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ORIGINAL ARTICLE

Promoter analysis reveals *cis*-regulatory motifs associated with the expression of the WRKY transcription factor *CrWRKY1* in *Catharanthus roseus*

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Abstract WRKY transcription factors (TFs) are emerging as an important group of regulators of plant secondary metabolism. However, the *cis*-regulatory elements associated with their regulation have not been well characterized. We have previously demonstrated that CrWRKY1, a member of subgroup III of the WRKY TF family, regulates biosynthesis of terpenoid indole alkaloids in the ornamental and medicinal plant, *Catharanthus roseus*. Here, we report the isolation and functional characterization of the *CrWRKY1* promoter. In silico analysis of the promoter sequence reveals the presence of several potential TF binding motifs, indicating the involvement of additional TFs in the regulation of the TIA pathway. The *CrWRKY1* promoter can drive the expression of a β-glucuronidase (*GUS*) reporter gene in native (*C. roseus* protoplasts and transgenic hairy roots) and heterologous (transgenic tobacco seedlings) systems. Analysis of 5′- or 3′-end deletions indicates that the sequence located between positions −140 to −93 bp and −3 to +113 bp, relative to the transcription start site, is critical for promoter activity. Mutation analysis shows that two overlapping *as-1* elements and a CT-rich motif contribute significantly to promoter activity. The *CrWRKY1* promoter is induced in response to methyl jasmonate (MJ) treatment and the promoter region between −230 and −93 bp contains a putative MJ-responsive element. The *CrWRKY1* promoter can potentially be used as a tool to isolate novel TFs involved in the regulation of the TIA pathway.

Keywords *Catharanthus roseus* · Hairy roots · Terpenoid indole alkaloids · WRKY transcription factor

Abbreviations

as-1	Activation sequence 1
MJ	Methyl jasmonate
TIA	Terpenoid indole alkaloids
TSS	Transcription start site

Introduction

Secondary metabolites, including alkaloids, flavonoids and terpenes, play diverse biological roles in plants. Moreover, some of these compounds have tremendous beneficial effects on human health. In addition to being one of the top annual bedding ornamental plants, *Catharanthus roseus* (Madagascar periwinkle), a member of the family Apocynaceae, is a natural source for over a hundred terpenoid indole alkaloids (TIAs) including ajmalicine, serpentine, vinblastine and vincristine. Ajmalicine and serpentine are used pharmaceutically for the treatment of hypertension

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whereas vinblastine and vincristine have proven invaluable in the treatment of cancers (Gantet and Memelink 2002; Murata et al. 2008). However, these compounds are produced in extremely low quantities in periwinkle plants. Despite their importance as potent anti-cancer drugs, the regulation, biosynthesis and transport of TIAs are not well understood (Glenn et al. 2013). The complexity of biochemical pathways and the lack of appropriate genetic tools are major factors that limit research progress in this area. Efforts made in the past few decades have led to the identification and characterization of a number of genes encoding key enzymes and regulatory proteins in the TIA pathway (Liu et al. 2007; Memelink and Gantet 2007; Yang et al. 2012). The expression of structural and TF genes in the TIA pathway is significantly induced by phytohormones, such as jasmonate (JA), and fungal elicitors. Jasmonate- and elicitor-responsive elements (JERE) have been identified in the promoter regions of a number of structural genes, such as *cytochrome P450 reductase* (*cpr*), *tryptophan decarboxylase* (*tdc*), *strictosidine synthase* (*str*) and *deacetylvindoline-4-O-acetyltransferase* (*dat*), and the TF gene, *octadecanoid responsive Catharanthus AP2-domain 3* (*ORCA3*) (Cardoso et al. 1997; Menke et al. 1999; Ouwerkerk and Memelink 1999; Vom Endt et al. 2007; Wang et al. 2010). Genes encoding AP2/ERF TFs, ORCA1 and ORCA2 and AT-hook proteins, have been isolated using the JERE of *str* and jasmonate responsive elements (JRE) of *orca3* promoters, respectively, in yeast one-hybrid experiments (Menke et al. 1999; Vom Endt et al. 2007), whereas the elicitor-responsive regions of the *tdc* and *str* promoters have been used to isolate the *zinc-finger C. roseus transcription factors* (*ZCTs*) and the *box-P binding factor* (*BPF-1*), respectively (Pauw et al. 2004; van der Fits et al. 2000). The bHLH TF gene, *CrMYC1*, was isolated using the G-box motif of the *str* promoter as a bait in a yeast one-hybrid screen (Chatel et al. 2003). Therefore, the *cis*-regulatory elements in promoters have proven invaluable in the isolation of a number of key positive or negative regulators in the TIA pathway. Investigating the interaction between specific *cis*-elements in the target promoters and activators and/or repressors in response to phytohormones and/or fungal elicitors provide insightful information about gene regulation in the TIA pathway.

WRKY TFs are emerging as key regulators of secondary metabolism in plants. WRKY TFs are characterized by the presence of a conserved amino acid sequence WRKYGQK followed by a $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$ zinc-finger motif, and comprise one of the largest families of TFs in plants. WRKY TFs recognize the canonical W-box motif (TTGAC/T) in the promoters of target genes (Chi et al. 2013; Rushton et al. 2010). WRKY TFs are involved in regulating the biosynthesis of a number of plant secondary metabolites including benzylisoquinolines in *Coptis japonica* (Kato et al. 2007), camalexin in *Arabidopsis thaliana* (Mao et al. 2011), artemisinin in *Artemisia annua* (Ma et al. 2009), gossypol in *Gossypium arboreum* (Xu et al. 2004) and paclitaxel in *Taxus chinensis* (Li et al. 2013). Recently, we have isolated and characterized a WRKY TF, CrWRKY1, from *C. roseus* for its role in TIA biosynthesis (Suttipanta et al. 2011). In addition to secondary metabolism, WRKY TFs are also involved in the regulation of diverse biological processes in plants such as plant defense, hormone signaling, responses to abiotic stress, trichome development and senescence (Chi et al. 2013). Expression of *WRKY* genes is influenced by a number of factors including phytohormones such as methyl jasmonate (MJ) and salicylic acid (Chi et al. 2013; Dong et al. 2003; Suttipanta et al. 2011); however, the mechanism by which phytohormones control *WRKY* expression is poorly understood. Recently, characterization of several *WRKY* gene promoters has revealed that W-box and activation sequence 1 (*as-1*) (TGACG) elements are involved in the regulatory activities of these genes (Chujo et al. 2009; Petitot et al. 2013). In this study, we isolated and functionally characterized the *CrWRKY1* promoter from *C. roseus* protoplasts, transgenic *C. roseus* hairy roots and tobacco seedlings. Deletion and mutation analysis of the *CrWRKY1* promoter identified *cis*-regulatory regions that are critical for its basal and MJ-responsive expression.

Materials and methods

Isolation of *CrWRKY1* promoter

The 5′-flanking region of the *C. roseus CrWRKY1* gene was isolated by a PCR-based genome walking method using the GenomeWalker Universal Kit (Clonetech, Mountain View, USA). Genomic DNA was extracted from *C. roseus* cv. First kiss seedlings using DNeasy plant mini kit (Qiagen, Chatsworth, USA). Two non-overlapping gene-specific primers (GSP1 and GSP2; Table S1) were designed based on *CrWRKY1* cDNA sequence information available in GenBank (Accession no. HQ646368). Genomic DNA, digested with, *Dra*I, *Eco*RV and *Ssp*I, was ligated to the GenomeWalker adapter. The primary PCR was carried out using an adapter primer (AP1) and a gene-specific primer (GSP1) followed by a second PCR using nested adapter primers (AP2) and a gene-specific primer (GSP2). All PCR reactions were performed using Advantage 2 Polymerase mix (Clonetech, Mountain View USA) as per manufacturer's instruction. The major nested PCR product obtained using an *Ssp*I-digested library was gel-purified and cloned into the pGEM-T Easy vector (Promega, Madison, USA) and sequenced. Based on this sequence information a forward primer, with an *Eco*RI restriction site, and a reverse

primer, with a *Xho*I restriction site, were designed (Table S1). To verify the specificity of the sequence obtained by genome walking, the promoter fragment was re-amplified from genomic DNA using these primers, cloned in pGEM-T easy and sequenced. The plasmid containing the *CrWRKY1* promoter was designated as pGEM-*CrWRKY1*.

Identification of transcription start site by 5′-RACE

The transcription start site (TSS) of the *CrWRKY1* promoter was identified by 5′-rapid amplification of cDNA ends (5′-RACE). Total RNA was isolated from *C. roseus* seedlings using the RNeasy Plant mini kit (Qiagen, Chatsworth, USA). RACE was performed following the protocols supplied with the 5′ RACE System (Invitrogen, Grand Island, USA). A gene-specific primer (WRKY-RACE 1; Table S1) was used for the synthesis of first-strand cDNA. For amplification of the dC-tailed first-strand cDNA, the adapter primer from the kit and two nested gene-specific antisense primers (WRKY-RACE 2 and 3; Table S1) were used in subsequent PCR. PCR products were cloned in the pGEM-T Easy vector and 10 positive clones were sequenced to determine the TSS.

Construction of plasmids for transient assay in *C. roseus* protoplasts

A series of promoter fragments of various lengths, depicted in Fig. 2, was PCR-amplified from the pGEM-*CrWRKY1* plasmid using appropriately designed primers. PCR products were gel-purified, digested with *Eco*RI and *Xho*I, and cloned into a modified pUC19 vector containing a β-glucuronidase (*GUS*) reporter gene and *rbcS* terminator (Suttipanta et al. 2007). The clones were sequenced to verify sequence integrity. A total of six plasmids were constructed, corresponding to coordinates (relative to TSS) −411 to +113 (WT), −323 to +113 (delA), −230 to +113 (delB), −140 to +113 (delC), −93 to +113 (delD), and −411 to −3 (delE). Five mutant promoter constructs, designated as M1 through M5, were generated by replacing the corresponding *cis*-element with a six nucleotide sequence (AGATCC) which does not create any known *cis*-regulatory motif. The mutant promoters were cloned in a modified pUC19 vector as mentioned earlier (Suttipanta et al. 2007).

Protoplast isolation and electroporation

Protoplast isolation from *C. roseus* cell suspension cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH no. 510) and electroporation with promoter/GUS plasmids were performed essentially as previously described (Suttipanta et al. 2007, 2011). Electroporation was carried out using the Gene Pulser II apparatus (BioRad, Hercules, USA) with the Capacitance Extender II (Model 165–2107). An electroporation cuvette (0.4 cm electrode gap) containing approximately a million protoplasts in a total volume of 750 μL was electroporated (200 V and 950 μF capacitance ~50 ms) with 5 μg of plasmid DNA. A plasmid containing the firefly luciferase coding sequence under the control of the *CaMV*35S promoter and *rbcS* terminator was co-electroporated as an internal control. After 20–22 h, protoplasts were harvested to measure GUS and luciferase activities (Suttipanta et al. 2007). Fluorometric assays to measure GUS activities in protoplast extracts or plant tissue were performed according to the protocols described previously (Jefferson et al. 1987; Suttipanta et al. 2011). Luciferase activity in transfected protoplasts was measured using the Luciferase Assay System (Promega, Madison, USA) and a luminometer (Model No. TD2020; Turner Designs, Sunnyvale, USA). GUS activity was normalized against luciferase activity and expressed as fold activation relative to control. All constructs were tested in at least three independent experiments.

Construction of plant expression vectors and plant transformation

The full-length promoter (WT) and three deletion fragments(delC, delD and delE)were isolated from protoplast expression vectors and cloned into the plant transformation vector pKYLX71 (Schardl et al. 1987). The resulting plant expression vectors were designated pKWT-GUS, pKdelC-GUS pKdelD-GUS and pKdelE-GUS. The plasmids were mobilized into *Agrobacterium tumefaciens* strain C58C1:pGV3850 and *Agrobacterium rhizogenes* R1000 by freeze–thaw method. Transformation of *C. roseus* 'Cooler Apricot' with *A. rhizogenes* for generation of transgenic hairy root cultures was performed essentially as previously described (Suttipanta et al. 2011). *Catharanthus roseus* seeds were surface-sterilized and germinated on Murashige & Skoog's (MS) medium. After removal of the roots, 10-day-old seedlings were immersed in bacterial suspension for 30 min and then transferred onto solid MS basal medium for co-cultivation in dark. After 48 h, explants were transferred to half-strength MS basal medium containing cefotaxime (400 mg/L). Hairy roots developed at the cut surface of explants after 3–4 weeks. After 6–7 weeks, roots were excised and transferred to one-third-strength Schenk and Hilderbrandt (SH) basal medium containing 100 mg/L kanamycin and 400 mg/L cefotaxime. Eight to ten independent lines were generated for each construct and two positive hairy root lines were chosen for detailed analysis. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were transformed with *A. tumefaciens* using the leaf disc transformation procedure (Pattanaik et al.

2008). Regenerated kanamycin-resistant tobacco plants were grown under greenhouse conditions. Histochemical assays to detect GUS activity in transgenic hairy roots or tobacco seedlings were performed essentially as described (Suttipanta et al. 2007). Stained plant materials were viewed by light microscope (Olympus BX51) and photographed with a DP50 camera. GUS-positive independent hairy root lines were transferred to liquid SH medium and kept at room temperature with continuous shaking (100 rpm) for 2–3 weeks. These roots were used in all subsequent experiments.

Molecular analysis of transgenic plants: RNA isolation, semi-quantitative RT-PCR and qRT-PCR

Total RNA was isolated from transgenic hairy roots or tobacco seedlings using the RNeasy Plant mini kit (Qiagen, Chatsworth, USA). About 1.5 μg RNA was treated with RNase-free DNase (Sigma, USA) and used for synthesis of first-strand cDNA in a total volume of 20 μL using Superscript III Reverse Transcriptase (Invitrogen, USA). To verify the presence of both pKYLX71 vector and Ri T-DNAs in the transgenic hairy root lines, neomycin phosphotransferase II (*nptII*), *GUS*, *rolB*, and *rolC* transcripts were amplified from cDNA using gene-specific primers. A gene outside of the Ri-plasmid T-DNA, *virC*, was used as a control to detect *A. rhizogenes* contamination of the hairy roots. To verify the presence of pKYLX71 T-DNA in transgenic tobacco lines, *nptII* and *GUS* transcripts were amplified from the cDNA made from seedling RNA. To compare the levels of *GUS* mRNA in different transgenic hairy root or tobacco seedlings, quantitative real-time PCR (qRT-PCR) was performed essentially as described earlier (Pattanaik et al. 2010; Suttipanta et al. 2011). The 40S ribosomal protein subunit 9 (*Rps9*) and glyceraldehyde phosphate dehydrogenase (GADPH) were used as normalization controls for hairy roots and tobacco, respectively. Each assay had two biological and three technical replicates. All primer pairs used in qRT-PCR are listed in Table S1.

Methyl jasmonate treatment

A 100 mM stock solution of MJ was made in absolute ethanol and was diluted to liquid MS medium to a final concentration of 100 μM. Transgenic hairy roots were incubated in the medium for 2 h. Only ethanol was added to the mock-treated samples. The MJ- and mock-treated samples were then used for quantifying the *GUS* transcripts and measuring GUS activity using the protocol described earlier (Suttipanta et al. 2007).

Results and discussion

CrWRKY1 promoter contains several *cis*-regulatory motifs

The *CrWRKY1* promoter was isolated by PCR-based genome walking. The TSS of the *CrWRKY1* promoter was determined by 5′-RACE using total RNA isolated from *C. roseus* seedlings followed by cloning and sequencing of amplified PCR products. The TSS was mapped to a thymidine (T) residue located 113 bp upstream of the translation initiation codon (Fig. 1). Analysis of the *CrWRKY1* promoter sequence using the PLACE database (http://www.dna.affrc.go.jp/PLACE) (Higo et al. 1999) revealed the presence of several well-characterized *cis*-regulatory elements including a MYB-recognition motif (CCAACC) at position −195 to −190, and an E-box (CAAATG) at position −189 to −184, relative to TSS, and multiple DOF

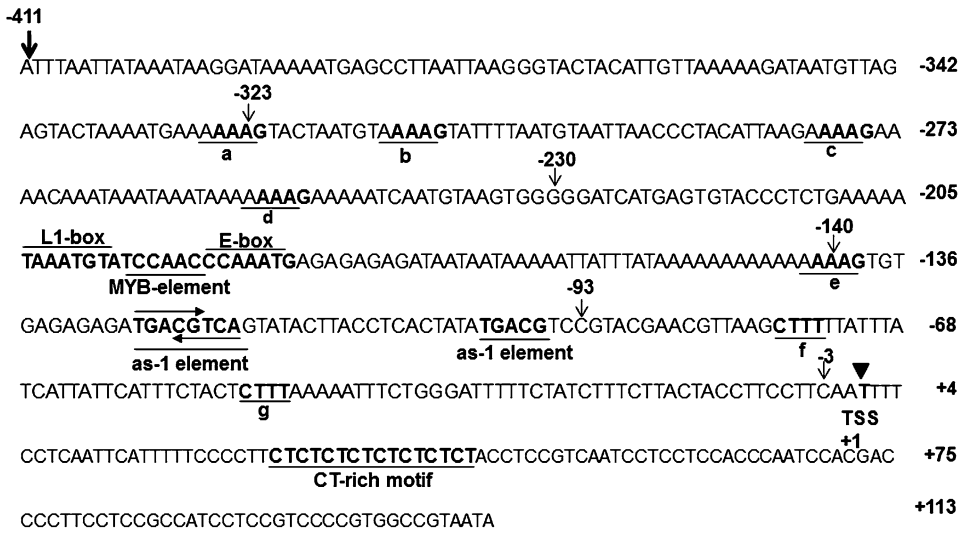

Fig. 1 The DNA sequence of the *CrWRKY1* promoter. A 524-bp fragment (−411 to +113) with respect to the transcription start site (TSS) is presented. The TSS is indicated with *reverse solid triangle* and designated as +1. An *arrow* above the sequence indicates the start point of different deletion fragments. Potential *cis*-elements are in *bold* and indicated by a *solid line* above or below the sequence. Putative Dof binding motifs are labeled as *a* through *g*

(DNA binding with one finger) recognition core sequences (AAAG and CTTT) (Fig. 1).

MYB and E-box motifs are found in a number of plant promoters and are bound by R2R3 MYBs and basic helix-loop-helix (bHLH) TFs, respectively. The G-box (an E-box derivative) motif present in the *C. roseus str* promoter has been shown to be recognized by the basic leucine zipper (bZIP) TFs, G-box binding factor (GBF) 1 and 2, and the bHLH TF, CrMYC1 (Chatel et al. 2003; Siberil et al. 2001). The T/G-box element (an E-box derivative) in the *C. roseus ORCA3* promoter is bound by the bHLH TF, CrMYC2, which regulates its expression in response to MJ (Vom Endt et al. 2007). The *C. roseus* MYB-like factor, BPF-1, interacts with the elicitor-responsive region of the *str* promoter (van der Fits et al. 2000). Whether these factors can interact with the putative E-box and MYB-binding motifs in the *CrWRKY1* promoter remains to be determined. DOF proteins are plant-specific TFs that have been identified in many monocots and dicot species. DOFs act as either activators or repressors and are involved in various biological processes in plants (Yanagisawa 2004). Although DOF recognition sites have been identified in other TIA pathway promoters, their biological significance remains to be elucidated (Ginis et al. 2012; Suttipanta et al. 2007).

In addition, the *CrWRKY1* promoter contains three *as-1* elements (TGACG), two of which overlap to create an imperfect palindrome (TGACGTCA) in the region −127 to −120, and the third is located between −100 and −95 relative to the TSS. *as-1* elements are found in the *Agrobacterium nopaline synthase* (*nos*) promoter, caulimovirus promoters, as well as in many plant promoters, and have been shown to respond to phytohormones such as MJ and salicylic acid (Kim et al. 1993; Kumar et al. 2012; Petitot et al. 2013; Rouster et al. 1997; Zhao et al. 2011). In *C. roseus*, *as-1* elements in the *dat* promoter have been reported to be involved in JA-responsive expression (Wang et al. 2010). TFs in the TGA family of bZIP proteins are also known to bind *as-1* elements (Lam and Lam 1995). In the *CrWRKY1* promoter, an L1-box (TAAATGTA) and a CT-rich element are located at −204 to −197 and +26 to +41, respectively, relative to the TSS. The L1-motif is found in the promoter of *Arabidopsis Protodermal Factor 1* (*PDF1*) that confers L1-layer specific expression and is targeted by the L1-specific homeodomain protein (Abe et al. 2001). CT-rich elements are present in certain caulimovirus promoters and have been shown to act as enhancers (Pauli et al. 2004).

WRKY gene promoters are enriched with W-box motifs (TTGAC/T), an indication of auto-regulation or cross-regulation for *WRKY* genes (Petitot et al. 2013). However, no W-box motifs are found in the *CrWRKY1* promoter. This explains our previous observation that overexpression of *CrWRKY1* in *C. roseus* protoplasts does not activate the co-transformed *CrWRKY1* promoter-reporter construct (data not shown). Analysis of the *CrWRKY1* promoter revealed unique organization of *cis*-regulatory sequences, suggesting the involvement of additional regulatory proteins in TIA pathway regulation.

A *cis*-element-rich region in *CrWRKY1* promoter is critical for its activity

To determine the promoter regions that are critical for activity, the *CrWRKY1* promoter was subjected to 5′- and 3′-end deletion analysis. Six promoter fragments were generated by PCR amplification and cloned into a modified pUC19 protoplast expression vector containing the *GUS* reporter gene. Activities of the promoter fragments were first assayed in *C. roseus* protoplasts. A schematic map of deletion constructs and the corresponding GUS activities of each construct are shown in Fig. 2. Deletion of the *CrWRKY1* promoter from the 5′-end to position −230 had no significant effect on promoter activity (comparing delA and delB with WT). However, deletion to −140 (delC) resulted in a 20 % decrease in promoter activity. Further deletion to position −93 (del D) decreased the promoter activity by 2.5-fold compared to that of the full-length promoter, suggesting that the regions between −230 to −140 and −140 to −93 determine the strength of the promoter. Sequence analysis revealed that these two regions contain several well-characterized potential TF binding sites which likely play roles in regulating the promoter. The construct delE (coordinates position −411 to −3 relative to TSS), devoid of the untranslated region (UTR), showed considerably lower GUS activity compared to the full-length promoter. These results suggest that *cis*-regulatory motifs present downstream of the TSS are also critical for *CrWRKY1* promoter activity.

as-1 and CT-rich motifs contribute significantly to *CrWRKY1* promoter activity

We next performed block mutagenesis to investigate the influence of individual *cis*-elements on *CrWRKY1* promoter activity. *cis*-regulatory elements in the promoter, either individually or in combinations, were replaced by an identical six nucleotide sequence (AGATCC). Activities of the mutant promoters on the GUS reporter were assayed in *C. roseus* protoplasts. A schematic map shows the positions of different elements and the relative GUS activities of the mutant promoters in Fig. 3. Mutation to the putative MYB-binding site (M1) or E-box (M2) individually had no significant effects on promoter activity. However, promoter activity decreased considerably (25–35 % of the WT) when the two overlapping *as-1* motifs (M3) or the CT-rich motif (M5) was mutated. To examine whether *as-1* (M3) and CT-rich (M5) motifs have synergistic effects on promoter activity, the two sites were mutated in combination. The activity of

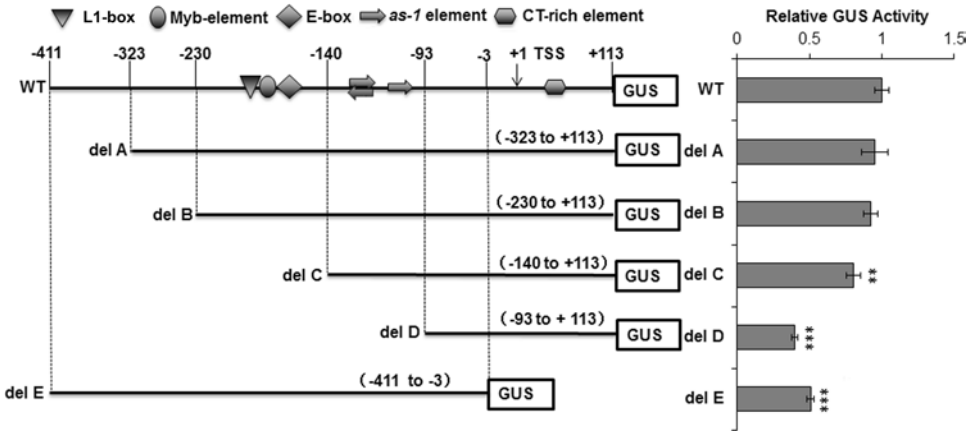

Fig. 2 Deletion analysis of the *CrWRKY1* promoter. The *left panel* shows a schematic map of the promoter deletion constructs. The 5′- and 3′-end coordinates of the deletion fragments are given in *parentheses*. At the *top*, the relative position of the transcription start site (TSS, +1) and the *CrWRKY1* promoter coordinates are indicated. The *right panel* shows the relative GUS activities of the corresponding construct. GUS activities were determined in the *C. roseus* protoplast transient assay. Each construct was assayed at least three times in three independent experiments. The relative GUS activity (GUS/LUC) with standard deviation is presented. Statistical significance was calculated using Student *t* test and were marked (**$P < 0.001$ and ***$P < 0.0001$)

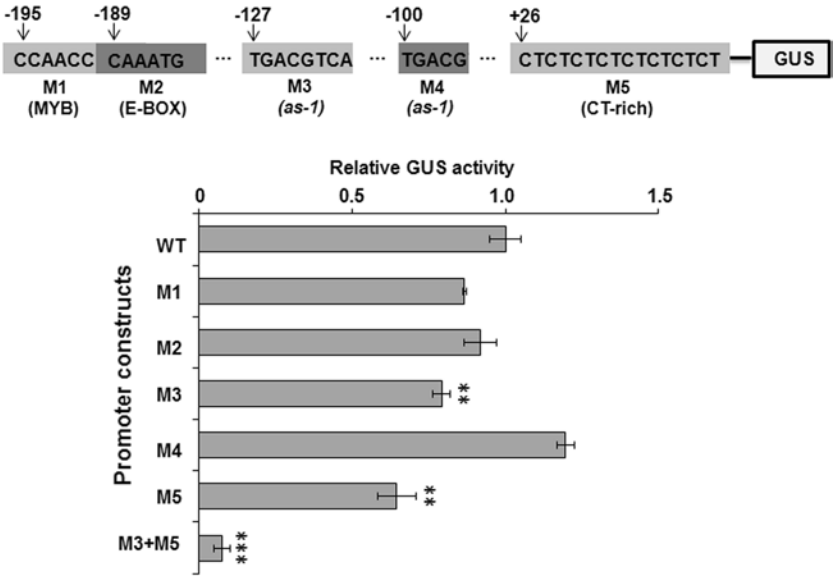

Fig. 3 Influence of different *cis*-elements on the activity of the *CrWRKY1* promoter. The *top panel* shows a schematic map with relative positions of the different *cis*-elements in the full-length promoter. Each *cis*-element was replaced by a hexa-nucleotide sequence, AGATCC. The *bottom panel* shows the corresponding relative GUS activities of each construct. GUS activities were determined in the *C. roseus* protoplast expression assay. Each construct was assayed at least three times in three independent experiments. The relative GUS activity (GUS/LUC) with standard deviation is presented. Statistical significance was calculated using Student *t* test and were marked (**$P < 0.001$)

the double mutant (M3 + M5) diminished more than 90 %, suggesting that these two elements are critical for *CrWRKY1* promoter activity (Fig. 3). *as-1* motifs and CT-rich elements of the *CaMV*35S promoter contribute to enhancer function and interaction with plant nuclear proteins (Lam et al. 1989; Pauli et al. 2004). Our results suggest that the *as-1* and CT-rich motifs in the *CrWRKY1* promoter also act as enhancers and likely interact with other regulatory factors.

The *CrWRKY1* promoter drives reporter gene expression in *C. roseus* hairy roots and tobacco seedlings

To evaluate the transcriptional activities of the full-length (WT) and truncated (delC, delD and delE; Fig. 2) promoters in stably transformed *C. roseus* cells, independent transgenic hairy root lines were generated with the *GUS* reporter gene under the control of the testing promoter. Expression of the

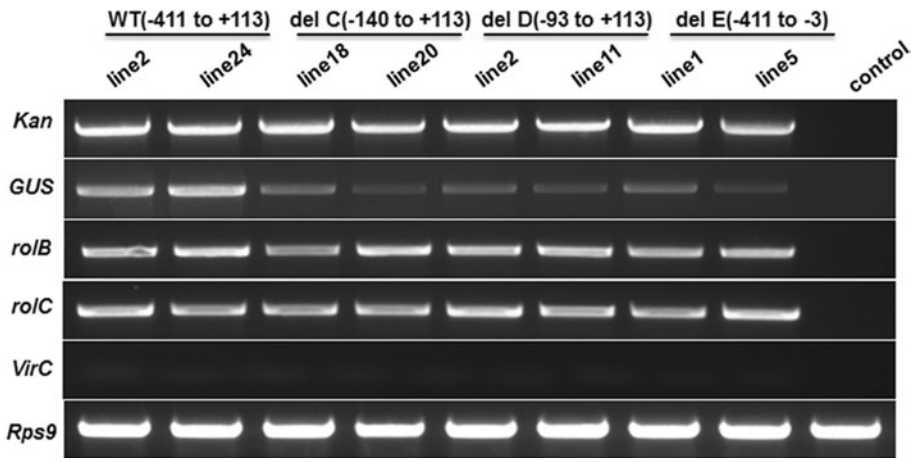

Fig. 4 RT-PCR analysis to detect the presence of transgenes in *C. roseus* hairy roots. For each construct two independent lines were used. The 5′- and 3′-end coordinates and the name of each construct are given at the *top* of each of the *two lines*. The roots from non-transgenic *C. roseus* seedlings were used as control. The ribosomal protein S9 (*Rps9*) gene was used as an internal control

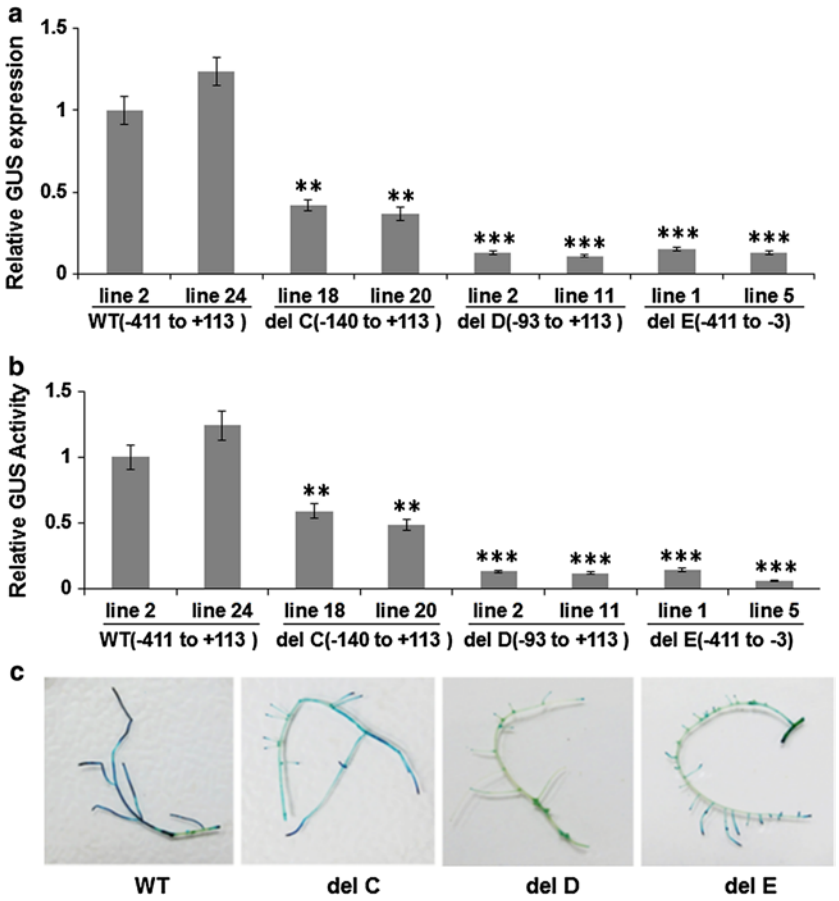

Fig. 5 Analysis of different *CrWRKY1* promoter deletion constructs in transgenic *C. roseus* hairy roots. **a** qRT-PCR, **b** quantitative GUS assay, **c** GUS histochemical staining. Two independent lines for each deletion construct were used in qRT-PCR and GUS assays. The 5′- and 3′-end coordinates of each construct are given in *parentheses*. The *Rps9* gene was used as an internal control. Each value represents the average of three measurements, with *error bars* representing standard deviation. Statistical significance was calculated using Student *t* test and were marked (**$P < 0.001$ and ***$P < 0.0001$)

transgenes in hairy roots was verified by semi-quantitative RT-PCR using gene-specific primers. For each construct, two independent positive hairy root lines were selected for further analysis (Fig. 4). *GUS* expression in transgenic hairy root lines was quantified using qRT-PCR. The highest *GUS* expression was detected in WT hairy roots. *GUS* transcript levels in delC lines were approximately 40 % of the WT. *GUS* expression in lines harboring the minimal promoter (delD) and delE was reduced by 80–90 % compared with WT (Fig. 5a). Quantitative GUS activity assay (Fig. 5b) and histochemical staining (Fig. 5c) in different transgenic lines correlated well with the accumulation of *GUS* transcripts in those lines. The highest *GUS* accumulation was detected in WT hairy roots but diminished in roots harboring the deletion fragments (Fig. 5b, c).

To determine whether the *CrWRKY1* promoter can drive gene expression in a heterologous system, independent tobacco transgenic lines harboring different promoter

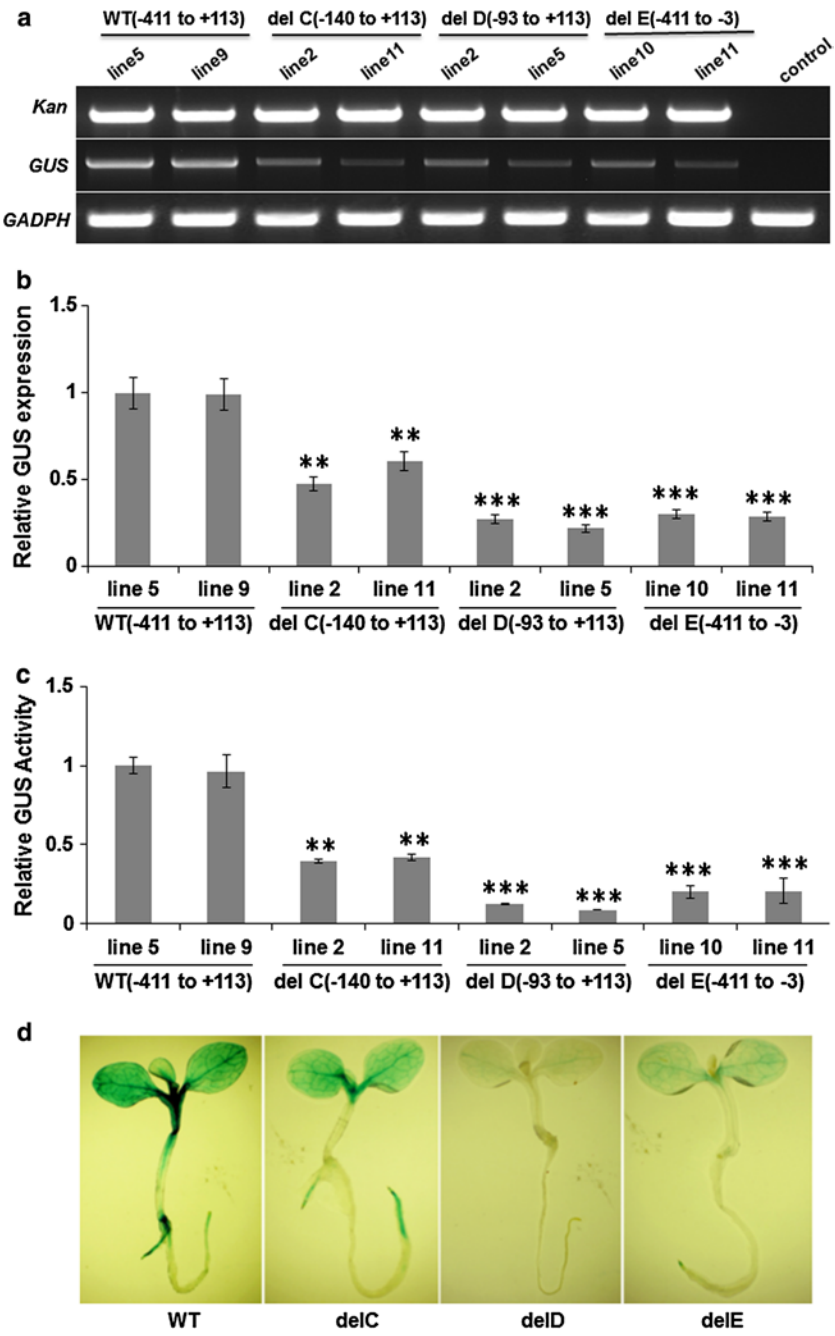

Fig. 6 Analysis of different *CrWRKY1* promoter constructs in transgenic tobacco seedlings. **a** RT-PCR analysis, **b** qRT-PCR, **c** quantitative GUS assay, **d** GUS histochemical staining. For each construct, two independent lines were used for RT-PCR, qRT-PCR and GUS assays. The 5′- and 3′-end coordinates of each construct are given in *parentheses*. The *GADPH* gene was used as an internal control. Non-transgenic tobacco seedlings were used as control. Each value represents the average of three measurements, with *error bars* representing standard deviation. Statistical significance was calculated using Student *t* test and were marked (**$P < 0.001$ and ***$P < 0.0001$)

fragments were generated. Expression of the transgenes was verified by semi-quantitative RT-PCR using gene-specific primers (Fig. 6a). For each construct, two independent lines were selected for further analysis. To quantify *GUS* transcripts in the different transgenic lines, qRT-PCR was performed using total RNA from 4-week-old seedlings. As in transgenic hairy roots, the accumulation of *GUS* transcripts was significantly higher in seedlings harboring the full-length promoter. *GUS* expression in lines harboring delC, delD or delE was 50–80 % lower compared with WT lines (Fig. 6b). Quantitative GUS activity assay (Fig. 6c) and histochemical staining (Fig. 6d) corroborated the expression levels of the *GUS* gene in seedlings. Histochemical staining of whole seedlings harboring the WT promoter showed that *GUS* expression was localized to leaves and roots. No visible GUS staining was observed in hypocotyls. In roots, GUS staining was restricted mainly to root tips. A tissue-specific expression pattern was also reported for *G10H* and HDS TIA pathway promoters (Ginis et al. 2012; Suttipanta et al. 2007). Experiments with young seedlings have shown that biosynthesis and accumulation of TIAs are developmentally regulated and genes associated with TIA biosynthesis are

expressed in actively growing tissues such as young leaves and roots (St-Pierre et al. 1999). The *CrWRKY1* promoter exhibits similar expression patterns even in a heterologous system. The intensities of GUS staining were significantly weakened in seedlings harboring the deletion fragments (delC, delD or delE; Fig. 6d). Although GUS staining was detected in leaf and roots of delE seedlings, it was significantly lower compared to WT seedlings. GUS staining was essentially undetectable in roots of the delD seedlings. The delD mutant is devoid of *as-1* motifs. A similar spatial expression pattern of the *CrWRKY1* promoter in *C. roseus* hairy roots and in a heterologous system such as tobacco indicates that regulatory proteins controlling transcriptional activity of the promoter are conserved across the species.

In the *CaMV*35S promoter, the *as-1* motifs (TGACG) have been implicated in tissue-specific expression. Lam et al. (1989) have shown that mutation in *as-1* elements caused a 50 % reduction in leaf expression of the *CaMV*35S promoter. Moreover, the same mutations attenuated stem- and root-specific expression of the *CaMV*35S promoter five to tenfold compared with the expression in leaves. The *as-1* motif in promoters is recognized and bound by nuclear proteins, such as TGAs, from both leaves and roots, and is important in determining tissue-specificity (Lam and Lam 1995). Our results suggest that the *as-1* elements in the *CrWRKY1* promoter are probably involved in controlling tissue-specific expression. Whether the TGA TFs play a role in *CrWRKY1* regulation is unclear. The delE mutant, which lacks the CT-rich motif, produces greatly reduced GUS activity. In the *CaMV*35S promoter, a repeated CT-rich motif present downstream of the TSS, referred to as stimulatory region 1 (S1), has been shown to act as an enhancer in a protoplast assay. Mutation of this motif significantly reduced promoter activity (Pauli et al. 2004). The lower GUS activity in delE seedlings indicates that the CT-rich element is critical for *CrWRKY1* promoter function.

Taken together, our findings suggest that: (a) the *CrWRKY1* promoter is capable of driving the expression of a reporter gene in native as well as heterologous systems, (b) the activity of the *CrWRKY1* promoter is most likely controlled by combinatorial or synergistic interaction between cognate trans-acting factors in the cells and *cis*-regulatory elements present both upstream and downstream of the TSS, and (c) the *as-1* and CT-rich motifs play significant roles in determining strength and tissue-specificity of this promoter.

CrWRKY1 promoter contains MJ-responsive sequences

It has been previously demonstrated that expression of *CrWRKY1* in *C. roseus* seedlings is induced in response to MJ-treatment (Suttipanta et al. 2011). To determine whether the *CrWRKY1* promoter contains MJ-responsive sequences, independent transgenic *C. roseus* hairy root lines, harboring full-length (WT) promoter or a truncated one in which *cis*-elements rich region was deleted (delD; Fig. 2), were treated with MJ and analyzed for *GUS* expression using qRT-PCR and quantitative GUS activity assay. Accumulation of *GUS* transcripts and relative GUS activity in MJ-treated lines were compared with corresponding mock-treated lines. The *CrWRKY1* promoter was moderately induced in response to MJ-treatment (Fig. 7). Similar induction patterns were observed for two other TIA pathway promoters, *G10H* and *HDS* (Ginis et al. 2012; Suttipanta et al. 2007). The response to MJ-treatment was completely abolished when the *cis*-element rich region was deleted (delD), indicating the presence of a putative MJ-responsive element between −230 and −93 bp in the promoter. This 137 bp region contains several known *cis*-regulatory elements including *as-1* elements (TGACG). We have previously shown that mutation in overlapping *as-1* elements (TGACGTCA) present in this region affects promoter activity (Fig. 3).

Phytohormone-responsive elements in plant gene promoters often lack positional and sequence consensus.

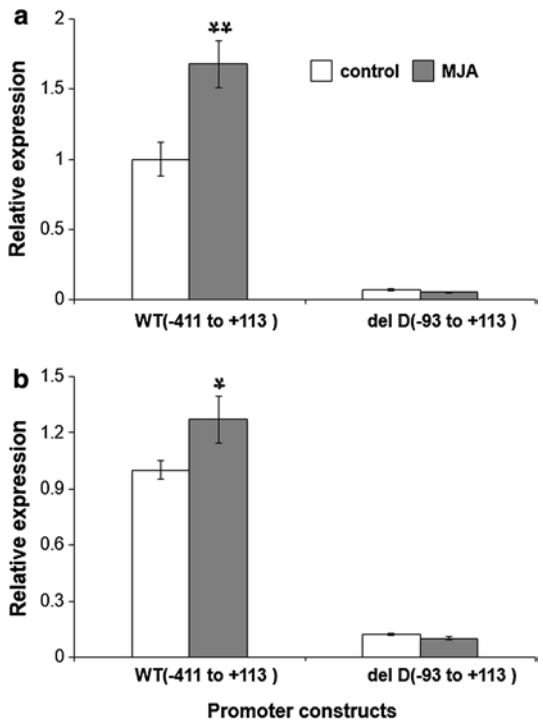

Fig. 7 Effect of methyl jasmonate (MJ) on GUS expression in transgenic hairy roots. **a** qRT-PCR, **b** quantitative GUS assay. Transgenic hairy roots were treated with MJ for 2 h. GUS transcripts were quantified using qRT-PCR and relative GUS activity was measured. Untreated hairy roots were used as control. The *Rps9* gene was used as an internal control for qRT-PCR. The relative GUS expression and GUS activity with standard deviation is presented. Statistical significance was calculated using Student *t* test and were marked (*$P < 0.01$ and **$P < 0.001$)

They usually contain multiple *cis*-elements that are shared by other stress responses, underscoring the complexity of transcriptional regulation. JERE have been identified in the promoters of *C. roseus* TIA pathway genes including *cpr*, *str*, *tdc*, *dat* and *orca3*. The JERE sequence varies greatly among these promoters. The JERE in the *str* promoter is a 24-bp sequence with a GCC-core sequence that is bound by the AP2/ERF TF, ORCA2 (Menke et al. 1999), whereas the JA-responsive expression of *ORCA3* is controlled by a 74-bp element that consists of a quantitative A/T-rich sequence and a qualitative T/G-box motif (Vom Endt et al. 2007). The *as-1* elements in *C. roseus dat* and barley *lipoxygenase 1* promoters have been implicated in MJ-responsive gene expression (Rouster et al. 1997; Wang et al. 2010). MJ-responsive expression of the *dat* promoter is controlled by three TGACG motifs and an inverted CGTCA sequence, whereas MJ-inducibility of the *lipoxygenase 1* promoter depends on two complementary *as-1* motifs (CGTCA and TGACG). Our results indicate that the *as-1* elements present in the 137 bp region are likely involved in MJ induction of the *CrWRKY1* promoter.

Conclusion

Utilizing promoter *cis*-elements to isolate regulatory factors is an effective approach to establish transcriptional hierarchy and network and has been successfully applied to identify several key regulatory factors of *C. roseus* TIA biosynthesis. In this report, we describe the functional characterization of the promoter of *CrWRKY1*, a regulator in the TIA pathway. We demonstrated that the promoter is active in both native and heterologous systems and that multiple *cis*-regulatory elements in the *CrWRKY1* promoter are responsible for regulating its strength. Two overlapping *as-1* motifs and a CT-rich element are especially critical for promoter activity. Some of these *cis*-regulatory elements have not been previously implicated in the regulation of any well-characterized TIA pathway genes. Accumulating evidence suggests that biosynthesis of secondary metabolites, including TIAs, is controlled at the transcriptional level. In *C. roseus*, transcriptional activators and repressors are simultaneously induced in response to phytohormones, fungal elicitor or environmental stimuli to form a dynamic regulatory network that fine-tunes the spatio-temporal expression of target genes and the subsequent accumulation of metabolites. We have previously shown that CrWRKY1 is a regulator in the TIA pathway and most likely acts upstream of ORCAs and MYC2 in the transcriptional cascade. In the present study, the identification of key *cis*-elements suggests that TFs binding to *as-1* and CT-rich sequences are involved further upstream of the transcriptional cascade. Furthermore, MYB-, bHLH-, and DOF-like TFs may regulate the *CrWRKY1* gene. We, therefore, propose that targeted gene isolation based on key *cis*-elements in the *CrWRKY1* promoter will lead to identification of novel regulators involved in TIA pathway regulation.

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