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Catharanthus roseus G-box binding factors 1 and 2 act as repressors of strictosidine synthase gene expression in cell cultures

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Abstract

The enzyme encoded by the strictosidine synthase (*Str*) gene catalyses a key step in the biosynthesis of therapeutically valuable terpenoid indole alkaloids. In *Catharanthus roseus* the *Str* gene was shown to be regulated by a wide variety of signals including auxin, methyl jasmonate and fungal elicitors in cell suspension cultures and by tissue-specific control in plant organs. The *Str* promoter contains a functional G-box (CACGTG) *cis*-regulatory sequence. In order to understand better the mechanisms involved in the regulation of *Str* gene expression, we isolated the *C. roseus* cDNAs encoding G-box binding factors *Crgbf1* and *Crgbf2*. The binding specificity of their protein products CrGBF1 and CrGBF2 was analysed by competitive electrophoresis mobility and saturation binding assays. CrGBF1 had a high binding specificity for class I G-boxes including the *Str* G-box. CrGBF1 showed a lower affinity for class II G-boxes and for the G-box-like element (AACGTG) found in the tryptophan decarboxylase (*Tdc*) gene which encodes another enzyme involved in TIA biosynthesis. CrGBF2 showed a high affinity for all types of G-boxes tested and to a lesser extent for the *Tdc* G-box-like element. Transient bombardment experiments demonstrated that both CrGBF1 and CrGBF2 can act *in vivo* as transcriptional repressors of the *Str* promoter via direct interaction with the G-box. These data indicate that GBFs may play functional role in the regulation of expression of the terpenoid indole alkaloid biosynthetic gene *Str*.

Introduction

Strictosidine is the universal precursor for therapeutically valuable terpenoid indole alkaloids (TIAs) which are produced by several plant species such as *Catharanthus roseus*, *Campthotheca accuminata, Cinchona ledgeriana* and *Rauvolfia canescens*. Strictosidine results from the condensation of the amino acid derivative tryptamine and the terpenoid secologanin. This reaction is catalysed by the key enzyme strictosidine synthase (EC 4.3.3.2) (Kutchan, 1993). In *C. roseus* complex molecular mechanisms are involved in the control of *Str* gene regulation by hormones, elicitors and during development (Meijer *et al.*, 1993). At the whole-plant level, *Str* gene expression and TIA biosynthesis are under organ- and tissue-specific regulation. *In situ* hybridization experiments have shown that *Str* gene expression is located in root tips and in the epidermal cell layer of young leaves (St-Pierre *et al.*, 1999).

In cell suspension cultures, auxin, which is necessary for maintaining growth in *C. roseus* suspension cells, is a powerful inhibitor of TIA biosynthesis

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers AF084971 (*Crgbf1*) and AF084972 (*Crgbf2*).

(Mérillon *et al.*, 1986; Morris, 1986). Auxin exerts its negative effect at the transcriptional level, since addition of auxin to cell suspensions grown in auxin-free medium rapidly reduces the *Str* mRNA steady-state level and transcription (Pasquali *et al.*, 1992). Treatment of *C. roseus* cells with fungal elicitors induces *Str* gene transcription (Pasquali *et al.*, 1992). Methyl jasmonate (MeJa) has been reported to be a strong inducer of TIA biosynthesis in *C. roseus* seedlings (Aerts *et al.*, 1994) and cell suspensions (Gantet *et al.*, 1998) and was shown to induce *Str* gene expression (Menke *et al.*, 1999b).

Functional analysis of the *Str* gene promoter has led to the identification of a *cis*-regulatory sequence involved in elicitor and MeJa responses (Menke *et al.*, 1999a). This sequence, located at −94 to −70 relative to the start of transcription, interacts specifically with the AP2/ERF-domain transcription factor ORCA2. This transcription factor is the terminal component of the elicitor/MeJa signal transduction pathway leading to stimulation of *Str* gene expression in *C. roseus* cell suspension cultures (Menke *et al.*, 1999a).

A search for additional consensus *cis*-regulatory sequences in the *Str* promoter revealed the presence of a G-box (CACGTG) at −108 to −103 (Pasquali *et al.*, 1999), close to the elicitor-responsive element binding to ORCA2. Interestingly, the *Tdc* gene promoter sequence also possesses a G-box-like sequence (AACGTG) at −98 to −93 (Ouwerkerk *et al.*, 1999). This gene, which encodes tryptophan decarboxylase (EC 4.1.1.28), an enzyme involved in TIA biosynthesis, is co-regulated with the *Str* gene in cell suspension cultures as well as at the whole-plant level (Pasquali *et al.*, 1992; Menke *et al.*, 1999b; St-Pierre *et al.*, 1999). Therefore, G-box (G-box-like) promoter sequences might be involved in the co-ordinate control of *Str* and *Tdc* gene expression patterns.

Other studies have suggested that G-boxes could participate in the co-ordinate activation of phenylpropanoid genes in response to elicitation of soybean and pea cells (Dröge-Laser *et al.*, 1997; Seki *et al.*, 1997). A G-box may also act as a repressor element in the E1 auxin-responsive domain of the soybean GH3 gene promoter (Liu *et al.*, 1994, 1997). G-boxes also participate in the MeJa responsiveness of the potato proteinase inhibitor II gene (Kim *et al.*, 1992) and the soybean *vspB* gene (Mason *et al.*, 1993). Moreover, G-box sequences were also found to determine tissue-specific expression patterns, for example in seeds and roots (Salinas *et al.*, 1992; Thomas, 1993).

The G-box element from the *Str* gene promoter binds *in vitro* to the tobacco G-box binding factor (GBF) TAF-1 (Pasquali *et al.*, 1999). A *Str* G-box tetramer is able to direct seed-specific expression of a *GusA* reporter gene in transgenic tobacco, independently of other regulatory sequences. Moreover, G-box-directed expression in transgenic tobacco leaves requires the presence of the region of the CaMV 35S promoter containing the *as-1* enhancer element (Ouwerkerk and Memelink, 1999). These data suggest that, in *C. roseus*, the G-box is likely to interact with GBFs and may be involved in *Str* gene regulation, either alone or by acting co-ordinately with other regulatory elements.

In order to better understand the molecular mechanisms regulating *Str* gene expression, GBF cDNA clones were isolated from *C. roseus*. Degenerated oligonucleotide primers based on GBF conserved sequences were used to amplify specific probes. A cDNA library was screened with these probes to isolate two *Crgbf* clones, *Crgbf1* and *Crgbf2*. Expression patterns of the corresponding mRNAs were analysed in various plant organs. The binding specificities of *C. roseus* CrGBF1 and CrGBF2 for the G-box of the *Str* gene promoter and for the G-box-like sequence from the *Tdc* gene promoter were evaluated *in vitro*. The ability of CrGBF1 and CrGBF2 to regulate *in vivo Str* gene expression was tested by transient transformation experiments of *C. roseus* cell suspension cultures.

Material and methods

Plant material

Whole plants of *C. roseus* (L) G. Don (cv. Pink Glory; Ducrettet Nursery, Thonon, France) were cultivated under natural conditions in the laboratory. Seedlings (cv. Pink Glory) were obtained as described (Aerts *et al.*, 1994).

Isolation of Crgbf *cDNA clones*

Total RNA was extracted from 7-day old *C. roseus* seedlings as described by Gantet *et al.* (1993). Total RNA (20 μg) was incubated with 1 unit of DNase RQ1 (Promega), 1.4 units of RNAsin (Promega) and 20 mM $MgCl_2$ in de-ionized DEPC (diethylpyrocarbonate)-treated (0.1% v/v) sterile water, for 30 min at 4 °C. The reaction was stopped by phenol/chloroform treatment.

cDNA was synthesized from 1 μg of DNA-free total RNA. RNA was denatured for 5 min at 65 °C and

reverse-transcribed with 22.5 μM of primer polyT-reo2 (5′-T(15)TGCAGGATCCCGGGG-3′), obtained from Genome Express, France, with 10 u of AMV reverse transcriptase (Promega) for 90 min at 37 °C. The excess polyT-reo2 primer was removed by filtration on a Sephacryl S-400 (Promega) column.

A nested PCR amplification was performed in two amplification steps with degenerate oligonucleotide primers (Genome Express) based on two different highly conserved GBF amino acid sequences: PHPYMWG in the proline-rich region for primers NP1 (5′-CCNCAYCCNTAYATG-3′) and NP2 (5′-YCCNTAYATGTGGGG-3′) and [N or D]ERELK in the basic region for primer NP3 (5′-YTTNARYTCN-CKYTCRT-3′).

PCR cycling conditions were: 94 °C for 4 min (1 cycle) and 94 °C for 1 min, an annealing step at various temperatures depending on the T_m of the primers used, for 1.5 min, and 72 °C for 1 min (30 cycles) with a 5 min final extension step at 72 °C. PCR was performed in a final volume of 25 μl with 0.25 u of *Taq* polymerase and 1 × $MgCl_2$-free buffer (Promega), 2 mM $MgCl_2$, 200 nM each dNTP, appropriate oligonucleotides and cDNA template.

The first amplification reaction was run with 2 μl of cDNA mixture, 1 μM primer NP1, 0.056 μM primer reo2 (5′-TGCAGGATCCCGGGG-3′), with an annealing step at 42 °C. The second amplification reaction was run with 2 μl of the first amplification reaction product, 0.24 μM primer NP2, 3 μM primer NP3, with an annealing step at 44 °C with 10 additional cycles.

The 0.7 kb amplified product was blunt-ended (Maniatis *et al.*, 1982) and cloned into the *Sma*I site of pBluescript II SK vector. Two clones, *Crgbf1* and *Crgbf2*, contained a cDNA insert of 0.7 kb encoding amino acid sequences highly homologous to GBFs. Complete cDNA clones for *Crgbf1* and *Crgbf2* were isolated from a *C. roseus* λZAP II cDNA library (Stratagene). The cDNA library was synthesized with a 1:1 mixture of poly(A)$^+$ RNA populations prepared from *C. roseus* MP183L cell cultures that were elicited with 0.05% yeast extract for 1 and 4 h, respectively. The library was amplified from 1.8×10^6 independent primary transformants. For screening, 300 000 plaques of the cDNA library were transferred onto positively charged nylon filters (ICN) and probed with *Crgbf1* and *Crgbf2* radiolabelled partial cDNA according to Church and Gilbert (1984). One clone of 2 kb for each probe was selected for further study.

Sequencing and sequence analysis

DNA sequencing of the PCR fragment and cDNA were performed on both strands by means of the dideoxy chain termination method (Sanger *et al.*, 1977) by commercial services (Eurogentec and Genome Express, France). Database searches were performed with the NCBI BLAST algorithm on the non-redundant protein or DNA sequence data banks. Motif searches were performed in the PROSITE database via the Infobiogen Web site (http://www.infobiogen.fr).

Northern and Southern blot analysis

Total RNA and genomic DNA were isolated from *C. roseus* cell suspension cultures and plants by using the Plant RNeasy mini extraction kit and the DNeasy mini extraction kit, respectively, according to the manufacturer's instructions (Qiagen).

Northern blots (20 μg total RNA) were hybridized with random-primed radiolabelled *Crgbf1* or *Crgbf2* cDNAs as probes, according to Gantet *et al.* (1993).

For the genomic Southern blot, 10 μg of total DNA was digested with restriction endonucleases *Eco*RI, *Bam*HI and *Hin*dIII (Promega), separated on a 0.8% agarose gel and blotted onto a Nylon N+ membrane (Appligen). Hybridization was carried out as described by Church and Gilbert (1984) with random-primed radiolabelled *Crgbf1* and *Crgbf2* cDNA probes. After hybridization, the membrane was washed twice in 2× SSC/0.1% SDS at 65 °C for 20 min and twice in 0.1× SSC/0.1% SDS at 65 °C for 20 min, before exposing to X-ray film (Fuji).

Construction of expression plasmids

For each *Crgbf* clone, the open reading frame was amplified by PCR. Oligonucleotide primers E1S (5′-CCGCTCGAGGGAAGTAGTGGAGAG-3′) and E2S (5′-CCGCTCGAGGGTAGCGTGAGATA-3′) were designed to start amplification just after the translation initiation codon of *Crgbf1* and *Crgbf2* respectively. In addition, primers E1S and E2S contain an *Xho*I site on their 5′ end for cloning in frame with the translation start codon contained in the pET14-b expression vector (Novagen). Oligonucleotide primers E1AS (5′-CCGCTCGAGTTAGTTAGTTACACAATGGCCTCC TG-3′) and E2AS (5′-CCGCTCGAGTTAGTTAGTTA TGATCATTCATGCAC-3′) were designed downstream of the stop codon of *Crgbf1* and *Crgbf2* re-

spectively. E1AS and E2AS primers contain additional stop codons and an *Xho*I site at their 5′ ends. The PCR conditions were as described for the isolation of *Crgbf* clones, except that *Tli* polymerase (Promega) was used instead of *Taq* polymerase. PCR was performed with 200 nM *Crgbf1* or *Crgbf2* plasmid, with 1 μM primer E1S or E1AS and 1 μM primer E2S or E2AS respectively. The temperature of the annealing step was 58 °C. The PCR products were purified by low-melting gel agarose electrophoresis and cloned in a pGEM-T easy vector (Promega). The inserts were liberated by *Xho*I restriction, purified by low-melting gel agarose electrophoresis and subcloned at the corresponding site in the expression vector pET14-b (Novagen). The expression vectors containing the *Crgbf1* or the *Crgbf2* open reading frame were named pETgbf1 and pETgbf2 respectively.

Protein expression and purification

Escherichia coli strain BL21 (DE3) containing pET14-b (control), pETgbf1 or pETgbf2 were incubated overnight in Luria broth (LB) medium with 100 μg/ml ampicillin. Subsequently, 4 ml of the overnight culture was added to 250 ml fresh LB medium containing 100 μg/ml ampicillin and grown at 37 °C until OD at 600 nm reached 0.4. Induction of recombinant proteins was performed by addition of 0.4 mM isopropylthiogalactoside (IPTG) for 4 h at 32 °C. The purification of His-tagged GBF fusion protein by metal Ni^{2+} affinity chromatography was performed according to the manufacturer's instructions (Novagen). Purified proteins were quantified according to Bradford (1976) and analysed by SDS-PAGE electrophoresis.

Electrophoresis mobility shift assay (EMSA) and measurements of DNA-binding constants

For EMSA analysis, 35 bp oligonucleotides were synthesized (Genome Express) related to the G-box-containing SR fragment of the *Str* gene promoter (5′-AGATCTAGATATTACTCCACGTGGTGGGGATCCG A-3′) or the SRE mutated fragment (5′-CTAGATATTAC TCCACggaattccGTGGTGGGGAT-3′) (Pasquali *et al.*, 1999), the G-box-like-containing GTDC fragment of the *Tdc* gene promoter (5′-TTTTGATATTTTTAAACG TGGAATTAGTTCTC-AGT-3′) (Ouwerkerk *et al.*, 1999), GBCI (5′-CTATTCTCATTCTGACACGTGGC ACCTTTCTT-GGA-3′) containing a class I G-Box element which is deduced from the tomato *rbcS* 3A promoter (Meier and Gruissem, 1994), GBCII (5′-ATCTAGATATTACGTCACGTGTCGGGTACCGTC GA-3′) which contains a class II G-box element (Hong *et al.*, 1995) and CB, an oligonucleotide containing a degenerated C-Box sequence (5′-ATCTGAAAGCTTANNGACGTCNNACTCGAGGT CGA-3′) (Martinez-Garcia *et al.*, 1998). Double-stranded oligonucleotides were end-labelled with γ^{32}P ATP (ICN, Biochemical) using T4 polynucleotide kinase from Promega, according to the manufacturer's instructions.

For EMSA experiments, all reactions (final volume 20 μl) contained 1 ng of labelled DNA probe, 0.1 μg of poly(dIdC)-poly(dIdC), 20 mM Tris-HCl pH 8, 100 mM KCl, 10% glycerol, 400 ng CrGBF1 or 1.1 μg CrGBF2 Ni^{2+} purified protein extracts, and a molar excess of specific competitor DNAs as described in the figures. Reactions were incubated for 30 min at 37 °C, and loaded on 5% acrylamide/bisacrylamide (30:1)-0.5× TBE gels. After electrophoresis at 170 V for 90 min, the gels were dried on Whatman 3MM paper and autoradiographed.

For saturation binding assays, binding reactions were performed as described above except that the concentrations of the ^{32}P-labelled DNA probes varied and the concentration of recombinant GBFs was kept constant. The percentage of unbound DNA and DNA bound to GBF in each reaction was determined by an instant-imager (Packard). Dissociation constants (K_d) were determined by the method of Scatchard (1949).

Transient transformation assays

Crgbf1 and *Crgbf2* cDNA clones were amplified by PCR with TS1 (5′-GAAGATACGGTGAAGG-3′) or TS2 (5′-GAGTTATAGTTCATTC-3′) sense oligonucleotide primers and TAS1 (5′-CACAATGGCCTCCT G-3′) or TAS2 (5′-TGATCATTCATGCAC-3′) antisense oligonucleotide primers (Oligo-Express) respectively. The PCR conditions were as already described for the isolation of *Crgbf* clones except that *Tli* polymerase (Promega) was used instead of *Taq* polymerase. PCR was performed with 200 nM *Crgbf1* or *Crgbf2* plasmid, with 1 μM primer TS1 or TAS1 and 1 μM primer TS2 or TAS2 respectively. The temperature of the annealing step was 44 °C for *Crgbf1* and 40 °C for *Crgbf2*. The PCR product was purified from low-melting gel agarose electrophoresis and cloned in pMOG-184 plasmid (Zeneca Mogen, Leiden, Netherlands) between the double CaMV 35S

promoter and the *nos* terminator. The plasmids containing *Crgbf1* or *Crgbf2* cDNA in sense orientation were named pGBF1-sense or pGBF2-sense, respectively, and pGBF1-anti or pGBF2-anti for the antisense orientation respectively. The reporter constructs BH-GusSH which contains a fragment of 339 bp of the *Str* gene promoter and BH^{-NR}-GusSH which contains the same promoter fragment with a deletion of the G-box from −145 to −100 were previously described in Menke *et al.* (1999a). *C. roseus* cells grown according to Pasquali *et al.* (1992) were transiently cotransformed by biolistics as described by Menke *et al.* (1999a) with a reporter construct, an over-expression vector carrying *Crgbf1* or *Crgbf2* in sense or antisense orientation, and a plasmid carrying the chloramphenicol acetyl transferase gene (*cat*) under the control of the CaMV 35S promoter in a ratio of 2:8:1. At 24 h after transformation, cells were harvested, proteins were extracted and GUS and CAT activity assays were performed as described by van der Fits and Memelink (1997). *GusA* reporter gene expression was related to *cat* expression to correct for transformation efficiency and depicted as relative expression compared with the reporter vector. Transient transformation assays were carried out in triplicate.

Figure 1. Comparison of deduced amino acid sequences of *Crgbf1* and *Crgbf2* cDNA clones. Proline residues in the proline-rich region are in bold. The basic domain is in bold italic characters, the amino acids representing the putative bipartite nuclear localization signal are underlined. The heptamer repeats of hydrophobic residues in the putative leucine zipper structure are in bold underlined characters. In the CrGBF2 sequence, the putative GBF-conserved box is indicated with underlined italic characters. Identical amino acid residues between CrGBF1 and CrGBF2 are indicated by an asterisk.

Results

Isolation of cDNAs encoding C. roseus *G-box binding factors*

Previous studies have shown that TIA biosynthesis is active during the first days of development of *C. roseus* seedlings (Aerts *et al.*, 1994). Therefore, in order to isolate transcription factors involved in *Str* gene regulation, template cDNA was prepared from total RNA purified from 7-day old seedlings of *C. roseus*. Degenerate oligonucleotides were designed based on two highly conserved GBF regions, located in the N-terminal proline-rich domain and in the basic domain of GBFs, isolated from various plant species. cDNAs encoding GBFs were amplified by nested PCR with these oligonucleotides as primers. Cloning and sequence analysis of amplification products led to the identification of two partial cDNAs encoding distinct *C. roseus* GBFs, *Crgbf1* (711 bp) and *Crgbf2* (723 bp) (data not shown). A *C. roseus* λZAP-II cDNA library was screened in order to obtain the complete sequence encoding the GBFs. Screening of 3×10^5 plaques with *Crgbf1* or *Crgbf2* partial cDNA probes resulted in the isolation of 1875 bp and 1904 bp full-length cDNAs respectively.

Deduced amino acid sequences for *Crgbf1* and *Crgbf2* cDNAs are shown in Figure 1. The putative ATG start codons were located for both *Crgbf1* and *Crgbf2* at nucleotides 290–293. In-frame stop codons were identified upstream of the proposed ATG start codon, indicating that the cDNAs carry the complete open reading frame. The 3′-untranslated region of both *Crgbfs* contained at least one putative polyadenylation signal characteristic of higher-plant nuclear genes (Wu *et al.*, 1995). The open reading frames encoded a 426 amino acid protein (CrGBF1) for *Crgbf1* and a 394 amino acid protein (CrGBF2) for *Crgbf2* with calculated molecular masses of 45 and 41.7 kDa respectively.

The analysis of deduced amino acid sequences revealed that both CrGBF1 and CrGBF2 contain a basic leucine zipper motif characteristic of bZIP transcription factors at the C-terminus (Landschulz *et al.*, 1988). Both GBFs contain putative bipartite nuclear

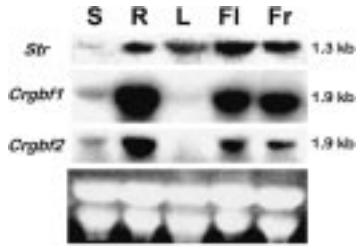

Figure 2. *Crgbf1*, *Crgbf2* and *Str* mRNA steady-state level analysis. Total RNA was extracted from various *C. roseus* plant material: fruit (Fr), flowers (Fl), leaves (L), roots (R) and 7-day old dark-grown seedlings (S). Northern blots were hybridized with *Crgbf1*, *Crgbf2* or *Str* radiolabelled probes. Total RNA is shown after staining by ethidium bromide and visualization under UV light.

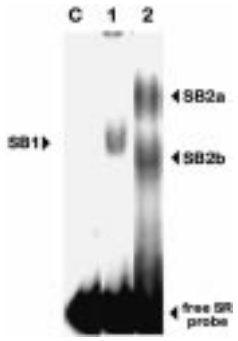

Figure 3. EMSA of the interaction between CrGBFs and the *Str* G-box. The labelled SR probe (1 ng) was incubated with Ni^{2+} affinity chromatography-purified protein extracts from *E. coli* containing pET14-b (C), pETgbf1 (1) or pETgbf2 (2) expression plasmids. The positions of CrGBF1/SR shifted band (SB1), CrGBF2/SR upper shifted band (SB2a) and CrGBF2/SR lower shifted band (SB2b) are indicated.

localization signals (NLSs) in the basic region. The RRKQ10RLRK of CrGBF1 (amino acids 283–308) and CrGBF2 (295–320) matched well with a consensus sequence for the NLS of other bZIP proteins (BRKX10RXBK), where B indicates a basic residue (Varagona and Raikhel, 1994). The two GBFs contain a RKQS sequence in the basic region (amino acids 292–295 for CrGBF1 and 304–307 for CrGBF2), reported to be a potential phosphoserine motif involved in GBF interaction with plant 14-3-3 regulatory protein homologues (Lu *et al.*, 1992; Aitken, 1996). The N-termini of CrGBF1 and CrGBF2 contain a proline-rich domain (21 and 27 proline residues within the first 123 amino acids for CrGBF1 and CrGBF2 respectively). The N-terminal proline-rich domain has been defined as a transcription activation or repression domain in other GBFs (Schindler *et al.*, 1992a; Liu *et al.*, 1997). In addition, CrGBF2 is characterized by a GBF-conserved box at 248–256 which is included in a potential *trans*-activation domain defined in other bZIP proteins as described by Okanami *et al.* (1996).

Southern and northern blot analysis

A Southern blot of *C. roseus* genomic DNA digested with three different restriction enzymes probed with *Crgbf1* and *Crgbf2* sequences at high stringency showed only one to three hybridizing bands indicating that CrGBF1 and CrGBF2 are probably encoded by a single copy of each gene (data not shown).

Northern blot analysis (Figure 2) revealed a 1.9 kb transcript for both *Crgbf1* and *Crgbf2*. In plant organs, the levels of both *Crgbf* mRNAs were high in roots, moderate in flowers and fruits, low in developing seedlings and not detectable in leaves. The relative mRNA accumulation seems to be higher in roots for *Crgbf1* than for *Crgbf2*. In comparison, *Str* mRNA steady-state levels appeared moderate in roots, flowers and fruits and low in leaves. In developing seedlings the *Str* mRNA steady-state level was very low.

Specificity analysis of CrGBF binding activity

The DNA binding activity of CrGBFs was evaluated by EMSA experiments with purified recombinant GBF proteins expressed in *E. coli*. The EMSA experiments were performed with a 100-fold excess of the non-specific competitor poly(dI-dC)-poly(dIdC). As shown in Figure 3, CrGBF1 formed a single shifted band (SB1) when incubated with the G-box-containing SR fragment from the *Str* promoter. Under the same experimental conditions, CrGBF2 formed two shifted bands (SB2a and SB2b, Figure 3) with the SR probe. The lower shifted band (SB2b) formed with CrGBF2 migrated faster than SB1. Because CrGBF1 has a higher predicted molecular mass than CrGBF2, we assumed that SB2b corresponded to a CrGBF2 dimer bound to the SR probe while SB2a may represent different oligomeric forms of CrGBF2 bound to the SR probe.

The specificity of CrGBF1 and CrGBF2 binding activity for SR and GTDC probes was determined by competition analysis, as shown in Figure 4. The GTDC probe contains a G-box-like element found in the *Tdc* promoter. The CrGBF1/SR complex was fully competed by a 50-fold excess of SR unlabelled DNA while it remained as a detectable amount when incubated with a 500-fold excess of unlabelled SRE, a derivative of SR mutated by insertion of an *Eco*RI linker in the G-box (Figure 4A). A 100-fold

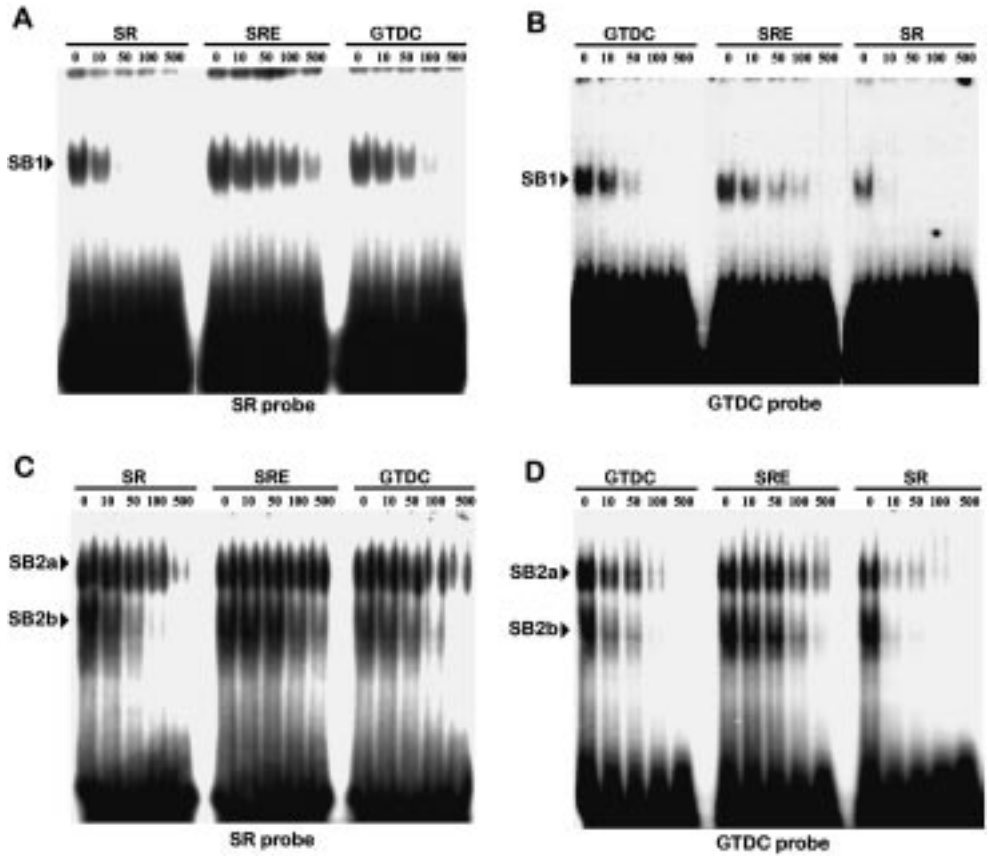

Figure 4. Competitive EMSA analysis of CrGBF binding to G-box-like sequences. A and C. SR probe from the *Str* gene promoter. B and D. GTDC probe containing the G-box-like sequence from the *Tdc* gene promoter. ^{32}P end-labelled SR or GTDC probes (1 ng) were incubated with Ni^{2+}affinity chromatography purified protein extract from *E. coli* containing pETgbf1 (CrGBF1) or pETgbf2 (CrGBF2) expression plasmid. Specific DNA competitors and fold molar excess relative to the amount of probe are indicated at the top of the figures. The SRE probe is a mutated SR probe obtained by insertion of an *Eco*RI restriction site in the G-box of the SR probe.

excess of unlabelled GTDC was sufficient to compete with the CrGBF1/SR complex (Figure 4A). The CrGBF1/GTDC complex was competed by a 10-fold excess of unlabelled SR, a 100-fold excess of unlabelled GTDC and a 500-fold excess of SRE unlabelled DNA probes (Figure 4B). These results attested that CrGBF1 binds specifically to the SR G-box-containing fragment of the *Str* gene promoter and to a lesser extent to the G-box-like sequence from the *Tdc* gene promoter.

Similar experiments were carried out for CrGBF2. In all experiments SB2b was more sensitive to competition with unlabelled DNA probes than SB2a (Figure 4C and 4D). Further measurements will focus only on the lower SB2b shifted band. The CrGBF2/SR complex was strongly competed by a 100-fold excess of SR, a 100-fold excess of GTDC but not fully competed with a 500-fold excess of SRE unlabelled DNA fragments (Figure 4C). CrGBF2/GTDC complex was strongly competed by a 10-fold excess of SR, a 100-fold excess of GTDC but only with a 500-fold excess of SRE unlabelled DNA fragments (Figure 4D). These data show that CrGBF2 bound specifically to the G-box-containing SR fragment of the *Str* gene promoter as well as to the G-box-like sequence of the *Tdc* gene promoter. In addition, the binding affinity of CrGBF2 seems to be significantly weaker for the GTDC DNA probe than for the SR DNA probe.

Estimation of binding constants for CrGBF-DNA interactions

In order to further study the DNA-binding activities of CrGBF1 and CrGBF2, saturation binding assays were performed with increasing amounts of DNA probe at constant protein concentration. It has previously been reported that the four nucleotides (n) flanking

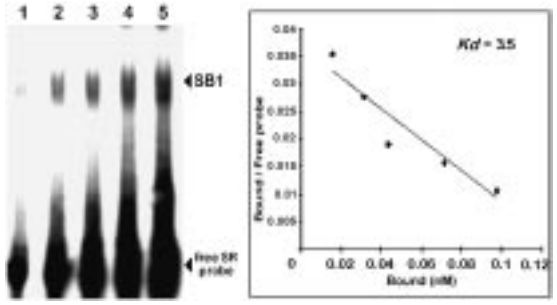

Figure 5. Estimation of the dissociation constant of CrGBF1 and the *Str* G-box. A (left). Saturation EMSA assay with increasing concentration of ^{32}P-labelled SR probe (lanes 1 to 5: 0.5 nM, 1.2 nM, 2.3 nM, 4.6 nM, 9.3 nM respectively) and a fixed amount of CrGBF1 (400 ng). B (right). Scatchard plot: the dissociation constant (K_d) is calculated ($K_d = -1$/slope).

Table 1. Estimated dissociation constant (K_d, expressed in nM) of complexes formed between CrGBF1 or CrGBF2 and the SR, GTDC, CBC I, CBC II or CB DNA probes, which contain the G-box element of the *Str* gene promoter, the G-box-like element of the *Tdc* gene, a class I G-box element, a class II G-box element, and a C-box element, respectively.

Protein	DNA probe				
	SR	GBC I	GBC II	GTDC	CB
CrGBF1	3.5	2	11.2	10.5	14
CrGBF2*	2.5	1.6	2.1	3.9	26

*For CrGBF2 only the lower shifted band (SB2b) was taken in account.

the hexameric G-box core nnCACGTGnn affected the specificity of protein binding. With regard to this, G-box elements were organized into two classes, class I and class II, depending on (1) the nature of the flanking sequences of the hexanucleotide core motif and (2) the type of EMSA patterns of complex formation with cauliflower nuclear protein (Williams *et al.*, 1992). In addition to SR probe which contains a class I G-box element of the *Str* gene promoter and GTDC probe which contains a G-box-like element, we used for these experiments two other G-box-containing probes: GBCI, which contains another class I G-box element (Meier and Gruissem, 1994), and GBCII, which contains a class II G-box element (Hong *et al.*, 1995). The CB probe is an oligonucleotide containing a degenerate C-box sequence (Martinez-Garcia *et al.*, 1998). The ratio of unbound DNA and DNA bound to GBF in each reaction was determined. An example of a Scatchard analysis of CrGBF1 binding to SR probe is shown in Figure 5.

Estimated binding constants are summarized in Table 1. Both CrGBF1 and CrGBF2 had a weak affinity for the CB DNA probe demonstrating that these GBFs do not have specific binding activity for C-boxes. CrGBF1 bound with higher affinity to class I G-boxes (SR and GBCI DNA probes) than to the class II G-boxes (GBCII DNA probe) or to the G-box-like element of the *Tdc* gene promoter (GTDC DNA probe). In contrast, the affinity of CrGBF2 for all the G-box probes tested (SR, GBCI, GBCII DNA probes) was high, although it was slightly lower for the G-box-like probe (GTDC probe). These data suggest that these two GBFs have different binding properties *in vitro* for the different DNA targets: CrGBF1 bound with a high affinity to class I G-boxes while CrGBF2 bound with high affinity to all types of G-boxes and to a lesser extent to the G-box-like element.

In vivo *transcriptional regulation of the Str promoter by CrGBF*

To test whether CrGBF1 and CrGBF2 can act as transcriptional regulators of the *Str* promoter, *C. roseus* cells were transiently co-transformed by particle bombardment with a *Str*-promoter-*gusA* construct, and an expression vector carrying *Crgbf1* or *Crgbf2* cDNAs fused in sense or antisense orientation to the double CaMV 35S promoter. Co-expression of *Crgbf1* or *Crgbf2* in sense orientation resulted in a 50% lower level of *Str* promoter activity compared with the empty expression vector or with expression of *Crgbf1* and *Crgbf2* in antisense orientation (Figure 6A).

To test whether CrGBF1 and CrGBF2 inhibit the *Str* promoter via interaction with the G-box, co-transformations were done using the BH^{-NR} fragment of the *Str* promoter which is deleted for the NR region containing the G-box. Co-expression of *Crgbf1* in sense orientation reduced the activity of the deleted *Str* promoter only marginally, while co-expression of *Crgbf2* in sense orientation had no effect (Figure 6B). The weak negative effect of CrGBF1 on the BH^{-NR} promoter could be caused by a low affinity to another sequence without obvious homology to a G-box in the construct. Some GBFs have relaxed DNA-binding specificity, as exemplified by soybean G/HBF-1 which binds the unrelated G- and H-boxes (Dröge-Laser *et al.*, 1997). Without expression vector carrying CrGBFs, expression levels of BH and BH^{-NR} reporter constructs were identical, suggesting that deletion of the G-box has no effect on the basal level of transient *Str* promoter activity in cell suspension cultures (Figure 6C).

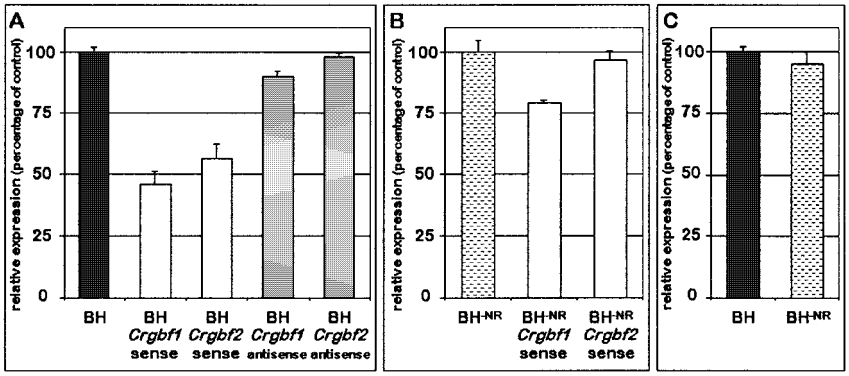

Figure 6. *Str* promoter *trans*-inhibition by CrGBF1 and CrGBF2. *C. roseus* cells were transiently co-transformed with wild-type (BH) (A) or G-box-deleted (BH^{-NR}) (B) *Str*-promoter-*gusA*, CaMV 35S promoter-*cat* and expression vectors containing *Crgbf1* or *Crgbf2* cDNA fused either in sense or antisense orientation to the CaMV 35S promoter (2:1:8 relative ratio). *GusA* reporter gene expression was related to *cat* expression to correct for transformation efficiency and depicted as relative expression compared with the reporter vector alone. C. Relative expressions of BH-NR reporter vector alone compared to BH reporter vector. Bars represent SE.

These results show that transient expression of *Crgbf1* and *Crgbf2* negatively regulates *Str* promoter activity. Deletion of the G-box had no effect on the basal activity of reporter constructs but affected *trans*-inhibition of the *Str* promoter *in vivo*, indicating that CrGBF1 and CrGBF2 inhibit *Str* promoter activity via direct interaction with the G-box.

Discussion

We cloned two cDNAs encoding GBFs from *C. roseus*. Analysis of deduced amino acid sequences shows that both proteins possess features of bZIP transcription factors. Amino acid sequence comparison showed that CrGBFs exhibited a greater difference between themselves (35% of identity) than with other GBFs characterized in other plant species. CrGBF2 is 61.2% identical at the amino acid level with tomato GBF9, a GBF characterized as binding to the G-box element of the rubisco small subunit gene (Meier and Gruissem, 1994). CrGBF1 is 54.5% identical with CPRF-1 from parsley, a GBF involved in light regulation of the chalcone synthase gene active in the phenylpropanoid pathway (Weisshaar *et al*, 1991). These data suggest that CrGBF1 and CrGBF2 belong to two divergent GBF families (Hong *et al.*, 1995), CrGBF1 being a member of GBF family II and CrGBF2 of GBF family I. Although these structural differences could not be assigned to a precise function, it has been suggested that each family may represent functionally distinct proteins that could be regulated by different signal transduction pathways (Hong *et al.*, 1995).

The N-terminal parts of CrGBF1 and CrGBF2 contain proline-rich regions. Similar proline-rich regions have already been described in other DNA-binding proteins and transcription modulating functions have been assigned to these domains. For example, the N-terminal proline-rich regions of the mammalian transcription factors CTF/NF-1 and AP-2 (Mermod *et al.*, 1989) and of *Arabidopsis* GBF1 (Schindler *et al.*, 1992b) mediated activation of transcription in mouse and plant cells respectively. The proline-rich region of soybean SGBF-2 has been reported to function as a repressor in carrot cells (Liu *et al.*, 1997). SGBF-2 binds to the G-box within the 47 bp E1 auxin-responsive element of the soybean GH3 promoter (Liu *et al.*, 1994). Another plant transcription factor that binds to this auxin-responsive element contained a proline-rich domain which acted as a repression domain in carrot cells but as an activation domain in yeast cells (Ulmasov *et al.*, 1997). This suggests that proline-rich regions might function as repressors or activators depending on the presence of specific co-activators and co-repressors in various cell types (Liu *et al.*, 1997). With regard to our data obtained by transient transformation assays, the proline-rich region of CrGBF1 and CrGBF2 may function as repressor domains in *C. roseus* suspension cells. This should be confirmed via *trans*-regulation experiments using truncated proteins lacking the proline-rich domain.

At the whole-plant level organ-specific expression patterns for *Crgbf1* and *Crgbf2* were similar, suggesting that *Crgbf* gene expression is regulated in an organ-specific way. This has already been reported for different *Arabidopsis* and tomato GBFs and TAF-1

from tobacco (Oeda *et al*, 1991; Meier and Gruissem, 1994; Schindler *et al.*, 1992a, b). An inverse correlation cannot be found between the organ-specific expression patterns of *Crgbf* genes and the *Str* gene as we would expect if the CrGBFs repress *Str* gene expression as suggested by transient transformation experiments in cell suspensions. One explanation could be that the influence of CrGBFs on *Str* gene expression depends on the presence or absence of other regulatory proteins in specific organs, tissues or cell types. For example, as discussed before, the proline-rich domain of GBFs has been reported to act as either inhibitor or activator of transcription depending on the cell type used for the experiments (Liu *et al.*, 1997; Ulmasov *et al.*, 1997). Post-transcriptional mechanisms could also be involved in the control of CrGBF accumulation in *C. roseus* cells. For instance, the rapid UV-induced increase of parsley GBF protein was shown to be insensitive to transcriptional inhibitor (Kircher et *al.*, 1998).

Str gene expression is under strict tissue-specific and developmental control and restricted to epidermal cells of young leaves and to epidermal and cortical cells of the root apex. Furthermore, *Str* gene expression was found to be down-regulated at a specific stage of epidermal cell development (St-Pierre *et al.*, 1999). To establish whether an inverse correlation exists between the tissue-specific expression patterns of *Crgbf* and *Str* mRNA and protein accumulation, northern gel blot studies need to be extended to RNA and protein *in situ* localization.

In order to further characterize the binding activities of CrGBF1 and CrGBF2, competitive gel shift experiments and saturation binding assays were carried out. CrGBF1 and CrGBF2 had a low affinity for the CB probe, indicating that both GBFs preferentially bind to G-boxes in contrast to some other bZip transcription factors that were found to bind G-boxes as well as C-boxes (Martinez-Garcia *et al.*, 1998). CrGBF1 had a higher binding affinity for class I G-boxes (SR, GBCI) while CrGBF2 bound to all types of G-boxes (SR, GBCI, GBCII) tested with comparable affinity constants. Moreover, our experiments showed that both GBFs have a higher affinity for the G-box element of the *Str* gene promoter than for the G-box-like element of the *Tdc* gene promoter.

For this reason, as a first step, only the regulatory activities of CrGBFs on the *Str* gene *in vivo* were investigated. Transient bombardment experiments showed that both CrGBFs act as transcriptional repressors of the *Str* promoter. *In vivo* co-expression of CrGBFs with wild-type (BH) and G-box-deleted mutant (BH^{-NR}) versions of the *Str* promoter demonstrated that *trans*-inhibition occurs via direct interaction with the G-box located in the NR region of the *Str* gene promoter. Nevertheless, when the reporter constructs were used alone, deletion of the G-box had no significant effect on transient *Str* promoter activity. One explanation for this result may be that the expression levels of the GBFs in the cells, if present, is too low to repress the excess of reporter plasmid introduced. Another possibility could be that the deletion of the NR region alters promoter strength by changing distances between *cis*-acting elements or by creating fortuitously new ones.

Most of the GBFs characterized from plants were reported to be activators of transcription (Menkens *et al.*, 1995). However, some of them have been described as inhibitors. This is the case, for example, for SGBF-2 which binds to the G-box of the 47 bp E1 auxin-responsive element of the soybean GH3 promoter (Liu *et al.*, 1994, 1997). Since the *Str* gene is transiently down-regulated by auxin in *C. roseus* cell suspension cultures (Pasquali *et al.*, 1992) the possibility that GBFs and the G-box are involved in the regulation of the *Str* gene by auxin should be further investigated.

In this paper we have characterized GBF transcription factors that, in transient transformation experiments, down-regulate expression of the *Str* gene, which encodes the key enzyme strictosidine synthase in TIA biosynthesis. Previous studies have suggested that the *Str* G-box could be involved in *Str* gene regulation by acting co-ordinately with other regulatory elements (Ouwerkerk and Memelink, 1999). Recently other transcription factors, the AP2/ERF-domain proteins ORCA2 and ORCA3 which regulate the expression of the *Str* gene in response to elicitor and/or MeJa treatment of *C. roseus* suspension cells, have been reported (Menke *et al.*, 1999a; van der Fits and Memelink, 2000). The G-Box of the *Str* promoter is located at -108 to -103 (Pasquali *et al.*, 1999), next to the GCC-box element binding to ORCAs at -94 to -70 (Menke *et al.*, 1999a). Interestingly, in tobacco, a G-box and a GCC-box spaced 4 bp apart have been shown to co-ordinately participate in the activation of the tobacco PRB-1b gene in response to ethylene (Sessa *et al.*, 1995). In addition, a direct protein/protein interaction between a bZIP transcription factor and an AP2-domain transcription factor has been described in *Arabidopsis thaliana* (Büttner and Singh, 1997). Preliminary data obtained by transient

co-bombardment experiments suggested that such an interaction between CrGBFs and ORCAs could exist in *C. roseus* and participate in the regulation of the *Str* gene (unpublished results). Possible interactions between CrGBFs and ORCAs can be investigated with the yeast two-hybrid system. In addition, the physiological role of CrGBFs in the regulation of the *Str* gene expression and TIA biosynthesis in *C. roseus* cell suspension cultures will be assessed by a transgenic strategy.

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