Transcriptional regulation of monoterpenoid indole alkaloid biosynthesis in Catharanthus roseus

Darehei, M.

Chapter 3

The role of F-box protein O2.51 in the regulation of alkaloid biosynthesis genes in *Catharanthus roseus*

Mina Darehei, Debora Vom Endt, Alessandra Biancucci, Angga Dwituti Lestari and Johan Memelink

Plant cluster, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

Abstract

Plants produce a large variety of secondary metabolites and many commercial pharmaceutical drugs are derived from these metabolites. The medicinal plant *Catharanthus roseus* produces two important monoterpenoid indole alkaloids (MIA) vincristine and vinblastine which are widely used for cancer treatment. Some genes in the MIA pathway including *strictosidine synthase* (*STR*) and *Tryptophan decarboxylase* (*TDC*), are regulated by the transcription factors Octadecanoid derivative-Responsive *Catharanthus* AP2-domain protein 2 and 3 (ORCA2 and ORCA3). Yeast two-hybrid screening identified a protein named O2.51 which interacted with ORCA2. It showed high similarity to *A. thaliana* F-box proteins EBF1/2, known for their interaction with the ethylene-responsive transcription factor ETHYLENE-INSENSITIVE 3 (EIN3) and the related EIL3. The F-box protein O2.51 could potentially function similarly in JA signaling, by interacting with ORCA2 and, probably, ORCA3. A hypothesis is that O2.51 may promote the degradation of ORCA2 and ORCA3 through the ubiquitin-26S proteasome system. The experiments detailed in this chapter aimed to explore the potential impact of O2.51 on ORCA activity and, subsequently, on MIA biosynthesis genes including *STR* and *TDC*. O2.51 interacted only with ORCA2 in yeast. However, the co-expression of O2.51 in transient trans-activation assays in *C. roseus* cells resulted in a negative impact on the activities of both ORCA2 and ORCA3. To investigate the effect of O2.51 on MIA biosynthesis genes, stable cell lines overexpressing *O2.51* or with silenced expression levels were generated. However, the number of replicate lines was too small and/or the gene expression was too variable to draw firm conclusions.

Keywords: *Catharanthus roseus*, MeJA, F-box protein, ORCA2, ORCA3

Introduction

Catharanthus roseus is one of the most studied medicinal plants because of the wide range of medicinal compounds it produces. *C. roseus* produces more than 130 monoterpenoid indole alkaloids (MIA), including vincristine and vinblastine. These compounds are commonly used in treating cancer (Hemmati et al., 2020). They are produced via the MIA pathway starting with the condensation of the indole moiety tryptamine with the monoterpene-seco-iridoid moiety secologanin (Oudin et al., 2007). This reaction is catalyzed by strictosidine synthase (STR).

Recently, there has been considerable progress in understanding the biosynthesis and the regulation of the MIA pathway. However, several regulatory steps of this pathway are still not well understood (Miettinen et al., 2014; Van Moerkercke et al., 2015; 2016). MIA biosynthesis is induced by the group of jasmonate plant hormones (JAs) (Memelink et al, 2001). JAs regulate plant responses to environmental and developmental cues (Memelink, 2009), such as the induction of secondary metabolites. (+)-7-iso-JA-L-Ile ((+)-7-iso-JA-Ile, JA-Ile), the endogenous active JAs molecule and coronatine (COR), a Pseudomonas syringae-produced phytotoxin that mimics JA-Ile, are able to efficiently trigger JA signaling (Yan et al., 2018). The MYC protein family, which are key regulators in JA signaling, are central TFs of the JA core signaling complex that interact with the Jasmonate ZIM Domain (JAZ) repressors and are maintained in a repressed state in the absence of JAs (Goossens et al., 2016). JA-Ile promotes the interaction between JAZs and the F-box protein Coronatine Insensitive1 (COI1). JAZs are ubiquitinated and degraded by the 26S proteasome, leading to the release of MYC2 and other downstream regulators and the transcriptional activation of target genes. The AP2/ERF transcription factors (TFs), Octadecanoid-Responsive Catharanthus AP2-domain protein 2 (ORCA2) and ORCA3 are major regulators of several MIA pathway genes. CrMYC2 has been shown to act upstream of ORCA2 and ORCA3, and to directly activate their transcription (Zhang et al., 2011). ORCA2 and ORCA3 subsequently induce the expression of several MIA genes including *STR*.

Here we used yeast two-hybrid screening to isolate proteins that interact with ORCA2, resulting in the F-box protein O2.51. This suggests that O2.51 is involved in the degradation of ORCA2. Although O2.51 interacted only with ORCA2 in yeast, it had a negative effect on the trans-activation activity of both ORCA2 and ORCA3. Another aim of this study was to generate stable transgenic cell lines with elevated or reduced expression of *O2.51* to study the effect on MIA biosynthesis.

Result

Yeast two-hybrid screening for ORCA2-interacting proteins

Since the full-length ORCA2 sequence resulted in strong auto-activation in yeast, the truncated version Δ5ORCA2 lacking an N-terminal acidic domain and without auto-activation activity was cloned in the yeast expression vector pAS2-1 creating a fusion protein between the truncated ORCA2 and the GAL4 DNA-binding domain. This construct was used to screen a cDNA library of elicitor-treated *C. roseus* suspension

cells in the yeast expression vector pACTII in yeast strain PJ69-4A. cDNA-encoded proteins are expressed as fusions with the GAL4 activation domain. PJ69-4A contains two nutritional markers (*HIS3* and *ADE2*) and a reporter gene (*MEL1)* driven by the GAL4-controlled *GAL1*, *GAL2* and *MEL1* promoters respectively (James et al., 1996). The use of different promoter-reporter genes eliminates false positives. The number of positive yeast transformants in each step of the screening is listed in Table 1. In the first round of screening, the transformed cells were plated on a medium lacking histidine. Colonies from these plates were then re-streaked on a medium lacking adenine. Growing yeast cells were then patched on plates containing X-α-Gal to detect α-galactosidase activity conferred by the GAL4-controlled *MEL1* gene. Plasmids were extracted from yeast colonies that turned blue on the last medium as a consequence of *MEL1* gene activation and transformed to *E.coli* for plasmid preparation. pACTII plasmids containing different cDNA inserts were then re-transformed to PJ69-4A together with the empty pAS2-1 plasmid, or with pAS2-1 containing Δ5ORCA2. Growth was compared and pACTII clones, which were able to confer growth only in the presence of the bait, were considered true positives (Table 1). Assuming that the mRNA population of *C. roseus* suspension-cultured cells has the standard complexity found in cells of higher eukaryotes, screening of 0.5 to 1.0 x 10^6 independent transformants with a unidirectional cDNA library results in a probability of $\geq$ 99% of screening every mRNA species (Klickstein, 1992). Thus, assuming that the cDNA library forms a faithful representation of the mRNA template, the yeast two-hybrid screening represented a near-complete screening of the mRNA population of elicitor-treated *C. roseus* cells (Table 1). Based on Southern blot cross-hybridization experiments positive clones were grouped in classes (Table 2). Unique clones and the longest clone from each cross-hybridization class were further characterized by sequencing, and sequence comparison with the NCBI DNA database (http://www.ncbi.nlm.nih.gov). A number of clones coding for proteins interacting with Δ5ORCA2 had homology to putative proteins in the database, which had no homology to other described proteins and are referred to as unknown proteins (Table 2).

Table 1. Number of yeast transformants screened using the yeast two-hybrid system and the number of positive clones that were isolated in each step of the screening with the Δ5ORCA2 bait.

Bait	**Δ5ORCA2**
Transformants screened	6.5 x 10^5
Histidine selection	448
Adenine selection	180
X-α-gal screening	159
Retransformation	19

Table 2. Classification of the positive clones found using Δ5ORCA2 as bait in yeast two-hybrid screening. Classes of cross-hybridizing clones are indicated. The column "# clones" represents the number of members of each class. For some classes, homology to proteins in the NCBI database is indicated. "Unknown protein" means that searches in NCBI database revealed homology to predicted proteins with no known function.

Classes	# clones	Homologous to
I	1	tRNA pseudouridine synthase
II	3	Glutathione S-transferase
III	3	Polyphenol Oxidase
IV	1	Unknown protein
V	1	Unknown protein
VI	7	Aspartic Proteinase
VII	1	Unknown Protein
VIII	1	F-box protein
IX	1	Pyruvate Kinase

Clone with homology to F-box proteins

Class O2.VIII was composed of one unique clone, numbered O2.51, which had similarity to a class of plant proteins bearing an N-terminal 60 amino acid F-box, followed by a variable number of Leu-rich repeats. The F-box interacts with the Skp1 component of the SCF (Skp1-Cullin-F-box) complex, whereas the Leu-rich repeats interact with the target protein, thereby targeting it for ubiquitination and degradation by the 26S proteasome. Clone O2.51 encoded only the Leu-rich repeat region. A full-length sequence, termed FL-O2.51, was retrieved from the ORCAE database (bioinformatics.psb.ugent.be/orcae; Van Moerkercke et al., 2013). The best *Arabidopsis* homologues of this *Catharanthus* F-box protein are the F-box proteins EBF1 and EBF2 (Fig. 1A). These two functionally redundant proteins target the transcription factors EIN3 and EIL1, key regulators of ethylene signaling, for degradation (Potuschak et al., 2003; Guo and Ecker, 2003; Gagne et al., 2004). Ethylene inhibits EBF-mediated EIN3 degradation resulting in nuclear accumulation of EIN3 protein which then activates ethylene-responsive genes (Fig. 1B). The ORCA2-interacting F-box protein FL-O2.51 could function in a similar way to regulate ORCA2 protein abundance, possibly in a JA-responsive manner.

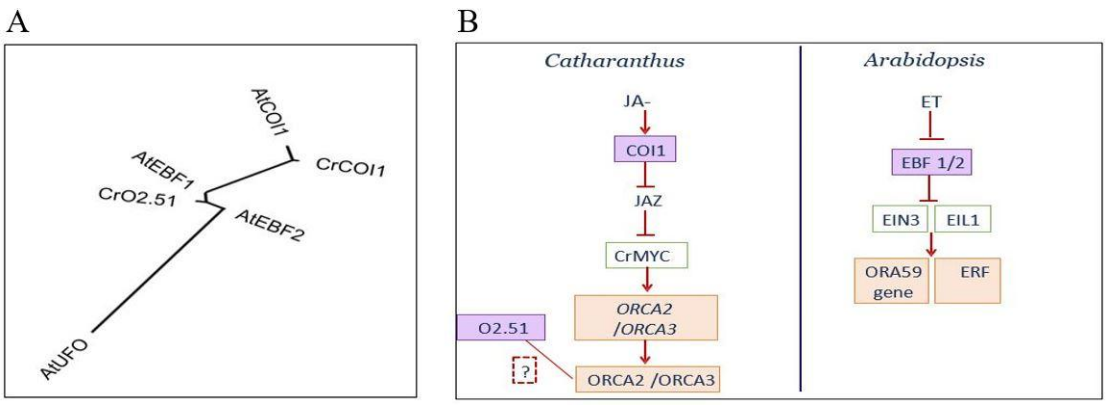

Figure 1. Phylogenetic relationship of related F-box proteins in *C. roseus* and *A. thaliana*. (A) Protein sequences of AtCOI1, CrCOI1, AtEBF1, AtEBF2, CrO2.51, and AtUFO were obtained from TAIR and ORCAE. They were aligned by using the Clustal W program and an unrooted phylogenetic tree was drawn by the neighbour-joining method. (B) Schematic representation of analogies between JA and ET hormonal signaling cascades. ET inhibits the ubiquitination of EIN3 via the $SCF^{EBF1/EBF2}$ complex, resulting in nuclear accumulation of the TFs EIN3 and EIL1. EIN3 and EIL1 activate genes encoding the TFs *ORA59* and several ERFs. JA results in degradation of JAZ repressors via the SCF^{COI1} complex resulting in the release of CrMYC2 from repression. CrMYC2 activates genes encoding TFs such as *ORCA2* and *ORCA3*. Purple boxed abbreviations indicate F-box proteins. Arrows depict activation, T-bars indicate inhibition, dashed line represents that the interaction still needs to be investigated.

FL-O2.51 interacts with Δ5ORCA2

The truncated clone O2.51 was found by interaction with Δ5ORCA2 and did not interact with Δ5ORCA3 (not shown). The interaction of FL-O2.51 with ORCA2 and ORCA3 was tested in yeast. FL-O2.51 was expressed as a fusion with the GAL4 activation domain from the plasmid pACTII. Deletion derivatives of ORCA2 and ORCA3, lacking the acidic activation domains, were expressed as fusions with the GAL4 DNA-binding domain (GAL4-BD) from yeast/*E.coli* shuttle vector pAS2-1. Six combinations of plasmids were tested in the yeast two-hybrid system. The BD and AD domains are brought together when the interaction between the bait protein and the prey protein occurs. This interaction activates the reporter genes and their transcription in yeast was evaluated by monitoring growth on the plates. All growth media lacked tryptophan and leucine (SD-LW), and depending on the selection, additionally lacked adenine (SD-LWA) or histidine and in the latter case also contained 3 amino-1,2,4-triazole (SD-LWH with 5 mM or 10 mM 3-AT). On non-selective growth media used as control, each combination of plasmids resulted in the growth of yeast transformants. FL-O2.51 interacted only with Δ5ORCA2 as evidenced by growth on the SD-LWA selection medium (Fig. 2). Yeast cells did not grow with combinations containing the empty vectors pAS2.1 or pACTII in any selection medium except for the non-selective control.

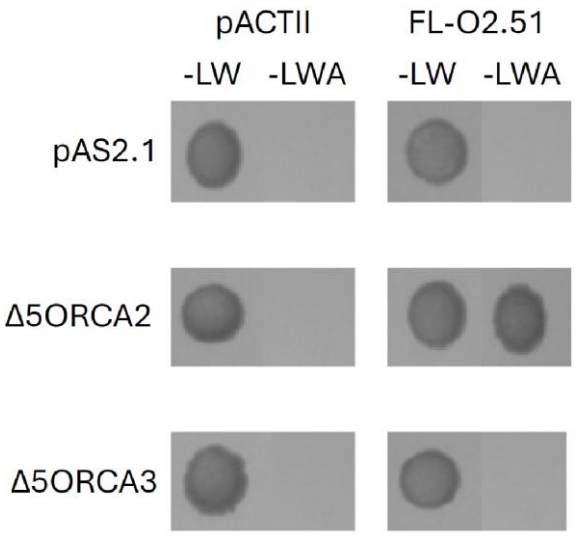

Figure 2. ORCA2 interacts with full-length O2.51. On the left side of each panel, yeast growth on a plate containing Synthetic Minimal Media (SD) lacking Leu, Trp (-LW). On the right side, yeast growth on plates containing SD medium lacking Leu, Trp, and Ade (SD-LWA) media. FL-O2.51 was cloned in pACTII, Δ5ORCA2 and Δ5ORCA3 were cloned in pAS2.1.

Generating *C. roseus* cell suspension lines overexpressing *O2.51*

C. roseus cell suspension lines overexpressing *O2.51* were generated using a construct with the full-length *O2.51* open reading frame fused to the CaMV *35S* promoter in the plasmid pRT101. The empty vector pRT101 was used to generate control cell lines. The *O2.51* overexpression construct and the empty control plasmid were introduced in cell line MP183L by particle bombardment together with a plasmid carrying a hygromycin resistance gene. The experience in the research group is that co-transformation frequency is in the order of 10-30 %. Around one month after the bombardment independent hygromycin-resistant calli were transferred onto new hygromycin selection plates. The calli were grown within around four weeks until a size of around 1-2 cm in diameter upon which they were transferred into liquid medium. After growth of the calli in the liquid medium was established after 2-3 weeks, the weekly transfer of the cell lines was started until stably growing cell lines were obtained (Fig. S1).

This bombardment experiment was relatively unsuccessful because we did not obtain any control suspension culture and only seven lines from the bombardment with *O2.51*. These lines were analysed for *O2.51* gene expression by Northern blot hybridization to select lines that effectively overexpress *O2.51* (Fig. 3). As a control for mRNA loading, the blot was probed with the *RPS9* gene encoding protein 9 of the small subunit of the cytoplasmic ribosome.

Out of the seven independent cell lines, two showed high *O2.51* mRNA levels, one line had a lower but elevated mRNA level, and four lines did not express *O2.51* at a measurable level. Lines 1 and 4 were kept as overexpression lines and lines 3 and 8 as control lines for further experiments.

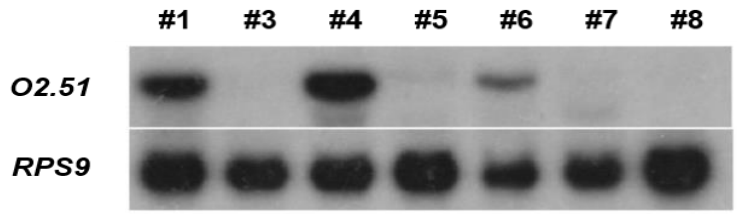

Figure 3. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an *O2.51* overexpression construct. Northern blots containing identical amounts of total RNA from each line were hybridized with the *O2.51* and *RPS9* cDNA probes. *RPS9* encodes the ribosomal protein 9 of the cytoplasmic small ribosomal subunit and was used as a loading control.

Effect of *O2.51* overexpression on expression of MIA biosynthesis genes

Using transgenic lines that constitutively overexpress the *O2.51* gene from the CaMV *35S* promoter, the role of O2.51 in MIA biosynthesis was assessed by measuring the mRNA levels of *STR* and *TDC*, since these two MIA biosynthesis genes are known to be regulated by ORCA2 and ORCA3 (Van der Fits and Memelink, 2000, Peebles et al., 2009; Li et al., 2013).

At 30 min of MeJA treatment no effect was observed on the expression of *TDC* and *STR* (Fig. 4) as reported previously (Van der Fits and Memelink, 2001). At 6 hrs of MeJA treatment increased expression of the *TDC* and *STR* genes was observed, whereas no effect was found on the expression of the control gene *RPS9*. In the two independent *O2.51* overexpression lines, the mRNA levels of *TDC* and *STR* were very similar between the two lines. In the control lines, however, the mRNA levels were quite different. In control line 3, high *TDC* and *STR* mRNA levels were observed without MeJA treatment, whereas in control line 8 without MeJA treatment the *TDC* and *STR* mRNA levels were extremely low. This difference in the expression of control cell lines was unexpected. The interpretation of the effect of *O2.51* overexpression on the expression of MIA biosynthesis genes was hindered by this high level of variability in the control lines.

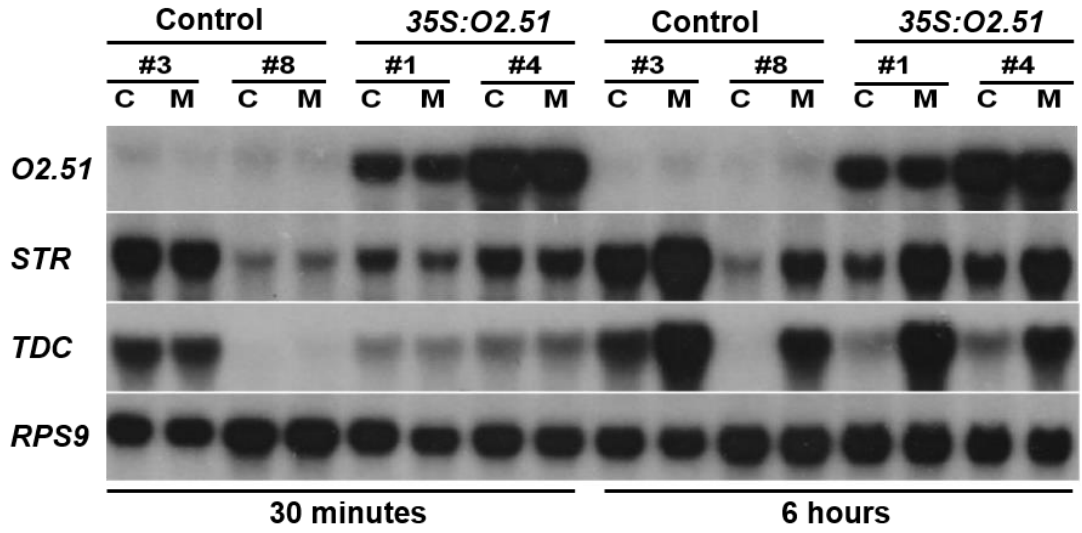

Figure 4. Effect of *O2.51* overexpression on MeJA-responsive expression of MIA biosynthesis genes. Two independent overexpression lines and two independent control lines were treated for 30 minutes and or 6 hours with 0.1% (v/v) DMSO and or with 10 µM MeJA/0.1% DMSO. Northern blots were hybridized with the *O2.51*, *STR*, *TDC* and *RPS9* cDNA probes. *RPS9* encodes the ribosomal protein 9 of the cytoplasmic small ribosomal subunit and was used as a loading control.

O2.51 gene expression is not induced by MeJA

Genes encoding the transcription factors ORCA2 and ORCA3 and MIA biosynthesis genes, including *TDC* and *STR* are induced by MeJA (Van der Fits and Memelink, 2001; Peebles et al., 2009; Li et al., 2013). Whether the gene *O2.51* responds to MeJA can be seen in the previous experiment (Fig. 4) by looking at the results for control lines 3 and 8. A detectable *O2.51* RNA band is observed, but the intensity does not change with longer MeJA treatment which showed that the *O2.51* gene was not induced by MeJA.

A new attempt to study the effect of *O2.51* overexpression on the expression of MIA biosynthesis genes

Given the high variability in the control lines and the low number of overexpression lines in the previous experiment, we made a new attempt to generate more lines.
In total, 92 transgenic cell lines were made from the bombardment with pRT101-*O2.51* and 8 lines were made with the empty vector. These lines were analysed for *O2.51* gene expression by Northern blot hybridization to select lines that effectively overexpress *O2.51*. Lines 3, 20, 32, 43, 64 and, 72 showed different expression patterns compared to controls and many lines did not express *O2.51* at a measurable level (Fig. S2). For confirmation of overexpression, a Reverse Transcriptase (RT) PCR was done. The RT products were then PCR amplified. The forward primer was designed based on the first 20 base pairs of the *O2.51* gene and the reverse primer was designed based on the CaMV *35S* transcriptional terminator of the pRT-101 plasmid. The PCR result showed that all cell lines express *O2.51* in comparison with controls (Fig. S3).

Overexpression cell lines together with 2 control cell lines were treated either with 0.1% (v/v) DMSO or 10 µM MeJA with 3 time points (0, 0.5, 6 h). Northern blots were hybridized with *O2.51*, *ORCA2*, *ORCA3*, *STR*, and *TDC* probes, with *RPS9* as a loading control (Fig. S4). All O2.51 lines except 72 overexpressed smaller RNAs that were too short for the full-length *O2.51* mRNA. Line 72 overexpressed a large RNA that could be the full-length *O2.51*. *ORCA3* gene expression was rapidly and transiently induced by MeJA with high expression level as early as 30 min after MeJA addition, in both overexpression and control cell lines. *ORCA2* was induced at the 6 hr timepoint but showed variable expression between lines. *TDC* and *STR* mRNA levels were relatively consistent with the highest expression at the 6 hr timepoint. However, there were no consistent differences between overexpression and control lines.

Attempt to study the effect of *O2.51* silencing on the expression of MIA biosynthesis genes

To determine whether decreased levels of *O2.51* expression affect the expression of ORCA target genes, we tried to knock down *O2.51* expression via RNA interference (RNAi). The silencing *O2.51* construct was made by selecting a 1558 bp fragment of *O2.51* (positions 463-2021) for insertion as an inverted repeat in the pHANNIBAL plasmid. Independent cell lines were transformed with either the empty pHANNIBAL vector or pHANNIBAL carrying the inverted *O2.51* repeat. Out of 39 generated stable cell lines 8 cell lines (9, 12, 17, 18, 25, 27, 31, 33) had different expression patterns in comparison with controls, so we kept them for further analysis (Fig. S5). Since silencing cannot be confirmed by Northern blot, an RT-PCR was done with *O2.51* cDNA-specific primers. A primer pair was designed based on the part of the *O2.51* gene that was not included in the pHANNIBAL-*O2.51* construct (position 1- 462). Screening of those 8 selected cell lines yielded a single line with lower expression of O2.51, i.e. RNAi-*O2.51* line 27 (Fig. S6).

The silenced cell line together with 2 control cell lines were also treated either with 0.1% (v/v) DMSO or 10 µM MeJA with 3 time points (0, 0.5, 6 h). *ORCA2* showed aberrant expression in all lines since it was not induced by MeJA. *ORCA3* showed aberrant timing of induction in one control line. The expression of *TDC* and *STR* was slightly but notably higher in the silenced line compared to both control lines (Fig. S7). While the replicate lines are missing to confirm this result, this finding is in line with the notion that the reduction of the O2.51 protein amount results in higher amounts of ORCA proteins. In the knockdown cell line there was an accumulation of *STR* and *TDC* after 6 hours which was a bit higher than controls.

O2.51 appears to have a negative effect on ORCA2 and ORCA3 activities in the transient trans-activation assay

The yeast-two hybrid experiment (Fig. 2) showed that FL-O2.51 interacted with Δ5ORCA2. Therefore, the effect of O2.51 on the activity of ORCA2 was tested in a transient transactivation assay. In addition, the possibility that O2.51 might also affect the activity of ORCA3 was investigated, because ORCA3 acts redundantly with

ORCA2 in the MIA pathway (Peebles et al., 2009; Li et al., 2013). To assess whether *O2.51* overexpression affects ORCA activity, *ORCA2* and *ORCA3* were transiently expressed in *C. roseus* cells in the absence or presence of *O2.51* co-expression. ORCA activity was measured by transactivation of the *STR* promoter, which was coupled to the *GUS* reporter gene to facilitate promoter activity measurements. Furthermore, all tested effector proteins were expressed using the CaMV *35S* promoter. Controls contained the empty vectors to equalize plasmid amounts in all co-bombardments. *ORCA2* showed a higher level of activation than *ORCA3*. O2.51 appeared to reduce the activities of ORCA2 and ORCA3. The experiment suggested that O2.51 has a negative effect on the activities of ORCA2 as well as ORCA3, although this effect of O2.51 was not significantly different compared to the control in statistical analysis using the Student *t*-test (Fig. 5). The experiment was repeated four times giving consistently similar results about the tendency of O2.51 to reduce the activities of ORCA2 and ORCA3 (Fig. S8).

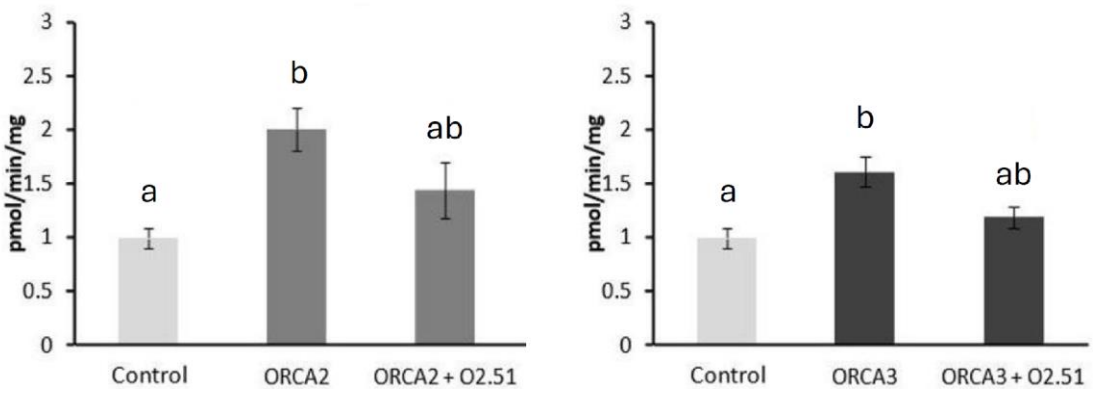

Figure 5. Effect of FL-O2.51 on ORCA2 and ORCA3 activities in transient assays. *C. roseus* MP183L cells were transiently co-transformed with 2 µg *STR-GUS* reporter construct and 4 µg of each effector plasmid. Total effector amount was adjusted to 8 µg in all transformations using pMOG184 vector plasmids (for *ORCA2* and *ORCA3*) and pRT101 vector plasmids (for FL-*O2.51*). Bars represent means +/- SEM (n=3). Letters indicate statistically significant values (P<0.05) according to one-way ANOVA with Tukey post hoc test. GUS activities are shown in pmol MU/min/mg protein.

Discussion

The alkaloids vincristine and vinblastine are derived from the JA-responsive MIA biosynthetic pathway in *C. roseus*. It has been shown that MeJA-responsive expression of alkaloid biosynthesis genes in *C. roseus* is controlled by a transcription factor cascade targeting the AP2/ERF-domain transcription factors ORCA2 and ORCA3, which regulate in turn a subset of alkaloid biosynthesis genes including *TDC* and *STR* (Zhang et al., 2011). These TFs are regulators of the middle part of the MIA pathway (Memelink and Gantet, 2007). Here, we found that the F-box protein O2.51 interacts with ORCA2 in yeast. Sequence analysis revealed that the F-box protein O2.51 shares 91% aa identity with the F-box proteins EBF1 and EBF2 from *A. thaliana*. This supported our hypothesis that O2.51 might facilitate the degradation of ORCA2 and ORCA3 through the 26S proteasome, much like how EBF1/2 target the transcription

factors EIN3/EIL1 for degradation (Hao et al., 2021). When ethylene is present, EBF1/2 become inactive, and EIN3/EIL1 are not subjected to degradation (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). A comparable mechanism could be in play for the interaction between O2.51 and ORCAs.

Full-length O2.51 interacted with Δ5ORCA2 but not with Δ5ORCA3 in yeast. In trans-activation assays, O2.51 had negative effects on the activities of both ORCAs. One option is that O2.51 does not interact with the truncated ORCA3 but does interact with the full-length ORCA3, which may be testable by swapping the vectors in the yeast two-hybrid assay. Another option is that ORCAs might need a post-translational modification. For example, an attractive theory is that phosphorylation by the casein kinases I studied in Chapter 4 increases the affinity for O2.51. This is testable by combining ORCAs, O2.51 and CKIs in the transactivation assay, or by doing a yeast tri-hybrid assay. The co-expression of O2.51 with either ORCA2 or ORCA3 resulted in a decreased activation of the *STR* promoter although this was not statistically significant in a Student *t*-test due to relatively high variation between replicates. This might be solvable by including a reference gene in the assay to correct for transformation and extraction efficiencies thereby reducing variation.

MeJA induces the expression of all known MIA biosynthesis genes and both ORCA2 and ORCA3 (Menke et al., 1999; Van der Fits and Memelink, 2000; Miettinen et al., 2014). MeJA treatment did not induce the expression of the *O2.51* gene. There is still a possibility that the activity of O2.51 is regulated by JAs at the protein level, for example, O2.51 activity could be negatively regulated by JAs, similar to how ethylene has a negative effect on EBF1/2 activity.

We did not succeed in the generation of reliable overexpression lines. Many lines appeared to express truncated versions of the *O2.51* gene. In addition, gene expression between overexpression and control lines was highly variable which made it difficult to draw any firm conclusions.

We were able to generate only a single silencing line. Interestingly the expression of the ORCA target genes *TDC* and *STR* seemed to be higher in line with our working hypothesis. Maybe knocking out *O2.51* with the Crispr-Cas technique is a more promising approach.

Material and methods

Plasmid constructs

For overexpression of the F-box protein O2.51, the *O2.51* open reading frame (ORF) was PCR amplified with the primers 5'-GGA ATT CAA ATG TCT AAA GTC TTT GAT TTC GC-3' and 5'-C CTC GAG GGA TCC TTA GTA AAG GAT ATC ACA CCT CC-3' using a pACTII cDNA library as template, digested with EcoRI/XhoI and cloned initially in pJET1.2 (ThermoScientific). Using pJET1.2 clone - #2 the *O2.51* ORF was excised with EcoRI/BamHI and cloned in pRT101 (Töpfer et al., 1987) and digested with EcoRI/BamHI. The *O2.51* RNAi construct consisted of an inverted repeat of an *O2.51* fragment (463-2021) in pHANNIBAL (Wesley et al., 2001) to produce

double-stranded RNA of the inserted sequence, triggering post-transcriptional silencing. First, a BglII (pos. 463)-XbaI (pos. 2021) fragment was isolated from pRT101-O2.51 and cloned in pHANNIBAL digested with BamHI/XbaI. Next, a BglII-XhoI (463-2021) fragment was isolated from pJET1.2-O2.51#2 and cloned in pIC-19R (Marsh et al., 1984) digested with BamHI-XhoI. The last step was the isolation of the EcoRI-XhoI fragment from pIC-19R-O2.51BX and cloning in pHANNIBAL-O2.51RI. For identification of plasmids containing the inverted repeat, minipreps were checked with EcoRI/XhoI and positive minipreps were additionally digested with XhoI/XbaI. The FL-O2.51 ORF was excised with EcoRI/XhoI from pJET1.2 clone #2 and cloned in the vector pACTII with EcoRI/XhoI and is referred to as plasmid construct pACTII-FL-O2.51. All the plasmid constructs were isolated by transformation into the *Escherichia coli* strain XL1-Blue and were purified according to the QIAGEN plasmid Midikit 100® Purification Protocol.

Cell culture, stable transformation, treatments

Catharanthus roseus cell suspension line MP183L was maintained by weekly 5-fold dilution in 20 ml of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM 1-NAA and 0.23 µM kinetin and was grown at 25˚C in a 16/8 hour light/dark regime at 200 µE m^{-2} S^{-1} at 60% relative humidity on a rotary shaker at 120 rpm. For stable transformation of cell line MP183L,the plasmid construct of interest was co-transformed with the plasmid pGL2 (Bilang et al., 1991) carrying a hygromycin selection gene driven by the CaMV *35S* promoter in a ratio of 4 to 1 by particle bombardment (Van der Fits and Memelink, 1997). Transgenic cells were selected on a solid LS medium containing 50 µg/mL hygromycin-B and individual transgenic calli were converted to cell suspensions. Cells were incubated overnight with either 10µl DMSO (D) or 10 µM Estradiol (E). Cells were then harvested by vacuum filtration, wrapped in aluminum foil, frozen in liquid nitrogen and stored at -80°C.

RNA isolation and Northern blot analysis

Total RNA was extracted from frozen cells by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% (v/v) ethanol and resuspended in water. Ten µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose, 1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization and subsequent washing of blots were performed as described (Memelink et al., 1994) with minor modifications. cDNAs used as probes were: *ORCA2* (GenBank acc. No. AJ238740), *ORCA3* (AJ251250), *STR* (X61932), *TDC* (M25151), *RPS9* (AJ749993).

RT-PCR analysis of *O2.51* overexpression

One µg of total RNA from independent cell lines was reverse transcribed (RT). The RT products were then PCR amplified with 5’-GATCTGCATTCCCAAACCCG-3’ as

forward primer and 5'-CAACACATGAGCGAAACCCTATAAGAACCC-3' as reverse primer. The expected size of the PCR product is 2200 bp (Fig. S3).

RT-PCR analysis of *O2.51* silencing

One µg of total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. The silencing construct for *O2.51* was made by selecting a 1558 bp fragment of *O2.51* (positions 463-2021) for insertion as an inverted repeat in pHANNIBAL. For doing the RT-PCR the part that was not inserted in pHANNIBAL (1-463) was used as a template. 5'-ACCTGGGGGACAAGAGAAGA-3' was used as forward primer and 5'-ACAGGATCAGCCACAACTCC-3' as reverse primer. PCR of the *RPS9* gene was also done. *RPS9* forward primer was TCCACCATGCCAGAGTGCTCATTAGG and reverse primer TCCATCACCACCAGATGCCTTCTTCG (Fig. S6).

Isolation of plasmids and probes

Plasmid DNA for the bombardment was isolated with the QIAGEN plasmid Midikit 100® according to the manufacturer's instructions. Plasmids for analysis during cloning procedures were isolated using a laboratory protocol. The plasmid miniprep protocol was modified from Birnboim and Doly (1979). The probes *ORCA2* and *ORCA3* were amplified with PCR and afterwards isolated with a Gel Purification Kit (Thermo Scientific). PCR was done with a Thermo Scientific Phusion High-Fidelity DNA Polymerase using M13 forward and M13 reverse primers.

Yeast two-hybrid screening

Saccharomyces cerevisiae strain PJ69-4A was grown on yeast extract peptone dextrose (YPD) agar for 3 days at 30°C. This agar was made with 20 g/l Difco peptone, 10 g/l yeast exact, 20 g/l glucose adjusted to pH 5.8 with HCl, with 18 g/l agar for solid medium. Yeast colonies were inoculated in YPD medium and grown overnight in a shaker at 30°C. The yeast transformation was done according to Gietz et al. (2007). Transformants were plated on Synthetic Minimal Media (SD) lacking Leu, Trp (-LT) solid medium and grown at 30°C for 6 days. Spot assays were done by inoculating 3 colonies per plate in 1 mL SD-LT liquid media and growth overnight at 30°C. Afterwards, cultures were 10 times and 100 times diluted in SD medium lacking Leu, Trp, and Ade (SD-LTA) media. Six combinations in 2 dilutions were spotted on an SD-LTA plate and an SD-LT plate as a control. Plates were incubated for 5 days at 29ºC.

Transient expression assay

Particle bombardment was used to transform the constructs into cells of *C. roseus* cell suspension line MP183L as described (Van der Fits and Memelink, 1997). Cells were co-bombarded with 2 µg of an *STR-GUS* reporter construct carrying the *STR* promoter derivative BH (Menke et al., 1999) and 8 µg of effector plasmids. Twenty hours after

bombardment, cells covering half of the Petri dish were harvested and frozen in liquid nitrogen. Co-transformation of the reporter plasmid with the empty overexpression vectors served as controls. The GUS activity in *C. roseus* cell suspension line samples was measured by a fluorometric assay.

Acknowledgements

We thank Guillaume Chatel for construction of pAS2-1-Δ5ORCA2, and initial auto-activation tests. M.D. was partially supported by the Iranian Ministry of Science, Research, and Technology.

References

Bilang R, Iida S, Peterhans A, Potrykus I, Paszkowski J (1991) The 3'-terminal region of the hygromycin-B-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. Gene 100: 247-250

Birnboim HC, Doly G (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513-1523

Gagne JM, Smalle J, Gingerich DJ, Walker JM, Yoo S, Yanagisawa S, Vierstra R (2004) *Arabidopsis* EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proc Natl Acad Sci USA 101: 6803-6808

Goossens J, Fernández-Calvo P, Schweizer F, Goossens A (2016) Jasmonates: signal transduction components and their roles in environmental stress responses. Plant Mol Biol 91: 673-689

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by $SCF^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. Cell 115: 667-677

Hemmati N, Azizi M, Spina R, Dupire F, Arouei H, Saeedi M, Laurain-Mattar D (2020) Accumulation of ajmalicine and vinblastine in cell cultures is enhanced by endophytic fungi of *Catharanthus roseus* cv. Icy Pink. Industrial crops and products 158: 112776

James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144: 1425-1436

Klickstein LB (1992) Production of a complete cDNA library. Current protocols in molecular biology. Greene publishing associates, New York, pp 5.8.1-5.8.8

Li CY, Leopold AL, Sander GW, Shanks JV, Zhao L, Gibson SI (2013) The ORCA2 transcription factor plays a key role in regulation of the terpenoid indole alkaloid pathway. BMC Plant Biol 13:1471-2229

Marsh JL, Erfle M, Wykes EJ (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32: 481-485

Memelink J (2009) Regulation of gene expression by jasmonate hormones. Phytochem 70: 1560-1570

Memelink J, Gantet P (2007) Transcription factors involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. Phytochem Rev 6: 353-362

Memelink J, Swords K, Staehelin A, Hoge J (1994) Southern, Northern and Western blot analysis. Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht pp F1-F23

Menke FLH, Champion A, Kijne JW, Memelink, J (1999) A novel jasmonate and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18: 4455-4463

Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, Van der Krol S, Lugan R, Ilc T, Verpoorte R, Oksman-Caldentey K, Martinoia E, Bouwmeester H, Goossens A, Memelink J, Werck-Reichhart D (2014) The seco-iridoid pathway from *Catharanthus roseus*. Nat Commun 5: 3606

Oudin A, Courtois M, Rideau M, Clastre M (2007) The iridoid pathway in *Catharanthus roseus* alkaloid biosynthesis. Phytochem Rev 6: 259-27

Peebles CAM, Hughes EH, Shanks JV (2009) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. Metab Eng 11: 76-86

Potuschak T, Lechner E, Parmentier Y, Yanagisawa S, Grava S, Koncz C, Genschik P (2003) EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F-box proteins: EBF1 and EBF2. Cell 115: 679-689

Topfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res 15: 5890

Van der Fits L, Memelink J (1997) Comparison of the activities of CaMV *35S* and FMV *34S* promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33: 943-946

Van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295-297

Van der Fits L, Memelink J (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25: 43-53

Van Moerkercke A, Fabris M, Pollier J, Baart GJE, Rombauts S, Hasnain G, Rischer H, Memelink J, Oksman-Caldentey KM, Goossens A (2013) CathaCyc, a metabolic pathway database built from *Catharanthus roseus* RNA-Seq data. Plant Cell Physiol 54: 673-685

Vom Endt D (2004) Regulation of jasmonate-responsive ORCA transcription factors in *Catharanthus roseus*. PhD thesis, Leiden University, The Netherlands

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM

(2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27: 581-590

Yan J, Yao R, Chen Li, Suhua Li S, Gu M, Nan F, Xie D (2018) Dynamic perception of jasmonates by the F-box protein COI1. Mol Plant 11: 1237-1247

Zhang H (2008) Jasmonate-responsive transcriptional regulation in *Catharanthus roseus*. PhD thesis, Leiden University, The Netherlands

Zhang H, Hedhili S, Montiel G, Zhang Y, Chatel G, Pre M, Gantet P, Memelink J (2011) The basic helix-loop-helix transcription factor CrMYC2 controls the jasmonate-responsive expression of the *ORCA* genes that regulate alkaloid biosynthesis in *Catharanthus roseus*. Plant J 67: 61-71

Supplementary information

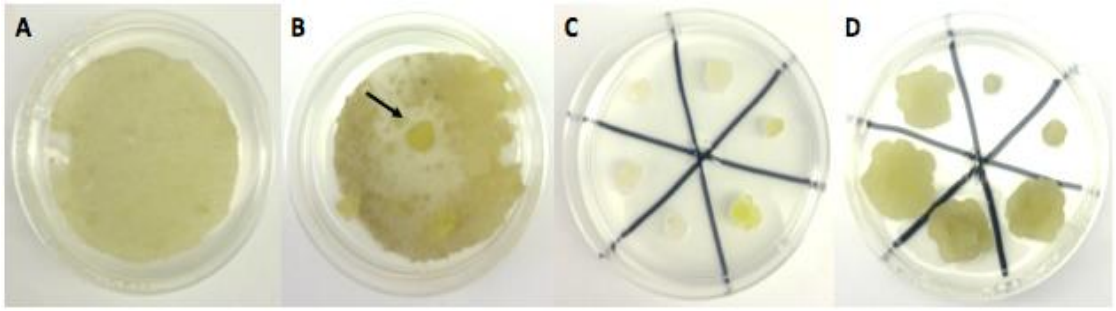

Figure S1. Generating transgenic *C. roseus* cell lines. (Diameter petri dish: 60 x 15 mm) (A) *C. roseus* cells on a filter paper one day after bombardment. (B) After 4 weeks calli had grown, arrow indicates a callus. (C) Grown calli were transferred to new media. (D) After another 4 weeks calli had grown big enough to be transferred to liquid media.

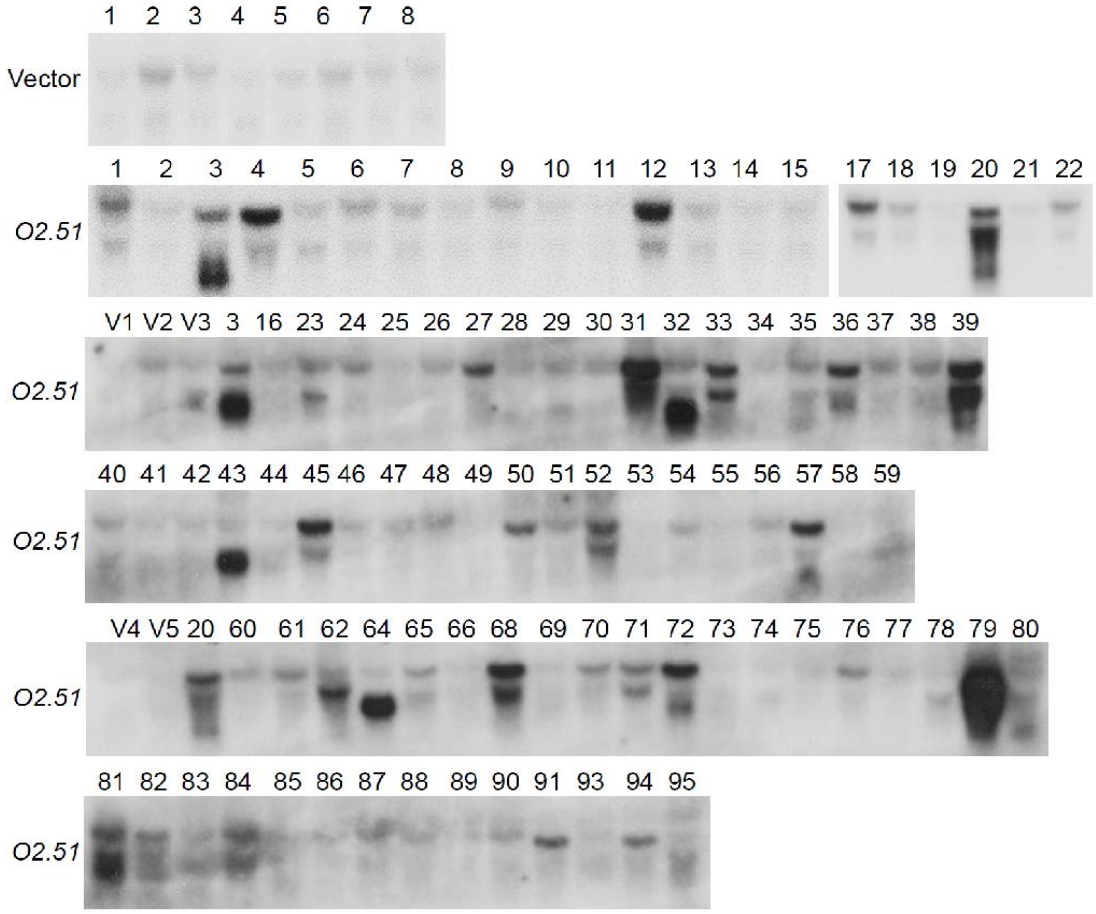

Figure S2. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an *O2.51* overexpression construct. Northern blots containing identical amounts of total RNA from independent control lines or lines transformed with *O2.51* cDNA overexpression construct, were hybridized with the *O2.51* cDNA probes. Vector lines were transformed with empty pRT101.

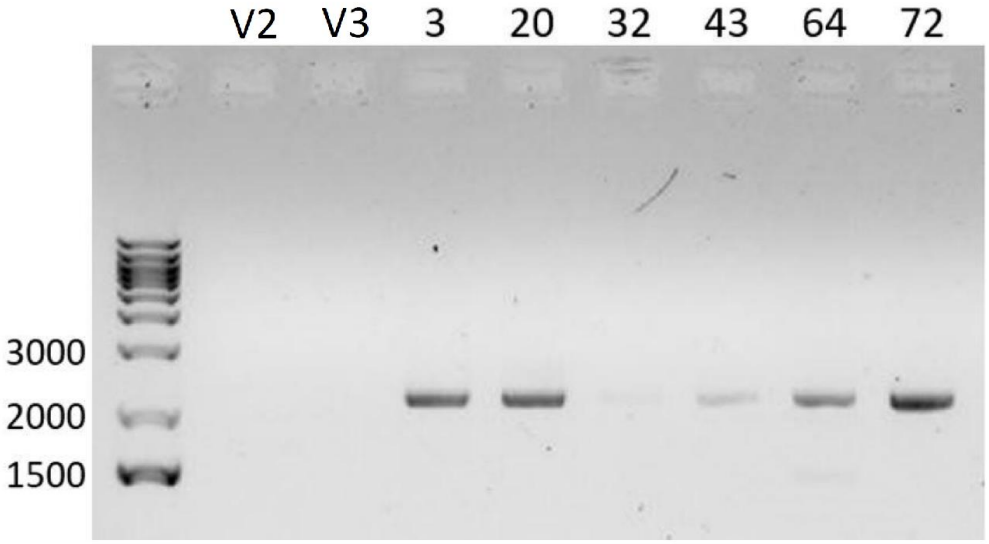

Figure S3. RT-PCR analysis of *O2.51* overexpression. Total RNA from independent cell lines were reverse transcribed (RT). The RT products were then PCR amplified. The expected size of the PCR product is 2200 bp. V indicates empty vector control lines.

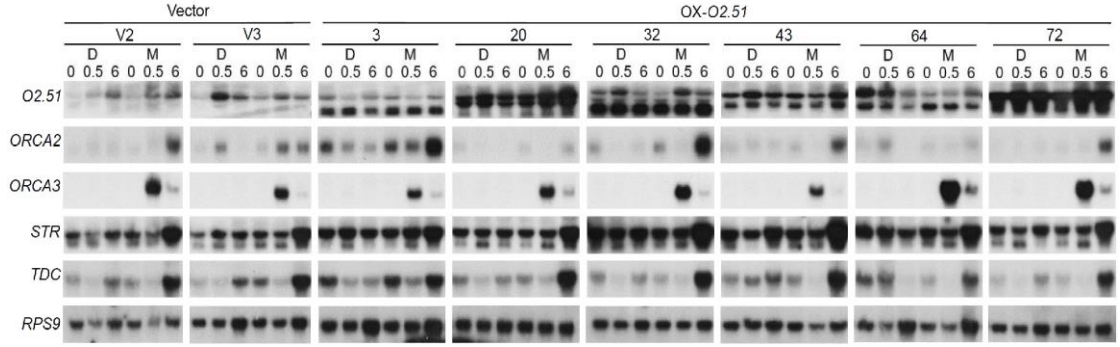

Figure S4. *O2.51* is not induced by MeJA**.** Northern blot showing *O2.51*, *ORCA2*, *ORCA3*, *STR*, *TDC* mRNA levels in independent transgenic control cell lines (V2, V3) and overexpression cell lines (3, 20, 32, 43, 64, 72). Cells were incubated for 0, 0.5, 6 h with either DMSO (D) or 10 µM MeJA (M). The Northern blot was sequentially probed with *O2.51*, *ORCA2*, *ORCA3*, *STR*, *TDC* cDNAs.

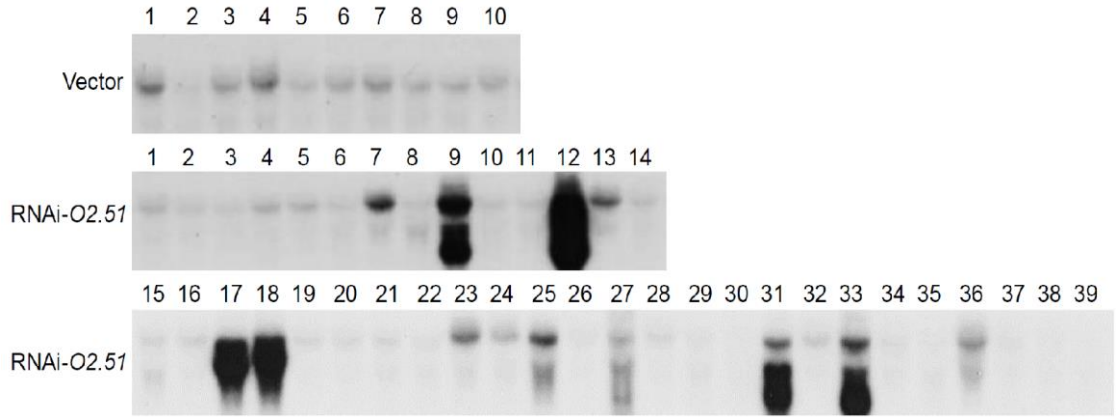

Figure S5. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an *O2.51* silenced construct. Northern blots containing identical amounts of total RNA from each line were hybridized with the *O2.51* cDNA probes. Vector lines were transformed with pHANNIBAL-empty.

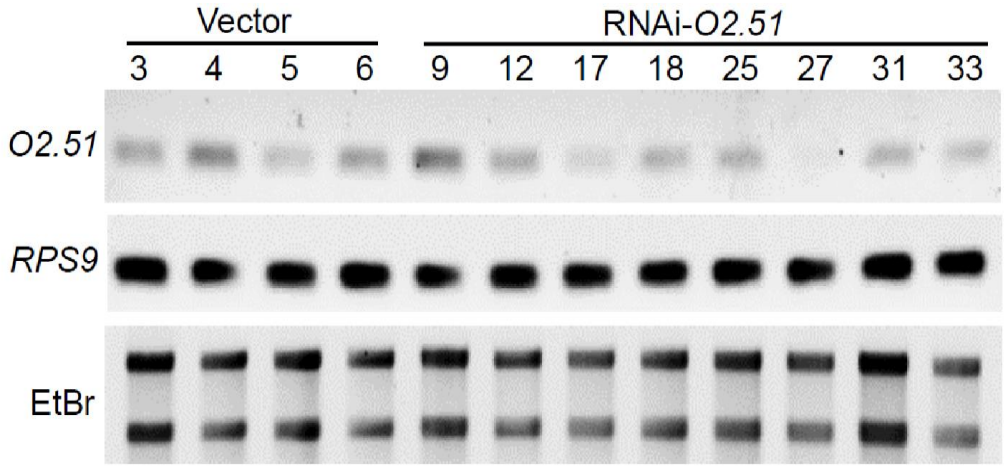

Figure S6. RT-PCR analysis of *O2.51* silencing. Total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. PCR of *RPS9* gene was included as a control. The Ethidium Bromide (EtBr) stained gel of input RNAs is shown as a loading control.

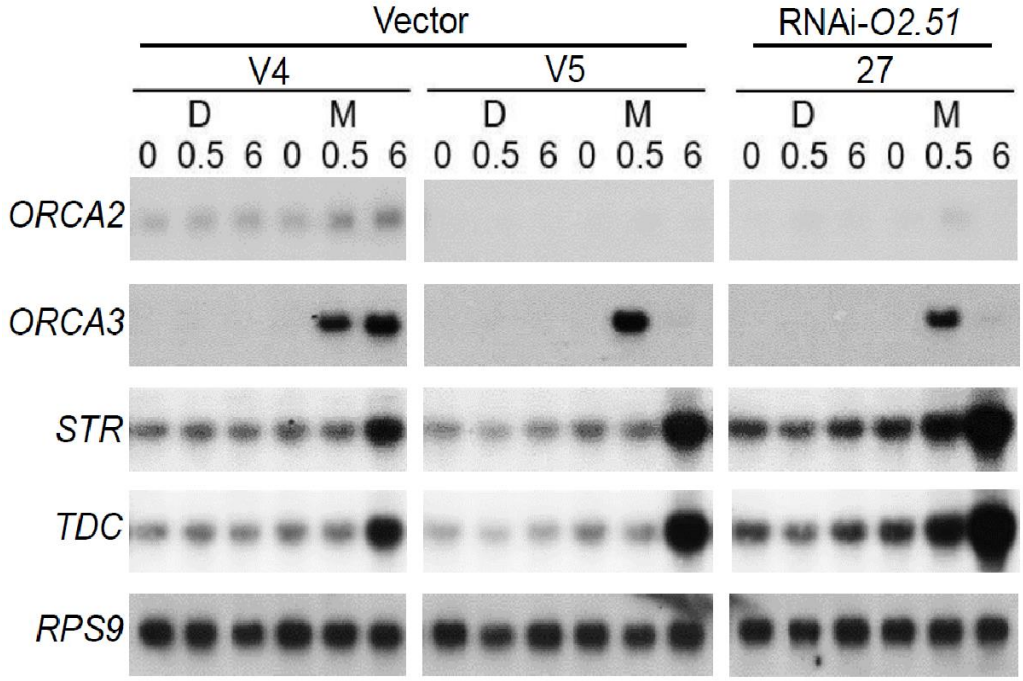

Figure S7. Time course analysis of gene expression in control cell lines V4 and V5, and in RNAi-*O2.51*. Northern blot showing *O2.51*, *ORCA2*, *ORCA3*, *STR*, *TDC* mRNA levels in independent transgenic cell lines. Cells were incubated for 0, 0.5, 6 h with either DMSO (D) or 10 µM MeJA (M). The Northern blot was sequentially probed with *O2.51*, *ORCA2*, *ORCA3*, *STR*, and *TDC* cDNAs.

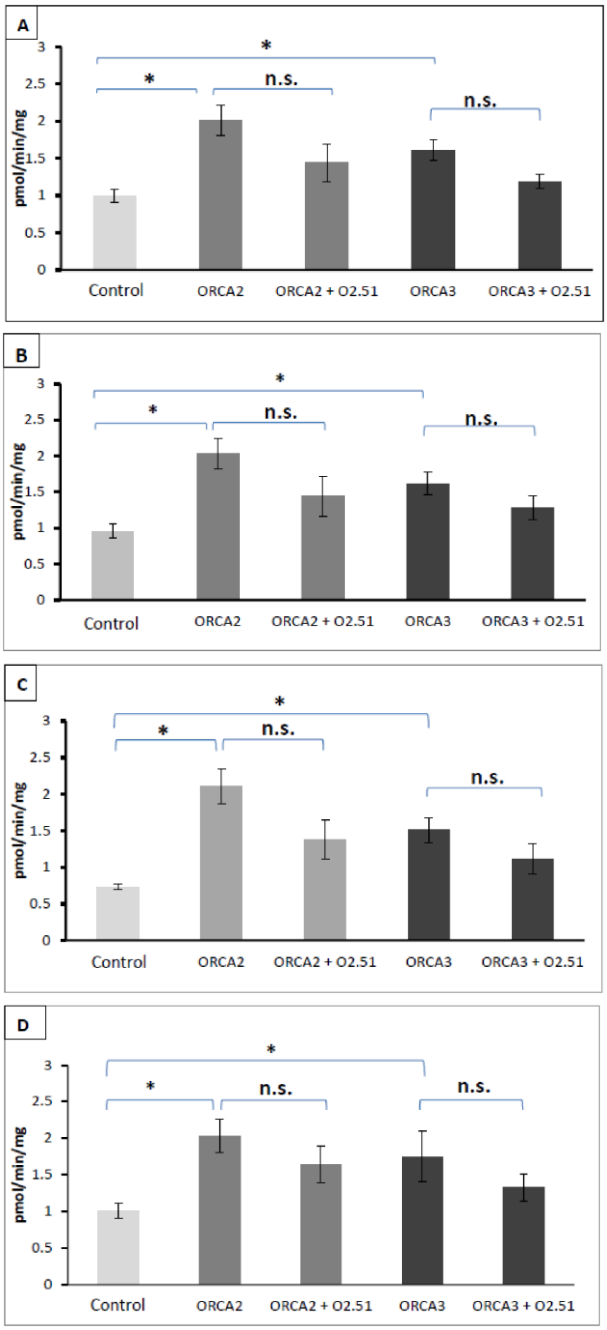

Figure S8. Effect of FL-O2.51 on ORCA2 and ORCA3 activities in transient activation assays. *C. roseus* MP183L cells were transiently co-transformed with 2 µg *STR-GUS* reporter construct and 4 µg of each effector plasmid. Total effector amount was adjusted to 8 µg in all transformations using pMOG184 vector plasmid (for ORCA2 and ORCA3) and pRT101 vector plasmid (for FL-O2.51). Bars represent means +/- SEM (n=3). Asterisks represent Student's *t*-test significance (*, $P<0.05$), while n.s. stands for not significant between pairs indicated with brackets. GUS activities are shown in pmol MU/min/mg protein. Panel A to D represent repetitions of the same experiment.