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 Cys2/His2-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), 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 PbBPF-1, called CrBPF1 (9). The jasmonate- and elicitor-responsive element interacts with two JA-responsive transcription factors 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 or the MYB-related protein CrBPF1 (9) do not bind to the DB element.
Zinc Finger Proteins Repress Alkaloid Biosynthesis Genes

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 promoter fragment fused to the HIS3 reporter gene in plasmid p601 (15). The tetramer-HIS3 fusion was inserted into a BamHI site of yeast strain Y187 (17). Recombinants were selected on YPD (yeast extract/peptone/dextrose) medium containing 150 μg/ml G418, and the occurrence of single recombinant events between the pUP04 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 described by Ref. 10. After transformation of the cDNA YE-induced secondary metabolism. transcription factors can act as repressors in the regulation of promoter activity in ZCT2, and ZCT3. were shown to act as transcriptional repressors of C2H2 zinc finger classes. representative members of each class resulted in the identification of three plasmids conferring His-Leu-independent growth upon isolation/re- transformation. Plasmid cross-hybridizations and sequencing of representative members of each class resulted in the identification of three C2H2 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'-CCCCACCAAAACCCAAAAG-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'-CTAAAGATTGGAGTAGATC-3' were digested with BamHI/HindIII and cloned in plasmid SK+ 3'. Sequencing of the cloned inserts yielded additional nucleotides. ZCT2 5' sequences amplified with the gene-specific primer 5'-CATACAAATTCTGACTTCCACC-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 pUC18. Then it was excised from pACTII with SmaI/XhoI, cloned in pIC-19R digested with SmaI/XhoI, and finally cloned into the expression vector pGEX-KG as a BamHI/XhoI fragment. The resulting plasmid was transferred as a BamHI fragment into the BclI site of integration vector pJP04, which is essentially similar to pINT1 (16). The resulting clone was excised from pACTII with SmaI/XhoI and first cloned into the vector pACTII. In total, 2.4 million Y187-4DB transformants were co-transformed with plasmids carrying different promoter parts fused to GUS and overexpression vectors carrying ZCT1, ZCT2, ZCT3, and/or ORCA 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 molar ratio of 1:1 (pMOG-ZCT/ORCA:pMOG). The ratio of 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 purified from yeast extract (YE) (Difeo), through ultrafiltration and a mono-Q fast protein liquid chromatography (FPLC). The 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 alkalization response assay as described before (8). Methyljasmonate (Bedoukian Research Inc.) was diluted in dimethyl sulfoxide (Me2SO).

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 32P-labeled cDNA fragments. ORCA2, ORCA3, RPS9, and STR probes were probed 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 HIS3 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
The ZCT Proteins Bind to Several Regions of the TDC and STR Promoters—The ability of the ZCT proteins to activate HIS3 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. 2C). 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. 2A). 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. 2D). However, ZCT3 bound to all fragments of the TDC promoter with highest affinity for HS and DB (Fig. 2D). 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 TDC promoter fused to the HIS3 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. 2B) showed that they indeed bound to the RV region and additionally to the BA and VH regions (Fig. 2D). ZCT3 bound additionally to the XD and DB fragments of the STR promoter (Fig. 2D). 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. 2B; 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. 2E). 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 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 (13).

Comparison of the DNA sequences to sequences in the NCBI database 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 are separated by a long spacer (Fig. 1).

- **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 from pACTII 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 kDa, respectively. Comparison of the deduced ZCT1 and ZCT2 amino acid sequences to sequences in the NCBI database 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 SCO1 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 LxLxL 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 HIS3 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. 2C). 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. 2A). 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. 2D). However, ZCT3 bound to all fragments of the TDC promoter with highest affinity for HS and DB (Fig. 2D). 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 TDC promoter fused to the HIS3 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. 2B) showed that they indeed bound to the RV region and additionally to the BA and VH regions (Fig. 2D). ZCT3 bound additionally to the XD and DB fragments of the STR promoter (Fig. 2D). 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. 2B; 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. 2E). The ZCT binding site is therefore distinct from but overlapping with the binding site for the ORCA proteins.
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
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 TDC-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 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. 4A). Co-expression of any of the ZCT proteins reduced STR promoter activity at least 5-fold (Fig. 4A). 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 LxLxL 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 vivo repressor activities, Catharanthus cells were co-transformed with GUS reporter plasmids carrying tetramers of the RV or BA fragment 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. 4B).

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
activated repression of transcriptional activity conferred by the RV fragment. Previous studies showed that ORCA2 and ORCA3 activators and the ZCT repressors can bind to the RV region of the RV fragment (10). Therefore, both the ORCA proteins to the RV fragment, also affected trans-repression of the RV fragment in vitro, 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 5A, 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. 2D). 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 5B, 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 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
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 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. 2B), 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 LxLxL 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 repressors of 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).
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.

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 LxLxL 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.

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 (8). 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.

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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