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Zinc Finger Proteins Act as Transcriptional Repressors of Alkaloid Biosynthesis Genes in *Catharanthus roseus**

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In *Catharanthus roseus* cell suspensions, the expression of several terpenoid indole alkaloid biosynthetic genes, including two genes encoding strictosidine synthase (STR) and tryptophan decarboxylase (TDC), is coordinately induced by fungal elicitors such as yeast extract. To identify molecular mechanisms regulating the expression of these genes, a yeast one-hybrid screening was performed with an elicitor-responsive part of the *TDC* promoter. This screening identified three members of the Cys_2/His_2-type (transcription factor IIIA-type) zinc finger protein family from *C. roseus*, ZCT1, ZCT2, and ZCT3. These proteins bind in a sequence-specific manner to the *TDC* and *STR* promoters *in vitro* and repress the activity of these promoters in *trans*-activation assays. In addition, the ZCT proteins can repress the activating activity of APETALA2/ethylene response-factor domain transcription factors, the ORCAs, on the *STR* promoter. The expression of the *ZCT* genes is rapidly induced by yeast extract and methyljasmonate. These results suggest that the ZCT proteins act as repressors in the regulation of elicitor-induced secondary metabolism in *C. roseus*.

Perception of stress signals or of pathogen-derived molecules, called elicitors, activates a number of signal transduction steps in plants, eventually leading to the transcriptional activation of numerous genes, and consequently to *de novo* synthesis of a variety of defense proteins and protective secondary metabolites (1). The biosynthesis of one or more secondary signals, such as jasmonic acid (JA),[1] salicylic acid, and ethylene, plays a crucial role in this stress response (2). In elicitor-induced accumulation of secondary metabolites, jasmonic acid and its volatile methyl-ester methyljasmonate (MeJA), have been shown to act as intermediate signals (3).

Knowledge about the molecular mechanisms regulating elicitor-responsive expression of secondary metabolite biosynthesis genes is limited. In parsley, a fungal elicitor induces the expression of the MYB-like transcription factor box P-binding factor (BPF)-1, which interacts with the promoter of a gene encoding the phenylpropanoid biosynthesis enzyme phenylalanine ammonia-lyase (4). Terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* is one of the best studied elicitor-induced secondary metabolic pathways. In suspension cells, the perception of yeast extract (YE) leads to the activation of terpenoid indole alkaloid biosynthesis (5). Two genes involved in terpenoid indole alkaloid biosynthesis, encoding strictosidine synthase (STR) and tryptophan decarboxylase (TDC), are coordinately regulated and their mRNAs accumulate transiently after YE treatment (6, 7). Induction of these genes by YE is mediated by protein phosphorylation, the influx of calcium, and the biosynthesis of JA via the octadecanoid pathway (3, 8). In the *STR* promoter, two elicitor- and jasmonate-responsive sequences have been identified; the so-called BA region and a sequence close to the TATA box, called jasmonate- and elicitor-responsive element, located in the RV region (see Fig. 8). The BA region was found to bind to a homologue of parsley PcBPF-1, called CrBPF1 (9). The jasmonate- and elicitor-responsive element interacts with two JA-responsive transcription factors called ORCA2 and ORCA3 (10, 11). Both ORCAs belong to the APETALA2/ethylene response-factor (AP2/ERF) family of transcription factors. ORCA3 was shown to regulate multiple genes involved in primary and secondary metabolism, including the *TDC* and *STR* genes (3, 11, 12). The NR region of the *STR* promoter, which is not required for responsiveness to elicitor or jasmonate (10), interacts with two G-box binding basic leucine zipper proteins (CrGBFs; Ref. 13).

The *TDC* promoter also contains a YE-responsive element, the so-called DB element (14). The ORCA transcription factors[2] or the MYB-related protein CrBPF1 (9) do not bind to the DB

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[1] The abbreviations used are: JA, jasmonic acid; MeJA, methyljasmonate; BPF, box P-binding factor; GBF, G-box-binding factor; YE, yeast extract; STR, strictosidine synthase; TDC, tryptophan decarboxylase; ORCA, octadecanoid-responsive *Catharanthus* AP2 domain; AP2, APETALA2; ERF, ethylene-response-factor; TFIIIA, transcription factor IIIA; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; ZCT, zinc finger *Catharanthus* transcription factor.

[2] J. Memelink, unpublished results.

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element, whereas CrGBFs have a weak affinity for a G-box-like sequence in the DB element *in vitro* (13). To isolate transcription factors that interact with the DB element, a yeast one-hybrid screening was performed. This screening identified three members of the transcription factor IIIA (TFIIIA-type; Cys_2/His_2-type) zinc finger protein family from *C. roseus,* ZCT1, ZCT2, and ZCT3. *In vitro* DNA binding studies showed that these proteins bind in a sequence-specific manner to the *TDC* and *STR* promoters. Furthermore, these zinc finger proteins were shown to act as transcriptional repressors of *STR* and *TDC* promoter activity in *trans*-activation assays. Finally, expression of these zinc finger genes is rapidly induced by YE and MeJA. Together these data show that TFIIIA-type zinc finger transcription factors can act as repressors in the regulation of YE-induced secondary metabolism.

EXPERIMENTAL PROCEDURES

Isolation of Zinc Finger Clones—cDNA fragments encoding zinc finger proteins ZCT1, ZCT2, and ZCT3 were isolated by a one-hybrid screening of a *C. roseus* cDNA library with the DB element of the *TDC* promoter as bait. Tetramerization of the DB element from the *TDC* promoter was described in (14). The DB tetramer was fused to the yeast *HIS*3 reporter gene in plasmid p601 (15). The tetramer-*HIS*3 fusion was transferred as a BamHI fragment into the BclI site of integration vector pJP04, which is essentially similar to pINT1 (16). The resulting plasmid was linearized with NcoI and introduced into yeast strain Y187 (17). Recombinants were selected on YPD (yeast extract/peptone/dextrose) medium containing 150 μg/ml G418, and the occurrence of single recombination events between the pJP04 derivative and the chromosomal *PDC6* locus was verified by Southern blot analysis. The pACTII cDNA library with a complexity of 3.5×10^6 independent transformants was prepared from elicitor-treated *C. roseus* cell suspension line MP183L as described by Ref. 10. After transformation of the cDNA library into the yeast strain, cells were plated on minimal medium lacking leucine and histidine. Screening of an estimated total number of 2.4×10^6 yeast transformants resulted in 188 colonies containing plasmids conferring His/Leu-independent growth upon isolation/retransformation. Plasmid cross-hybridizations and sequencing of representative members of each class resulted in the identification of three C_2H_2 zinc finger classes.

Construction of Full-length cDNA Clones—To construct full-length clones, 5′ sequences were isolated by PCR with a gene-specific primer and the vector primer 5′-CCCCACCAAACCCAAAAAAAG-3′ using the pACTII cDNA library as a template. ZCT1 appeared to be a full-length clone. To confirm this notion, 5′ sequences amplified with the gene-specific primer 5′-CTAAAGATTGATGGAGTAGATC-3′ were digested with BamHI/HindIII and cloned in pBluescript SK+. Sequencing of the longest PCR fragment yielded additional sequence information of 10 nucleotides. ZCT2 5′ sequences amplified with the gene-specific primer 5′-CATCAACAATATTCGACTTCTTCACC-3′ were digested with BamHI/NdeI and cloned in pUC28. The insert from the pACTII-ZCT2 clone was excised with BamHI/XhoI and first cloned into the vector pIC-19R (18) digested with BglII/SalI, after which it was transferred as a NdeI/SmaI fragment to the pUC28 plasmid containing the PCR fragment digested with NdeI/EcoRV, resulting in a full-length ZCT2FL cDNA. ZCT3 5′ sequences amplified with the gene-specific primer 5′-CTAAAGATTGATGGAGTA-3′ were digested with BamHI/SacI and cloned in pBluescript SK+. The insert from the pACTII-ZCT3 clone was excised with EcoRI/EcoRV, and first cloned into the vector pIC-19H after which it was transferred as a SacI fragment to the pBluescript SK+ plasmid containing the PCR fragment, resulting in a full-length ZCT3FL cDNA.

Construction of Escherichia coli Expression Plasmids—The ZCT1 insert was excised from the pACTII vector with SmaI/XhoI and inserted in pIC-19R digested with EcoRV/SalI. The resulting plasmid was used as template with primers 5′-CGGGATCCTCGAGATGGGCGTGAAGAGATTCAGAG-3′ and M13–40 in a PCR, and the product was digested with BamHI and cloned in pACTII. From there it was excised with XhoI and introduced in pGEX-KG (19). The ZCT2FL insert was amplified with the primers 5′-CGCGGATCCGCGATGGTGATGATTAATATA-3′ and 5′-CCCAAGCTTGGGT$_{15}$-3′, and after digestion with BamHI/HindIII, was introduced in pGEX-KG. The ZCT3FL insert was amplified with the primers 5′-CGCGGATCCGCGATGGCACTTGAAGCTTTG-3′ and T3, and following digestion with BamHI/XhoI introduced in pGEX-KG. Expression plasmids were introduced in *E. coli* strain BL21 (DE3) pLysS, and proteins isolated using glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions were dialyzed against electrophoretic mobility shift assay (EMSA) binding buffer.

EMSAs—*STR* promoter fragments, RV wild-type and mutant fragments (10), and *TDC* promoter fragments (20) were isolated and labeled as described. DNA-binding reactions contained 0.1 ng of end-labeled DNA probe, 500 ng of poly(dA-dT)-poly(dA-dT), binding buffer (25 mM HEPES-KOH, pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% glycerol), and protein extract in a 10-μl volume. For analysis of the requirement of zinc for binding, ZCT proteins were pre-incubated for 5 min in binding buffer containing 3 mM EDTA, 3 mM EGTA, 10 mM 1,10-phenanthroline (Sigma)/1% ethanol or 1% ethanol before addition of probe DNA. Binding reactions were incubated for 30 min at room temperature before loading on 5% acrylamide/bisacrylamide (37:1)-0.5× Tris-borate-EDTA gels under tension. After electrophoresis at 125 V for 1 h, gels were dried on Whatman DE81 paper and autoradiographed.

Construction of Plant Expression Vectors—The ZCT1 insert was excised from pACTII with SmaI/XhoI, cloned in pIC-19R digested with EcoRV/SalI, and then cloned in pMOG463 as a BamHI fragment. The ZCT2 insert was excised from pUC28-ZCT2FL with BamHI and cloned in pMOG183. The pMOG vectors are pUC18 derivatives carrying a double-enhanced CaMV 35 S promoter and the *nos* terminator separated by a BamHI site. The full-length ZCT3FL cDNA was cloned as a BamHI/BglII fragment in SK+-35 S-nos. This pBluescript derivative carries a double-enhanced CaMV 35 S promoter and the *nos* terminator separated by a BamHI site.

Cell Cultures—*C. roseus* cell suspension line MP183L was grown as described (6).

Transient Expression Assays—Cells of *C. roseus* cell line MP183L were co-transformed with plasmids carrying different promoter parts fused to *GUSA* and overexpression vectors carrying *ZCT1*, *ZCT2*, *ZCT3*, and/or *ORCA2* or *ORCA3* cDNAs fused to the CaMV 35 S promoter. Co-transformations of the promoter-*GUS* constructs with an empty overexpression vector (pMOG184) served as controls. Cells were transformed with a total of 10 μg of plasmid DNA through particle bombardment as described before (21), using the two constructs in a ratio of 1:4 (*GUS*:*ZCT*/*ORCA*). In the case of co-bombardment with both *ORCA* and zinc finger cDNAs, the ratio was 1:4:4 (*GUS*:*ZCT*:*ORCA*). Each plasmid combination was bombarded in triplicate, where each replicate consisted of an independent DNA coating of tungsten particles. Twenty-four hours after transformation, cells were harvested and frozen in liquid nitrogen. β-Glucuronidase (GUS) activity assays were performed as described (21). GUS reporter activity was related to total protein amounts to correct for the amount of cells used in each transformation. GUS activity was depicted as relative activity compared with the vector control. Statistical analysis of the results was done using the nonparametric Wilcoxon-Mann-Whitney test.

Elicitor and Jasmonate Treatment—Partially purified elicitor was prepared from yeast extract (YE) (Difco), through ultrafiltration and a number of chromatographic steps, as described in Ref. 8. The amount of purified elicitor used for induction experiments was calibrated to correspond to a final concentration of 400 μg/ml of crude YE using a semi-quantitative alkalinization response assay as described before (8). Methyljasmonate (Bedoukian Research Inc.) was diluted in dimethyl sulfoxide (Me_2SO).

RNA Extraction and Northern Blot Analysis—RNA extraction and Northern blot analysis were performed as described before (8), loading 20-μg RNA samples onto the gels. All Northern blots were probed using ^{32}P-labeled cDNA fragments. *ORCA2*, *ORCA3*, *RPS9*, and *STR* probes were described before (8).

RESULTS

Isolation of Zinc Finger Proteins ZCT1, ZCT2, and ZCT3—To identify DNA-binding proteins that interact with the YE-responsive DB element of the *TDC* promoter, a yeast one-hybrid screening was performed with this element. A derivative of yeast strain Y187, containing a tetramer of DB fused to the *HIS*3 selection marker, was used in a screen to isolate DNA-binding proteins from a cDNA library of *C. roseus* cloned in a fusion with the GAL4 activation domain in yeast expression vector pACTII. In total, 2.4 million Y187–4DB transformants were screened for reporter gene activation. A total of 188 cDNA clones, belonging to several classes, were isolated from yeast colonies that showed growth on medium lacking histidine. No cDNAs encoding ORCA or CrBPF1 proteins were

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```
     1                                                50
ZCT1 ..................................MGVKRFREEQS.....
ZCT2 .............................MVMINIPMKRTREANDFDSIT
ZCT3 MALEALNSPTTPTPVFQYENAATLRYLDQPWAKGKRSKRPRSVEPPPPQH
                                     B-box

     51                                              100
ZCT1 ............ANCIMLLSKVGLLSEM.........EKSAVPALKPGAG
ZCT2 TM..........ANCIMLLSQ.NRSGEF.........IDSTTSNSSNLNS
ZCT3 QQQQQPTEEEYLALCLIMLARGGAPSTTTLPLPPPPQQHNIPSSSSSDPP
            L-box

     101                                             150
ZCT1 GRIYECKTCKKQFLTFQALGGHRASHKKLRLMAA........DLLHQ.SL
ZCT2 NRVFECKTCNRQFPSFQALGGHRASHKRPRLGGD........LTLSQIPV
ZCT3 KLLYKCSVCDKAFSSYQALGGHKASHRKLSTASAGGGDDQSTTSTSTTTA

     151                                             200S
ZCT1 AVTKPKTHACSICGLEFPLGQALGGHMRRHRGAALDGEKPVVVSDKPVAK
ZCT2 AAAKPKTHECSICGLEFAIGQALGGHMRRHRAAMSDSASGNSASPPRDDR
ZCT3 AAATGRTHECSICHKCFPSGQALGGHKRCHYEGGAGAVGSTGNAASGVTS

     201                                             250
ZCT1 AVPFLMRSNSSKRIFGFEVDGLDLNLTPEDNDPDERD.KFPPLLEFFV..
ZCT2 TV.VVKKSNIVDDDNDRRVWGLDLNLTPFENHLEFQLGKIAPTVDCFL..
ZCT3 SEGMGSTNTHSNRD.......FDLNLPALPEFWLAAAAGDDEVSSPHPAK
                        LxLxL motif

     251            266
ZCT1 ................
ZCT2 ................
ZCT3 RSRFALPIKVENHQIN
```

FIG. 1. **Protein alignment of ZCT1, ZCT2, and ZCT3.** Gaps introduced to maximize alignment are indicated by *dots*. Identical amino acids are *boxed* in *black* and conserved cysteines and histidines of the zinc fingers are *boxed* in *gray*. The B-box, L-box, and L*x*L*x*L motif are indicated.

recovered, which is consistent with the fact that these proteins do not bind DB *in vitro*. In addition, no clones encoding CrGBFs were found, despite the fact that CrGBFs have a weak affinity for a G-box-like sequence in the DB element *in vitro* (13).

Comparison of the DNA sequences to sequences in the NCBI data base revealed that three cDNA classes encoded proteins with two Cys_2/His_2-type (TFIIIA-type) zinc fingers. In a TFIIIA-type zinc finger protein, two cysteines and two histidines, in a conserved sequence motif ($CX_{2-4}CX_3FX_5LX_2HX_{3-5}H$), tetrahedrally coordinate a zinc atom to form a compact structure that interacts with the major groove of DNA in a sequence-specific manner (22, 23). All three *Catharanthus* classes possess the typical characteristics of plant TFIIIA-type two-fingered proteins (24). Both fingers have the QALGGH sequence in the putative DNA-contacting surfaces, and the two fingers are separated by a long spacer (Fig. 1).

We called the three encoded proteins ZCT1, ZCT2, and ZCT3, for zinc finger *Catharanthus* transcription factor. The ZCT3 class was isolated 14 times, the ZCT1 class 8 times, and *ZCT2* was a single clone. The longest clone from the *ZCT1* class was full-length, whereas all *ZCT2* and *ZCT3* clones appeared to be partial. The missing portions of *ZCT2* and *ZCT3* were isolated via PCR and fused to the partial cDNAs, to construct complete clones. An alignment of the deduced amino acid sequences of ZCT1, ZCT2, and ZCT3 is shown in Fig. 1. The ZCT1, ZCT2, and ZCT3 proteins have predicted molecular masses of 19.6, 21, and 27.4 kDa, respectively. Comparison of the deduced ZCT1 and ZCT2 amino acid sequences to sequences in the NCBI data base showed highest homology to ZPT2-5, ZPT2-14, ZPT2-12, and ZPT2-13 from *Petunia hybrida*. One of the closest homologues of ZCT3 is the SCOF-1 protein from soybean, which is involved in cold tolerance (25).

Besides the two zinc fingers, the ZCT proteins contain several conserved regions. Near their N termini, they contain a short basic region (B-box; Ref. 26), which may function as a nuclear localization signal (Fig. 1). Between the B-box and the first zinc finger, the ZCT proteins contain a short region of hydrophobic residues rich in leucines (L-box). The motif has been found in several other Cys_2/His_2 zinc finger proteins, and has been suggested to play a role in protein-protein interactions or in maintaining the folded structure of the proteins (26, 27). In their C-terminal region, the ZCT proteins have an L*x*L*x*L motif (Fig. 1), which is a potent repression domain found in most TFIIIA-type zinc finger, several AP2/ERF (28), and in all *Arabidopsis* AUX/IAA (29) transcriptional repressors. In AP2/ERF proteins this motif has also been called the ERF-associated amphiphilic repression domain (28).

The ZCT Proteins Bind to Several Regions of the TDC and STR Promoters—The ability of the ZCT proteins to activate *HIS*3 gene expression via the DB region in yeast and the presence of two zinc finger DNA-binding domains, indicated that they are DNA-binding proteins. To directly test the DNA binding of the zinc finger proteins, recombinant GST-ZCT fusion proteins were isolated from *E. coli* and EMSAs were performed. Incubation of the ZCT proteins with labeled DB fragment from the *TDC* promoter showed that they can bind to this fragment (Fig. 2*C*). ZCT1 and ZCT2 showed a similar binding pattern consisting of two bands, whereas ZCT3 formed a single shifted band. To test whether the ZCT proteins can also bind to other parts of the *TDC* promoter, EMSAs were performed with probes covering a 535-bp region of the *TDC* promoter upstream of the TATA box (Fig. 2*A*). ZCT1 and ZCT2 bound with highest affinity to the HS and DB regions of the *TDC* promoter, with little binding to the other fragments tested (Fig. 2*D*). However, ZCT3 bound to all fragments of the *TDC* promoter with highest affinity for HS and DB (Fig. 2*D*). Recombinant GST did not bind to any of the fragments used in EMSAs (data not shown).

Because the *TDC* and *STR* genes are coordinately regulated by YE and MeJA, the binding of the ZCT proteins to the *STR* promoter was also determined. Transformation of the zinc finger clones in pACTII to a yeast strain carrying a tetramer of the RV region of the *STR* promoter fused to the *HIS*3 selection gene (10) indicated that the ZCT proteins were also able to bind to the elicitor- and jasmonate-responsive RV region of the *STR* promoter *in vivo* (results not shown). Incubation of the ZCT proteins with probes covering a 583-bp region of the *STR* promoter *in vitro* (Fig. 2*B*) showed that they indeed bound to the RV region and additionally to the BA and VH regions (Fig. 2*D*). ZCT3 bound additionally to the XD and DB fragments of the *STR* promoter (Fig. 2*D*). The RV region of the *STR* promoter contains the binding site for the ORCA transcriptional activators. In a previous study, a mutation scanning of the RV fragment, which comprised changing blocks of six adjacent nucleotides into their complementary nucleotides (Fig. 2*B*; Ref. 10), demonstrated that the ORCA binding site is located in the M2-M3-M4 region. To determine the specific binding site of the ZCT proteins in the RV fragment, the different RV mutant fragments were used as probes in EMSAs. Because the ZCT proteins showed little or no binding to mutated RV fragment M2, but did bind to the other mutated RV fragments, it can be concluded that the main binding determinant for the ZCT proteins is located in the M2 region (Fig. 2*E*). The ZCT binding site is therefore distinct from but overlapping with the binding site for the ORCA proteins.

To determine whether the interaction of the ZCT proteins with DNA requires the binding of a zinc atom to their zinc fingers, the DNA binding affinity of the ZCT proteins was analyzed in the presence of the zinc-chelating agents EDTA or 1,10-phenanthroline. Fig. 3 shows that under standard experimental conditions the ZCT proteins can bind to the RV fragment. However, the presence of EDTA or 1,10-phenanthroline inhibits the binding of the ZCT proteins to the RV fragment, indicating that zinc is

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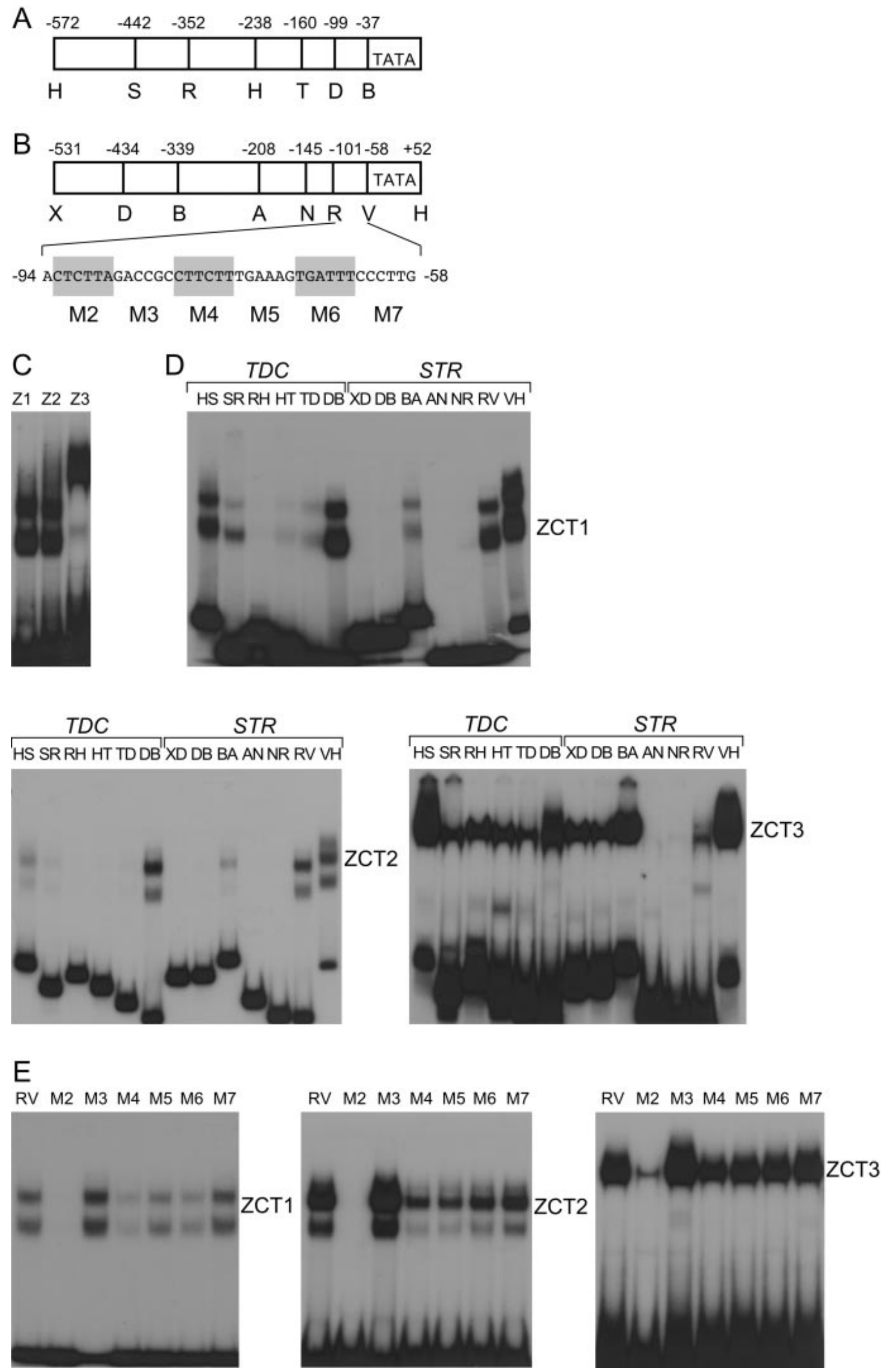

FIG. 2. **Binding of ZCT proteins to different fragments of the *TDC* and *STR* promoters.** *A*, schematic representation of the *TDC* promoter fragments used in EMSAs. *Letters* indicate restriction sites used for isolation of the fragments. *Numbers* indicate the position relative to the transcriptional start site. The fragment containing the TATA box is indicated. *B*, schematic representation of the *STR* promoter fragments used in EMSAs. Part of the wild-type sequence of the RV region is shown, and the numbering of block mutations is given *below* the sequence. In each block mutation, six adjacent nucleotides were changed into their complementary nucleotides. *C*, binding of ZCT1, ZCT2, and ZCT3 to the DB fragment. *D*, binding of ZCT1, ZCT2, and ZCT3 to fragments of the *TDC* and *STR* promoters. *E*, sequence-specific binding of ZCT1, ZCT2, and ZCT3 to the RV region. Fragments used as probes are indicated at the *top* of the panels. *M2–M7*, mutant RV fragments; *Z1*, ZCT1; *Z2*, ZCT2; *Z3*, ZCT3.

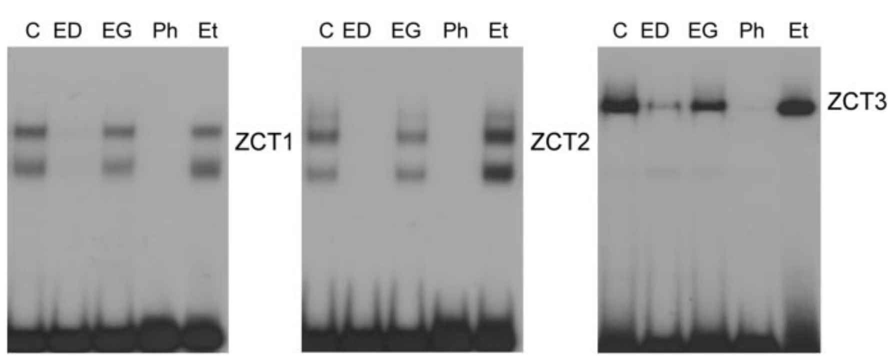

FIG. 3. **Zinc is required for interaction of the ZCT proteins with DNA.** Assay of ZCT1, ZCT2, and ZCT3 binding to the RV fragment from the *STR* promoter in the presence of 3 mM EDTA, 3 mM EGTA, 10 mM 1,10-phenanthroline/1% ethanol, or 1% ethanol. *C*, control (no treatment); *ED*, EDTA; *EG*, EGTA; *Ph*, 1,10-phenanthroline; *Et*, ethanol.

required for binding (Fig. 3). The presence of EGTA, which has a chemical structure similar to EDTA but specifically binds calcium, or the solvent ethanol did not influence the binding of the ZCT proteins to RV (Fig. 3) indicating the specificity of the inhibition by EDTA and 1,10-phenanthroline. A similar experiment using the DB fragment of the *TDC* promoter showed that

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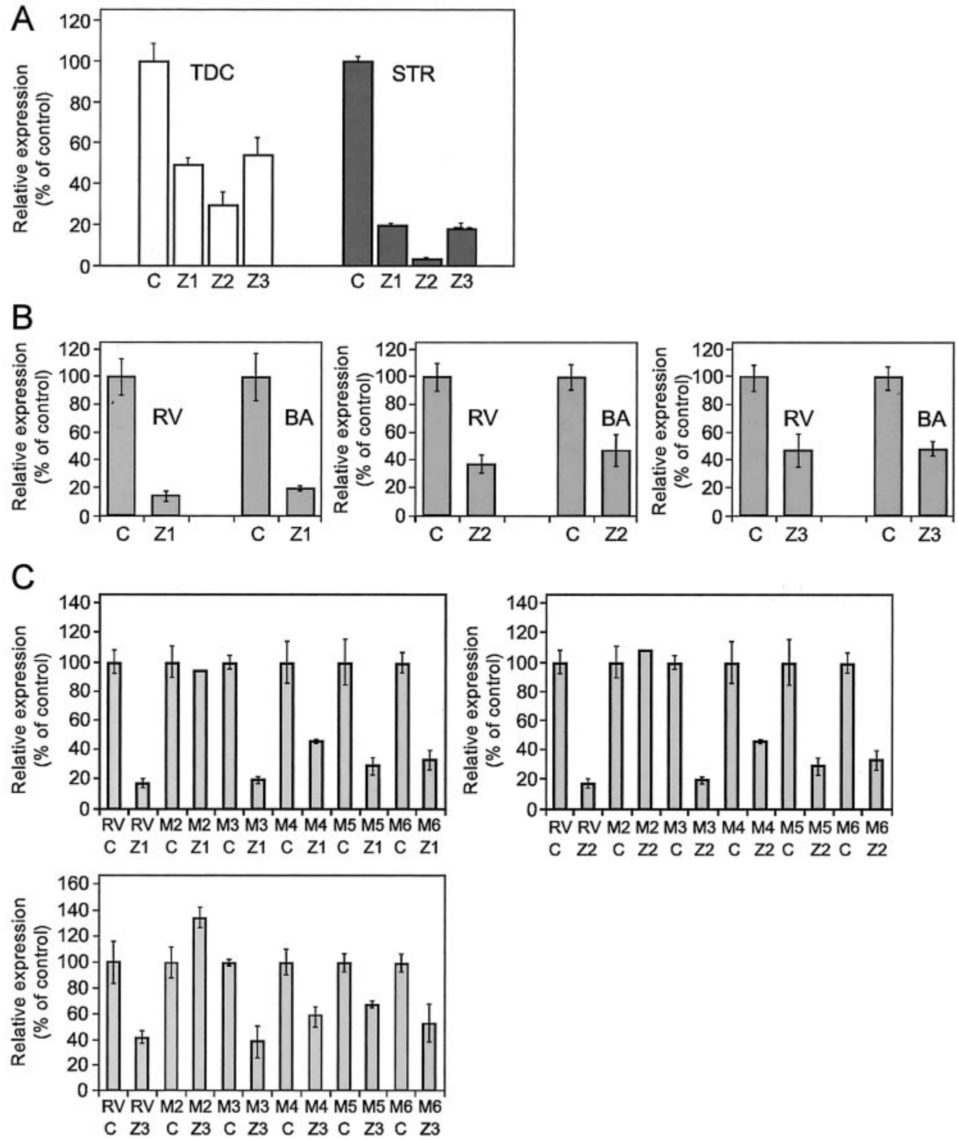

FIG. 4. ***STR* and *TDC* promoter activities are repressed by ZCT zinc finger proteins.** *A*, *C. roseus* cells were co-transformed with plasmids carrying *STR*-promoter-*GUSA* (−339 to + 52) or *TDC* promoter-*GUSA* (−99 to +198) and an overexpression vector containing *ZCT1*, *ZCT2*, or *ZCT3* cDNA driven by the CaMV 35 S promoter. *C. roseus* cells were co-transformed with a GUS reporter plasmid carrying a tetramer of the RV or BA fragment fused to the minimal CaMV 35 S promoter (−47 to +27) (*B*) or tetramers of RV wild-type and mutant fragments fused to the minimal CaMV 35 S promoter, and an overexpression vector with or without the *ZCT1*, *ZCT2*, or *ZCT3* cDNA fused to CaMV 35 S promoter (*C*). *Bars* represent means + S.E. (n = 3). GUS activities are shown as percentages of the vector controls. *C*, vector control (empty expression vector); *Z1*, ZCT1; *Z2*, ZCT2; *Z3*, ZCT3; *BA*, *RV*, different *STR* promoter fragments (see legend to Fig. 2); *M2–M6*, different RV mutants (mutations as in the legend to Fig. 2).

zinc is also essential for the binding of the ZCT proteins to this fragment (results not shown).

The ZCT Proteins Act as Transcriptional Repressors of STR and TDC Promoter Activity—Binding of the zinc finger proteins to both the *TDC* and *STR* promoters suggested that these proteins might be involved in the coordinated regulation of the expression of these genes. To test whether the ZCT proteins can regulate these promoters *in vivo*, *C. roseus* cells were co-transformed with a *TDC*-promoter-*GUSA* construct and an overexpression vector carrying a *ZCT* cDNA fused to the CaMV 35 S promoter. Co-expression of any of the ZCT proteins reduced *TDC* promoter activity ~2-fold compared with the vector control (Fig. 4*A*). Co-expression of any of the ZCT proteins reduced *STR* promoter activity at least 5-fold (Fig. 4*A*). These results show that the ZCT proteins can act as transcriptional repressors of both the *TDC* and *STR* promoters. The repressor activity of the ZCT proteins is consistent with the presence of the L*x*L*x*L motif within these proteins.

We focused our *in vivo trans*-regulatory studies on the *STR* promoter, because its structure with regard to *cis*-acting elements and their interaction with *trans*-acting factors has been elucidated in more detail than for the *TDC* promoter (30). As shown above, the ZCT proteins can bind to the BA and RV regions of the *STR* promoter *in vitro*. To test whether the *in vitro* binding affinities are reflected in *in vivo* repressor activities, *Catharanthus* cells were co-transformed with GUS reporter plasmids carrying tetramers of the BA or RV fragments fused to the minimal CaMV 35 S promoter (−47 to +27), and an overexpression vector carrying a *ZCT* cDNA fused to the CaMV 35 S promoter. All three ZCT proteins could repress the activity of both the RV and BA promoter fragments (Fig. 4*B*).

A repressor protein can inhibit transcription via different mechanisms, requiring promoter binding (*e.g.* competition with activators for DNA binding sites or recruitment of chromatin-modifying or remodeling complexes) or not requiring promoter binding (*e.g.* sequestration of basal transcription factors or

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activators). To determine whether the repression by the ZCT proteins occurs via a direct interaction with the DNA, co-bombardment experiments were performed using the different RV mutants fused to a minimal promoter-*GUS* gene. The RV mutants affected in the ORCA binding site have reduced basal transcriptional activity (11) but still enhanced minimal promoter activity 5-fold (data not shown). This may be because of residual binding of endogenous ORCA proteins or because of binding of another unidentified transcription factor. In any case, the RV mutants were sufficiently active to measure a reduction as a result of repression. As shown before, expression conferred by a tetramer of the wild-type RV fragment was significantly repressed by the ZCT proteins. The expression conferred by mutant constructs 4M3–4M6 was also repressed by the ZCT proteins in a statistically significant manner (Fig. 4*C*), whereas the expression conferred by mutant construct 4M2 was not significantly affected by these proteins ($p = 0.05$). As shown above, EMSAs demonstrated that the ZCT proteins were unable to bind to the RVM2 mutant fragment. Because the M2 mutation, which abolished *in vitro* binding of the ZCT proteins to the RV fragment, also affected *trans*-repression of the RV fragment *in vivo*, it can be concluded that ZCT-mediated repression of transcriptional activity conferred by the RV fragment occurs via direct binding.

Interactions between the ORCA Activators and the ZCT Repressors—Previous studies showed that ORCA2 and ORCA3 activate the *STR* promoter via binding to the M2, M3, and M4 region of the RV fragment (10). Therefore, both the ORCA activators and the ZCT repressors can bind to the RV region of the *STR* promoter. To test the effect of overexpression of a combination of activators and repressors on RV activity, *C. roseus* cells were co-transformed with a plasmid carrying a 4RV-*GUS* reporter construct and ZCT and/or ORCA effector constructs. Co-transformation of the ORCA2 or ORCA3 effector plasmids with any of the ZCT plasmids, resulted in RV-mediated expression levels that were not statistically significantly different from levels obtained upon transformation with the ORCA2 or ORCA3 effector plasmids alone (Fig 5*A*, $p = 0.05$). This indicates that with these ratios of effector plasmids, ORCA-mediated transcriptional activity conferred by the RV fragment is not negatively affected by the zinc finger repressors.

EMSAs showed that besides the RV fragment, the −339 *STR* promoter contains two other binding sites for the zinc finger repressors within the BA and the VH fragments (Fig. 2*D*). To test the effect of overexpression of a combination of activators and repressors on the activity of the −339 *STR* promoter, *C. roseus* cells were co-transformed with a GUS reporter plasmid carrying the *STR* promoter and ZCT and/or ORCA effector plasmids. In this promoter context, the co-transformation of ORCA2 or ORCA3 effector plasmids and any of the ZCT plasmids resulted in activity levels that were significantly lower than levels obtained upon transformation with the ORCA2 or ORCA3 effector plasmids alone (Fig 5*B*, $p = 0.1$). These results show that in a more natural *STR* promoter context, zinc finger proteins are able to counteract activation of this promoter by ORCAs. It is likely that in this promoter context, the zinc finger proteins repress gene expression via binding to the BA and/or VH fragments. This is confirmed by an experiment in which the repression of −339 *STR* promoter derivatives, containing the different RV mutations M2–M6, by ZCT1 was tested (Fig. 6). ZCT1 repressed the activity of all *STR* promoter derivatives, including the M2 mutant version, showing that repression of *STR* promoter activity by ZCT1 does not require binding to the RV fragment.

Elicitor and MeJA Rapidly Induce ZCT mRNA Accumulation—The binding of the ZCT proteins to the YE-responsive DB

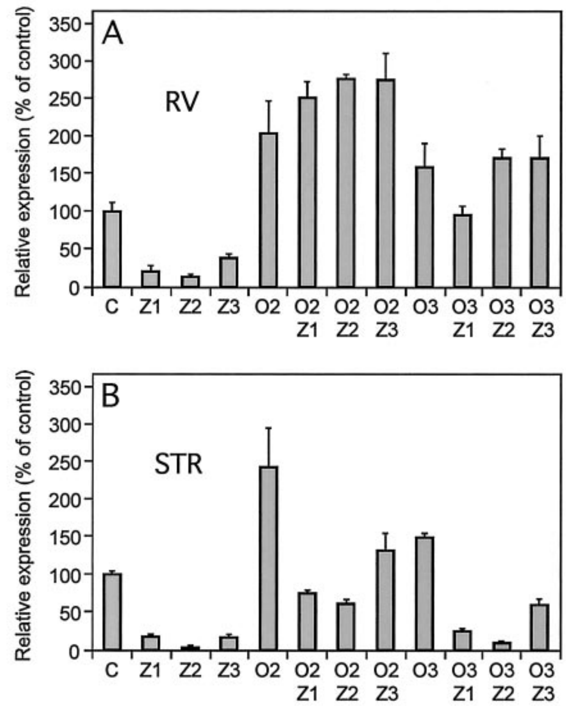

FIG. 5. **Zinc finger proteins do not affect RV-mediated activation by ORCAs but negatively affect activation of an extended *STR* promoter derivative by ORCAs.** *C. roseus* cells were co-transformed with a *GUS* reporter plasmid carrying a tetramer of the wild-type RV fragment fused to the minimal CaMV 35 S promoter (−47 to +27) (*A*) or the *STR*-promoter (−339 to +52) and an overexpression vector containing *ZCT1*, *ZCT2*, or *ZCT3* cDNA fused to the CaMV 35 S promoter and/or an overexpression vector with or without *ORCA2* or *ORCA3* cDNA fused to CaMV 35 S (*B*). *Bars* represent means + S.E. ($n = 3$). GUS activities are shown as percentages of vector controls. *C*, vector control (empty expression vector); *O2*, ORCA2; *O3*, ORCA3; *Z1*, ZCT1; *Z2*, ZCT2; *Z3*, ZCT3.

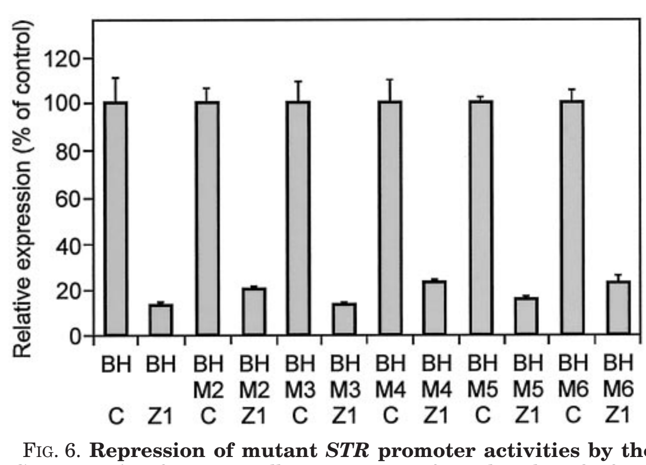

FIG. 6. **Repression of mutant *STR* promoter activities by the ZCT1 protein.** *C. roseus* cells were co-transformed with a *GUS* reporter plasmid carrying wild-type (−339 to +52) or mutant *STR* promoter derivatives and an overexpression vector with or without the *ZCT1* cDNA fused to the CaMV 35 S promoter. *Bars* represent means + S.E. ($n = 3$). GUS activities are shown as percentages of the vector controls. *C*, vector control (empty expression vector); *Z1*, ZCT1; *BH*, wild-type *STR* promoter (−339 to +52); *BHM2–BHM6*, different *STR* promoter mutants (see legend to Fig. 2).

region of the *TDC* promoter and the YE- and MeJA-responsive RV and BA regions of the *STR* promoter suggested that these proteins might be involved in the regulation of *TDC* and *STR* expression in response to elicitors and jasmonic acid. To establish whether *ZCT* mRNA levels are modulated by YE or jasmonic acid, expression levels were analyzed after the treatment of *C. roseus* cells with these compounds. *ZCT* mRNA

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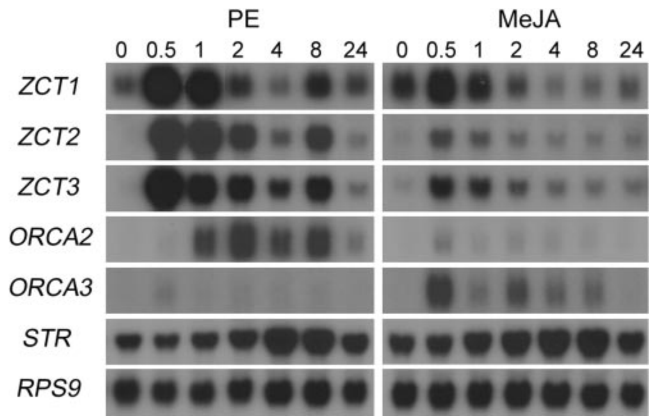

FIG. 7. ***ZCT* mRNA levels are rapidly induced by YE and MeJA.** Cells of *C. roseus* cell line MP183L were exposed to partially purified elicitor or MeJA (10 μM) for a number of hours indicated at the *top* of the figure. Northern blots were hybridized with cDNAs as indicated on the *left*.

levels were rapidly and transiently induced by YE, with a peak after 0.5 h of exposure to YE (Fig. 7). At 24 h of YE treatment, the *ZCT* mRNA levels returned to the basal levels. Furthermore, *ZCT* mRNA levels were also transiently induced by MeJA treatment, with maximum accumulation after 0.5 h of MeJA treatment. The accumulation of *ZCT* mRNAs was much more rapid than *STR* mRNA accumulation, which peaked at 4–8 h. *ZCT* mRNA accumulation in response to MeJA was significantly lower than following YE treatment. *STR* mRNA levels increased similarly in response to YE or MeJA, indicating that the low accumulation of *ZCT* mRNA after MeJA treatment, compared with the accumulation after YE treatment, is not because of a concentration effect (Fig. 7). *ZCT* mRNA levels were compared with *ORCA* mRNA levels in the same samples (Fig. 7). *ORCA2* mRNA accumulated preferentially in response to YE and was in this respect qualitatively similar to *ZCT* mRNA accumulation, whereas *ORCA3* mRNA accumulated preferentially in response to MeJA. *ZCT* mRNA accumulation in response to YE was faster than *ORCA2* mRNA accumulation, which peaked at 2 h. In response to MeJA, *ZCT* and *ORCA* mRNAs accumulated with similar kinetics with a peak at 0.5 h and returning to basal levels at 24 h.

DISCUSSION

In this report, we described the isolation of three members of the Cys_2/His_2-type (TFIIIA-type) zinc finger gene family in *C. roseus*, encoding ZCT1, ZCT2, and ZCT3. We showed that these proteins can directly bind in a zinc-dependent manner to the promoters of two elicitor- and jasmonate-responsive secondary metabolite biosynthesis genes *in vitro* and can repress the activity of these promoters in transient expression assays *in vivo*. We also demonstrated that *ZCT* mRNA levels were rapidly induced by elicitor and jasmonic acid. These data suggested that TFIIIA-type zinc finger transcription factors act as repressors in the regulation of elicitor- and jasmonate-induced secondary metabolism in *C. roseus*.

The ZCT proteins contain two Cys_2/His_2-type zinc fingers and belong to the EPF subfamily of TFIIIA-type zinc finger proteins in plants. Members of this subfamily are characterized by the highly conserved sequence QALGGH in their zinc finger motifs, which is essential for DNA binding (31). In the EPF protein family, the number of zinc fingers ranges from one to four and the zinc fingers are separated by long spacers of diverse lengths (24). The length of the spacers between the zinc finger motifs is important for target site recognition (24). Based on our results, the three ZCT proteins seem to be functionally equivalent in the repression of *STR* and *TDC* expression. This raises the possibility that they are redundant in function. However, there are some structural differences between the three proteins. ZCT3 (27.4 kDa) is larger than ZCT1 and ZCT2 (19.6 and 21 kDa, respectively). Also, the spacer between both zinc fingers of ZCT3 is longer than the spacers of ZCT1 and ZCT2, which may indicate that it has the ability to bind different target DNA sequences compared with ZCT1 and ZCT2. Furthermore, *ZCT3* mRNA is expressed at a higher level than *ZCT1* and *ZCT2* mRNAs (results not shown). Therefore, the possibility exists that each ZCT protein has specific functions as well.

We showed that the ZCT proteins can bind to different fragments of the *TDC* and *STR* promoters. DNA binding by plant EPF zinc fingers proteins *in vitro* is documented for a few other members of this family. It was found that two two-fingered proteins of the petunia EPF family, ZPT2-1 and ZPT2-2, can bind to two tandemly repeated AGT core sites (32). More recently, the optimal binding sequence for ZPT2-2 was determined. For the N-terminal finger, the optimal binding sequence is AGC(T) or AGG, and for the C-terminal finger it is CAGT (33). The *Arabidopsis* SUPERMAN protein, which only contains one zinc finger, can also bind to the AGT core sequence (34). In our experiments, the M2 mutation within the RV region of the *STR* promoter abolished binding of the ZCT proteins, suggesting that this mutation destroyed the binding site for one of the fingers in the RV region. The first two nucleotides of the wild-type M2 block and the nucleotide directly preceding it form an ACT sequence (Fig. 2*B*), which reads as an AGT sequence on the complementary strand. It seems likely that this is the actual binding site for the ZCT proteins, based on the optimal binding sites for the ZPT proteins. It is unclear whether the RV fragment contains a binding site for a second zinc finger or whether the ZCT proteins bind RV with a single finger.

In this report, we showed that the ZCT proteins can repress the activity of the promoters of the terpenoid indole alkaloid biosynthetic genes *STR* and *TDC*. We also demonstrated, via *in vivo* co-expression of ZCT proteins with wild-type and mutant versions of the *STR* promoter, that repression by the ZCT proteins occurred via direct DNA binding. All three ZCT proteins contain the L*x*L*x*L motif, which has been demonstrated in other zinc finger transcription factors, including proteins that are highly similar in amino acid sequence to the ZCTs, to be involved in active repression (28, 35). It seems likely that this L*x*L*x*L motif is responsible for the repressor activity of the ZCT proteins. The petunia two-fingered protein ZPT2-3 (36), and the *Arabidopsis* two-fingered proteins ZAT10, ZAT11 (28), and the one-fingered protein SUPERMAN (37) fused to the yeast GAL4 DNA-binding domain were shown to repress an artificial promoter containing GAL4 binding sites in *Arabidopsis* leaves. Removal of the L*x*L*x*L motif abolished the repressing activity of these proteins. In addition, the ZAT10, ZAT11, or SUPERMAN L*x*L*x*L motifs fused to the GAL4 DNA-binding domain can repress the activity of an artificial promoter carrying both GAL4 binding sites as well as binding sites for AP2/ERF-domain transcription factors in the presence of an activating AP2/ERF-domain transcription factor. We showed here that two natural promoters of the *TDC* and *STR* genes actually contain such an arrangement of binding sites for both activating AP2/ERF-domain activators and zinc finger repressors. We also showed that within the natural *STR* promoter context, the ZCT proteins can repress the activating activity of the ORCAs without competing for the same binding sites.

ZCT mRNA levels were increased by YE and MeJA. The expression of two other EPF-family genes, the petunia *ZPT2-2* and *ZPT2-3* genes, is also induced by JA (36, 38).

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However, the expression of *Arabidopsis ZAT6* and *STZ/ZAT10* is not induced by JA (39), indicating that the induction of gene expression by JA is restricted to specific members of the EPF family. YE induced *ZCT* gene expression after 30 min (Fig. 5), and JA biosynthesis was induced after 2 h (8). Therefore, the induction of *ZCT* gene expression by YE seems to be upstream or independent of the induction of JA biosynthesis. This is confirmed by the finding that the inhibitor of JA biosynthesis diethyldithiocarbamic acid did not affect YE-responsive *ZCT* expression levels.[2]

Although many members of the EPF subfamily of TFIIIA-type zinc finger transcription factors have been identified, no target genes are known, and only for a few of them biological functions have been described. The fact that the ZCT repressors can bind to YE- and JA-responsive regions of the *STR* and *TDC* promoters, and the fact that *ZCT* expression levels were induced by YE and MeJA treatment, indicates that these proteins are involved in regulation of *TDC* and *STR* expression by elicitor and JA. A few other members of the EPF family have also been reported to be involved in the regulation of stress responses. The soybean SCOF-1 protein is one of the closest homologues of ZCT3 and also contains a C-terminal L*x*L*x*L motif (25). Surprisingly, its overexpression in *Arabidopsis* induced the expression of cold-responsive genes, resulting in enhanced cold tolerance (25). Transgenic *Arabidopsis* plants overexpressing the *RHL41/ZAT12* gene showed an increased anthocyanin and chlorophyll content and increased tolerance to high intensity light (40). Constitutive overexpression of ZPT2-3 in petunia increased the tolerance to dehydration (36). However, for none of the latter zinc finger regulators is it known via which natural target genes they exert their biological effects.

There are several mechanisms by which the ZCT proteins could actively repress transcription of the *STR* and *TDC* promoters (41). The ZCT proteins could prevent the association of a transcriptional activator with these promoters or could suppress the function of a DNA-bound transcriptional activator protein. Alternatively, ZCT proteins could have negative effects on the basal transcription machinery or could induce the formation of an inactive chromatin structure at the sites of the *STR* and *TDC* promoters. Because the ZCT proteins can repress the activity of the BA fragment, to which ORCA proteins do not bind, it seems unlikely that the repression by the ZCT proteins would function via the modulation of ORCA activity or binding to the *STR* and *TDC* promoters. Therefore, the ZCT proteins may act on another unidentified transcriptional activator, on the general transcription machinery, or they may affect chromatin structure.

Many genes are regulated by multiple transcriptional regulators by virtue of having a specific set of protein binding sites in their promoters (42). Both ORCA activators and ZCT repressors can bind to the RV element of the *STR* promoter. When both proteins were overexpressed, the ORCA-mediated transcriptional activity of the RV fragment was not negatively affected by the ZCT proteins. However, the ORCA-mediated transcriptional activity of a longer *STR* promoter derivative was repressed by the ZCT proteins when both proteins were co-expressed. This indicates that in the larger promoter context, the ZCT proteins repressed *STR* promoter activity via binding to the BA and/or VH fragments. However, in a natural situation, it is probable that ORCA and ZCT proteins have different expression levels at a certain time, as is also suggested by the differential kinetics of *ORCA* and *ZCT* mRNA accumulation in response to YE and MeJA. This makes it difficult to draw conclusions about the *in vivo* stoichiometry and interactions between these proteins under natural conditions.

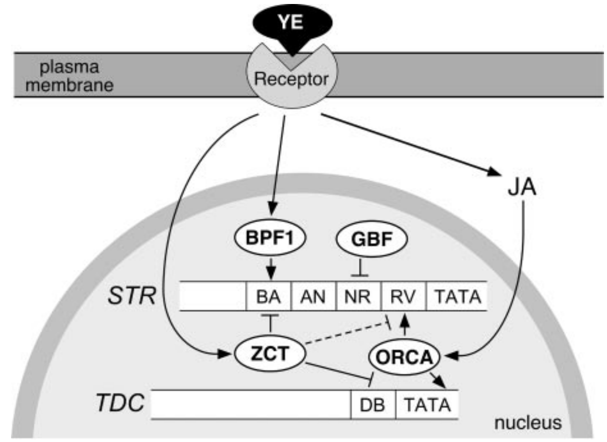

FIG. 8. **Overview of transcription factors that can interact with the *STR* and *TDC* promoters.** Perception of YE leads to an increase in JA levels, which is necessary for the activation of the ORCA transcription factors. Although the cellular location of the YE receptor is unknown, it is tentatively placed in the plasma membrane. The ORCA transcription factors can activate gene expression via interaction with the *TDC* promoter and the RV fragment of the *STR* promoter. Although the ORCA binding site in the *TDC* promoter has not been precisely mapped, it is tentatively indicated downstream of the DB fragment. In addition, YE rapidly induces the accumulation of mRNAs encoding ZCT proteins, which can repress gene expression via binding to the DB fragment of the *TDC* promoter and the BA and, to a lesser extent, the RV fragments of the *STR* promoter. Also, YE induces accumulation of mRNA encoding CrBPF1, which is putatively involved in regulation of *STR* via interaction with the BA region. CrGBF transcription factors can repress *STR* promoter activity via binding to the NR region.

In conclusion, perception of YE activates the octadecanoid pathway, which leads to an increase in JA levels (8). JA induces the expression of the *ORCA* genes, especially the *ORCA3* gene, and activates pre-existing ORCA proteins via post-translational modification (11). The ORCA proteins can activate gene expression via interaction with the *TDC* promoter and the YE- and JA-responsive RV fragment of the *STR* promoter (Fig. 8, Refs. 10–12). In addition, YE rapidly induces the expression of the zinc finger proteins (Fig. 7), which can repress gene expression via binding to the YE-responsive DB fragment of the *TDC* promoter and the YE- and JA-responsive BA and RV fragments of the *STR* promoter (Fig. 8). Also, YE induces accumulation of mRNA encoding CrBPF1, which is putatively involved in the regulation of *STR* via interaction with the BA region (9). Finally, CrGBF transcription factors can repress *STR* promoter activity via binding to the NR region (Fig. 8, Ref. 13).

The functional importance of the induction of both activators and repressors of *STR* and *TDC* gene expression by YE remains unclear. The simultaneous induction of repressors and activators may serve to fine tune the amplitude and timing of gene expression. Such a fine tuning may in part be achieved by the differential effect of YE and (Me)JA on the amplitude and kinetics of *ORCA* and *ZCT* mRNA accumulation. Alternatively, in an analogy to models used to explain switch-like transcriptional control by developmental signals (43), the induction of a combination of activators and repressors may be necessary to achieve a switch-like on/off state of gene expression in response to stress signals.

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Zinc Finger Proteins Act as Transcriptional Repressors of Alkaloid Biosynthesis Genes in *Catharanthus roseus*

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