Transcriptional regulation of monoterpenoid indole alkaloid biosynthesis in Catharanthus roseus

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Chapter 4

Roles of *casein kinase I* in the regulation of monoterpenoid indole alkaloid biosynthesis genes in *Catharanthus roseus*

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Abstract

The medicinal plant *Catharanthus roseus* produces more than 130 monoterpenoid indole alkaloids (MIA) including the well-known antineoplastic compounds vinblastine and vincristine. Jasmonic acid (JA) is a plant signaling molecule that stimulates the expression of MIA biosynthesis genes. The transcription factors Octadecanoid derivative-Responsive *Catharanthus* AP2-domain protein 2 and 3 (ORCA2 and ORCA3) regulate the middle part of the MIA pathway including the *Strictosidine synthase* (*STR*) and *tryptophan decarboxylase* (*TDC*) genes. A yeast two-hybrid screening resulted in the identification of ORCA3 interacting proteins that belong to the casein kinase I (CKI) family. *C. roseus* expresses a small family of four related CKIs. Here, we investigated the role of CKIs, especially CKI-1, in the regulation of ORCA transcription factors. CKIs were able to phosphorylate the ORCAs *in vitro*. CKI-1 had a strong inhibiting effect on the trans-activating activity of the ORCAs *in vivo*. GFP fusion studies showed that all four CKIs were localized in the nucleus. Based on these observations, we hypothesize that ORCA activity is modulated by CKIs.

Keywords: *Catharanthus roseus*, Casein kinase I, ORCA2, ORCA3

Introduction

Plants engage in the synthesis of a diverse array of secondary metabolites as adaptive responses to challenging environmental conditions. These metabolites serve not only as defensive agents but also contribute significantly to the overall growth and development of plants (Raina et al., 2012).

Catharanthus roseus, commonly known as Madagascar periwinkle, is recognized for the production of approximately 200 alkaloids, predominantly monoterpenoid indole alkaloids (MIAs). Notable among these are the anticancer drugs vinblastine and vincristine (Paul et al., 2016; Yang et al., 2023).

Jasmonates (JAs), including Jasmonic acid (JA) and related oxylipins, function as crucial signaling molecules in plant stress responses and diverse developmental processes. Additionally, they play a role in the regulation of ecological interactions (Wasternack and Strnad, 2019; Li et al., 2021). JAs exert their influence on the MIA pathway by activating regulatory transcription factors, where members of the APETALA2/Ethylene Response Factor (AP2/ERF)-domain transcription factor family have emerged as key regulators in JA-responsive gene expression of the middle part of the MIA pathway (Liu et al., 2015; Memelink, 2009). Octadecanoid-Responsive *Catharanthus* AP2-domain protein 2 (ORCA2) (Menke et al., 1999) and ORCA3 (Van der Fits and Memelink, 2000) belong to a small family of related AP2/ERF-domain transcription factors that also includes ORCA4, ORCA5 (Paul et al., 2017) and ORCA6 (Singh et al., 2020). They are believed to be partially functionally redundant but also have specific functions and they regulate the JA-responsive expression of the middle part of the MIA pathway genes, including *tryptophan carboxylase* (*TDC*) and *strictosidine synthase* (*STR*). The *ORCA* genes are transcriptionally regulated by JA via the key regulators CrMYC2 (Zhang et al., 2011) and the JAZ repressors (Patra et al., 2018).

Another important mechanism for controlling transcription factor activity is post-transcriptional regulation (Vom Endt et al., 2002). Protein phosphorylation, catalyzed by protein kinases, is one of the major posttranslational modifications involved in the activity, stability and localization of transcription factors.

Here we report the identification of a small family of four related Casein Kinase I (CKI) proteins in *C. roseus* which can interact with ORCA3 in yeast and can phosphorylate ORCA2 and ORCA3 *in vitro*. CKI-1, the family member studied in more detail, had a strong inhibitory effect on the activity of the ORCAs, suggesting that phosphorylation by CKIs plays a pivotal role in modulating ORCA activity.

Results

Yeast two-hybrid screening for ORCA3-interacting proteins

Since the full-length ORCA3 sequence resulted in strong auto-activation in yeast, the truncated version Δ5ORCA3 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 ORCA3 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 Δ5ORCA3. Growth was compared and 48 pACTII clones, which were able to confer growth only in the presence of the bait, were considered true positives (Table 1). Based on Southern blot cross-hybridization experiments positive clones were grouped in classes (Table 2). The unique clone 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) (Table 2). Two classes of Δ5ORCA3 interacting clones, O3.III and O3.IV, showed homology to casein kinase I (CKI). These two classes did not cross-hybridize, indicating that they code for different members of this family of serine/threonine protein kinases.

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 Δ5ORCA3 bait.

Bait	**Δ5ORCA3**
Transformants screened	6.0×10^5
Histidine selection	313
Adenine selection	173
X-α-gal screening	156
Retransformation	48

Table 2. Classification of the positive clones found using Δ5ORCA3 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. Homologies to proteins in the NCBI database are indicated.

Classes	**# clones**	**Homologous to**
I	25	Centromere/Kinetochore protein ZW10
II	8	Glutathione S-transferase
III	5	Casein Kinase I
IV	9	Casein Kinase I
V	1	Homeodomain-like Protein

Sequence analysis of CKI

The *C. roseus* RNAseq database at ORCAE (bioinformatics.psb.ugent.be/orcae) was searched for sequences with homology to CKIs. It turned out that this database contained four expressed members of the CKI gene family, termed CKI-1 to 4, with about 70% amino acid identity. All nine members of class IV were derived from the same mRNA sequence encoding CKI-1. Class III consisted of the members CKI-2 and CKI-3. CKI-4 was not found in this Y2H screening. Nevertheless, this shows that interaction with ORCA3 is a reproducible and common feature of the CKI protein family. Conserved protein kinase domains are present in the deduced CKI aa sequences, which show strong alignment with a consensus casein kinase domain (KOG1164) from the NCBI conserved domain database (Marchler-Bauer et al., 2003), indicating that the four *C. roseus* CKI proteins are casein kinases.

CKI-1 interacts with the Ser-rich domain of Δ5ORCA3

Both CKI classes III and IV identified in this screening were found to interact with Δ5ORCA3, but not with Δ5ORCA2 (not shown). ORCA2 and ORCA3 share a highly similar AP2/ERF-domain and an acidic domain in the N-terminus. A difference between these proteins is found in the C-terminus, where a serine-rich domain is found in ORCA3 but not in ORCA2 (Fig. 1A). To investigate whether CKI-1 interacts with this part of Δ5ORCA3, a deletion derivative was constructed in which a 24 amino acid region containing 11 serine residues was deleted from the C-terminus (Δ5Δ3ORCA3, Fig. 1A). Interaction between CKI and ORCA derivatives was tested in yeast two-hybrid assays. The longest partial clone from class IV was tested for interaction with Δ5ORCA2, Δ5ORCA3 and Δ5Δ3ORCA3. CKI-1 was able to interact with Δ5ORCA3, but it lost its interacting ability when the serine-rich domain was absent (Δ5Δ3ORCA3, Fig. 1B).

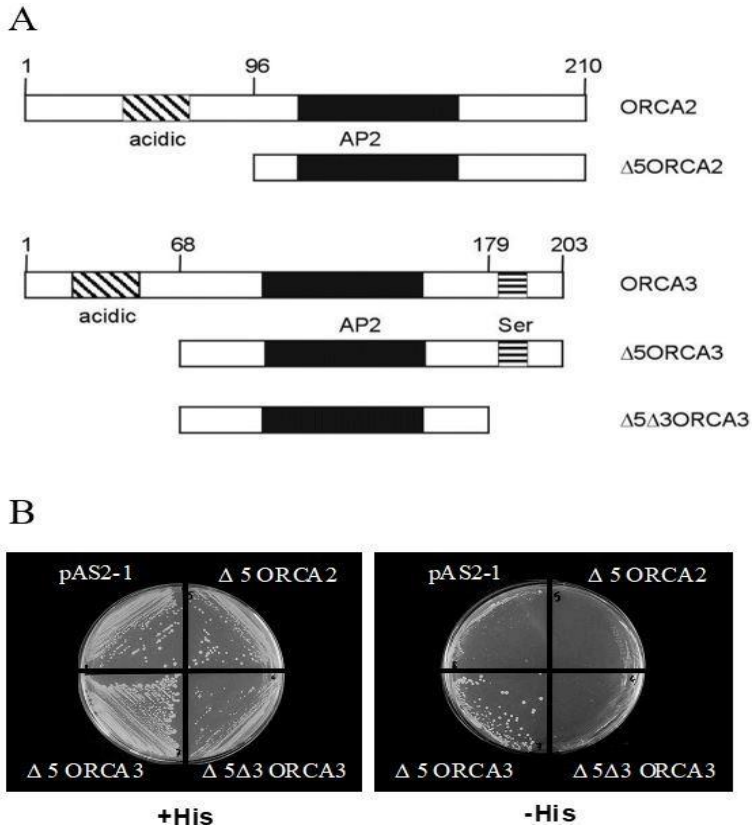

Figure 1. CKI-1 interacts with the Ser-rich domain of Δ5ORCA3. (A) Schematic representation of ORCA2 and ORCA3 and their deletion derivatives. Numbers indicate amino acid positions. AP2/ERF-domains are indicated as black boxes, and acidic domains and the serine-rich domain as hatched boxes. (B) Transformants of yeast strain PJ69-4A containing pACTII-CKI-1 (partial clone IV-113) plus empty pAS2-1 or pAS2-1 containing ORCA derivatives were grown on minimal medium with or without histidine for 5 days.

CKI phosphorylates ORCA2, ORCA3 and Δ3ORCA3

Full-length CKI-1 fused to GST was used to assay phosphorylation of His-tagged ORCA2 and ORCA3 and the Δ3ORCA3 deletion derivative. Protein mixtures of CKI-1 and ORCAs were incubated in the presence of γ-^{32}P ATP and subjected to SDS-PAGE. Gels were stained with coomassie brilliant blue and then dried and autoradiographed. The results showed that CKI-1 phosphorylated ORCA2, ORCA3 and to a lesser extent Δ3ORCA3. This indicates that the Ser-rich domain is a major phosphorylation target in ORCA3. Although ORCA2 lacks such a Ser-rich domain and did not interact with CKIs in Y2H assays, it was phosphorylated to a similar high degree as full-length ORCA3 indicating that it must have other effective phosphorylation sites. The CKI-1 preparation was strongly degraded as shown by the large amount of protein bands in lanes containing the protein. The putative full-length protein migrating at an apparent size of around 110 kDa and the two larger degradation products showed autophosphorylation activity and were probably the only active forms responsible for ORCA phosphorylation. Control incubations with GST did not lead to protein phosphorylation (Fig. 2).

The other CKIs were also tested for their ability to phosphorylate the ORCAs by incubating the full-length proteins fused to GST with full-length His-tagged ORCAs. The results showed that all four CKIs were able to phosphorylate ORCA2 (not shown) and ORCA3 (Fig. 3) to similar

levels. All CKI preparations showed extensive degradation, and only the larger fragments displayed autophosphorylation activity.

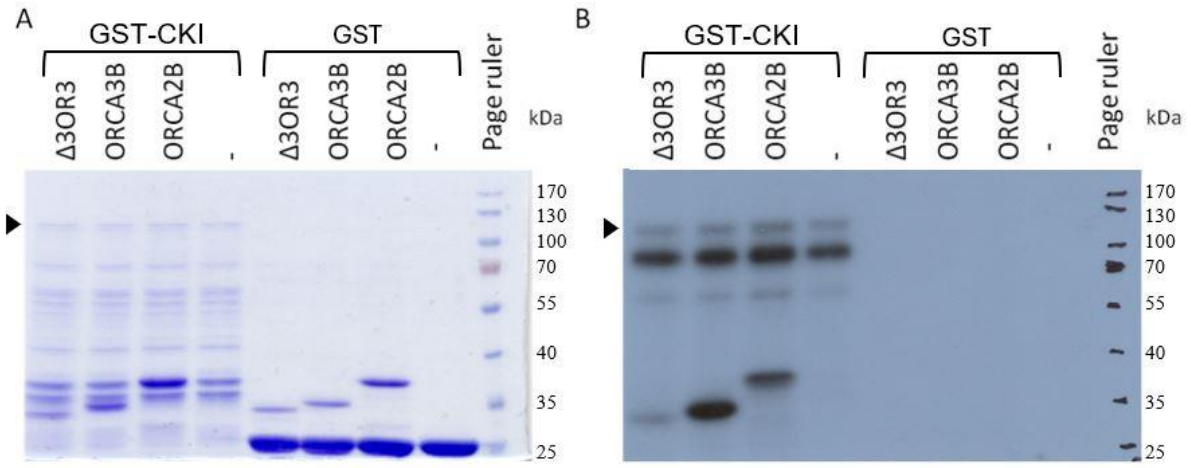

Figure 2. CrCKI phosphorylates ORCA2, ORCA3 and Δ3ORCA3. (A) Amounts of protein equal to 5 µg of ORCA and 10 µg of GST-CKI or GST as control, were incubated in the presence of γ-^{32}P ATP for 40 minutes. Samples were subjected to 10% SDS-PAGE. Staining was done with Coomassie Brilliant Blue. (B) Autoradiography of SDS-PA gel. X-ray film was exposed to the dried gel for 16 hours with a Tungstate intensifying screen at -80 °C. Protein size markers are indicated in kilo Daltons (kDa). The predicted sizes for each protein are: GST=25 kDa, ORCA2=24 kDa, ORCA3=22.3 kDa, Δ3ORCA3=14.7 kDa, GST-CKI=104.1 kDa. The putative CKI full-length protein is indicated with a black triangle.

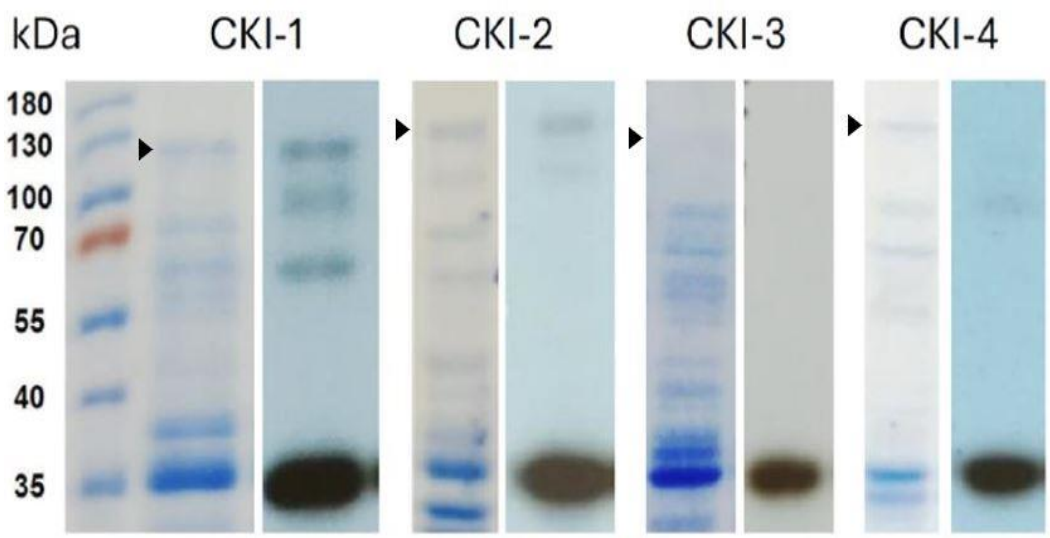

Figure 3. Phosphorylation assays of ORCA3 with GST-CKI-1/2/3/4. Reactions contained 5 µg GST-CKI-2/3/4 or 4 µg GST-CKI-1 and 3 µg ORCA3 and proceeded at room temperature for 40 min. Protein mixtures were then separated by 10% (w/v) SDS-PAGE and gels were CBB stained (left lanes in each CKI panel). Afterwards the PAA gels were dried on Whatman 3MM paper and autoradiographed using X-ray films (right lanes). Protein size markers are indicated in kilo Daltons (kDa). The predicted sizes for each kinase are 105.1 kDa (GST-CKI-1), 109 kDa (GST-CKI-2), 104.6 kDa (GST-CKI-3) and 105.3 kDa (GST-CKI-4). Putative CKI 1-4 full-length proteins are indicated with a black triangle.

Generation and analysis of stable *CKI* overexpression lines

We wanted to generate stably transformed *C. roseus* cell suspension lines overexpressing *CKI-1* and *CKI-3* to study the role of CKIs in MIA biosynthesis. The plasmid pRT101 carrying the CaMV *35S* promoter and either *CKI-1* or *CKI-3* were introduced in cell line MP183L by particle bombardment together with a plasmid carrying a hygromycin resistance gene. To generate control lines, cells were bombarded with the empty pRT101 vector. Hygromycin-resistant cell lines were analysed for *CKI-1* and *CKI-3* gene expression by Northern blot hybridization of extracted RNA to select lines that effectively overexpress *CKI-1* (Fig. S1) or *CKI-3* (Fig. S4). For *CKI-1* lines, successful overexpression lines should express a transcript with a size of 2800 nt. Some lines overexpressed a large RNA that might correspond to the expected size, while several lines expressed also smaller, possibly truncated RNAs (Fig. S1). Seven *CKI-1* cell lines were selected for further analysis. For further checking of overexpression of a full-length transcript, an RT-PCR analysis was done with a forward primer based on the first 20 bp of *CKI-1* and a reverse primer based on the CaMV terminator on the pRT101 plasmid (Fig. S2). Only CKI-1 cell lines 18 and 39 had the expected PCR product size. These cell lines together with two control cell lines were treated either with 0.1% (v/v) DMSO or 10 µM MeJA with 4 time points (0, 0.5, 3, 6 h) and analysed for expression of the *ORCA2* and *ORCA3* genes and the ORCA target genes *TDC* and *STR* (Fig. S3). Based on the hypothesis that ORCA protein phosphorylation by CKIs modulates their activities, the expectation is that *CKI* overexpression does not affect *ORCA* RNA levels, but affects the RNA levels of ORCA target genes. The analysis showed that the *ORCA* genes and their target genes were induced by MeJA as shown numerous times before. The timing of *ORCA2* gene expression was similar to the *TDC* and *STR* genes, whereas the *ORCA3* gene was expressed much earlier. The results from the Northern blot hybridization experiment showed some seemingly random variability, but we concluded that there were no consistent differences between control lines (V) and the selected overexpression lines. The *CKI-1* RNA levels also showed variable levels without a clear trend, but we concluded that the selected lines did not actually overexpress *CKI-1*. In addition, we concluded that the endogenous *CKI-1* gene was not consistently or reproducibly induced by MeJA.

For *CKI-3*, successful overexpression lines should express a transcript of 2300 nt .Several lines overexpressed a large RNA that might correspond to the expected size, while several lines expressed also smaller, possibly truncated RNAs (Fig. S4). Six *CKI-3* cell lines were selected for further analysis. RT-PCR analysis showed that all these *CKI-3* cell lines except line 15 had the expected PCR product size (Fig. S5). Analysis of *ORCA* and target gene expression did not show differences between control cell lines and the selected overexpression lines (Fig. S6). The latter lines overexpressed smaller RNAs as already observed in the initial screening but did not overexpress a larger *CKI-3* RNA of the expected size. Looking at the control cell lines we concluded that *CKI-3* expression is not reproducibly and consistently induced by MeJA.

CKI inhibits ORCA2 and ORCA3 trans-activation activity

To test the effect of CKI-1 on the activity of the ORCAs, co-bombardment experiments were performed on *C. roseus* cells with effector plasmids carrying *ORCA2, ORCA3,* Δ*3ORCA3* (Fig.

4A) and *CKI* fused to the CaMV *35S* promoter and a reporter construct consisting of the *STR* promoter derivative NH fused to the *GUS* reporter gene.

First the effective concentrations of effector plasmids and the effective ratio of CKI:ORCA3 were determined (Fig. 4B). The results show that 1 µg of pRT101-ORCA3 provided a better trans-activation than 3 µg. Co-bombardment with pRT101-CKI-1 resulted in strong inhibition of reporter gene expression even below the basal level, with similar effectiveness of the three different pRT101-CKI-1 amounts. Based on these results, 1 µg amounts of all effectors were used in Figures 4C and 4D. As reported before (Van der Fits and Memelink, 2001), the Δ3ORCA3 deletion derivative had a higher trans-activation activity than full-length ORCA3 (Fig. 4C), indicating that the Ser-rich domain has an inhibitory effect. Co-bombardment with CKI-1 still reduced reporter gene activity, but not below the basal level, indicating that CKI-1 had a lower inhibiting effect on the ORCA3 deletion derivative. Co-bombardment with CKI-1 also inhibited the trans-activation activity of ORCA2, resulting in a reporter gene expression level slightly below the basal level (Fig. 4D).

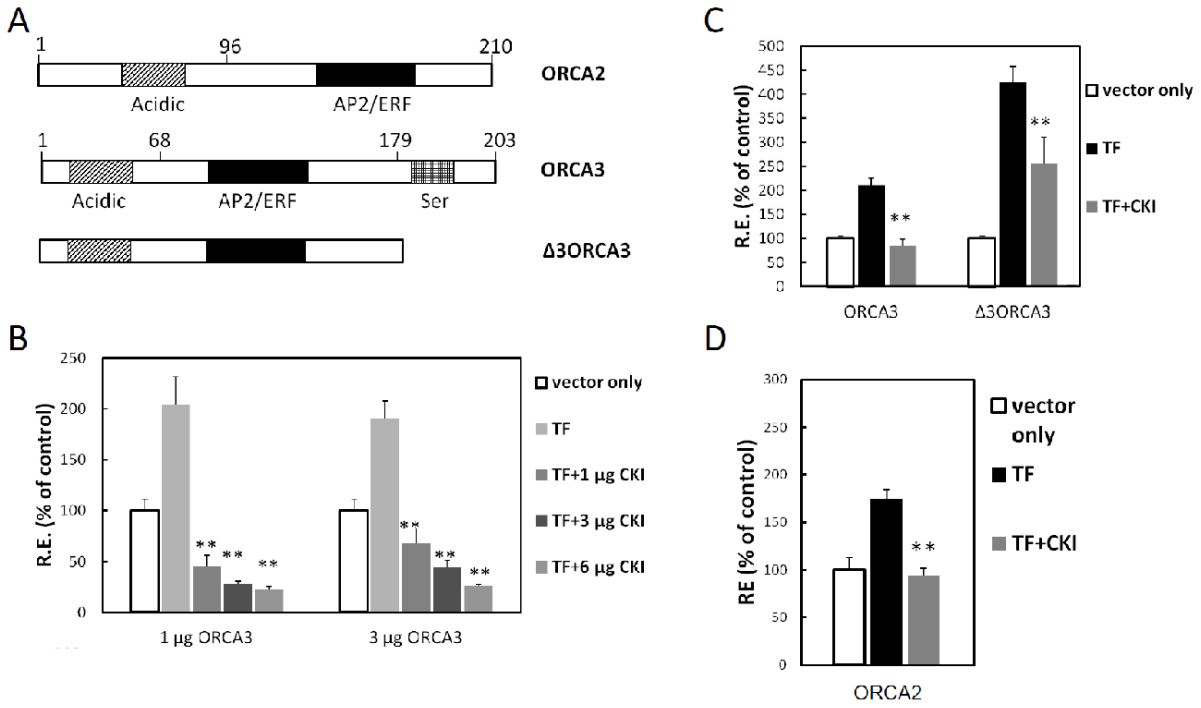

Figure 4. CKI inhibits ORCA2 and ORCA3 trans-activation activities. (A) Schematic representation of ORCA2, ORCA3 and the Δ3ORCA3 derivative. Numbers indicate amino acid positions. The AP2/ERF domain is indicated as a black box, and the acidic domain and the serine-rich domain as hatched boxes. (B) Determination of the effective ORCA3:CKI ratio. *C. roseus* cells were transiently co-transformed with *35S-ORCA*, *35S-CKI-1* and the *STR* NH promoter derivative fused to the *GUS*. Vector only indicates substitution of *ORCA* and *CKI* with the empty *35S*-plasmid pRT101. (C) Effect of CKI on ORCA3 and Δ3ORCA3 activities. *C. roseus* cells were transiently co-transformed with 1 µg pRT101-*ORCA3* or pRT101-Δ*3ORCA3* and 1 µg of pRT101-*CKI-1*. (D) Effect of CKI on ORCA2 activity. *C. roseus* cells were transiently co-transformed with 1 µg pMOG184-*ORCA2* and 1 µg of pRT101-*CKI-1*. Vector only indicates substitution of ORCA2 and CKI with the empty *35S*-plasmid pMOG184. R.E. stands for relative expression with all values normalized to % of control. Bars represent means ± standard error (n=3). ** denotes significance ($P<0.05$ with pairwise comparison using paired *t*-tests).

CKIs are localized in the nucleus of *C. roseus* cells

If CKIs are really modulators of ORCA activity, one would expect them to be localized in the nucleus. To determine the subcellular location of CKI-1/2/3/4 by fluorescence localization, we constructed the plasmids pTH2-CKI-1/2/3/4 and pTH2BN-CKI-1/2/3/4 to express the C- and N-terminal fusions with green fluorescent protein (GFP) CKI-GFP and GFP-CKI, respectively. Through polyethylene glycol (PEG) transformation, we successfully expressed the fusion proteins in *C. roseus* protoplasts. All the fusion proteins were detected in the nucleus while GFP alone was in both the cytoplasm and the nucleus (data not shown). However, compared to protoplasts from *A. thaliana*, the transformation efficiency of *C. roseus* protoplasts was much lower at about 10^{-3} to 10^{-4}, indicating that the method needs further optimization. To further check whether the GFP fusions of CK-I1/2/3/4 are indeed located in the nucleus, we co-transformed the GFP fusion plasmids with a marker plasmid which directs the expression of a red fluorescent protein (DsRED) fusion protein in the nucleus (Jasinski et al., 2002). Due to the low efficiency of transformation and for unknown other reasons, we only obtained *C. roseus* protoplasts co-transformed with pTH2-CKI1/2/3/4 and the marker plasmid. The results of confocal microscopy showed that the GFP fluorescence from CKI-GFP fusions was exactly overlapping the DsRED fluorescence from NtKIS1a-DsRED (Fig. 5), which confirmed that the location of CKI-GFP fusions was in the nucleus. Together, the fluorescence results showed that GFP fusions of CKI-1/2/3/4 were situated in the nucleus of *C. roseus* cells.

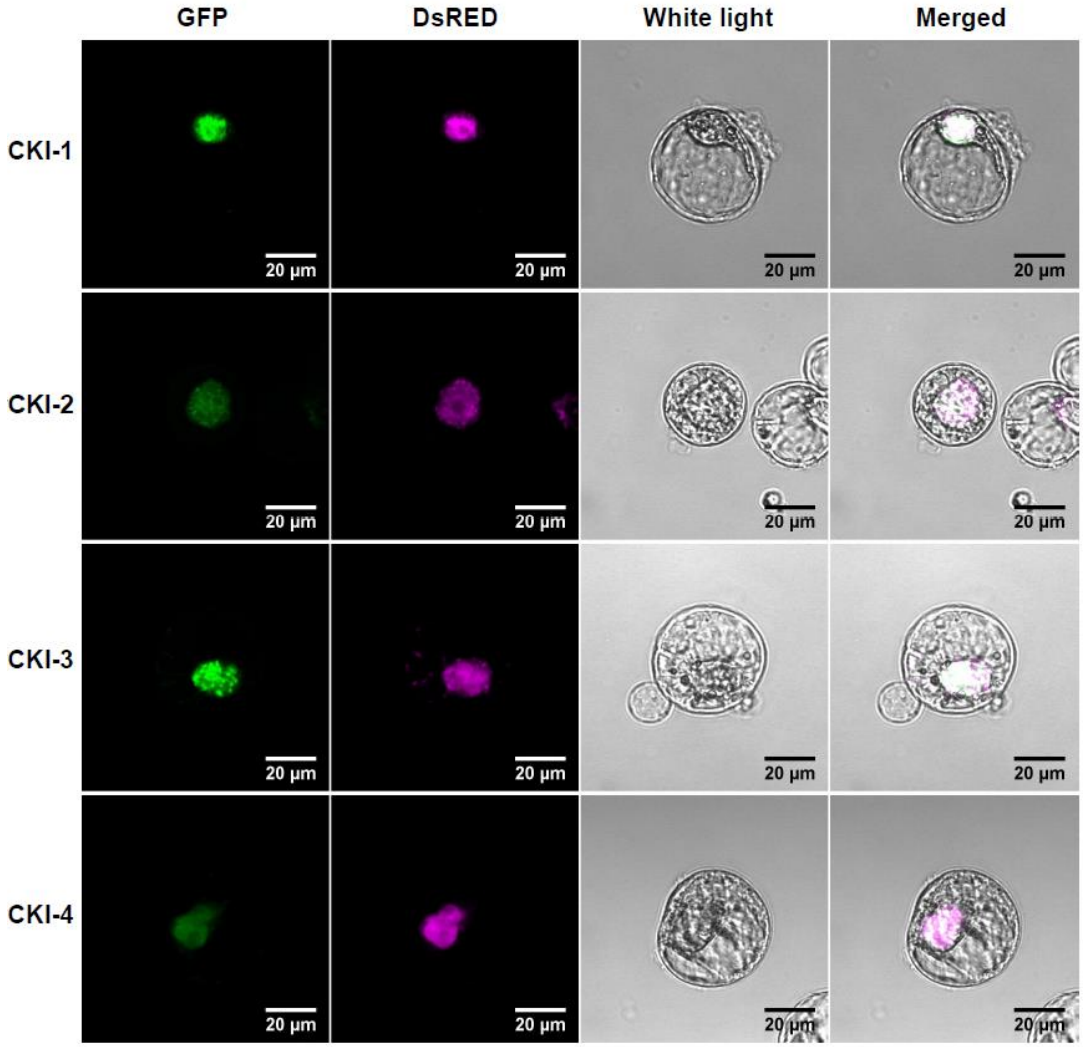

Figure 5. Fluorescence localization of CKI1/2/3/4 in protoplasts of *C. roseus*. Plasmids carrying CKI-GFP fusions were co-transformed with the NtKIS1a-DsRED marker plasmid.

Discussion

Using yeast two-hybrid screening we found a small family of related casein kinase I proteins that can interact with ORCA3. Although they do not interact with ORCA2 in the yeast two-hybrid system, they are able to phosphorylate both ORCA2 and ORCA3 *in vitro*. A Ser-rich domain in ORCA3 is necessary for interaction with CKI-1, and its removal significantly reduces the ability of ORCA3 to become phosphorylated by CKI-1. Trans-activation assays show that CKI-1 strongly inhibits the activity of both ORCA2 and ORCA3 *in vivo*. Presumably, this is due to phosphorylation of the proteins, although this remains to be demonstrated for example by using ORCA mutants that cannot be phosphorylated. However, the observation that deletion of the Ser-rich domain in ORCA3 resulted in increased trans-activation of the *STR* promoter in co-bombardment experiments (Fig. 4C; Van der Fits and Memelink, 2001) combined with the observation that this deletion derivative is less phosphorylated strongly suggest that CKI modulates the repressor activity of the Ser-rich domain in ORCA3 via phosphorylation. However, the removal of the Ser-rich domain resulted in a modest reduction in the phosphorylation level and did not abolish the inhibiting activity of CKI-1. This indicates that

ORCA3 contains other target sites for CKI. ORCA2 lacks a Ser-rich region but nonetheless is strongly inhibited *in vivo* and strongly phosphorylated *in vitro* by CKI-1 indicating that it has other efficient target sites for CKI phosphorylation.
CrCKIs were found to be localized in the nucleus, consistent with a role in transcription factor phosphorylation. The CrCKIs are about 700 aa in size and contain a large (around 280 aa) central kinase domain with the ATP binding site in the N-terminal part of the domain. Just the C-terminal of the kinase domain a monopartite nuclear localization signal (NLS) is located.
CK1 is found in all eukaryotes ranging from yeast to humans and plants, with plants having, as usual, more members. They function as monomeric proteins. In mammals, seven CK1 isoforms were identified (alpha, beta, gamma1-3, delta and epsilon), which are implicated in multiple cellular processes such as circadian rhythm, DNA repair, chromosome segregation, morphogenesis, cell cycle, intracellular trafficking and Wnt signaling.
Due to an ancient lineage duplication event, plants have evolved two subclasses of CKI1, the casein kinase 1-like (CKL) class with higher similarity to CKIs in other organisms, and a unique plant-specific group containing the Mut9p-LIKE KINASES (MLKs) (Kang and Wang, 2020). The CrCKIs belong to this plant-specific MLK subclass. The *A. thaliana* genome encodes 4 MLK members (Kang and Wang, 2020), an identical number to the four expressed CrCKIs found in *C. roseus*. CrCKI has 83% aa identity to for example AtMLK1. AtMLK1 was reported to interact in yeast with the DELLA protein REPRESSOR of *ga1-3* (RGA) and with CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), a MYB-domain TF which is an integral part of the circadian clock (Zheng et al, 2018). *mlk1* mutant plants have short hypocotyls and are hyposensitive to gibberellic acid. Strangely enough, the study does not contain any phosphorylation assays. However, the rice MLK EARLY FLOWERING1 (EL1), was shown to phosphorylate the DELLA protein SLENDER RICE 1 (SLR1) to negatively regulate gibberellin signaling (Dai et al., 2010).
One question is why phosphorylation of ORCAs leads to a reduction in their activity. Phosphorylation could result in exclusion from the nucleus. Phosphorylation could reduce the affinity of ORCAs to bind their DNA target site. This was reported for the basic leucine zipper (bZIP) TF TGA5 in rice upon phosphorylation by CKII, resulting in compromised expression of defense-related genes (Niu et al., 2022). Phosphorylation could also result in degradation. An attractive theory is that phosphorylation of the ORCAs increases their affinity for the F-box protein O2.51 described in Chapter 3 of this thesis, leading to degradation via the ubiquitin-proteasome pathway.

Material and methods

Plasmid constructions

The complete sequences of four *CKI* genes (*CKI1/2/3/4*) were cloned into pRT101 (Töpfer et al., 1987). *CKI1/2/3/4* were PCR amplified using pRT101-CrCKI1/2/3/4 as templates with appropriate primers (Table S1) and then ligated to pJET1.2/blunt cloning vector (Thermo Scientific). *CKI1/2/3/4* fragments were transferred using restriction enzymes (Table S1) to pTH-2 (Chiu et al., 1996; Niwa et al., 1999) and pTH-2BN (a derivative of pTH-2 without the GFP stop codon) vectors. SalI partial digestion was used for excising the fragment of *CKI4*

from pJET1.2. All constructed pJET1.2, pTH-2 and pTH-2BN plasmids were verified by sequencing.
The coding sequences of *ORCA2* and *ORCA3* were cloned in pET-16b (Novagen) for His-tagged protein isolation according to the Novagen protocol.
For isolating GST-tagged CKI1/2/3/4 proteins, full-length sequences of *CKI1/2/3/4* were cloned into pGEX-J2 vector, a derivative of pGEX-KG (Guan and Dixon, 1991) with modified multiple cloning sites.

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, plasmid constructs of interest were 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. For RNA isolation, cells were 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).
Deletion derivatives of ORCA2 and ORCA3 lacking the acidic domain in the pAS2-1 vector were used as baits for the screening. The pACTll library was prepared from *C. roseus* suspension cells treated with yeast extract for one and four hours as described by Menke et al. (1999). Co-transformation of bait and cDNA library at a ratio 1:1 was performed into yeast strain PJ69-4A according to a yeast transformation protocol modified from Gietz et al. (1992). Transformed cells were plated on a medium containing 5 mM 3AT and lacking histidine. Colonies were re-streaked on medium lacking adenine, and patched on medium containing X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside; BD Biosciences – Clontech)

Fluorescence localization

Protoplasts of *C. roseus* cell line MP183L were made using a modified version of a previously described protocol for *A. thaliana* (Schirawski and Planchais, 2000). Linsmaier and Skoog medium (LS medium) was used instead of the B5 medium. For PEG transformation, the

plasmids of pTH2-CKI1/2/3/4 and pTH2BN-CKI1/2/3/4 were co-transformed with a nucleus marker plasmid NtKIS1a-DsRED (Jasinski et al., 2002), using the protocol as previously described (Sheen, 2002). The transformed protoplasts were incubated for 22 hours at 25℃ in the dark.

After incubation, the fluorescence of transformed protoplasts was examined with a confocal microscope Zeiss LSM5 Exciter/Axio Observer. For GFP visualization, the excitation wavelength was 488 nm and the emission wavelength ranged from 505 nm to 530 nm. An excitation wavelength of 543 nm and an emission wavelength ranging from 560 nm to 700 nm was used for viewing the fluorescence of DsRED.

Protein isolation

For inducing the expressions of proteins, a single colony of transgenic *E. coli* was shaken in a liquid LC medium with antibiotics at 37℃ until OD600 reached 0.5. Then 0.15 g solid isopropyl-β-d-thiogalactoside (IPTG) was added and the bacteria were grown for 3 h at 29℃ until OD600 reached around 0.8. The bacteria were collected by centrifugation and resuspended in 20 ml His binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0) for His-tagged proteins or 20 ml PBS binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) for GST-fusion proteins. Resuspended cell samples were transferred to 50 ml SS34 polypropylene tubes and then stored at -80℃. When isolating proteins by column chromatography, the samples were thawed at 37℃ and sonicated on ice until the viscosity was low. The samples were subsequently centrifuged for 20 min at 18,000 rpm in a SS34 rotor at 4℃. The supernatant was filtered with a 0.45 μm membrane (Nalgene, Catalog #190-2545) and ready for loading into the column.

His-tagged ORCA2 and ORCA3 proteins were purified using plastic poly-prep columns (Bio-Rad Catalog #731-1550) containing Ni-NTA Agarose (Qiagen). After loading the samples, the columns were washed with His binding buffer and His wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). His-tagged proteins were eluted by His elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). For isolating GST and the GST-fusion proteins of CKI1/2/3/4, Gluutathione-Sepharose 4B (Amersham Biosciences) was used for preparing the columns and purifying the proteins. GST and GST-fusion proteins loaded into columns were washed with PBS binding buffer and then eluted with glutathione elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0). The isolated proteins were separated by 10% (w/v) SDS-PAGE and checked by CBB staining for quality analysis.

Phosphorylation assay

ORCA2 and ORCA3 (resuspended in His elution buffer) were incubated with GST or GST-CKI proteins (resuspended in glutathione elution buffer) in phosphorylation buffer (1 μCi [γ-^{32}P]-ATP, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5) at 25℃ for 40 min. After incubation, samples were run on 10% (w/v) SDS-PAGE until the front line containing the 15-kDa marker bands of the Pageruler (Thermo Scientific) just ran out of the gel. The gel was then stained with CBB, destained and photographed and then completely dried on Whatman 3MM filter paper at 80℃ using a Model 583 Gel Dryer (Bio-Rad). The dried gels were exposed to the Fuji medical

X-ray film (Fujifilm) in a cassette with Lighting Plus intensifying screens (DuPont Cronex) at -80°C for the appropriate time depending on the radioactivity level.

RT-PCR analysis of *CKI1* overexpression

One µg of total RNA from independent cell lines was reverse transcribed (RT). The RT products were then PCR amplified with 5'-ATGCCAGAGCTTCGTAGTGG-3' as the forward primer and 5'-CAACACATGAGCGAAACCCTATAAGAACCC-3' as the reverse primer. The expected size of the PCR product is 2800 bp (Fig. S2).

RT-PCR analysis of *CKI3* overexpression

One µg of total RNA from independent cell lines was reverse transcribed (RT). The RT products were then PCR amplified with 5'-ATGCCGGAGTTACGAAAGGG-3' as the forward primer and 5'-CAACACATGAGCGAAACCCTATAAGAACCC-3' as the reverse primer. The expected size of the PCR product is 2300 bp (Fig. S5).

Accession numbers

The sequences of *CrCKI1/2/3* can be found in the ORCAE database (Van Moerkercke et al., 2013) as Caros005148.3 (*CrCKI1*), Caros007013.3 (*CrCKI2*) and Caros009329.1 (*CrCKI3*). A partial sequence of *CrCKI4* corresponds to Caros007013.1 in the ORCAE database with the 5'part completed by the sequence of MAGPIE:cro_CRO1L1VD_velvet--Contig1393 from the Phytometasyn transcriptome database (Xiao et al., 2013). The sequences of *ORCA2* and *ORCA3* can be found in GenBank as AJ238740 and AJ251250 respectively.

Acknowledgements

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Supplementary information

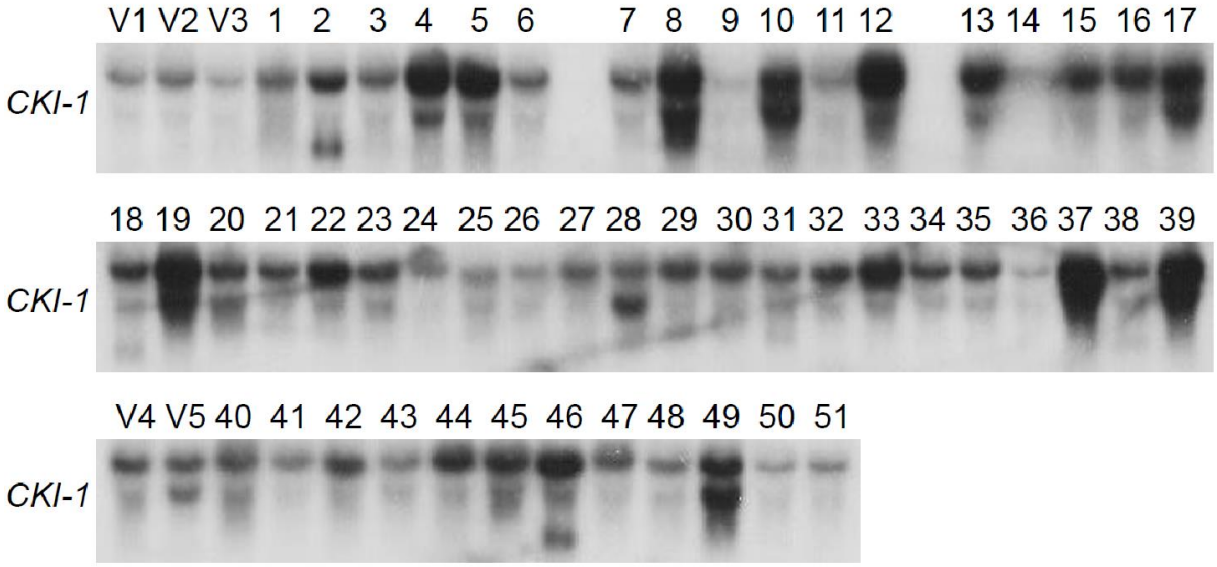

Figure S1. Expression analysis of *C. roseus* cell lines generated following particle bombardment with a pRT101-*CKI-1* construct, where *CKI-1* expression is driven by the *35S* promoter. A Northern blot containing identical amounts of total RNA from each line were hybridized with the *CKI-1* cDNA probe. All lanes were on the same blot.

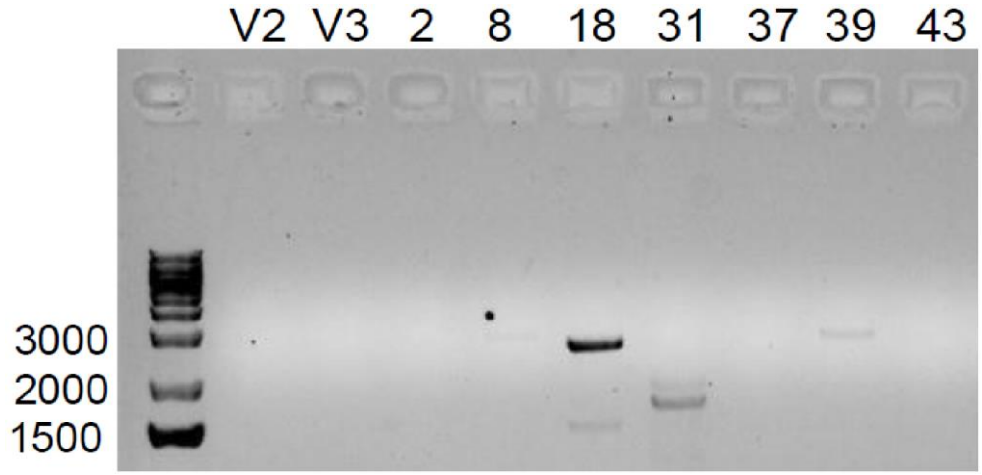

Figure S2. RT-PCR analysis of *CKI-1* overexpression. Total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. The expected size of the PCR product is 2800 bp. V are vector control lines.

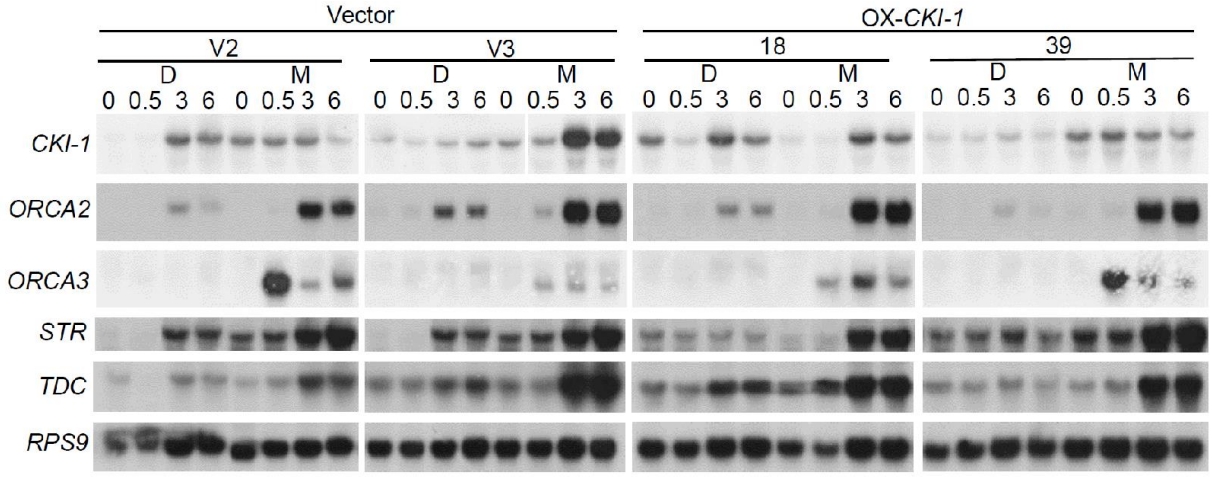

Figure S3. Time course analysis of gene expression in control cell lines V2 and V3, and OX-*CKI-1* lines. Northern blot showing *CKI-1*, *ORCA2*, *ORCA3*, *STR*, *TDC*, *RPS9* mRNA levels in independent transgenic control cell lines (V2, V3) and *CKI-1* overexpression cell lines (18, 39). Cells were incubated for 0, 0.5, 3, 6 h with either DMSO (D) or 10 µM MeJA (M). Replicate Northern blots were probed with *CKI-1*, *ORCA2*, *ORCA3*, *STR*, *TDC*, and *RPS9* cDNAs. All groups of four horizontally arranged panels were on the same blot. The white line in panel V3-*CKI-1* between M0 and M0.5 is due to removal of a leaky lane. All lanes on the group of four horizontally arranged panels were on the same blot.

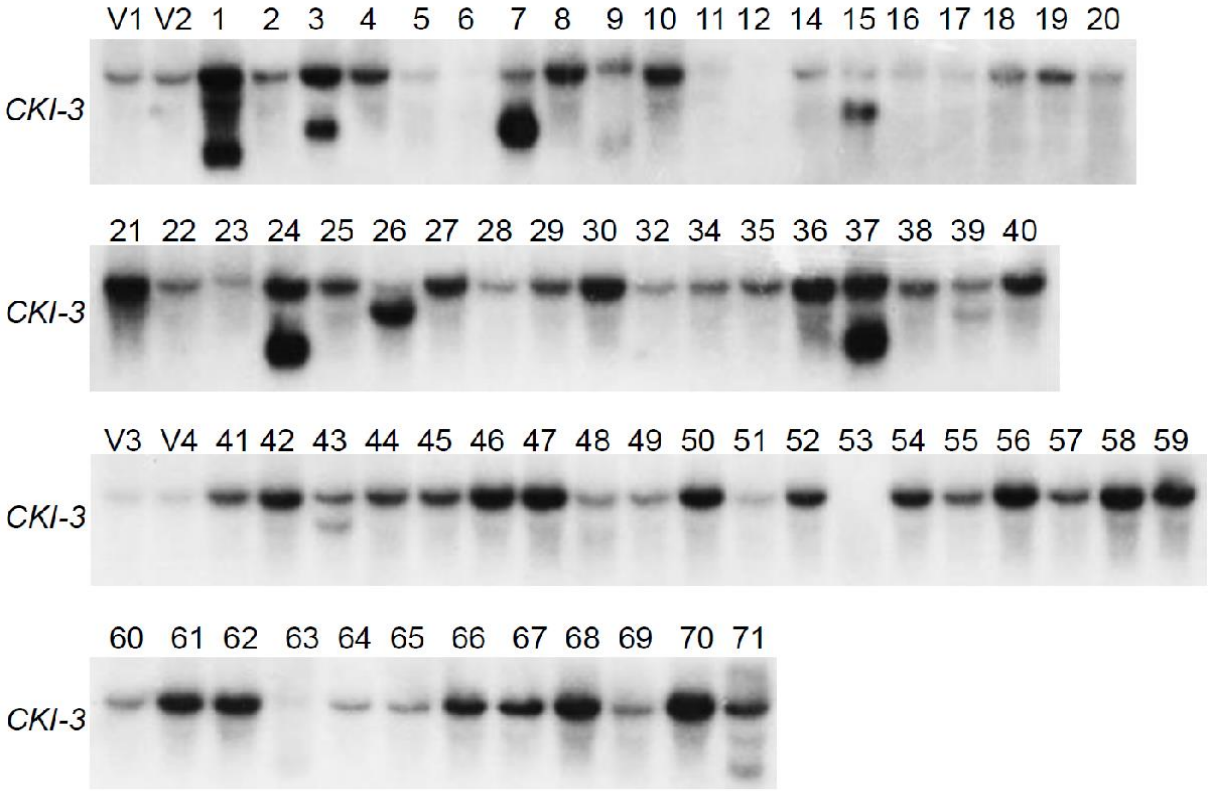

Figure S4. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an pRT101-*CKI-3* construct, where *CKI-3* expression is controlled by the *35S* promoter. Northern blots containing identical amounts of total RNA from each line were hybridized with the *CKI-3* cDNA probe.

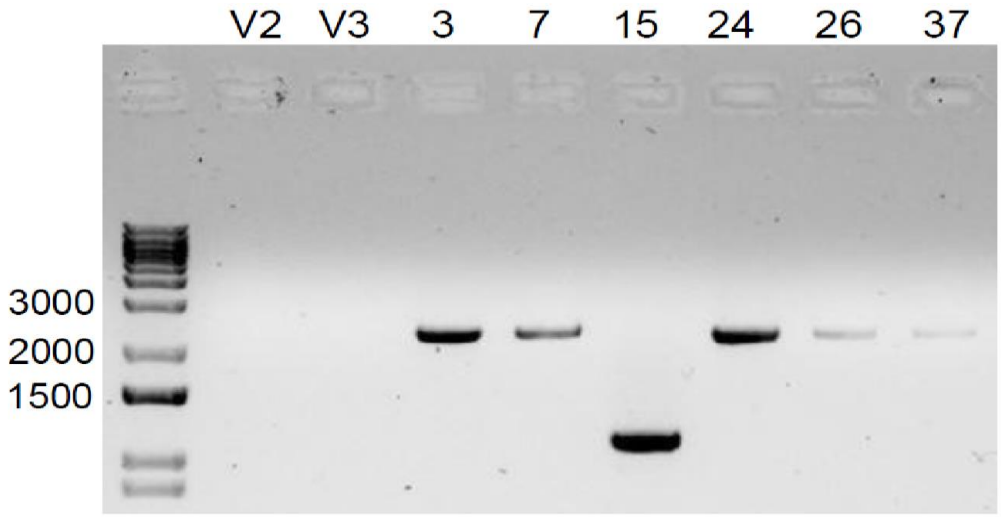

Figure S5. RT-PCR analysis of *CKI-3* overexpression. Total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. The expected size of the full-length PCR product is 2300 bp. V indicates vector control lines.

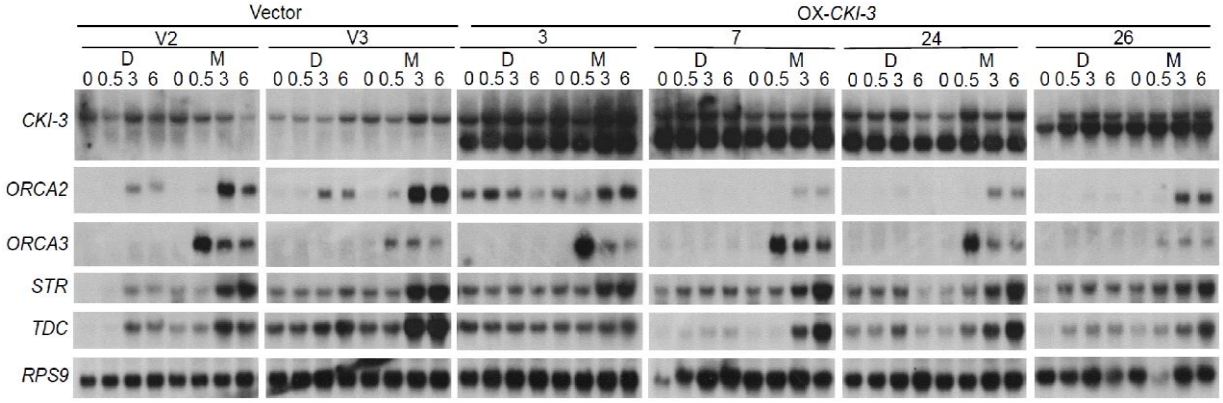

Figure S6. Time course analysis of gene expression in control cell lines V2 and V3, and OX-*CKI-3* lines. Northern blots showing *CKI-3*, *ORCA2*, *ORCA3*, *STR*, *TDC*, *RPS9* mRNA levels in independent transgenic control cell lines (V2, V3) and *CKI-3* overexpression cell lines (3, 7, 24, 26). Cells were incubated for 0, 0.5, 3, 6 h with either DMSO (D) or 10 µM MeJA (M). The Northern blot was sequentially probed with *CKI-3*, *ORCA2*, *ORCA3*, *STR*, *TDC*, *RPS9* cDNAs. All horizontally arranged panels were on the same blot.

Table S1. Templates, primers, vectors, and restriction enzymes used for constructing the plasmids for fluorescence localization.

names	templates	PCR primers and restriction enzymes	Vectors
pTH2-CKI1	pRT101-*CKI1*	5'-GCCTCGAGAAAATGCCAGAGCTTCGTAGTGG-3' 5'-ATAGTCGACGCACACCGTGCGTCCGTAACAG-3' *Xho*I/*Sal*I	pTH-2 *Sal*I
pTH2-CKI2	pRT101-*CKI2*	5'-GCGTCGACAAAATGCCTCAACTGCGTAGCGGAG-3' 5'-GCCCATGGAGGACACAGTCCGACCATAACAAAC-3'	pTH-2 *Sal*I/*Nco*I
pTH2-CKI3	pRT101-*CKI3*	5'-GCCTCGAGAAAATGCCGGAGTTACGAAAGGG-3' 5'-GCGTCGACACTCATAGTCCGTCCATAGCAG-3' *Xho*I/*Sal*I	pTH-2 *Sal*I
pTH2-CKI4	pRT101-*CKI4*	5'-GCGTCGACAAAATGCCGGTGCTGCGTAACGG-3' 5'-GCGTCGACTGACACTGTGCGACCATAAC-3' *Sal*I partial digestion	pTH-2 *Sal*I
pTH2BN-CKI1	pRT101-*CKI1*	5'-GCCTCGAGAAAATGCCAGAGCTTCGTAGTGG-3' 5'-GAACTAGTCAGCACACCGTGCGTCCGTAAC-3'	pTH-2BN *Xho*I/*Spe*I
pTH2BN-CKI2	pRT101-*CKI2*	5'-GCGTCGACAAAATGCCTCAACTGCGTAGCGGAG-3' 5'-GAACTAGTCAGGACACAGTCCGACCATAAC-3' *Sal*I/*Spe*I	pTH-2BN *Xho*I/*Spe*I
pTH2BN-CKI3	pRT101-*CKI3*	5'-GCCTCGAGAAAATGCCGGAGTTACGAAAGGG-3' 5'-GATCTAGATCAACTCATAGTCCGTCCATAG-3' *Xho*I/*Xba*I	pTH-2BN *Xho*I/*Spe*I
pTH2BN-CKI4	pRT101-*CKI4*	5'-GAGAATTCATGCCGGTGCTGCGTAACGG-3' 5'-GAACTAGTCTATGACACTGTGCGACCATAAC-3'	pTH-2BN *Eco*RI/*Spe*I