circadian-regulated elements, LTRE, skn-1 along with some unknown *cis*-elements present in -1616 to -1377 region of the *STP4* were associated with the aphid-responsive promoter activity of proBjSTP4. However, further study based on a greater number of deletions, tetramerization of putative motifs, use of synthetic promoters, etc. needs to be carried out for identifying the minimal promoter region associated with the aphid responsive activity (Ali and Kim 2019).

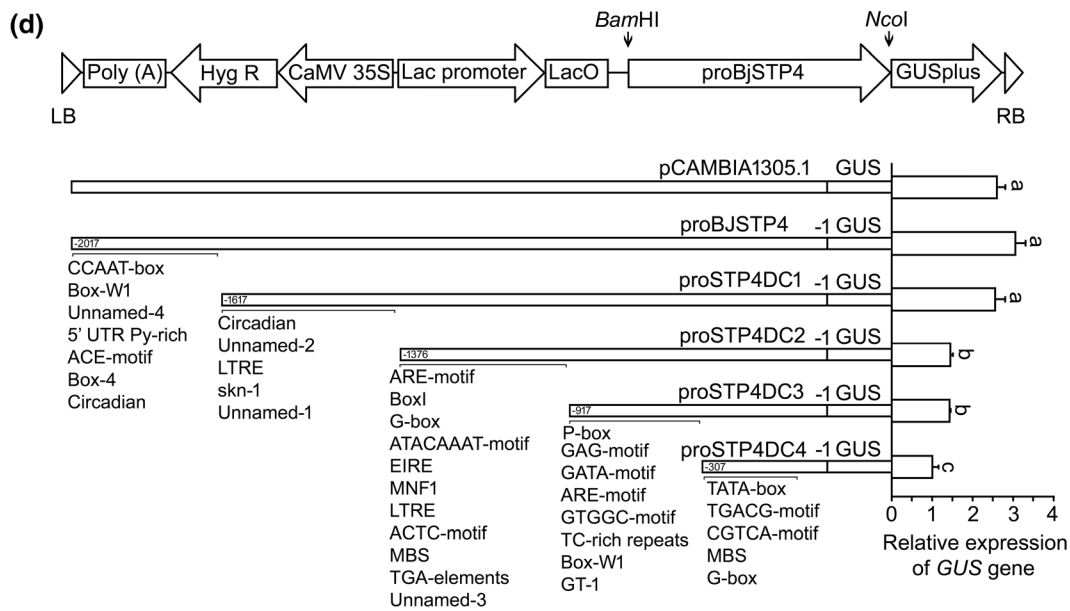
Conclusions

Developing varietal resistance against aphids is constrained due to unavailability of resistance genes within the crossable germplasms in case of many of the major crop species including *B. juncea*. Thus, transgenic strategy has been considered as a potential alternative for developing aphid resistance. Use of an inducible promoter with temporal specificity instead of constitutive promoters for transgene expression can potentially decrease metabolic burden on the host plants and pleiotropic effects. Gene expression pattern of *STP4* conformed its aphid-inducible expression in the major aphid-feeding tissues of the oilseed crop, *B. juncea*. Cloning of the upstream intergenic region of *STP4* and further deletion analysis demonstrated that the 1617 bp upstream region of *STP4* can be potentially used as a promoter for driving aphid-responsive transgene-expression. With much lower basal activity, this promoter can potentially reduce undesirable constitutive expression of the transgene and can be activated by aphid-attack leading to high level gene-expression, comparable to constitutive gene-expression by CaMV 35S. The promoter shall remain useful for engineering inducible expression of plant immunity genes in reversing host-defense suppression by aphids in susceptible hosts.


(a)
Negative control
pCAMBIA1305.1 (35S::GUS)
24 h control (proBjSTP4::GUS)
24 h aphid (proBjSTP4::GUS)
48 h control (proBjSTP4::GUS)
48 h aphid (proBjSTP4::GUS)


(b)
Relative expression
0
1
2
3
4
a
c
a
b
b
c
Negative control
pCAMBIA1305.1 (35S::GUS)
24 h control (proBjSTP4::GUS)
24 h aphid (proBjSTP4::GUS)
48 h control (proBjSTP4::GUS)
48 h aphid (proBjSTP4::GUS)


(c)
pCAMBIA1305.1 (35S::GUS)
proBjSTP4::GUS
proSTP4DC1
proSTP4DC2
proSTP4DC3
proSTP4DC4


(d)
BamHI
NcoI
Poly (A)
Hyg R
CaMV 35S
Lac promoter
LacO
proBjSTP4
GUSplus
LB
RB
pCAMBIA1305.1 GUS
proBJSTP4 -1 GUS
proSTP4DC1 -1 GUS
proSTP4DC2 -1 GUS
proSTP4DC3 -1 GUS
proSTP4DC4 -1 GUS
-2017
-1617
-1376
-917
-307
CCAAT-box
Box-W1
Unnamed-4
5' UTR Py-rich
ACE-motif
Box-4
Circadian
Circadian
Unnamed-2
LTRE
skn-1
Unnamed-1
ARE-motif
BoxI
G-box
ATACAAAT-motif
EIRE
MNF1
LTRE
ACTC-motif
MBS
TGA-elements
Unnamed-3
P-box
GAG-motif
GATA-motif
ARE-motif
GTGGC-motif
TC-rich repeats
Box-W1
GT-1
TATA-box
TGACG-motif
CGTCA-motif
MBS
G-box
a
a
a
b
b
c
0 1 2 3 4
Relative expression of GUS gene

◀**Fig. 7** Aphid-responsive promoter activity of proBjSTP4 (**a**, **b**) and its deletion constructs (**c**, **d**). The promoter activity was assessed through histochemical analysis of GUS-activity and qRT-PCR based quantification of GUS-transcripts. Mean was derived from three biological replicates with three technical replicates each time and different lower-case letters indicate significant difference in mean, evaluated by student's t-test at $P < 0.05$

Acknowledgements This work was supported by Post Graduate School, IARI through a Ph.D fellowship to CR and DST-SERB grant (EMR/2017/003463) to RB.

Supporting information

Supplementary Fig. 1 Assay of pCAMBIA-proBjSTP4 construct for bacterial expression. The Agrobacterium cells harbouring the pCAMBIA-proBjSTP4 construct was incubated at 37 °C for 1 h along with GUS assay buffer. Agrobacterium strain harbouring pBI121 (35S::GUS) and pORER2 (35S::GUS) were taken as positive control. **Supplementary Table 1** Details of gene-specific primers used for qRT-PCR analysis. **Supplementary Table 2** List of primers used for amplification of proBjSTP4 and deletion fragments. **Supplementary Table 3** Characteristics features of putative *cis*-regulatory elements in proBjSTP4.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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