ORCA3, a Jasmonate-Responsive Transcriptional Regulator of Plant Primary and Secondary Metabolism

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Biosynthesis of many classes of secondary metabolites in plants is induced by the stress hormone jasmonate. The gene for ORCA3, a jasmonate-responsive APETALA2 (AP2)-domain transcription factor from *Catharanthus roseus*, was isolated by transferred DNA activation tagging. *Orca3* overexpression resulted in enhanced expression of several metabolite biosynthetic genes and, consequently, in increased accumulation of terpenoid indole alkaloids. Regulation of metabolite biosynthetic genes by jasmonate-responsive AP2-domain transcription factors may link plant stress responses to changes in metabolism.

Metabolism comprises coordinate series of coupled enzymatic conversions in living organisms. Secondary metabolites are not vital to cells that produce them, but contribute to the overall fitness of the organism. Functions of these compounds in plants include protection against pests and pathogens. For man, plant secondary metabolites are useful as pharmaceuticals, dyes, fragrances, insecticides, or flavors. Production of secondary metabolites is controlled at the level of expression of the biosynthetic genes by developmental and tissue-specific factors or by external signals (*1*, *2*). Furthermore, accumulation of some metabolites is induced by (methyl)jasmonate [(Me)JA] (*3*), a plant hormone produced in response to stress.

One group of secondary metabolites are the anthocyanin flower pigments. Expression of genes encoding anthocyanin biosynthetic enzymes is controlled by transcription factors with homology to mammalian MYB and basic helix-loop-helix (bHLH) proteins, respectively [reviewed in (*1*)]. Knowledge about regulation of other secondary metabolic pathways is more limited.

For the biosynthesis of secondary metabolites, plants must accommodate their primary metabolic pathways. Coordinate regulation between these processes has been observed (*4*), but the regulatory mechanisms are unknown.

MeJA induces the production of terpenoid indole alkaloid (TIA) secondary metabolites (*3*). To identify regulators of this metabolic pathway (Fig. 1) in the plant species *Catharanthus roseus* (Madagascar periwinkle), we used a transferred DNA (T-DNA) activation tagging approach (*5*, *6*). Enhancers carried on the T-DNA of a plant transformation vector can deregulate a gene flanking the T-DNA integration site in the plant genome, resulting in a dominant mutation. We used tryptophan decarboxylase (TDC) as a selectable marker (*7*). TDC catalyzes the first step in TIA biosynthesis starting from tryptophan (Fig. 1). In addition, TDC is able to detoxify 4-methyltryptophan (4-mT). *Agrobacterium*-mediated transformation of *C. roseus* cell suspensions (*8*) with the activation tagging construct (Fig. 2A) resulted in roughly 4×10^5 to 5×10^5 stable transformants that were screened for 4-mT resistance at a 0.4 mM concentration. This yielded 180 resistant cell lines screened for *Tdc* expression levels by Northern blot analysis to identify mutant lines with a transcriptional regulator activated by the T-DNA insertion. Among 20 lines with increased *Tdc* expression (*9*), six lines also showed increased expression of a second gene involved in TIA production (i.e., *Str*), suggesting that the tag activated a regulator of multiple genes involved in TIA biosynthesis. From one of these six lines (line 46), 1.6 kilobase pairs (kbp) of plant DNA flanking the T-DNA insertion site were isolated by plasmid rescue (*10*).

An open reading frame (ORF) was located ~600 bp downstream of the T-DNA that predicted a protein of 203 amino acids with a conserved AP2-domain (Fig. 2B). This DNA-binding domain is found in the AP2/EREBP class of plant transcription factors, many of which are involved in the regulation of responses to stress (*11*). Because of its similarity to ORCA2 (Octadecanoid-derivative Responsive *Catharanthus* AP2-domain protein 2) (*12*), we named the protein encoded by the rescued plant DNA ORCA3. The *Orca3* gene contains no introns (*13*), and the T-DNA integration did not induce any DNA rearrangements (*14*). Expression of *Orca3* is activated by the T-DNA tag (Fig. 3A, upper row). The overexpressed transcript and the endogenous mRNA have identical sizes, indicating that full-length mRNA and protein are produced, which is consistent with the tagged gene structure (Fig. 2B).

Expression of TIA biosynthetic genes *Tdc*, *Str*, *Sgd*, *Cpr*, and *D4h* was increased in the *Orca3*-tagged line (*15*) (Fig. 3A). TIA biosynthetic genes *G10h* and *Dat* were not induced, suggesting that these genes are not controlled by ORCA3. Genes encoding the alpha subunit of AS (ASα) and DXS, enzymes involved in primary metabolism leading to TIA precursor synthesis, were induced by *Orca3* overexpression, whereas two other primary metabolic genes not involved in production of TIA precursors (*Ggpps* and *Ics*) were not regulated by ORCA3 (Fig. 3A). Except for *Sgd*, which shows variable expression in control cell lines (Fig. 3B), expression patterns observed in cell lines that were independently transformed by particle bom-

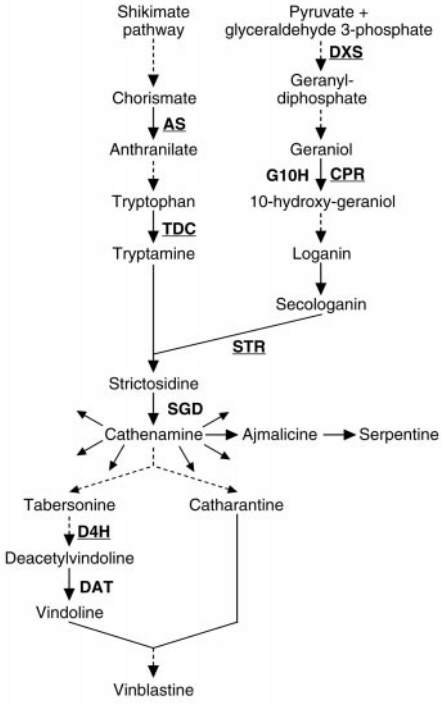

Fig. 1. Biosynthesis of TIAs in *C. roseus*. Solid arrows indicate single enzymatic conversions, whereas dashed arrows indicate multiple enzymatic conversions. Abbreviations of enzymes in the figure are as follows: AS, anthranilate synthase; DXS, D-1-deoxyxylulose 5-phosphate synthase; G10H, geraniol 10-hydroxylase; CPR, cytochrome P450-reductase; TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, strictosidine β-D-glucosidase; D4H, desacetoxyvindoline 4-hydroxylase; and DAT, acetyl-CoA: 4-O-deacetylvindoline 4-O-acetyltransferase. Genes regulated by ORCA3 are underlined.

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bardment with the rescued *Orca3* ORF (Fig. 3, B and D) or cDNA (*9*) under control of cauliflower mosaic virus (CaMV) 35*S* promoter elements (O3-OX lines) were identical to that of line 46. These results indicate that ORCA3 is a regulator of primary as well as secondary metabolite biosynthetic genes involved in TIA biosynthesis. Although previous studies report differential regulation of genes involved in early (i.e., *Tdc* and *Str*) and late (i.e., *D4h*) steps in vindoline biosynthesis in periwinkle plants (*16*), genes of both classes are controlled by *Orca3* in suspension-cultured cells.

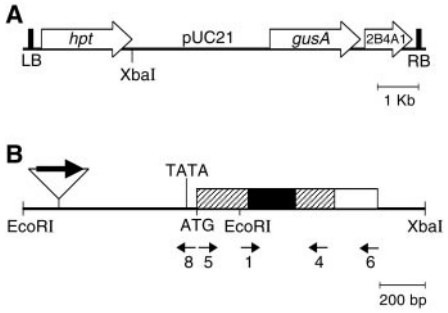


Fig. 2. (**A**) Schematic representation of the T-DNA tagging construct. *Hpt*, hygromycin resistance marker; pUC21, *E. coli* pUC21 vector; *gusA*, β-glucuronidase reporter gene; 2B4A1, CaMV 35*S* promoter enhancer sequences (*18*) without TATA-box or ATG start codon; LB and RB, left and right T-DNA border sequences. (**B**) Genomic structure of *Orca3* as isolated by plasmid rescue (*10*) and I-PCR (*14*). Boxed region, the transcribed region determined by PCR (*13*); hatched box, the ORF; black box, the DNA-binding AP2-domain. Positions of the TATA box and ATG start codon are shown. The T-DNA integration site in line 46 is indicated as an inverted triangle. Arrows indicate primers used for PCR (*13*) and I-PCR (*14*). The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AJ251249 (cDNA sequence) and AJ251250 (genomic sequence).

Expression of *Orca3*, as well as of all other genes used in this study, was induced by MeJA (Fig. 3C). However, not all these genes were induced by *Orca3* overexpression. Combinations of different jasmonate-responsive transcription factors, such as ORCA2 (*12*) and ORCA3, may regulate TIA biosynthesis by selective activation of target genes.

Production of TIAs in untransformed *C. roseus* cell suspensions is low or absent. TIAs were not detected (*17*) in the O3-OX lines. Tryptamine and tryptophan synthesis was increased upon *Orca3* overexpression (Fig. 3E), showing that the metabolic pathway leading to indole precursors was induced. A possible bottleneck in production of the terpenoid moiety of TIAs is G10H because *G10h* expression was undetectable (Fig. 3B). To provide an excess of terpenoid precursor, we added loganin extracellularly. Upon loganin feeding, *Orca3* overexpression caused significantly increased TIA production (3.2-fold increase compared with controls) (Fig. 3E). O3-OX lines accumulated strictosidine (8.11 ± 0.87 μmol/g dry weight of *C. roseus* cells), ajmalicine (0.25 ± 0.10 μmol/g dry weight), and an unidentified lochnericine-like TIA (1.21 ± 0.66 area units per gram dry weight). Identical TIA patterns as observed in O3-OX lines after feeding with loganin were detected in untransformed cell suspensions after MeJA treatment (*9*), demonstrating that the JA-induced conversions of TIAs are regulated by ORCA3.

Electrophoretic mobility shift assays (EMSA) (*12*) and transient expression assays following particle bombardment (*18*) showed that ORCA3 functions as a DNA-binding protein which is directly involved in transcriptional activation of *Tdc*, *Str*, and *Cpr*. ORCA3 formed sequence-specific complexes with *Tdc*, *Str*, and *Cpr* promoter fragments (Fig. 4A). Expression from *Tdc*, *Str*, and *Cpr* promoter fragments was activated by coexpressed ORCA3 in *C. roseus* cells, whereas activation was not observed with unrelated T*cyt* elements of T-DNA cytokinin gene pro-

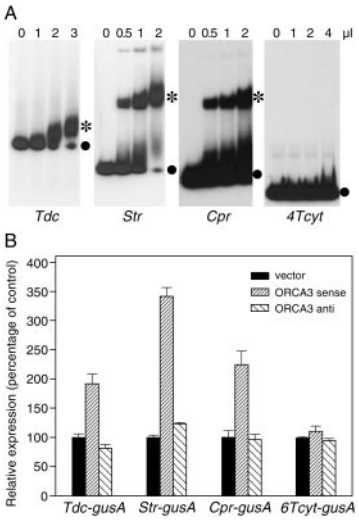
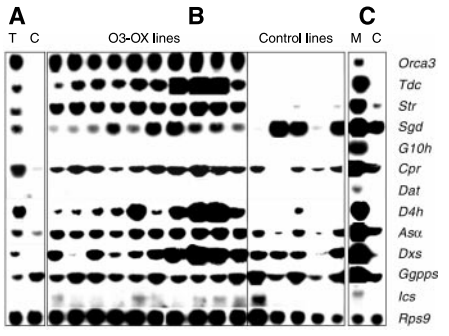


Fig. 4. *Tdc*, *Str*, and *Cpr* promoter fragments are sequence-specifically bound in vitro and *trans*-activated in vivo by ORCA3. (**A**) EMSAs were performed with the indicated amount of ORCA3 protein expressed in *E. coli* with *Tdc* (−99 to +198), *Str* (−145 to +52), *Cpr* (−114 to –36), and 4T*cyt* (*19*) promoter fragments as probes. The free probe and the ORCA3 complex are marked by circles and asterisks, respectively. (**B**) *C. roseus* cells were transiently cotransformed (*18*) with *Tdc* (−99 to +198), *Str* (−145 to +52), *Cpr* (−87 to +272), or T*cyt* (six copies fused to CaMV 35*S* −47 to +27 minimal promoter) promoter fragments fused to the *gusA* reporter gene; overexpression vectors containing the *Orca3* cDNA fused to the CaMV 35*S* promoter in sense or antisense orientation; and CaMV 35*S* promoter-*chloramphenicol acetyl transferase* (*cat*) (ratio 1:3:1). B glucuronidase (GUS)/CAT activity ratios were determined (*18*) and expressed as percentage of empty vector controls. Bars represent means ± standard error ($n = 6$ replicate experiments, except for antisense where $n = 3$).



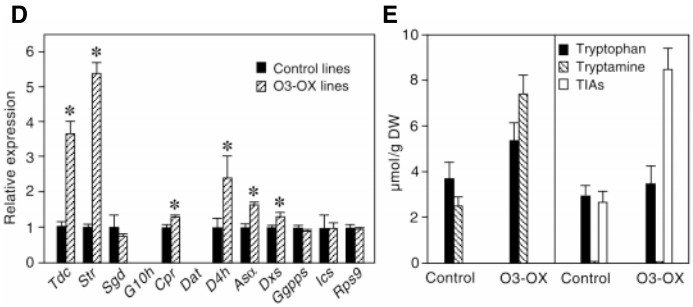


Fig. 3. *Orca3* overexpression causes induction of expression of primary and secondary metabolic genes and increased accumulation of TIAs. Complementary DNAs of the genes indicated were used as probes on (**A**) RNA extracted from *C. roseus* BIX cell line 46 (T) and from a representative control line (C), (**B**) RNA extracted from ten O3-OX lines and five control cell lines generated by particle bombardment in a genetic background of *C. roseus* cell line MP183L, and (**C**) RNA extracted from wild-type MP183L cells exposed for 6 hours to 50 μM MeJA (M) or 0.1 % dimethyl sulfoxide (C). Analysis of a control gene (*Rps9*) showed equal loading of RNA. (**D**) Expression levels in (B) were quantified. Mean expression of the control lines is set at 1. Bars indicate means ± standard error. Asterisks above the bars indicate statistically significant differences in expression levels as determined with the nonparametrical Wilcoxon–Mann-Whitney test ($P < 0.05$). (**E**) Tryptophan, tryptamine, and TIAs [expressed in μmol per gram dry weight (DW)] were extracted from 8-day-old *C. roseus* MP183L cultures (five control lines and five O3-OX lines), either without loganin supply (left panel) or 24 hours after addition of 1 mM loganin to the culture medium (right panel). Bars indicate means ± standard error.

moter (*19*) or with antisense *Orca3* expression (Fig. 4B).

Thus, plants can regulate primary metabolic pathways coordinately with secondary metabolism using a single transcription factor. Because the biosynthesis of many secondary metabolites is induced by jasmonate, the identification of an AP2-domain protein as regulator of several genes involved in JA-responsive metabolism uncovers a control mechanism that may be operative in other stress-responsive plant metabolic pathways as well.

References and Notes

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8. Cell cultures of *C. roseus* grown as described in (*18*) were transformed by particle bombardment (*C. roseus* cell line MP183L) (*18*) or with an improved *Agrobacterium tumefaciens* strain (*C. roseus* cell line BIX) (*9*).
9. L. van der Fits and J. Memelink, data not shown.
10. Ten micrograms of genomic DNA from line 46 were digested with Xba I, self-ligated, and electroporated into *Escherichia coli* strain NM554. Cells were selected on carbenicillin.
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13. Polymerase chain reactions (PCR) were performed on a *C. roseus* root cDNA library in lambda ZAPII (Stratagene) with primers based on *Orca3* genomic sequences (Fig. 2B). Primer combinations OR1 with M13-40 and OR4 with T3 primer were used to isolate 3′ and 5′ cDNA parts, respectively. Primers OR5 and OR6, based on cDNA sequences, were used to generate a complete *Orca3* ORF.
14. One microgram of genomic *C. roseus* DNA was digested with Eco RI and self-ligated and inverse-PCR (I-PCR) was performed with primers OR5 and OR8 (Fig. 2B).
15. Northern blots were hybridized with *C. roseus* cDNAs as follows (see Fig. 1 legend for abbreviations; GenBank accession numbers are in parentheses): *Tdc* (Acc. No. X67662), *Str* (Acc. No. X61932), *Sgd* (Acc. No. AF112888), *G10h* (Acc. No. AJ251269), *Cpr* (Acc. No. X69791), *D4h* (Acc. No. U71604), *Dat* (Acc. No. AF053307), *Dxs* (Acc. No. AJ011840), *Asα* (Acc. No. AJ250008), *Geranylgeranyl pyrophosphate synthase* (*Ggpps*) (Acc. No. X92893), *Isochorismate synthase* (*Ics*) (Acc. No. AJ006065), and *40S* ribosomal protein S9 (*Rps9*) (*12*).
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21. The authors thank S. Whitmer and A. Hermans for assistance with high-performance liquid chromatography analysis; P. Hock for preparing the figures; W. de Winter for assistance with tissue culturing; D. Vom Endt for I-PCR; and J. Kijne, R. Verpoorte, and F. Menke for valuable discussions. Supported by the Ministry of Economic Affairs; the Ministry of Education, Culture, and Science; and the Ministry of Agriculture, Nature Management, and Fishery in the framework of an industrial relevant research program of the Netherlands Association of Biotechnology Centres in the Netherlands (ABON).

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An Inherited Functional Circadian Clock in Zebrafish Embryos

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Circadian clocks are time-keeping systems found in most organisms. In zebrafish, expression of the clock gene *Period3* (*Per3*) oscillates throughout embryogenesis in the central nervous system and the retina. *Per3* rhythmic expression was free-running and was reset by light but not by the developmental delays caused by low temperature. The time of fertilization had no effect on *Per3* expression. *Per3* messenger RNA accumulates rhythmically in oocytes and persists in embryos. Our results establish that the circadian clock functions during early embryogenesis in zebrafish. Inheritance of maternal clock gene products suggests a mechanism of phase inheritance through ovogenesis.

Circadian rhythms in physiology and behavior allow living organisms to anticipate daily environmental changes (*1*). These rhythms are driven by endogenous circadian clocks synchronized to external time cues. All known circadian clocks, in organisms ranging from cyanobacteria to mammals, involve clock genes that interact to generate a molecular oscillator regulating output clock-controlled genes (*2, 3*). Studies in mammals have suggested that the circadian clock starts to function during late fetal and postnatal life (*4*). However, in lower vertebrate embryos, external development may require an earlier onset of the clock. In addition, although the mammalian fetal circadian rhythm is synchronized to that of the mother through maternal signals such as melatonin (*5*), such a mechanism cannot operate in embryos developing externally.

To analyze the development of the circadian clock in zebrafish, we isolated a homolog of the *Drosophila* clock gene *Period* (*dPer*), which encodes an essential component of the circadian clock (*6, 7*). Mammals have three homologs of *dPer* (*Per1*, *Per2*, and *Per3*), one of which, *Per2,* controls the mouse circadian clock (*6–11*). The isolated zebrafish cDNA contained an open reading frame predicted to encode a protein with a length of 1281 amino acids (Fig. 1A) (*12*). *Drosophila* and mammalian PER proteins share several conserved regions, including a PER-ARNT-SIM (PAS) dimerization domain, a cytoplasmic localization domain (CLD), a short region downstream of the *dPer^s* mutation, and a COOH-terminal serine/threonine–glycine repeat (*7, 8*). These regions were also conserved in the zebrafish protein (Fig. 1B). Phylogenetic analysis indicated that the zebrafish protein was most closely related to mammalian PER3 and was thus designated zebrafish PER3 (Fig. 1C) (*10*).

Expression of *Per3* was determined in zebrafish embryos raised under a cycle of 14 hours of light and 10 hours of dark (LD 14:10). Adults were entrained to the same LD 14:10 cycle, and overnight crosses resulted in synchronous spawning and fertilization upon lights on [zeitgeber time 0 (ZT 0)], thus time 0 of embryogenesis corresponded to ZT 0. A robust circadian expression of *Per3* was detected in the central nervous system and the retina of embryos throughout development from 40 to 128 hours postfertilization (hpf), with maximum mRNA expression during the early light phase from ZT 0 to ZT 4 (Figs. 2 and 3A). This suggests that embryonic *Per3* expression is controlled by a transcriptional feedback loop similar to that described in *Drosophila* and mice (*13, 14*).

We also analyzed the expression of the clock-controlled gene *Rev-erbα*, a transcriptional repressor that is expressed in a strong circadian rhythm in the adult rat liver (*15–17*). In contrast to *Per3*, *Rev-erbα* showed a developmentally regulated circa-

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