Funct Integr Genomics
DOI 10.1007/s10142-016-0516-x

ORIGINAL ARTICLE

Characterization of *VvPAL-like* promoter from grapevine using transgenic tobacco plants

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Received: 18 September 2015 / Revised: 2 August 2016 / Accepted: 8 August 2016

Abstract A 2000-bp 5′-flanking region of *VvPAL-like* was isolated from 'Summer Black' grapevine by PCR amplification, named *pVvPAL-like*. To gain a better understanding of the expression and regulatory mechanism of *VvPAL-like*, a chimeric expression unit consisting of the β-glucuronidase (GUS) reporter gene under the control of a 2000-bp fragment of the *VvPAL-like* promoter was transformed into tobacco via *Agrobacterium tumefaciens*. Histochemical staining showed that the full-length promoter directs efficient expression of the reporter gene in cotyledons and hypocotyls, stigma, style, anthers, pollen, ovary, trichomes, and vascular bundles of transgenic plants. A series of 5′ progressive deletions of the promoter revealed the presence of a negative regulatory region (−424 to −292) in the *VvPAL-like* promoter. Exposure of the transgenic tobacco plants to various abiotic stresses demonstrated that the full-length construct could be induced by light, copper (Cu), abscisic acid (ABA), indole-3-acetic (IAA), methyl jasmonate (MeJA) (*N*-1-naphthylphthalamic acid), ethylene, and drought. Furthermore, the ethylene-responsive region was found to be located in the −1461/−930 fragment, while the element(s) for the MeJA-responsive expression may be present in the −424/−292 region in the *VvPAL-like* promoter. These findings will help us to better understand the molecular mechanisms by which *VvPAL-like* participates in biosynthesis of flavonoids and stress responses.

Keywords Phenylalanine ammonia-lyase-like · Promoter · Transgenic tobacco · Spatial and temporal expression · Abiotic stress

Abbreviations

TIBA	2,3,5-Triiodobenzoic acid
2,4-D	2,4-Dichlorophenoxya-cetic acid
MU	4-Methylumbelliferone
MUG	4-Methylumbelliferyl glucuronide
X-Gluc	5-Bromo-4-chloro-3-indyle-β-D-glucuronide
ABA	Abscisic acid
ARFAT	Auxin response element
BG	Big green berries
BSA	Bovine serum albumin
CTAB	Cetyltriethylammnonium bromide,
FR	Full red berries
Genoscope	Grape Genome Browser
HR	Harvesting red berries
IAA	Indole-3-acetic
IBA	Indole-3-butyric acid
IR	Initial red berries
IPA	Isopentenyladenosine
MRE	Metal response element
MeJA	Methyl jasmonate
NPA	*N*-1-naphthylphthalamic acid

Electronic supplementary material The online version of this article (doi:10.1007/s10142-016-0516-x) contains supplementary material, which is available to authorized users.

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Published online: 25 August 2016

NAA	1-Naphthaleneacetic acid
NCBI	National Center for Biotechnology Information
PR	Partial red berries
PAL	Phenylalanine ammonia-lyase
qRT-PCR	Quantitative real-time polymerase chain reaction
SA	Salicylic acid
SG	Small green berries
TSS	Transcriptional start sites
T-DNA	Transfer DNA
GUS	β-Glucuronidase gene

Introduction

Color development of flowers and fruit has been an important evolutionary trait in plant growth. Fruit color is one of the characteristics that are important to the value of fruit commercially and aesthetically. The color of flowers and fruit is a blend of chlorophyll, carotenoids, and flavonoids. Flavonoids are secondary metabolites widespread among plants and act as UV and pathogen protectants as well as signal molecules in the interaction between bacteria and plants (Dixon and Steele 1999; Harborne and Grayer 1994). Anthocyanins, a subclass of flavonoids, are the main pigments in flowers and fruits, where they serve as insect and animal attractants, respectively, thus playing an important role in the ecology of pollination and seed dispersal (Harborne 1993; Holton and Cornish 1995). Anthocyanin biosynthesis has been extensively studied in many plants such as maize (*Zea mays*), petunia (*Petunia hybrida*), and snapdragon (*Antirrhinum majus*) (Holton and Cornish 1995), and the biosynthetic pathway is one of the well-known pathways in plants (Dixon and Steele 1999).

Phenylalanine ammonia-lyase (*PAL*) catalyzes the conversion of phenylalanine to *trans*-cinnamic acid in the initial step of anthocyanin biosynthetic pathway, thus controlling the flux of primary metabolites into this major secondary metabolic pathway. *PAL* was one of the first identified plant "defense genes" and could be induced by several factors, including pathogen infection, wounding, nutrient depletion, UV irradiation, extreme temperatures, and other stress conditions (Cramer et al. 1985; Dixon and Paiva 1995; Edwards et al. 1985; Kuhn et al. 1984; Lawton et al. 1983; Liang et al. 1989a; Liang et al. 1989b). *PAL* genes have been isolated and sequenced from a number of plant species, including gymnosperm, monocots, and herbaceous and woody dicots. In most plant species, *PAL* genes occur in small families of two to six members (Cramer et al. 1989; Frank and Vodkin 1991; Gowri et al. 1991; Lee et al. 1992; Lois et al. 1989; Minami and Tanaka 1993; Minami et al. 1989; Ohl et al. 1990; Subramaniam et al. 1993; Tanaka et al. 1989). *PAL* activity is modulated in plants during the development process of different tissues and in response to environmental signals. Organ-specific *PAL* messenger RNA (mRNA) expression patterns have been described in parsley, *Arabidopsis thaliana*, potato, bean, alfalfa, and poplar (Gowri et al. 1991; Jots and Hahlbrock 1992; Liang et al. 1989a; Liang et al. 1989b; Lois and Hahlbrock 1992; Ohl et al. 1990), and some *PAL* gene family members are induced by fungal elicitor or fungal infection of plant tissues in bean, parsley, pea, potato, and *A. thaliana* (Collinge and Slusarenko 1987; Jots and Hahlbrock 1992; Kawamata et al. 1992; Liang et al. 1989a; Lois et al. 1989; Yamada et al. 1992), and by bacterial pathogens (Dong et al. 1991; Keith 1991; Wanner et al. 1993). In summary, *PAL* has been studied extensively by molecular and genetic approaches during plant growth and development and in response to environmental stresses.

As an important component of eukaryote gene expression regulation, the promoter plays an important role in lighting, spatial, and temporal regulation of gene expression via controlling the efficiency and frequency of start. In order to adapt to different environments, various *cis*-elements in the promoter respond to elicitors outside to control the expression of genes. It has been reported that numerous *cis*-elements are involved in the response of signal molecule, stimuli of light, and invading bacteria (Boter et al. 2004; Kiran et al. 2006; Wang et al. 2004a, 2004b). Previous findings indicated that *PAL* promoters played important roles during the various developmental stages and stress conditions in plants. The results of transgenic experiments have showed that the GUS gene is expressed in a spatial and temporal pattern under the control of *PAL* promoters (Gray-Mitsumune et al. 1999; Osakabe et al. 2009; Wong et al. 2012). In addition, *PAL* promoter of sugar beet is involved in recognition of the repression signal from fungal pathogens. As the expression and enzyme activity level of *PAL* reduced, plant defense reactions were repressed resulting in plant diseases (Schmidt et al. 2004). *PAL* promoter in *Pinus taeda* significantly increased the activity of genes for lignin biosynthesis under bending stress (Osakabe et al. 2009). Moreover, the study on *Arabidopsis PAL* promoter demonstrated that it could also be induced by wounding, $HgCl_2$ stress, and light (Subramaniam et al. 1993).

In this study, a gene (*GSVIVG00013928001*) shared same domain (PLN02457) and high homology with the reported *PAL* from *Vitis vinifera* (GenBank: EF192469.1) and was named *VvPAL-like*. To the best of our knowledge, the systematic study of *VvPAL-like* in grapes has not been previously reported. Therefore, considering the significance of the promoter in gene expression regulation, it is necessary to investigate the regulatory mechanism and functional properties of *VvPAL-like* promoter. The aims of the present work were to elucidate the nature of the *cis*-acting elements within the *VvPAL-like* promoter and to evaluate the functions of *VvPAL-like* promoter using the reporter GUS gene in transgenic tobacco plants. Additionally, a series of different lengths of 5′-franking region of *VvPAL-like* gene were

fused to the GUS reporter gene to identify the critical regions for the *VvPAL-like* promoter activity.

Materials and methods

Plant materials, growth conditions, and extractions of genomic DNA

Based on the changes of fruit size and color, the fruit development processes of grapevine were divided into six different visual stages: small green berries (SG), big green berries (BG), initial red berries (IR), partial red berries (PR), full red berries (FR), and harvesting red berries (HR) at about 20, 40, 55, 70, 85, and 100 days after flowering, respectively. They were collected in 2014 from 5-year-old 'Summer Black' grapevine (hybrids of *V. vinifera* and *Vitis labrusca*) trees grown under common field conditions at the Nanjing Agricultural University fruit farm, Nanjing, China. In addition, roots, stems, leaves (30 days), tendrils, and flowers (unopened) were also collected. The leaves used in this study were prepared by removing and discarding the central and parallel veins. All the samples were immediately frozen in liquid nitrogen and stored at −80 °C until used. Genomic DNA was isolated from 100 to 200 mg of grapevine leaves using Plant Genome DNA Extraction Kit of Beijing Sunbiotech Co. Ltd according to the manufacturer's instructions. The DNA concentration was measured by gel electrophoresis using spectrophotometer. Then, the DNA extract was kept at −20 °C until use. The genomic DNA was used as templates for PCR of the 5′-flanking regions of *VvPAL-like* gene.

Tobacco seeds (*Nicotiana tabacum* L. cv. Xanthi) were sterilized in 50 % bleach solution for 10 min followed by three rinses with sterilized water and then planted on culture bottles containing Murashige and Skoog (MS) medium and sprouting. The sterile seedlings were cultured in a constant-temperature room at 22 °C and 70 % humidity under 16-h light/8-h dark cycles in our laboratory. Tobacco leaves were collected and used for transformation and plant regeneration according to Burrow et al. (1990). Transgenic tobacco plants were transplanted into soil pots (20 cm × 20 cm) and grown in a greenhouse under the normal growth conditions by irrigating them daily until they reached the reproductive stage.

RNA isolation and qRT-PCR

Total RNA was extracted using an RNA extraction kit (SV Total RNA Isolation System; Promega, Madison, WI, USA) according to the manufacturer's instructions. Genomic DNA was removed by 15-min incubation at 37 °C with RNase-Free DNase (TaKaRa, Otsu, Japan) followed by an RNA Clean Purification Kit (BioTeke, Beijing, China). The purity and integrity of RNA were analyzed both by agarose gel electrophoresis and by the A260/A230 and A260/A280 ratios. To generate first-strand cDNA, 3 μg of total RNA was reverse-transcribed using a SMARTTM RACE cDNA Synthesis Kit (TaKaRa) according to the manufacturer's protocol. Quantitative real-time (qRT)-PCR was carried out as described (Jia et al. 2015). Primers used for real-time PCR, designed using Primer 5 software (http://www.premierbiosoft.com/), were P1 (forward, 5′-TTAA AATGGCTGGGATCG AG-3′) and P2 (reverse, 5′-CCTGCATCACTTCAGCAAAA-3′). *Actin* was used as an internal control gene using the following primers: forward, 5′-TCACCACTACTGCT GAACG-3′; reverse, 5′-CTTCTGGACAACGGAATC-3′. The primer pairs were tested by PCR.

Real-time PCR reactions (20 μL) contained 10 μL SYBR Premix ExTaq (perfect real-time buffer contained dNTPs, $MgCl_2$, and DNA polymerase; Takara, Japan), 0.4 μL 10 μm forward specific primer, 0.4 μL 10 μm reverse specific primer (Sangon, Shanghai, People's Republic of China), and 2 μL cDNA template. The mixture was placed in a Bio-Rad iQ5 Sequence Detector (Bio-Rad, Hercules, CA, USA), and DNA amplification was conducted using the following program: 1 cycle of 95 °C for 2 min, and 40 cycles of template denaturation at 94 °C for 20 s, primer annealing at 56 °C for 20 s, and primer extension at 72 °C for 30 s, and 71 cycles increasing from 60 to 95 °C at 0.5 °C per cycle for 30 s. The sequence detector was programmed to measure fluorescence only during the annealing step. At this temperature, no incorporated uniprimer was in the hairpin conformation contributing to fluorescence measurements.

Bioinformatics analysis

All the sequence data were released at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/) and Grape Genome Browser (Genoscope) (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis). To detect putative *cis*-acting regulatory elements of *VvPAL-like* promoter sequence cloned, the database PLACE (Higo et al., 1999) and PlantCARE (Lescot et al. 2002) were used. The databases are accessible at http://www.dna.affrc.go.jp/PLACE/ and http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, respectively. Multiple sequence alignment was carried out in ClustalX 2.1 software (http://www.clustal.org/clustal2/) and BoxShade online software(http://www.ch.embnet.org/software/BOX_form.html).

The Conserved Domain database (CDD) was used for predicting the conserved domain of PAL family (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The consequential alignment was used to create a phylogenetic tree in MEGA version 4.0 (http://www.megasoftware.net/mega.html). The sequence analysis was undertaken using the BLAST computer program at http://www.ncbi.nlm.nih.gov.

PCR cloning of the *VvPAL-like* gene promoter region

The grapevine genomic DNA obtained above was used as a template for amplifying the 5′-flanking regions of *VvPAL-like* gene with forward primers pF0: 5′-TGACCCAAA CCACAAAGT-3′ and the reverse primer pR0: 5′-GAAT CCGCACAAAGAAAC-3′. The reactions were performed in a final volume of 20 μL, containing grapevine genomic DNA 1 μL, forward primer 0.6 μL, reverse primer 0.6 μL (Sangon, Shanghai, People's Republic of China), dNTPs (10.0 mmol/L) 0.8 μL, *ExTaq* 0.2 μL, 10× GC buffer 2 μL, and ddH_2O 14.8 μL. The conditions of the PCR were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, with a final extension of 72 °C for an additional 10 min. The PCR products were detected by electrophoresis on a 1 % agarose gel, and the target band was cut and purified by AxyPrep™ DNA Gel Extraction Kit [Axygen Biosciences (Hangzhou Co., Ltd.)].

Construction of plant transformation vectors harboring different deletion constructs

The binary vector pCAMBIA1304 was used as tobacco transformation in this study. A 2000-bp fragment upstream of the translational start codon of the *VvPAL-like* gene and its four shorter promoter fragments of progressive 5′ upstream deletions were generated by PCR with five forward primers (pF1, pF2, pF3, pF4, and pF5) in addition to pF0 and one reverse primer (pR1). These primer sequences are as follows: pF1, 5′-CTGCAGTGACCCAAACCACAAAGT-3′; pF2, 5′-CTGCAGAGAAGTGTCCATAATAGTGG-3′; pF3, 5′-CTGCAGTAGCCACCCACGAAACACTC-3′; pF4, 5′-CTGCAGGGCTAACAACCTCCACATCC-3′; pF5, 5′-CTGCAGCATCCAGCCCACCTACCAGT-3′; and pR1, 5′-AGATCTGAATCCGCACAAAGAAAC-3′ (introduced *Pst*I and *Bgl*II sites are underlined). After digestion, these DNA fragments were ligated into the *Pst*I and *Bgl*II sites of pCAMBIA1304 and a series of pCAMBIA1304-p*VvPAL-like* vectors were produced under the names of P2000 (−1999/+1), P1461 (−1460/+1), P930 (−929/+1), P424 (−423/+1), and P292 (−291/+1). Ligation reactions were carried out using T4 DNA ligase (Promega, USA). These ligation products were transferred into *Escherichia coli* (*E. coli*) DH5a, and positive clones were selected. Afterward, the recombinant plasmids harboring different promoter fragments were extracted by using AxyPrep Plasmid Miniprep Kit 250-prep [Axygen Biosciences (Hangzhou Co., Ltd.)] and firstly screened out through restriction digestion and PCR (Fig. 1). All of the new recombinant plasmids were also sequenced by Invitrogen (Shanghai, China). Additionally, the plasmid pCAMBIA1304 harboring CaMV35S promoter-GUS was used as positive control vector.

Tobacco transformation and verification by PCR

The plasmid DNA was transferred to *A. tumefaciens* EHA105 by freeze-thaw method (Walker-peach and Velten 1994). Then, the *A. tumefaciens*-positive clones were used to transform leaf discs of tobacco (*N. tabacum* L. cv. Xanthi) (Horsch et al. 1985). The infected tobacco leaf discs were placed on the inductive differentiation medium with 0.05 mg/mL kanamycin and 0.2 mg/mL cefotaxime to regenerate plantlet. The method on the screening of transgenic tobacco was similar to that in Jain et al. (2012). The putative transformants were selected on MS medium with 0.05 mg/mL kanamycin. The genomic DNA was isolated from leaf tissues of putative transformants according to the method of cetyltriethylammnonium bromide (CTAB) (Wang et al. 1996). PCR analysis was carried out with specific primers (forward, 5′-CAACGAACTGAACTGGCAGA-3′; reverse, 5′-TTTTTGTCACGCGCTATCAG-3′), and the wild type (WT) was used as control.

Southern blotting

Genomic DNA was isolated from leaves of transgenic tobacco plants in T1 generation as previously described (Wang et al. 1996). The genomic DNA was digested with *Hind*III, separated on 0.8 % agarose gel, and transferred to nylon membranes as described by Sambrook and Russell (2001). A 638-bp fragment from GUS gene was amplified by PCR from pCAMBIA1304 with the pair of primers (forward, 5′-CAGGAAGTGATGGA GCATCAG-3′; reverse, 5′-TCGTGCACCATCAGCACGTT A-3′) and separated on 0.8 % of agarose gel. This fragment was used as probe and labeled with ^{32}P-dCTP.

Stress treatments

Abiotic stress treatments were applied to 3-week-old transgenic tobacco seedlings in a growth chamber at 25 °C in the dark for 24 h. For chemical treatments, whole seedlings were floated in MS medium solution containing 100 μM indole-3-acetic (IAA), 100 μM abscisic acid (ABA), 100 μM ethylene, 150 μM $CuSO_4$, and 100 μM methyl jasmonate (MeJA). MS without any additions was used as a control. Drought was induced by removing tobacco plants from the pots and keeping them in a Petri dish to allow slow evaporation until the weight was decreased by about 25 % (Stockinger et al. 1997). Then, the stress-treated samples were harvested and frozen quickly in liquid nitrogen, and their GUS activities were determined. All data are the mean ± SE of at least four replicates.

Histochemical localization and fluorometric measurement of GUS activity

GUS activity was visualized histochemically using the chromogenic substrate 5-bromo-4-chloro-3-indyle-β-D-glucuronide

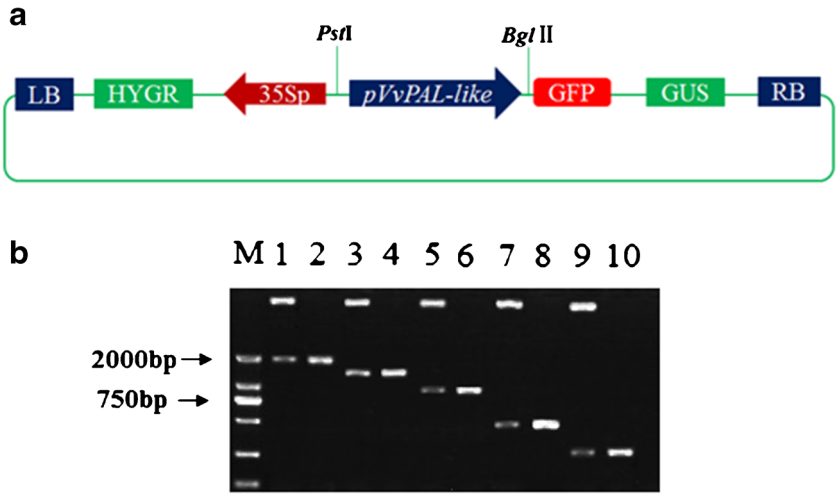


Fig. 1 Construction of pCAMBIA1304-p*VvPAL-like* expression vector. **a** Schematic illustration of pCAMBIA1304-*pVvPAL-like* expression vector. **b** Identification of the pCAMBIA1304-*pVvPAL-like* expression vector by PCR and digestion. *M*: DL2000 molecular marker; *1*, *3*, *5*, *7*, and *9*: P2000, P1461, P930, P424, and P292 were respectively digested by *Pst* I and *Bgl* II; *2*, *4*, *6*, *8*, and *10*: PCR product

(X-Gluc) staining, according to Meisel and Lam (1996) and Jefferson et al. (1987), with some modifications. Transgenic tobacco plants were incubated in GUS staining solution [1 mM X-Gluc, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 10 mM EDTA, 0.1 % (*v*/*v*) Triton X-100, 100 mg/mL chloramphenicol in 50 mM NaH_2PO_4 buffer (pH 7.0)] for 2–3 days at 37 °C. Plant material was then fixed in 4 % formaldehyde, 4 % acetic acid, and 28.5 % ethanol for 30 min. Subsequent incubation in 70 % ethanol for 1 h, 100 % ethanol for 1 h, and 70 % ethanol for 1 h, followed by incubation in distilled water, was performed to remove chlorophyll from the plant material.

Fluorometric GUS assays were performed based on a method described by Jefferson et al. (1987). The leaf tissues of tobacco transgenic lines were ground in the presence of liquid nitrogen in a mortar and transferred to a microtube. One milliliter of the extraction buffer [50 mM NaH_2PO_4, pH 7.0, 1 mM EDTA, 0.1 % Triton X-100, 0.1 % (*w*/*v*) sodium lauryl sarcosine, and 5 mM dithiothreitol] was added and mixed. The supernatant, after being centrifuged at 12,000*g* for 10 min at 4 °C, was assayed for GUS activity with 4-methylumbelliferyl glucuronide (MUG) substrate using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at the excitation/emission wavelengths of 365 nm/455 nm, as described by Jefferson et al. (1987). The protein concentrations were quantified using bovine serum albumin (BSA) as a standard, according to Bradford (1976), and the GUS enzyme activity was expressed as picomoles of 4-methylumbelliferone (MU) produced per milligram protein per minute.

Result

Spatial and temporal expression of the *VvPAL-like* gene

In this study, a gene (*GSVIVG00013928001*) in grape, which was highly homologous with the *PALs* in other species by multiple sequence alignment and phylogeny evolution analysis (Supplementary Figs. 1 and 2), and shared same domain (PLN02457), along with 98.17 % (2094/2133) nucleotide sequence and 98.59 % (700/710) protein sequence identity with the reported *PAL* from *V. vinifera* (GenBank: EF192469.1), and was named *VvPAL-like*. To verify the expression levels of the *VvPAL-like* gene in the root, stem, leaf, tendril, flower, and fruit tissues of grapevine, qRT-PCR experiments were performed by using primer (forward, 5′-TTAAAATGGCTGGGATCGAG-3′; reverse, 5′-CCTGCATCA CTTCAGCAAAA-3′) for each of the cDNAs. The results showed that the mRNA expression levels of *VvPAL-like* gene were extremely low in small green fruit, increased with the maturity of fruit, and finally remained slowly increasing at extremely high levels at the FR and HR stages (Fig. 2). In other words, the *VvPAL-like* activity was increasing with the fruit coloring. This also illuminates that *VvPAL-like* gene is closely related to the grape fruit coloring. For the different organizations, the *VvPAL-like* is constitutively expressed in all of the tested tissues, with the highest expression in stem, medium in root and leaf, and lowest in tendril (Fig. 2). Our data of abundant transcript accumulation in stem of grapevine were consistent with that of the study of *Phyllostachys edulis* (Gao et al. 2012) and *Ginkgo biloba* (Xu et al. 2008) but differ from the studies in *A. thaliana* (Wanner et al. 1995), *Solanum tuberosum* (Jots and Hahlbrock 1992), *Hordeum vulgare* (Kervinen et al. 1997), and *Isatis indigotica* (Lu et al. 2006), which showed that mRNA of *PAL* was typically high in root and low in leaf.

Isolation and characterization of the grapevine *VvPAL-like* promoter

A 2000-bp promoter fragment of *VvPAL-like* gene, designated as *pVvPAL-like*, was obtained by PCR amplification from ‘Summer Black’ grapevine with *ExTaq* DNA polymerase. The nucleotide sequence of *VvPAL-like* promoter identified from the grapevine genome (see Fig. 3), along with several

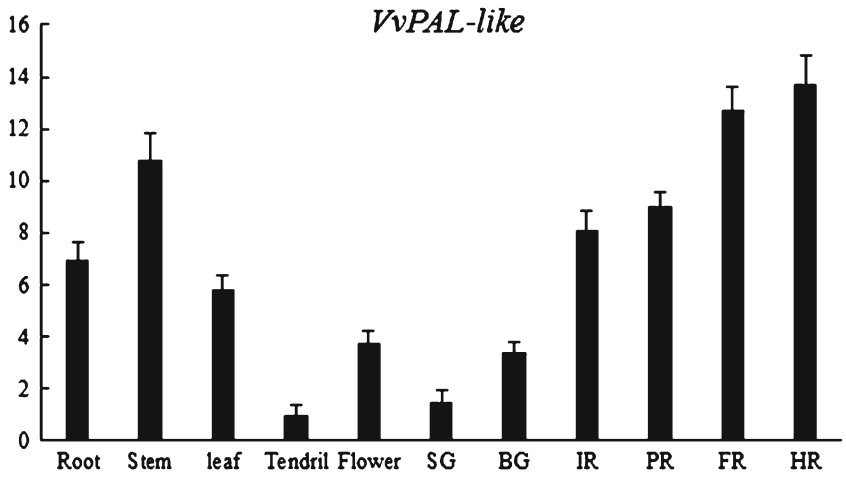


Fig. 2 Spatial and temporal expression of the *VvPAL-like* gene. *SG* small green berries, *BG* big green berries, *IR* initial red berries, *PR* partial red berries, *FR* full red berries, *HR* harvesting red berries

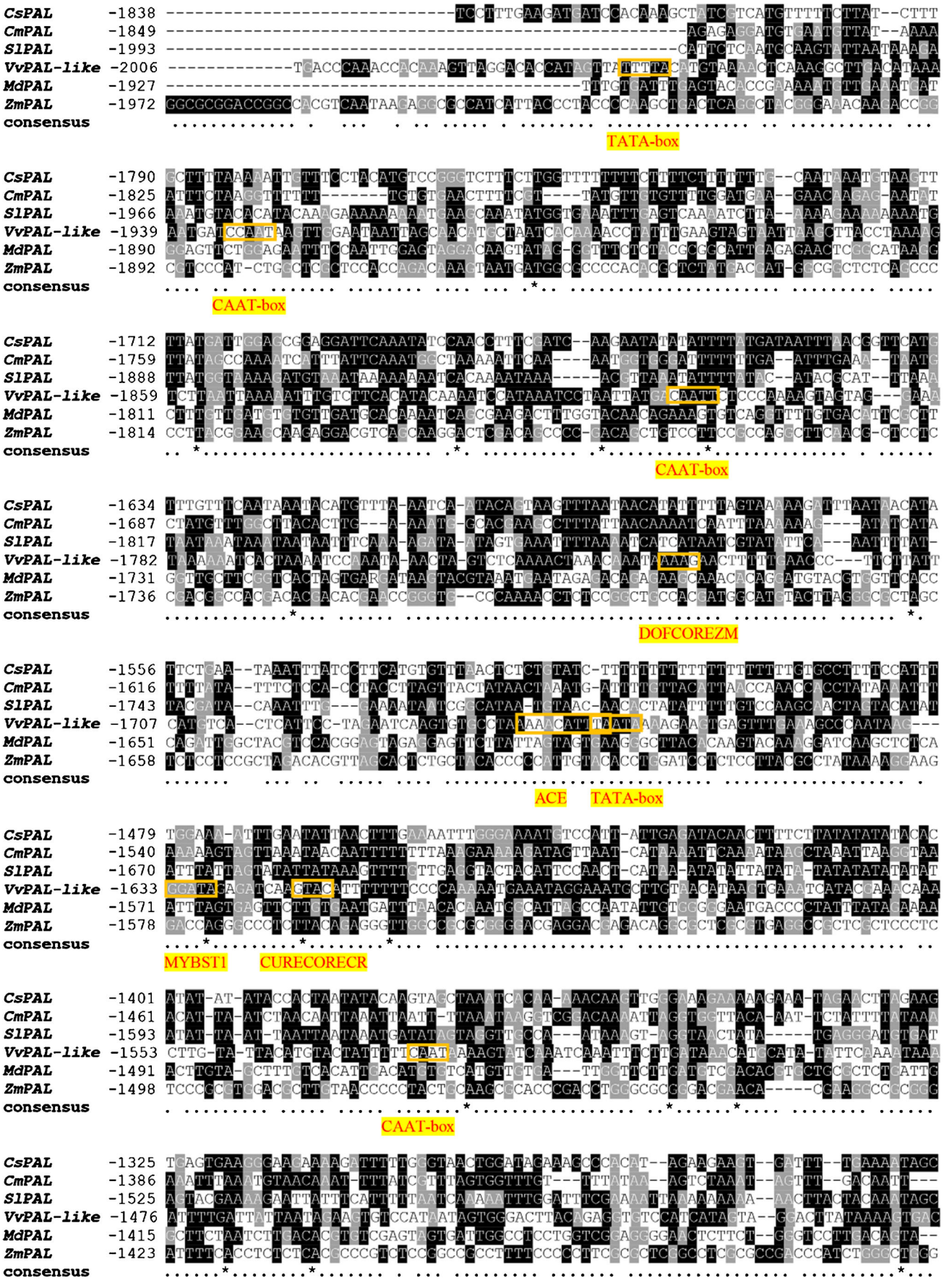
previously characterized *PAL* gene promoters from *Cucumis sativus*, *Cucumis melo*, *Z. mays*, *Solanum lycopersicum*, and *Malus domestica*, was analyzed to identify putative regulatory elements. To facilitate sequence numbering, the A of the translation initiation codon (ATG) of the *VvPAL-like* gene was defined as +1 (Fig. 3). The putative transcriptional start sites (TSS) of *VvPAL-like* were determined for the A and located 138 bp upstream from the translation initiation codon. A TATA-box (TATA), which serves as a basal promoter element for the transcription of eukaryotic genes, was present at 28 bp upstream of the TSS in the *VvPAL-like* promoter. A CAAT-box was present at 273 bp upstream of the TATA-box sequence and −441 bp relative to the initiation codon. It was reported that the distance between putative CAAT-box and TSS was greater than 80 bp for several plant promoters, consistent with our prediction (An et al. 1993). To further evaluate the sequence structure-function characteristics, the promoter motif search using the software programs PLACE (version 30.0) and PlantCARE showed that a number of putative *cis*-acting regulatory elements, which were potential binding sites for transcription factors with different numbers of copies and distribution patterns, were present, such as MBS, ERE, W-box, G-Box, Q-element, TCA element, ARFAT, ABRE, CURECORECR, CGTCA-motif, and AGAAA-motif (Table 1). Additionally, the comparison of putative *cis*-elements of *PAL* gene promoter from different species was performed to identify the conservation of some regulatory mechanisms (Table 2).

Production and molecular analysis of transgenic tobaccos

Approximately 200 leaf discs of *N. tabacum* L. cv. Xanthi were transformed using *A. tumefaciens* EHA105 with each of the recombinant constructions and placed on the LB plate with kanamycin and rifampicin to regenerate plantlet. As a result, 37, 28, 35, 26, and 31 independent putative transgenic lines are resistant to kanamycin and rifampicin. PCR analysis of genomic DNA from the regenerated tobacco lines showed that there were 17, 15, 18, 13, and 16 PCR-positive transgenic lines harboring pCAMBIA1304-P2000, pCAMBIA1304-P1461, pCAMBIA1304-P930, pCAMBIA1304-P424, and pCAMBIA1304-P292, respectively. Additionally, 28 positive transgenic lines harboring pCAMBIA1304 were screened as positive control. Furthermore, the morphology and growth of positive transgenic plants were observed, and visible differences between the transgenic plants and wild type (WT) were not found.

A 638-bp fragment from the GUS gene was used as the specific probe to determine the insertional copy numbers of the transgenic plants by Southern blotting analysis, and two transgenic tobacco lines for each of the recombinant constructions were randomly chosen for analysis. The hybridization results showed that the target fragments had integrated into tobacco genome and one to three insertions were present in selected transgenic lines, as well as most lines had one or two copies (Fig. 4). Meantime, the result of Southern blotting confirmed that PCR with specific primers targeting the transfer DNA (T-DNA) region of the Ti plasmid was a rapid and easy method to screen the positive transgenic plants. Only one *Hind*III was present within the fragment containing promoter, GUS gene, NOS terminator, and left border of T-DNA. After digestion of genomic DNA with *Hind*III, the length of restriction fragments hybridized with the probe was determined by plant sequences near the left border of T-DNA (Fig. 4). Different integration sites within transgenic genome produce different lengths of restriction fragments.

Fig. 3 Multiple alignment of the 5′ flanking regions of *VvPAL-like* and five plant *PALs* preformed in ClustalX. The nucleotides are numbered on the basis of the ATG initiation codon (+1). The putative transcriptional initiation site for the A is shown by the *yellow boxes*. The conserved nucleotides are *shaded*. The *PALs* included in the alignment were from *Cucumis sativus* (Gene ID: 101218856), *Cucumis melo* (Gene ID: 103501962), *Zea mays* (Gene ID: 100127011), *Solanum lycopersicum* (Gene ID: 101261892), and *Malus domestica* (Gene ID: 103450046), respectively ▶


CsPAL -1838
CmPAL -1849
SlPAL -1993
VvPAL-like -2006
MdPAL -1927
ZmPAL -1972
consensus
TATA-box
CsPAL -1790
CmPAL -1825
SlPAL -1966
VvPAL-like -1939
MdPAL -1890
ZmPAL -1892
consensus
CAAT-box
CsPAL -1712
CmPAL -1759
SlPAL -1888
VvPAL-like -1859
MdPAL -1811
ZmPAL -1814
consensus
CAAT-box
CsPAL -1634
CmPAL -1687
SlPAL -1817
VvPAL-like -1782
MdPAL -1731
ZmPAL -1736
consensus
DOFCOREZM
CsPAL -1556
CmPAL -1616
SlPAL -1743
VvPAL-like -1707
MdPAL -1651
ZmPAL -1658
consensus
ACE
TATA-box
CsPAL -1479
CmPAL -1540
SlPAL -1670
VvPAL-like -1633
MdPAL -1571
ZmPAL -1578
consensus
MYBST1
CURECORECR
CsPAL -1401
CmPAL -1461
SlPAL -1593
VvPAL-like -1553
MdPAL -1491
ZmPAL -1498
consensus
CAAT-box
CsPAL -1325
CmPAL -1386
SlPAL -1525
VvPAL-like -1476
MdPAL -1415
ZmPAL -1423
consensus

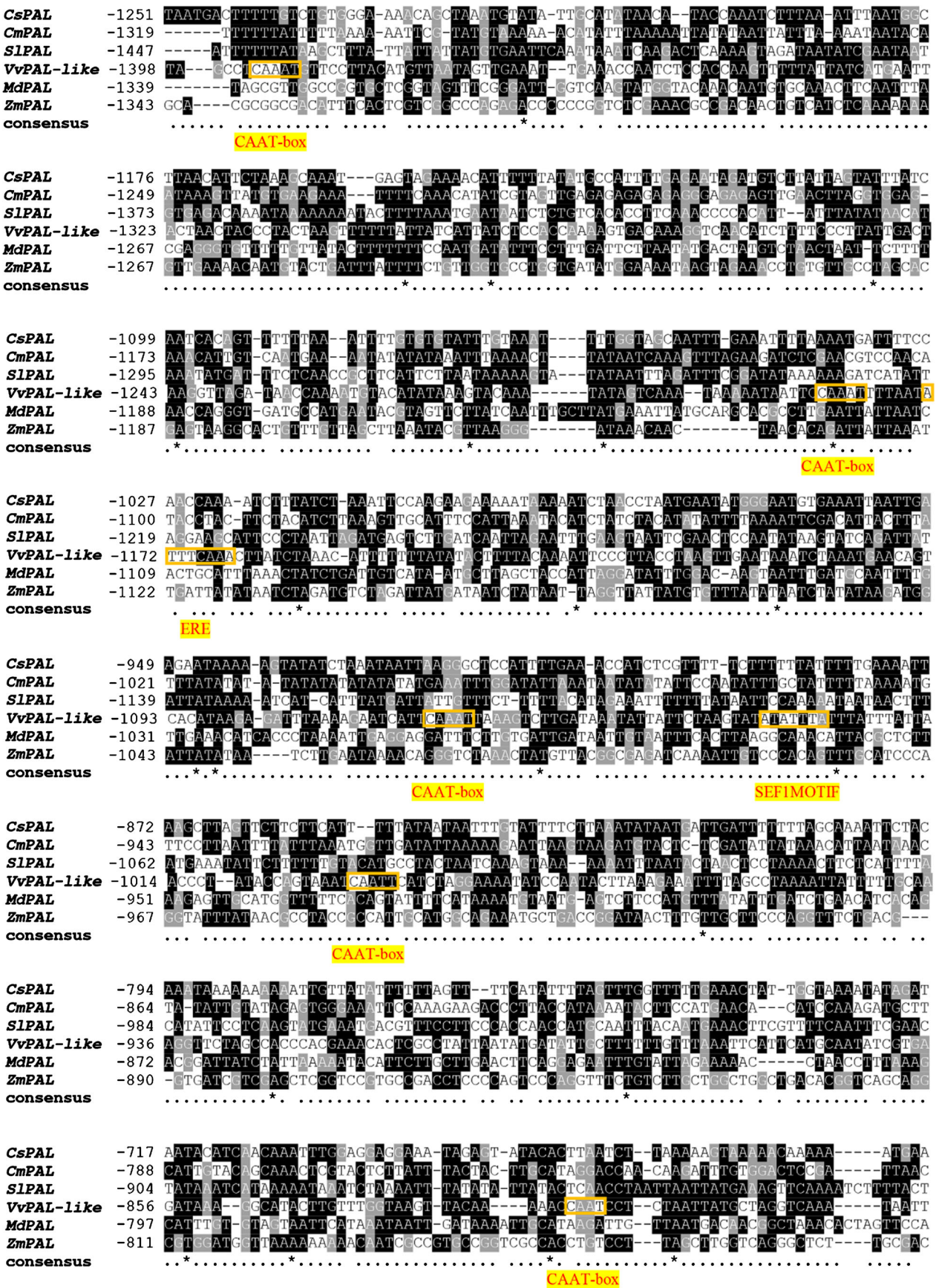


Fig. 3 continued.

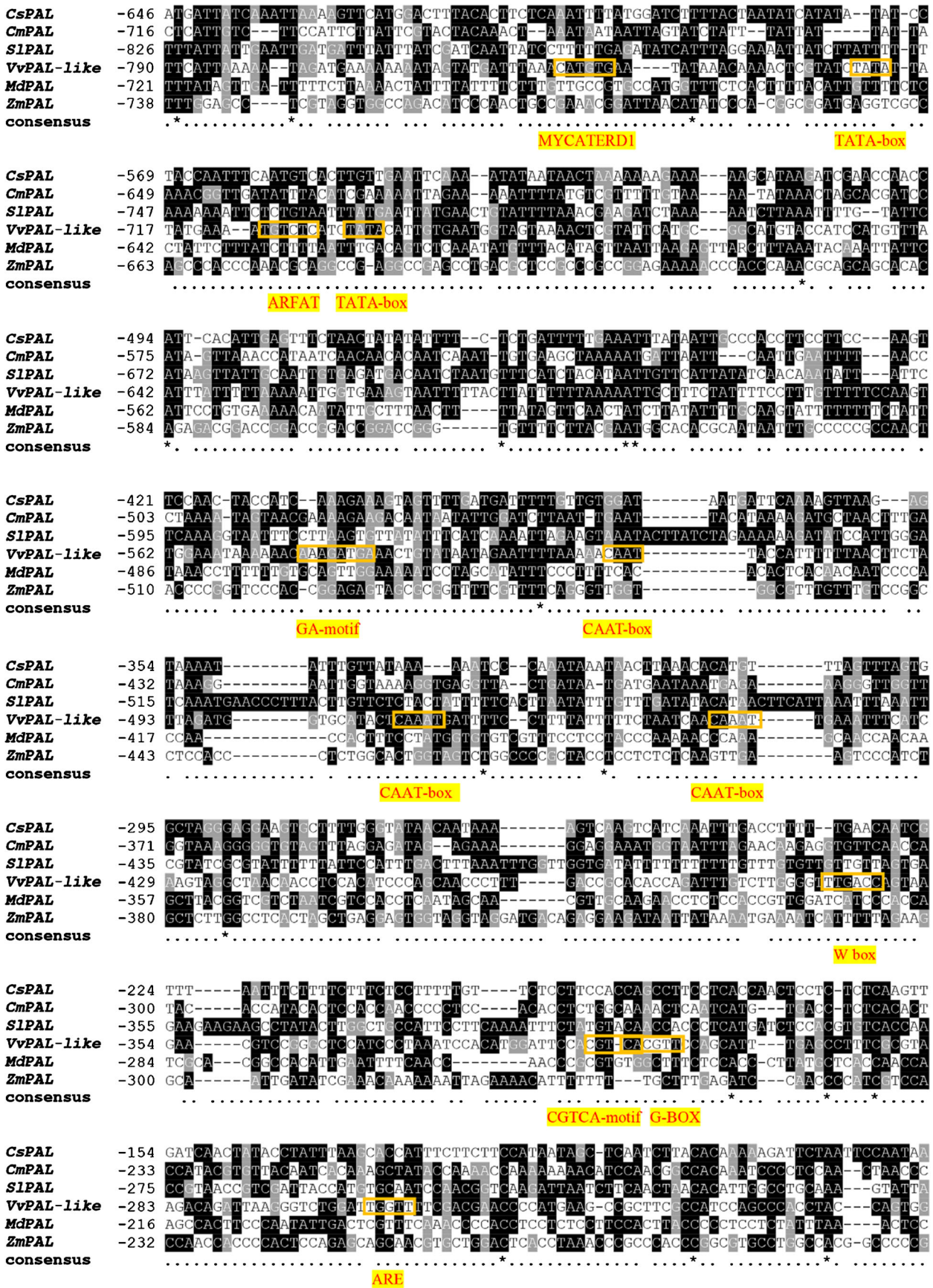


Fig. 3 continued.

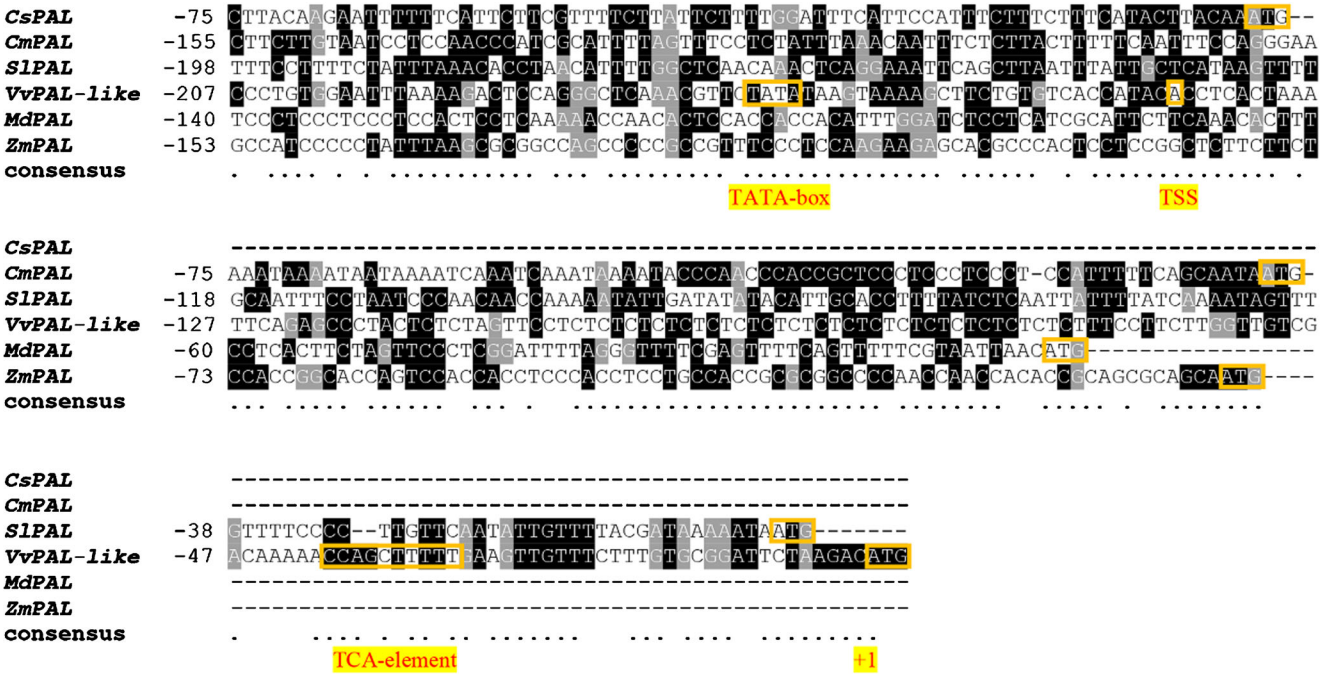


Fig. 3 continued.

Spatial and temporal expression patterns of *VvPAL-like* promoter in transgenic tobaccos

To investigate the spatial-temporal regulation directed by the *VvPAL-like* promoter region, transgenic tobacco plants harboring the GUS reporter gene fused to the 2000-bp fragment were grown in the greenhouse. Plantlets germinated from wild-type seeds were used as negative controls, and transgenic tobacco plants expressing GUS under the control of the CaMV35S promoter were used as positive controls. As shown in Fig. 5a, the root and shoot of a 3-day-old tobacco seedling grown in MS medium, including cotyledons and hypocotyls, were strongly stained and GUS activity was also observed in the root apex. Similar GUS activities were maintained in aerial parts of 5-day-old (Fig. 5b) and 10-day-old seedlings (Fig. 5c). Interestingly, the blue staining in the root apex gradually reduced with the passage of time. Additionally, histochemical localization of GUS expression was carried out in different organs (fruit, root, stem, and leaf) of transgenic tobacco plants grown in soil to determine the tissue-specific expression pattern directed by *VvPAL-like* promoter. As shown in Fig. 5d–e, in 40-day-old transgenic plants grown in soil, strong GUS staining was only observed in xylem elements of the vascular tissues by transverse and longitudinal sections of the stem. Figure 5f shows that strong GUS staining was detected at the leaf vein of 40-day-old transgenic plants. GUS expression was also detected in fruit (ovary), with stronger GUS expression in the ovule but relatively weaker staining in the ovary wall (Fig. 5g). As shown in Fig. 5h, strong GUS staining was detected in the root, particularly at the wounding site.

However, histochemical analysis does not permit the detection of low levels of GUS activity, nor can it precisely measure an increase in the level of GUS expression. Therefore, *pVvPAL-like* activity was also monitored by a quantitative GUS assay using transgenic tobacco lines T1 that had formed ten leaves. As shown in Fig. 6, the highest GUS activity was detected in the stem and similar GUS activity was detected in the root of 60-day-old transgenic tobacco plants, while the lowest expression was observed in the first leaf. The GUS activity of seventh leaf is the highest in ten expanded leaves. These results demonstrated that the GUS activity in stems and roots, mostly, was higher than that in the leaves, consistent with GUS staining of transgenic tobacco plants, indicating that the *VvPAL-like* promoter directed reporter expression mainly in vascular tissues in the later stages of development. No GUS activity was detected in a negative control leaf, whereas strong GUS activity was detected in a positive control harboring pCAMBIA1304 (35S::GUS).

In the reproductive growth stage, localization of GUS expression in the aerial tissues was observed in the trichomes (Fig. 7a–c). In addition, GUS staining was observable in the stomatal guard cells in enlarged petals (Fig. 7d). Furthermore, GUS staining was observed in anthers, pollen grains, style, ovary, and pigmented part of petals (Fig. 7e–g), but relatively weak staining was present in the pedicels, sepals, and receptacles. After counting the populations of pollen grains, the average proportion of pollen grains with blue staining was found to be about 58.33 %, which was close to the 1:1 ratio expected in the first-generation plants, indicating that the analyzed transgenic plants contained a single functional

Table 1 The main *cis*-acting regulatory elements found in the *VvPAL-like* promoter

Motif	Location	Sequence	Function	Source
TATA-box	−1491, −1410, −1219, −1147, −1035, −1010, −743, −724, −700, −535, −169	TATA	Core promoter element	PlantCARE
CAAT-box	−1932, −1807, −1640, −1530, −1355, −997, −976, −867, −821, −516	CAAT	Common *cis*-acting element in promoter and enhancer regions	PlantCARE
MBS	−387	CGGTCA	MYB binding site involved in drought inducibility	PlantCARE
ERE	−1166	ATTTCAAA	Ethylene-responsive element	PlantCARE
Q-element	−798, −1265	AGGTCA	*cis*-acting regulatory element involved in pollen expression	PlantCARE
AGAAA-motif	−962	AGAAA	Pollen responsive, pollen expression	PlantCARE
CGTCA-motif	−311	CGTCA	*cis*-acting regulatory element involved in the MeJA responsiveness	PlantCARE
DOFCOREZM	−1993, −1995, −1863, −1796, −1733, −1661, −1406, −1280, −1272, −621, −548, −159	AAAG	Core site required for Dof proteins binding with only one zinc finger, which are unique to plants	PLACE
W-box	−361, −390, −1245, −1945	TTGACC	WRKY proteins bind to this motif in response to wounding and pathogens	PlantCARE
ARFAT	−704	TGTCTC	Auxin-responsive element	PLACE
ABRE	−311, −309	ACGTG	Abscisic acid responsive element	PLACE
MYB1AT	−1359, −1234, −825, −43	WAACCA	MYB-like transcription factors	PLACE
MYBPLANT	−220	MACCWAMC	MYB-like transcription factors	PLACE
MYBPZM	−218	CCWACC	MYB-like transcription factors	PLACE
MYBST1	−1678, −980	GGATA	MYB-like transcription factors	PLACE
CURECORECR	−1617, −1538, −1221, −1210, −655	GTAC	*cis*-acting regulatory element involved in the copper responsiveness	PLACE
Box 4	−901, −1462	ATTAAT	Part of a conserved DNA module involved in light responsiveness	PlantCARE
Box I	−1166, −1644	TTTCAAA	Light-responsive element	PlantCARE
Box-W1	−42, —824	TTGACC	Fungal elicitor responsive element	PlantCARE
ARE	−262	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction	PlantCARE
TC-rich repeats	−1293	ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness	PlantCARE
TCA-element	−40	CCATCTTTTT	*cis*-acting element involved in salicylic acid responsiveness	PlantCARE
G-box	−322, −312, −307, −311	CACATGG/CACGTC/TGACGTGG/CACGTT	*cis*-acting regulatory element involved in light responsiveness	PlantCARE
G-Box	−307	CACGTT	*cis*-acting regulatory element involved in light responsiveness	PlantCARE
GA-motif	−541	AAAGATGA	Part of a light-responsive element	PlantCARE
GAG-motif	−110	AGAGAGT	Part of a light-responsive element	PlantCARE
TCT-motif	−281, −353	TCTTAC	Part of a light-responsive element	PlantCARE
I-box	−1230	TAGATAACC	Part of a light-responsive element	PlantCARE
L-box	−214	ATCCCACCTAC	Part of a light-responsive element	PlantCARE
GT1-motif	−1012, −352, −1012	GGTTAAT/AATCCACA/GGTTAA	Light-responsive element	PlantCARE
ACE	−1150, −313, −1665	AAAACGTTTA/ACGTGGA	*cis*-acting element involved in light responsiveness	PlantCARE
Sp1	−918	CC(G/A)CCC	Light-responsive element	PlantCARE
circadian	−1160	CAANNNNATC	*cis*-acting regulatory element involved in circadian control	PlantCARE

N indicates A or G or C or T, W means A/T, and M means A/C

Table 2 Comparison of putative *cis*-elements of *PAL* gene promoter from different species

Motif	Sequence	*Vitis vinifera*	*Cucumis sativus*	*Cucumis melo*	*Solanum lycopersicum*	*Malus domestica*	*Zea mays*	Source
TATA-box	TATA	√	√	√	√	√	√	PlantCARE
CAAT-box	CAAT	√	√	√	√	√	√	PlantCARE
MBS	CGGTCA	√	√		√	√	√	PlantCARE
ERE	ATTTCAAA	√	√	√	√			PlantCARE
Box-W1	TTGACC	√	√	√	√	√		PlantCARE
Q-element	AGGTCA	√						PlantCARE
CGTCA-motif	CGTCA	√			√		√	PlantCARE
ARE	TGGTTT	√	√	√		√		PlantCARE
GARE-motif	TCTGTTG						√	PlantCARE
LTR	CCGAAA					√	√	PlantCARE
TATC-box	TATCCCA						√	PlantCARE
TC-rich repeats	ATTTTCTCCA	√	√	√	√		√	PlantCARE
TCA-element	CCATCTTTTT	√			√		√	PlantCARE
TGACG-motif	TGACG	√	√		√		√	PlantCARE
TGA-box	TGACGTGGC						√	PlantCARE
AGAAA-motif	AGAAA	√						PlantCARE
DOFCOREZM	AAAG	√	√		√	√	√	PLACE
W-box	TTGACC	√	√		√	√		PlantCARE
ARFAT	TGTCTC	√				√		PLACE
ABRE	ACGTG	√		√	√	√	√	PLACE
CURECORECR	GTAC	√			√	√	√	PLACE
Box 4	ATTAAT	√	√	√	√	√		PlantCARE
Box I	TTTCAAA	√	√	√	√	√		PlantCARE
G-box	CACATGG/CACGTC/TGACGTGG/CACGTT	√		√	√	√	√	PlantCARE
G-Box	CACGTT	√		√	√	√	√	PlantCARE
GA-motif	AAAGATGA	√	√			√		PlantCARE
GAG-motif	AGAGAGT	√	√	√				PlantCARE
TCT-motif	TCTTAC	√	√	√			√	PlantCARE
I-box	TAGATAACC	√	√			√	√	PlantCARE
L-box	ATCCCACCTAC	√			√	√	√	PlantCARE
GT1-motif	GGTTAAT/AATCCACA/GGTTAA	√	√	√		√	√	PlantCARE
ACE	AAAACGTTTA/ACGTGGA	√		√	√	√	√	PlantCARE
Sp1	CC(G/A)CCC	√	√	√	√	√	√	PlantCARE
circadian	CAANNNNATC	√	√	√	√	√	√	PlantCARE

N indicates A or G or C or T. "√" represents the presence of motif, whereas blank represents absence of motif in the promoter from all selected species

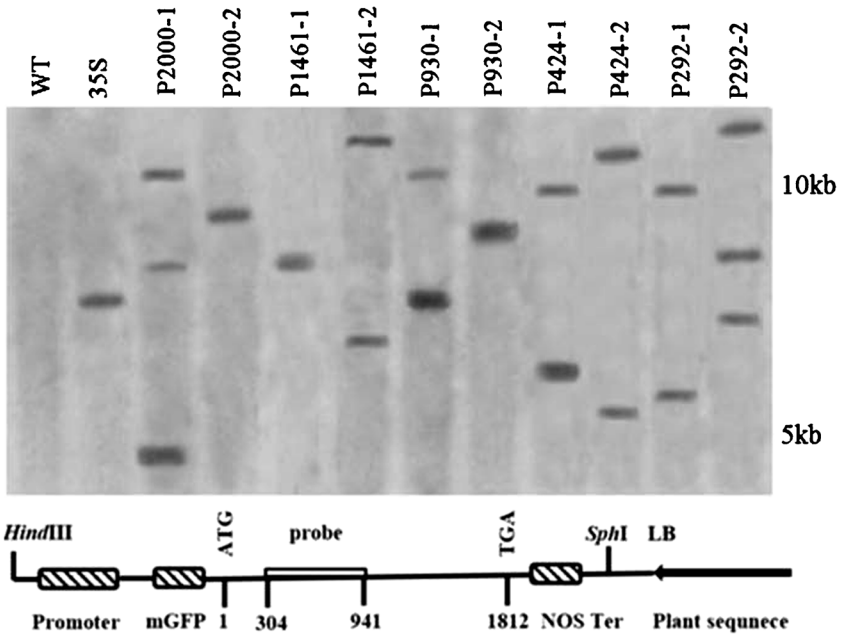


Fig. 4 Southern hybridization analyses of transgenic tobacco plants. *Lane 1*, nontransgenic tobacco; *lane 2* (CaMV35S::GUS transgenic tobacco), *lanes 3–4* (two transgenic tobacco lines harboring P2000), *lanes 5-6* (two transgenic tobacco lines harboring P1461), *lanes 7–8* (two transgenic tobacco lines harboring P930), *lanes 9–10* (two transgenic tobacco lines harboring P424), *lanes 11–12* (two transgenic tobacco lines harboring P292). The schematic representation of restriction enzyme map of transgenic tobacco genomes near the left border of T-DNA is shown below the Southern blotting by using sequencing information of pCAMBIA1304. The *open box* represents the probe fragment, and the site is numbered from the initiation code ATG of the GUS reporter gene

transgene. Consistent with the GUS staining, the quantitative GUS assay showed the high level of expression in the ovaries and styles and the low level in the pedicel and receptacles (Fig. 7i). Almost no GUS activity was detected in the corresponding tissues in negative control plants.

Expression of *pVvPAL-like*-GUS in response to light

Inspection of the *VvPAL-like* promoter sequence revealed a few motifs identical or similar to those well characterized in inducible promoters in response to light, suggesting that the regulation of the *pVvPAL-like* may be controlled by light. To verify this hypothesis, GUS activity was carefully compared between the transgenic seedlings grown under high light (1100 μmol m^{-2} s^{-1}), weak light (400 μmol m^{-2} s^{-1}), and dark conditions. For 4-day-old transgenic seedlings with *VvPAL-like* promoter, strong GUS staining was detected in full transgenic seedling, but the root apex had weak GUS activity grown under high light intensity (Fig. 8a). This might be due to tobacco seedlings grown in MS medium, in which the roots were shielded from light. Strong GUS activity was detected in cotyledons and hypocotyls, but the young root barely had no GUS activity grown under weak light condition (Fig. 8b), and virtually weaker GUS activity was detected in hypocotyls and only weak GUS staining appeared in cotyledons grown under dark condition (Fig. 8c). Additionally, no GUS staining was detected in wild tobacco (negative control) grown under high light condition (Fig. 8d). It is evident that GUS expression was significantly restrained under the dark conditions. Sequence analysis showed that many kinds of light-responsive elements located in the promoter region of *VvPAL-like* gene (Table 1). Therefore, we believed that some of the light-responsive *cis*-elements found in this region played a critical role in the light response for GUS gene expression in transgenic seedlings. To precisely measure the level of GUS expression under different lighting conditions, quantitative GUS assay was carried out using 4-day-old transgenic tobacco seedlings under high light, weak light, and dark conditions. As shown in Fig. 8e, the result of fluorometric GUS assay is consistent with the GUS staining. Interestingly, GUS activity of the root is activated by the high light, but GUS expression in cotyledon and hypocotyl is also activated under the weak light.

Expression of *pVvPAL-like*-GUS highly responsive to abiotic stresses

To identify the control regions of the *VvPAL-like* promoter in response to abiotic stresses, the 50-day-old transgenic tobacco plants that harbored the GUS reporter gene fused to the full-length (2000 bp) promoter fragment were subjected to various stress treatments and their reporter levels were assayed. Tobacco plants transformed with GUS gene under the control of CaMV35S promoter were used as positive control. As shown in Fig. 9, the GUS activity with ABA treatment was

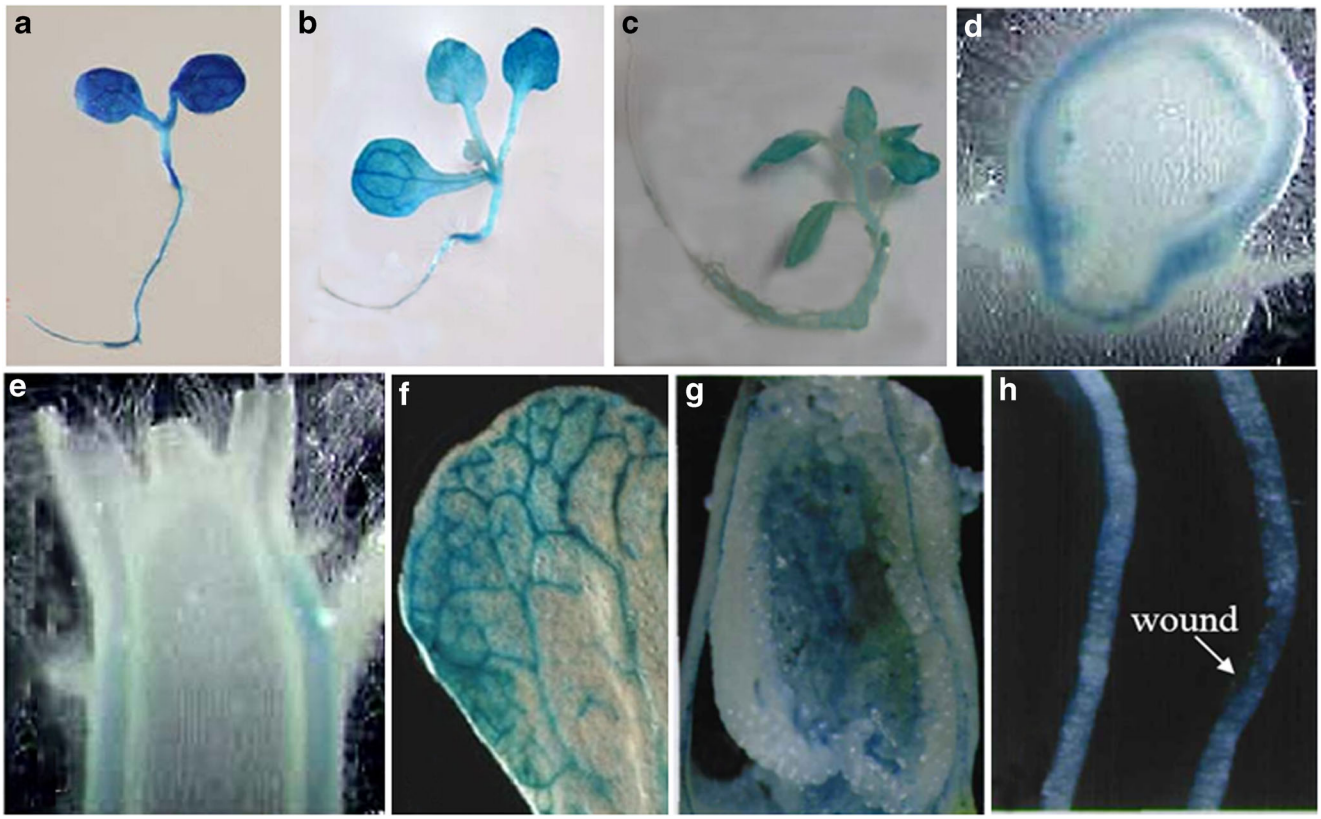


Fig. 5 Histochemical localization of GUS activity in representative transgenic tobacco plants carrying p*VvPAL-like*-GUS constructs. GUS staining of transgenic seedlings grown on MS medium at day 3 (**a**), day 5 (**b**), and day 10 (**c**). Cross section through a mature stem of transgenic tobacco. GUS staining of transverse section (**d**) and longitudinal section (**e**) of the stem in 40-day-old transgenic plants grown in soil. GUS staining in leaf of a 40-day-old transgenic tobacco (**f**). GUS staining in fruit of transgenic tobacco with longitudinal section (**g**). GUS staining in the root of transgenic tobacco grown in soil pots, particularly the wounding site (**h**)

the highest level of all the treatments (743.62 pmol MU/min/mg protein) and was 1.91-fold greater than that of the plants treated with the MS medium as control. Drought and ethylene also highly enhanced the GUS activity by 85.1 and 75.3 %, respectively, compared with the control, while the GUS activity with $CuSO_4$, MeJA, and IAA treatments only had 1.62-fold, 1.61-fold, and 1.50-fold greater than that of the plants treated with control, respectively (Fig. 9). These findings suggest that a possible reason for the inducibility phenomenon may be due to the relative substrate specificities of some promoter elements.

As the *VvPAL-like* promoter could be induced by ABA, an attempt was made to investigate the effects of time and dose on ABA induction. The transgenic tobacco plants that harbored the GUS reporter gene fused to the full-length (2000 bp) promoter fragment were treated with ABA, and GUS activities were measured at different concentrations

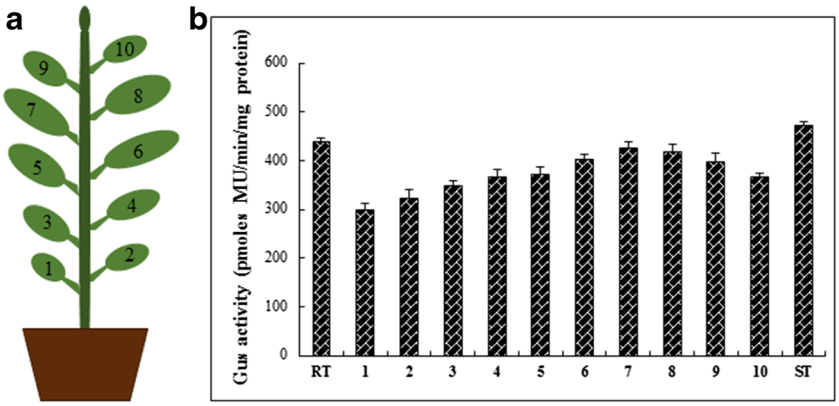


Fig. 6 β-Glucuronidase activity assay in transgenic tobacco lines T1. **a** Leaves (1–10: from the first leaf to tenth leaf) are indicated in the schematic drawing for the tobacco plants. **b** The roots (RT), stems (ST), and leaves (1–10) were assayed for GUS activity. Mean values are expressed in picomole MU per milligram protein per minute with data from independent lines. *Error bars* indicate SD values

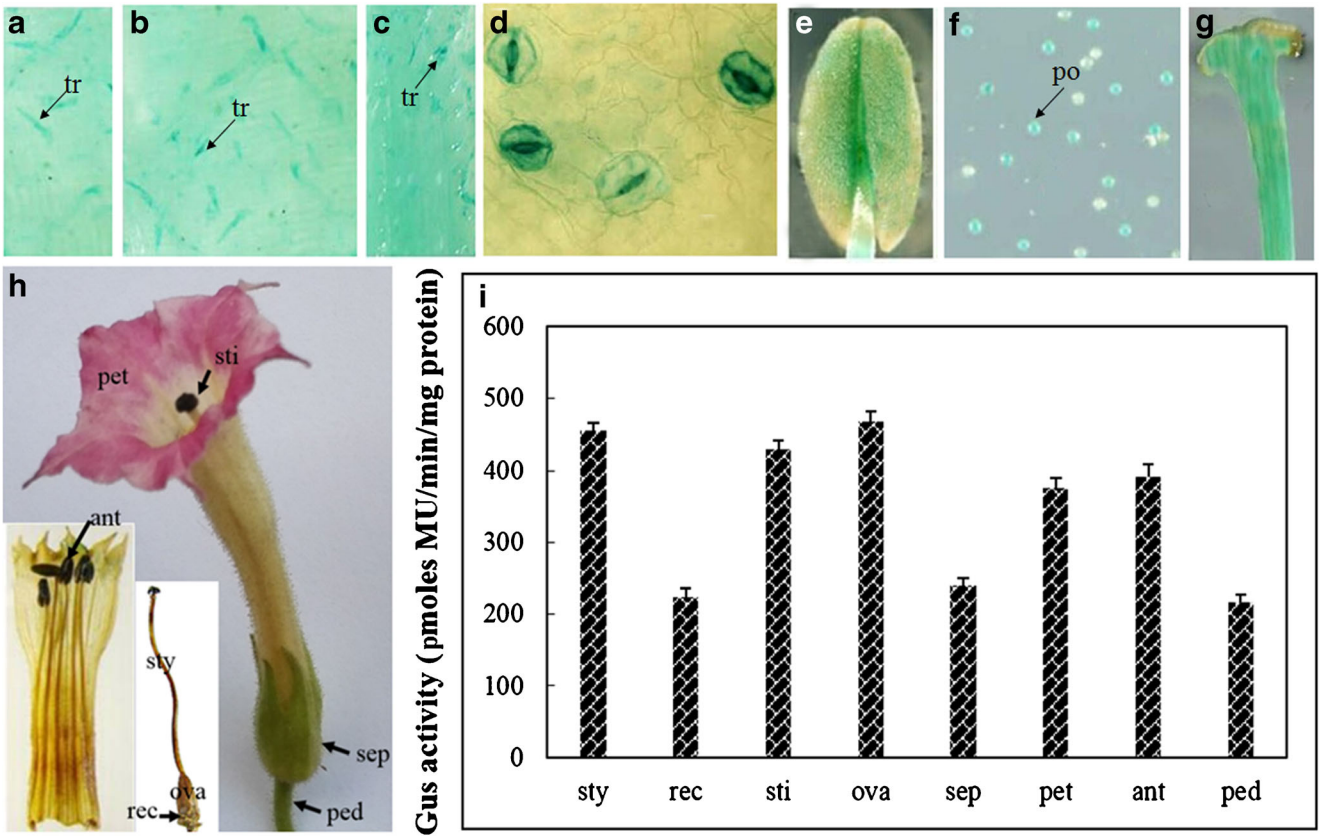


Fig. 7 Localization of GUS expression in trichomes (*tr*) on the leaf (**a**), pedicel (**b**), and stem (**c**) of transgenic tobacco plants. GUS staining in the stomatal guard cells in enlarged petals (**d**). Activity in the anthers (**e**) and pollen grains (*po*) (**f**). GUS staining in the style (sty) and stigmas (sti) with the longitudinal section (**g**). Anthers (*ant*), stigmas (*sti*), styles (*sty*), sepals (*sep*), petals (*pet*), ovaries (*ova*), receptacles (*rec*), and pedicels (*ped*) from the mature tobacco plant (**h**) were sampled and assayed for GUS activity (**i**). Mean values are expressed in picomole MU per milligram protein per minute with data from independent lines. *Error bars* indicate SD values

and time periods of treatment. After treatment with different concentrations for 12 h, reporter gene expression was induced at ABA concentration levels as low as 10 nM and reached the highest level of expression at 100 μM ABA and then decreased inducible effect with ABA concentration increasing (Fig. 10a), demonstrating an ABA dose-dependent pattern of change. Furthermore, GUS activity was induced immediately after treatment with 100 μM ABA, reached its peak at 4 h, and then slowly decreased (Fig. 10b). However, GUS activity gradually increased after treatment with 1 μM ABA and reached a high level at 14 h, and then, a high level was maintained for up to 24 h (Fig. 10b). A control without ABA treatment did not show any changes during the period of incubation.

As shown in Fig. 9, *VvPAL-like* promoter could be induced by IAA. Furthermore, IAA as an important auxin antagonized the stimulatory effect of ABA. Therefore, we also attempt to investigate the effects of time and dose on IAA induction. The GUS activities of transgenic tobacco plants carrying *pVvPAL-like*-GUS construct were measured under the conditions of MS medium for 12 h containing 100 μM IAA, 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxya-cetic acid (2,4-D), isopentenyladenosine (IPA), *N*-1-naphthylphthalamic acid (NPA), and 2,3,5-triiodobenzoic acid (TIBA). TIBA and NPA are known to be specific inhibitors of the polar transport of auxin in hypocotyl and coleoptile segments (Lomax et al. 1995). As shown in Fig. 11a, the highest GUS activity (around 634.6 pmol MU/min/mg protein) was detected in the transgenic tobacco plants treated with 2,4-D and was about 1.63-fold greater than that of the control plants. NAA, IAA, IBA, and IPA also enhanced GUS activity by 54.1, 50.2, 46.4, and 33.2 %, respectively, when compared with the control, while TIBA and NPA treatments decreased GUS activity by about 28.1 and 23.9 %, respectively (Fig. 11a). After treatment with different concentrations for 12 h, reporter gene expression was induced at IAA concentrations as low as 10 nM and reached the highest level of expression at 100 μM IAA and then decreased the inducible effect with IAA concentration increasing (Fig. 11b), demonstrating an IAA dose-dependent pattern of change. Furthermore, GUS activity was induced immediately after treatment with 100 μM IAA, reached its peak at 2 h, and quickly decreased in the following 2 h and then kept a stable level (Fig. 11c). However, GUS activity gradually increased after treatment with

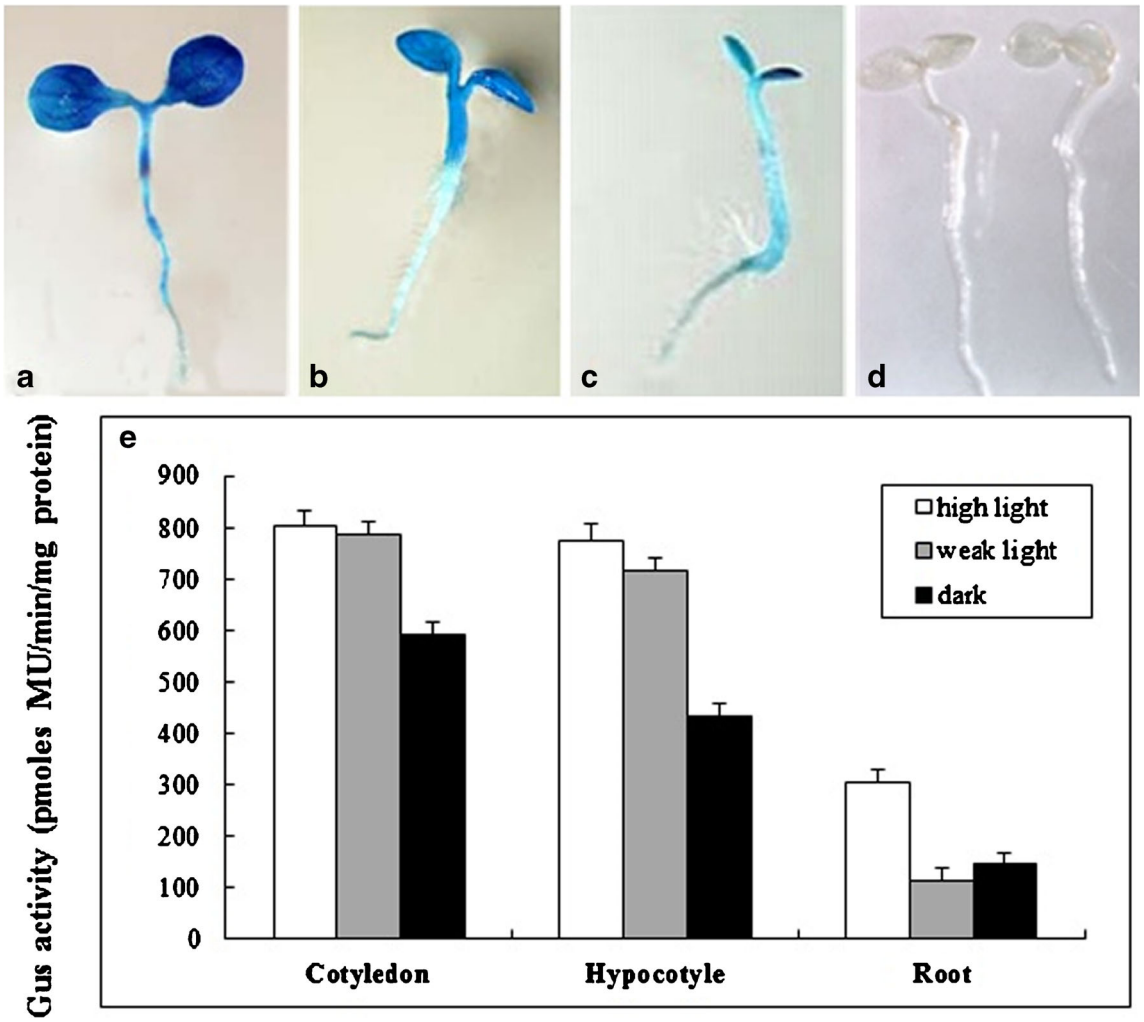


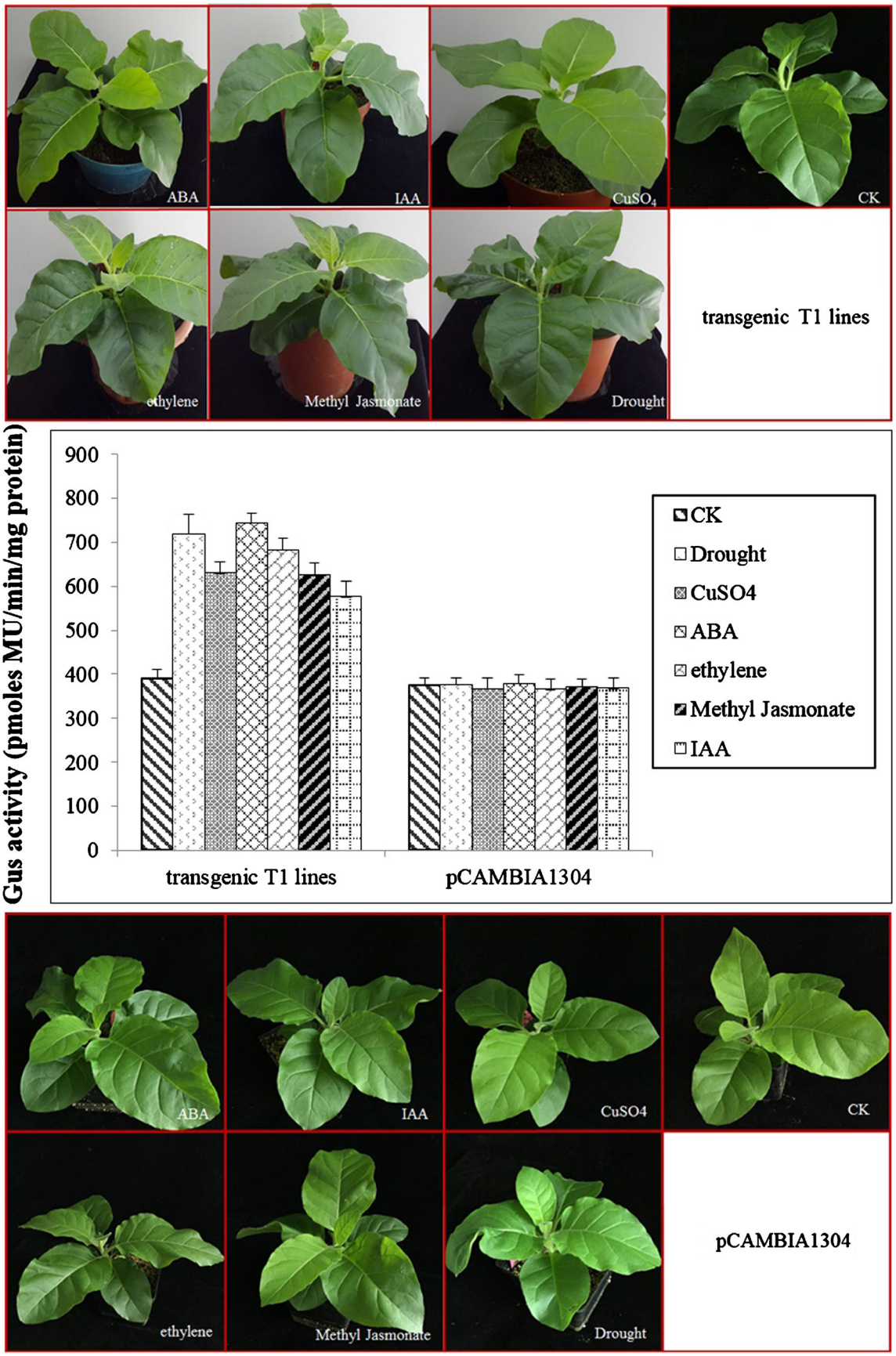
Fig. 8 Localization of GUS expression in 4-day-old transgenic seedlings under high light (**a**), weak light (**b**), and dark conditions (**c**). GUS staining in wild tobacco (negative control) grown under high light condition (**d**). Fluorometric GUS activity of 4-day-old transgenic seedlings under high light, weak light, and dark conditions (**e**). Mean values are expressed in picomole MU per milligram protein per minute with data from independent lines. *Error bars* indicate SD values

1 μM IAA and reached a high level at 8 h. This high level was maintained for up to 24 h (Fig. 11c). A control without IAA treatment did not show any changes during the period of incubation.

Deletion analysis of the *VvPAL-like* promoter in transgenic tobacco

To examine promoter regions potentially involved in the transcriptional control of *VvPAL-like*, the full-length promoter (P2000) and a series of 5′-truncated fragments (P1461, P930, P424, and P229) were transcriptionally fused to the GUS reporter gene (Fig. 12a). More than five individual 30-day-old transgenic tobacco plants were selected for each construct; the fourth leaf from the top, the middle stem (from one third to two thirds of the stature), and whole root of each plant were sampled for GUS activity assays. As shown in Fig. 12b, the full-length promoter P2000 exhibited the highest GUS activity (about 489.32 pmol MU/min/mg protein) in the stems. The ratio of stem, root, and leaf activity was 1.37:1.25:1, indicating that the *VvPAL-like* promoter is more active in stems than in roots, and low activity was detected in leaves. A progressive decline in GUS activity was observed in the constructs from P2000 to P424, implying that some positive regulatory elements are likely to be located

Fig. 9 Fluorometric GUS assay of p*VvPAL-like* -GUS constructs in response to various stresses. Fifty-day-old transgenic plants and positive controls were treated with 150 μM $CuSO_4$, 100 μM ABA, 100 μM ethylene, 100 μM MeJA, 100 μM IAA, and drought without water for 10 days, respectively. Total proteins were extracted, and GUS activity was measured. The pCAMBIA1304 was the CaMV35S promoter-GUS transgenic line and used as positive control


ABA
IAA
CuSO4
CK
ethylene
Methyl Jasmonate
Drought
transgenic T1 lines
Gus activity (pmoles MU/min/mg protein)
900
800
700
600
500
400
300
200
100
0
CK
Drought
CuSO4
ABA
ethylene
Methyl Jasmonate
IAA
transgenic T1 lines
pCAMBIA1304
ABA
IAA
CuSO4
CK
ethylene
Methyl Jasmonate
Drought
pCAMBIA1304

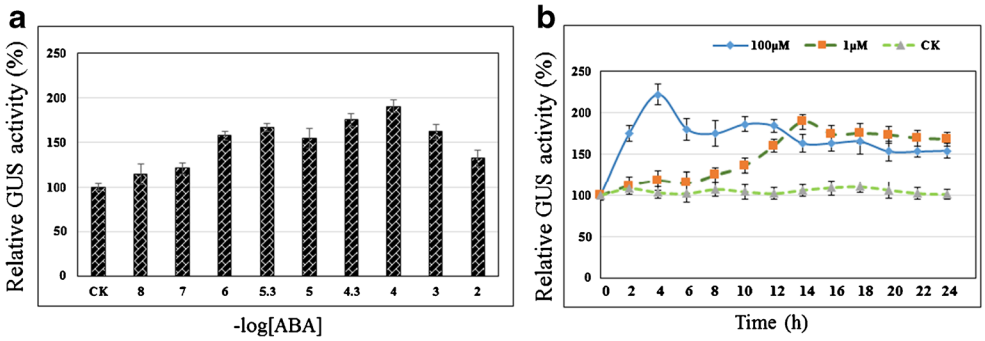


Fig. 10 ABA-induced GUS activities in transgenic tobacco plants. *Bars* denote standard deviation from the mean of independent replicate determinations with four transgenic individuals. **a** GUS activities were measured with different concentrations of ABA, which were 10^{-8}, 10^{-7}, 10^{-6}, 5×10^{-5}, 10^{-5}, 5×10^{-4}, 10^{-4}, 10^{-3}, and 10^{-2} M, after 12-h treatment. Activities were expressed relative to the MS control value, which was defined as 100 % (CK). **b** Temporal curves of GUS activity were induced by 100 and 1 μM ABA, while MS treatment was used as a control

within the −2000/−425 region. However, further 5′ truncation to −292 (P292) resulted in a 1.98-fold increase in GUS activity over P424 in the stems, indicating the presence of a negative regulatory element(s) of promoter activity in the −424 to −292 region. Interestingly, the GUS activity level of P292 was comparable to that of P1461 and P930, demonstrating that a fragment of only 292 bp of sequence could control sufficiently strong expression of the reporter gene in model plants and might serve as a basic activation unit for gene transcription.

Ethylene and MeJA-responsive regions in the *VvPAL-like* promoter

As mentioned above, the *VvPAL-like* promoter was induced by ethylene and MeJA treatments. An investigation to identify the *cis*-regulatory elements or regions responsible for ethylene- and MeJA-induced expression within the *VvPAL-like* promoter was conducted. For each promoter-GUS fusion construct, five individual transgenic tobacco plants were selected and assayed for GUS activity. As shown in Fig. 13, the GUS activity illustrated that only two constructs (P2000 and P1461) were induced by ethylene treatment, clearly indicating that the ethylene-responsive element of the *VvPAL-like* promoter is located in the region −1461 to −930. This result is consistent with that of sequence analysis in which one ERE (ATTTCAAA) was found at position −1173 to −1166. As for MeJA induction, four constructs (P2000, P1461, P930, and P424) were induced (Fig. 13), implying that the MeJA-responsive element may be located in the −424 to −292 region. The positive control (pCAMBIA1304) did not show any induction by ethylene and MeJA. These data collectively suggested that the −1461/−930 region harbors *cis*-element(s) for the ethylene-responsive expression and the −424/−292 fragment contains *cis*-element(s) for the MeJA-responsive expression in the *VvPAL-like* promoter.

Discussion

Previous studies have demonstrated that the *PAL* activity plays important roles in the biosynthesis of flavonoids, lignins, and phytoalexins in plants (Whetten and Sederoff 1992; Campbell and Ellis 1992). In many plant species, such as bean (Cramer et al. 1989), parsley (Lois et al. 1989), rice (Minami et al. 1989), tomato (Lee et al. 1992), and pea (Yamada et al. 1992), *PAL* enzyme has been shown to be encoded by a small multigene family. Additionally, the previous reports also suggested the possibility of various members of *PAL* family being differentially regulated either in a tissue-specific manner or in response to stress conditions (Shufflebottom et al. 1993). As a new-found member of the *PAL* gene family in grapevine, the significant values of the grape *VvPAL-like* gene provoked our interest in exploring the spatial and temporal expression patterns. The promoter, an important component of gene expression regulation, plays an important role in spatial-temporal, lighting, and stress-related regulation of gene expression via controlling the efficiency and frequency of start. Therefore, it is very meaningful and necessary to investigate the regulatory mechanism and functional properties of *VvPAL-like* promoter.

To circumvent difficulties in the regeneration of plants, many promoters have generally been analyzed in model plants such as tobacco (Hsu et al. 1999; Liu et al. 2000; Wu et al. 2007) or *Arabidopsis* (Kim and Triplett 2001; Shangguan et al. 2008; Wang et al. 2004a, 2004b). In this study, the 5′-franking region of *VvPAL-like* gene was transcriptionally fused to the GUS coding sequence and these constructs were studied in transgenic tobacco to identify its spatial-temporal expression pattern, light, and stress inducibility of *VvPAL-like* promoter. The activity conferred by the *VvPAL-like* promoter was assessed by histochemical localization of GUS expression in transgenic tobacco plants containing the full-length promoter construct. In young tobacco seedlings, GUS expression occurred throughout the seedling. Additionally, our full-length

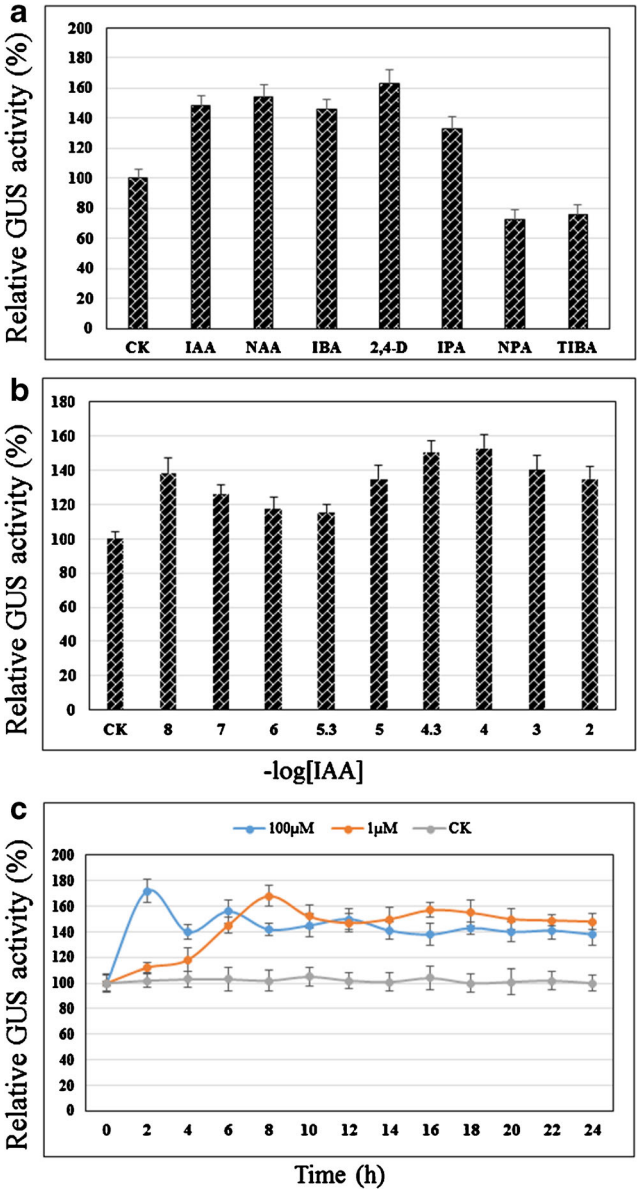


Fig. 11 IAA-induced GUS activities in transgenic tobacco plants. *Bars* denote standard deviation from the mean of independent replicate determinations with four transgenic individuals. **a** GUS activities were assayed under the conditions of MS medium for 12 h containing 100 μM IAA, NAA, IBA, 2,4-D, IPA, NPA, and TIBA. Activities were expressed relative to the MS control value, which was defined as 100 % (CK). **b** GUS activities were measured with different concentrations of IAA, which were 10^{-8}, 10^{-7}, 10^{-6}, 5×10^{-5}, 10^{-5}, 5×10^{-4}, 10^{-4}, 10^{-3}, and 10^{-2} M, after 12-h treatment. The MS control value was defined as 100 %. **c** Temporal curves of GUS activity were induced by 100 and 1 μM IAA, while MS treatment was used as a control

(2000 bp) *VvPAL-like* promoter was found to be highly active in vascular tissues of stem and leaf. This is consistent with the role of *PAL* in catalyzing the first step in the pathway leading to the synthesis of lignin.

Sequence analysis showed that many tissue-specific regulatory elements, such as the Q-element and AGAAA motif, were present in the *VvPAL-like* promoter. Both the Q-element and AGAAA motif were previously reported to be present in pollen expression genes, while pollen tube elongation is a tip growth process and is often compared with other tip growth processes, such as trichome development or root hair elongation in plants (Heath 1990). In transgenic tobacco plants harboring the *VvPAL-like* promoter-GUS construct, GUS staining and fluorometric measurement can be observed in the anthers and pollen, which is in agreement with motif analysis. It suggests that the *VvPAL-like* gene might play an important role in pollen development. It is well known that regulation of trichome development in plants is associated with a number of MYB-like transcription factors (Glover 2000). For example, an *Arabidopsis* MYB protein GLABROUS1 (GL1) governs leaf trichome formation in *Arabidopsis* (Oppenheimer et al. 1990), while ectopic expression of the MIXTA MYB gene could also promote trichome differentiation and produce excessive numbers of multicellullar trichomes on the leaves and floral organs of tobacco plants (Glover 2000). In the *VvPAL-like* promoter, four categories of MYB-like recognition sites [MYB1AT (WAACCA), MYBPLANT (MACCWAMC), MYBPZM (CCWACC), MYBST1 (GGATA); W means A/T, M means A/C] were found, and the GUS activity was also observed in the trichomes on the leaf, pedicel, and stem of transgenic tobacco plants (Fig. 7a–c). It is reasonable to believe that MYB-like recognition sites may play an important regulatory role in reporter gene expression in the tobacco trichomes.

Additionally, sequence analysis also shows that the *VvPAL-like* promoter contains the W-box (TTGAC), which is related to wounding/pathogen response to some extent. As shown in Fig. 5h, stronger GUS staining was observed in the wound of roots. This result is consistent with that of sequence analysis. However, the promoters of *PAL2* and *PAL3* of bean were activated by wounding in nature but not in transgenic plants. Probably, *trans*-regulatory elements required for the wound response can activate the promoter of *VvPAL-like* but cannot activate the promoters of bean *PALs*. One possible reason is ascribed to the difference of promoter activity between *VvPAL-like* and bean *PALs*. Another possibility is that there are several *PALs* in the bean; one is expressed transiently and another expressed later and increases gradually. Light is a well-known environmental factor that induces *PAL* expression. Sequence analysis showed that many kinds of light-responsive elements were located in the promoter region of *VvPAL-like* gene. As shown in Fig. 8, GUS activity was induced in the upper part of hypocotyl and cotyledons, indicating that this *VvPAL-like* gene participates in anthocyanin synthesis induced by light.

The 5′ flanking region of *VvPAL-like* contains a putative copper response element CURECORECR (GTAC), suggesting the possibility of metal-regulated transcription. As shown in Fig. 9, the GUS activities were highly enhanced under the Cu treatment stress. The results presented here also demonstrate

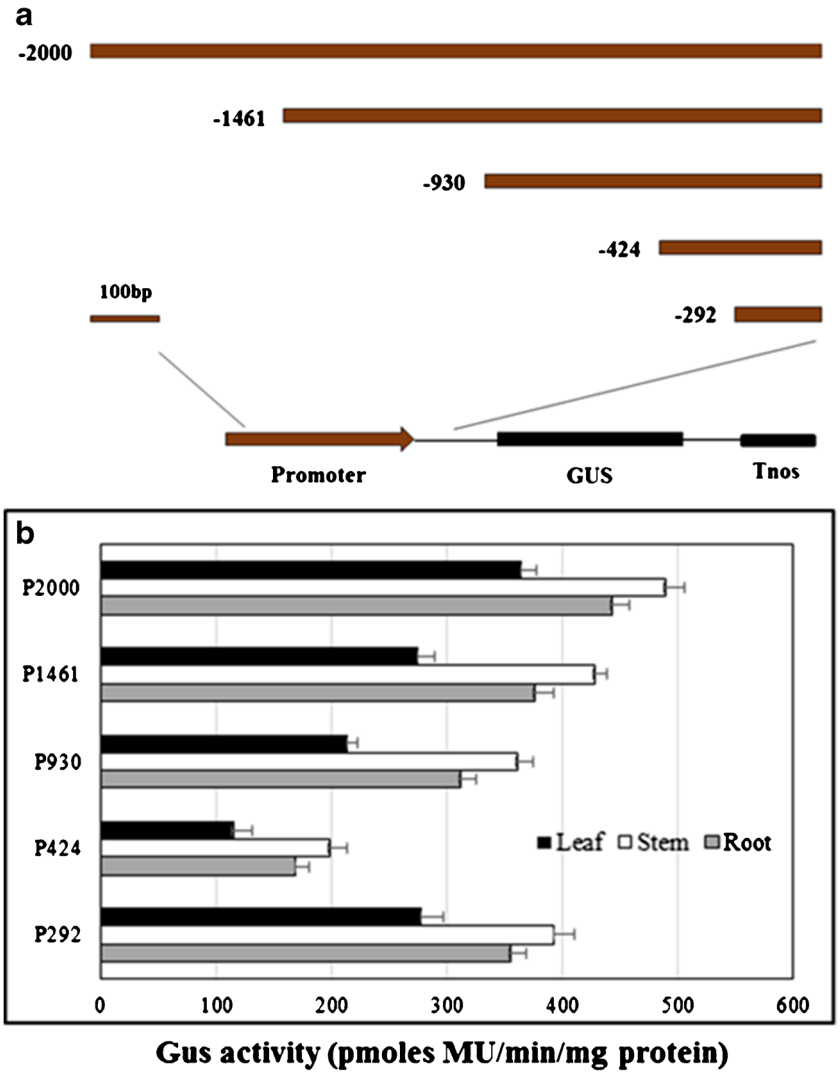


Fig. 12 Deletion analysis of the *VvPAL-like* promoter in transgenic tobacco. **a** Different deletions of the 5′ upstream regions of the *VvPAL-like* promoter were fused to the GUS reporter gene constructs, and the corresponding GUS activities in transgenic tobacco plants were determined. The *numbers on the left* indicate the distance between the 5′ end points of the promoter fragments and the translation initiation codon (ATG). **b** Activities of the transgenic tobacco plants were measured in leaves, roots, and stems. Means of the results from four independent lines are shown with SD

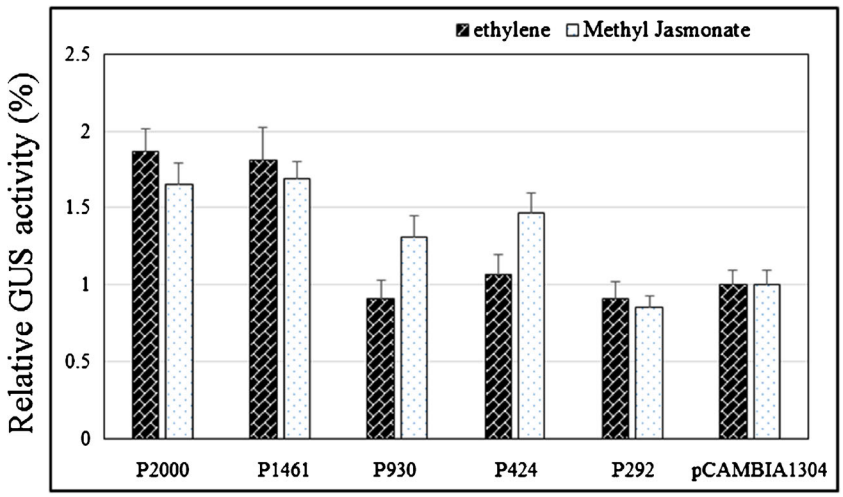


Fig. 13 Induction of GUS activity by ethylene and MeJA in transgenic tobacco plants carrying different *VvPAL-like* promoter fragments. GUS activity for each of the different upstream deletions of the *VvPAL-like* promoter fused to GUS was determined in MS medium alone (nontreatment) or supplemented with 100 μM ethylene or 100 μM MeJA (treatment). The relative GUS activity for the five deletion constructs is shown. The *bars* indicate the relative GUS activity (mean ± SD) in the treatment versus nontreatment. Four independent lines were analyzed for each construct. Additionally, the relative GUS activity was also measured in plants carrying 35S::GUS (pCAMBIA1304, positive control)

that the putative CURECORECR element of the *VvPAL-like* promoter is indeed required for copper-activated expression of the reporter GUS gene in transgenic tobacco plants. To reveal more insights into how this intricate metal ion-responsive reaction operates within the cells encountering unfavorable heavy metal ions, continuing work on plant metal response element (MRE) identification and a thorough investigation of interaction between the MREs and their binding factors are of great importance.

Many reports have indicated that auxin plays a role in vascular bundle formation since early physiological studies showed that auxin transport originating from the shoot apex is necessary for the formation of vascular tissues (Aloni 1987; Sachs 1991). Other evidences also indicate the auxin response element (ARFAT), which consists of a TGTCTC sequence and its reverse element (GAGACA), is found to exist in the promoter regions of many auxin-induced genes (Goda et al. 2004). In this study, a putative auxin response element (ARFAT) was found to exist in the *VvPAL-like* promoter. As shown in Fig. 11, the GUS activities were enhanced under the auxin treatment. The results presented here demonstrate that the putative ARFAT element of the *VvPAL-like* promoter is indeed required for auxin-activated expression of the reporter GUS gene in transgenic tobacco plants. ABA as an important phytohormone antagonized the stimulatory effect of IAA. As shown in Fig. 9, the *VvPAL-like* promoter could be induced by ABA. Therefore, we also made an attempt to investigate the effects of time and dose on ABA induction. Our results showed that *VvPAL-like* promoter may contain some putative ABA-responsive elements. Additionally, our results also revealed that the *VvPAL-like* promoter was inducible in transgenic tobacco plants in response to ethylene treatment (Fig. 9), and a series of 5′ progressive deletions of that full-length promoter revealed the presence of a regulatory region (−1461 to −930) important for ethylene-inducible expression (Fig. 13). This is consistent with the result that ERE (ATTTCAAA), involving in ethylene response, was found at position −1173 to −1166. Therefore, the putative ERE element of the *VvPAL-like* promoter is indeed required for ethylene-activated expression of the reporter GUS gene in transgenic tobacco plants. As for MeJA induction, four constructs (P2000, P1461, P930, and P424) were induced (Fig. 13), implying that the MeJA-responsive element may be located in the region −424 to −292. This result is consistent with that of sequence analysis in which one CGTCA-motif, involving in the MeJA responsiveness, was found at position −315 to −311. The result also showed that the CGTCA-motif of the *VvPAL-like* promoter is indeed required for CGTCA-motif-activated expression of the reporter GUS gene in transgenic tobacco plants. The above results demonstrate that a 2000-bp fragment of the *VvPAL-like* promoter possesses a complicated transcriptional regulation mechanism with distinct functional regions in a model plant. Although the roles and functions of *PAL* in plants have been well defined, the diversity of the sites of their expression and different stimuli used to activate them have enabled us to recognize that the plant *PAL* could be involved in many biological processes, including growth, development, and defense. It is also expected that this work will provide an important insight into the regions that control the temporal- and spatial-specific expression and stress responses of the *VvPAL-like* gene. Therefore, our results here will provide a framework for continued studies on identification of regulatory elements in the *VvPAL-like* promoter. On the other hand, the regulatory sequences of promoters regulate gene expression both quantitatively and qualitatively in plants.

Acknowledgments The study was funded by the Important National Science & Technology Specific Projects (No. 2012FY110100-3) and the Fundamental Research Funds for the Central Universities of China (KYZ201411). We thank all laboratory members for help, advice, and discussion.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests. All authors have read and approved the final manuscript.

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