Short title: Intra-cluster and mutual regulation of AP2/ERFs

Corresponding authors: Ling Yuan and Sitakanta Pattanaik, Department of Plant and Soil Sciences and Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY, USA.

Email: lyuan3@uky.edu and spatt2@uky.edu

Phone: 859-257-4806; 859-257-1976

Fax: 859-323-1077

Title: Mutually Regulated AP2/ERF Gene Clusters Modulate Biosynthesis of Specialized Metabolites in Plants

Mutually regulated APETALA2/ETHYLENE RESPONSE FACTOR clusters modulate specialized metabolite biosynthesis

Author names and affiliations:

Priyanka Paul†, Sanjay Kumar Singh†, Barunava Patra, Xiaoyu Liu, Sitakanta Pattanaik, and Ling Yuan

aDepartment of Plant and Soil Sciences and the Kentucky Tobacco Research and Development Center, University of Kentucky, 1401 University Drive, Lexington, KY 40546 USA

bKey Laboratory of South China Agricultural Plant Molecular Analysis and Genetic Improvement, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China 510650

*College of Life Sciences, Shanxi Agricultural University, Shanxi, China 030801

† These authors contributed equally to this work.

One-sentence summary:

Intra-cluster and mutual regulation of jasmonate-responsive transcription factor gene clusters is evident in the biosynthesis of many plant specialized metabolites.
Author contributions:
L.Y. and S.P. designed the research; P.P., S.K.S., B.P., X.L. and S.P. performed experiments; P.P., S.K.S. and S.P. analyzed data; and P.P., S.K.S., S.P. and L.Y. wrote the paper.

Funding information:
This work is supported partially by the Harold R. Burton Endowed Professorship to L.Y. and by the National Science Foundation under Cooperative Agreement no. 1355438 to L.Y.

ABSTRACT
APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) gene clusters regulate the biosynthesis of diverse specialized metabolites, including steroidal glycoalkaloids in tomato (Solanum lycopersicum) and potato (S. tuberosum), nicotine in tobacco (Nicotiana tabacum), and pharmaceutically valuable terpenoid indole alkaloids (TIAs) in Madagascar periwinkle (Catharanthus roseus). However, the regulatory relationships between individual AP2/ERF genes within the cluster remain unexplored. We uncovered intra-cluster regulation of the C. roseus AP2/ERF regulatory circuit, which consists of ORCA3, ORCA4, and ORCA5. ORCA3 and ORCA5 activate ORCA4 by directly binding to a GC-rich motif in the ORCA4 promoter. ORCA5 regulates its own expression through a positive auto-regulatory loop, and indirectly activates ORCA3. In determining the functional conservation of AP2/ERF clusters in other plant species, we found that GC-rich motifs are present in the promoters of analogous AP2/ERF clusters in tobacco, tomato, and potato. Intra-cluster regulation is evident within the tobacco NICOTINE2 (NIC2) ERF cluster. Moreover, overexpression of ORCA5 in tobacco and of NIC2 ERF189 in C. roseus hairy roots activates nicotine and TIA pathway genes, respectively, suggesting that the AP2/ERFs are functionally equivalent and are likely to be interchangeable. Elucidation of the intra-cluster and mutual regulation of transcription factor gene clusters advances our understanding of the underlying molecular mechanism governing regulatory gene clusters in plants.
Keywords: AP2/ERF gene cluster, intra-cluster and mutual regulation of transcription factor cluster, terpenoid indole alkaloids, nicotine, transcriptional regulation, *Catharanthus roseus* (Madagascar periwinkle)

INTRODUCTION

Plants produce a vast array of bioactive specialized metabolites in response to various biotic and abiotic stresses. Many specialized metabolites with nutritional and medicinal values are beneficial to animals and humans. While significant progress has been made in discovering the genes encoding key enzymes in biosynthesis of specialized metabolites, molecular regulatory mechanisms controlling the metabolic pathways are insufficiently understood. Biosynthesis of specialized metabolites is primarily regulated at the transcriptional level (Colinas and Goossens, 2018). The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family transcription factors (TFs) have emerged as key regulators of specialized metabolite biosynthesis, including nicotine in tobacco (*Nicotiana tabacum*) (Shoji et al., 2010; De Boer et al., 2011), terpenoid indole alkaloids (TIAs) in Madagascar periwinkle (*Catharanthus roseus*) (van der Fits and Memelink, 2000; Paul et al., 2017) and *Ophiiorrhiza pumila* (Udomsom et al., 2016), artemisinin in *Artemisia annua* (Yu et al., 2012; Lu et al., 2013), and steroidal glycoalkaloids (SGA) in tomato (*Solanum lycopersicum*) and potato (*S. tuberosum*) (Cardenas et al., 2016; Thagun et al., 2016; Nakayasu et al., 2018). AP2/ERFs are subdivided into 12 phylogenetic groups (Nakano et al., 2006). Several group IX AP2/ERFs form physically linked gene clusters that regulate biosynthesis of specialized metabolites. TF gene clusters have been characterized in a limited number of plant species, including tobacco (Shoji et al., 2010; Kajikawa et al., 2017), tomato (Cardenas et al., 2016; Thagun et al., 2016; Nakayasu et al., 2018), potato (Cardenas et al., 2016), and *C. roseus* (Paul et al., 2017). The tobacco *NICOTINE2* (*NIC2*) locus comprises at least 10 AP2/ERFs that are homologous to the *C. roseus* ORCAs. Not all NIC2 ERFs are equally effective in regulating nicotine biosynthesis; *ERF189* and *ERF221/ORC1* play major roles in nicotine biosynthesis (Shoji et al., 2010; De Boer et al., 2011). The AP2/ERF-gene cluster in tomato and potato comprise five and eight ERFs, respectively. GLYCOALKALOID METABOLISM 9 (GAME9)/JASMONATE-RESPONSIVE ERF4 (JRE4), a member of the AP2/ERF gene clusters in tomato and potato, is key to biosynthesis of SGAs and the upstream
isoprenoids. Knockdown, knockout, or overexpression of the GAME9 genes in tomato and potato affect the SGA pathway gene expression and SGA production (Cardenas et al., 2016; Thagun et al., 2016; Nakayasu et al., 2018). In C. roseus, the ORCA cluster consists of at least three AP2/ERFs, ORCA3, ORCA4, and ORCA5, and of which ORCA3 and ORCA4 are known to regulate the biosynthesis of the pharmaceutically valuable TIAs (Figure 1A) (van der Fits and Memelink, 2000; Paul et al., 2017).

In addition to the group IX AP2/ERFs, TF gene clusters have been identified in the group III AP2/ERFs, C-repeat Binding Factors (CBFs) (Gilmour et al., 1998; Zhang et al., 2004), Auxin Response Factors (ARFs) (Hagen and Guilfoyle, 2002), R2R3 MYBs (Zhang et al., 2000; Zhang et al., 2019) and basic helix-loop-helix (bHLH) factors (Sanchez-Perez et al., 2019). TF gene clusters are likely originated from tandem gene duplication events (Shoji et al., 2010; Kellner et al., 2015). Unlike the operon-like, non-homologous metabolic gene clusters (Boycheva et al., 2014; Nützmann and Osbourn, 2014; Nützmann et al., 2016), TF gene clusters encode homologous TFs with overlapping or unique functions. It has been suggested that gene duplication offers the opportunity for mutual regulation among the duplicated genes (Shoji et al., 2010); however, mutual regulatory relationships among the members of any TF cluster remained unconfirmed. Furthermore, the ORCA, NIC2, and GAME9/JRE locus ERFs are phylogenetically related and commonly respond to the phytohormone, jasmonic acid (JA), suggesting the evolution of similar regulatory mechanism in diverse metabolic pathways (Shoji et al., 2010; Thagun et al., 2016). Question thus arose as to whether AP2/ERFs from different clusters are functionally equivalent and interchangeable. Elucidation of the mutual regulatory relationship among the ERF gene clusters implies an evolutionarily conserved molecular mechanism that controls the biosynthesis of functionally and structurally diverse specialized metabolites.

In this study, we discovered a regulatory relationship among the members of the ORCA cluster. The direct activation of ORCA4 by ORCA3 and ORCA5, as well as self-regulation of ORCA5, highlight the presence of feed-forward and auto-regulatory loops in the ORCA cluster. We also demonstrated the intra-cluster regulation among the tobacco NIC2 ERFs. Moreover, ORCA5 overexpression in tobacco hairy roots upregulated nicotine biosynthetic genes and nicotine accumulation, and reciprocal overexpression of NIC2 ERF189 in C. roseus hairy roots induced
the TIA biosynthetic genes, suggesting that the ORCAs and NIC2 ERFs are functionally equivalent and are likely interchangeable.

RESULTS

Phylogenetic analysis positions ORCAs, GAME9, and NIC2 ERFs in the same clade

AP2/ERFs are divided into 12 groups based on domain structure and other conserved motifs. The group IX AP2/ERFs are involved in phytohormone signaling and defense response (Nakano et al., 2006). Phylogenetic analysis of group IX ERFs from tomato, tobacco, potato and C. roseus showed that ORCAs are grouped together with NIC2 and GAME9 ERFs, which are involved in nicotine and SGA biosynthesis in tobacco and tomato, respectively (Shoji et al., 2010; Cardenas et al., 2016) (Supplemental Figure S1). Interestingly, this clade does not include ERFs from Arabidopsis thaliana, suggesting that the ERFs in this clade are possibly evolved for the biosynthesis of structurally complex specialized metabolites.

ORCA gene cluster is differentially induced by MeJA and ethylene

MeJA is a key elicitor of biosynthesis of a number of specialized metabolites, including nicotine (Shoji et al., 2000), beta-thujaplicin (Zhao et al., 2004), artemisinin (Shen et al., 2016), taxol (Mirjalili and Linden, 1996) and SGAs (Thagun et al., 2016; Nakayasu et al., 2018). Ethylene (ET) acts synergistically with MeJA to promote biosynthesis of taxol in Taxus cuspidate (Mirjalili and Linden, 1996), beta-thujaplicin in Cupressus lusitanica (Zhao et al., 2004), and hydroxycinnamic acid amides (HCAAs) in Arabidopsis thaliana (Li et al., 2018), while ET attenuates the effects of MeJA on nicotine and SGA biosynthetic pathway gene expression in Nicotiana species (N. tabacum and N. attenuata) and tomato, respectively (Shoji et al., 2000; Winz and Baldwin, 2001; Shoji et al., 2010; Nakayasu et al., 2018). In C. roseus, MeJA induces expression of ORCA3, ORCA4, and ORCA5 as well as their targets (van der Fits and Memelink, 2000; Paul et al., 2017). To determine the effects of ET alone or in combination with MeJA on ORCA gene expression, C. roseus seedlings were treated with MeJA, ACC, or both for 2h, and transcript accumulation were measured by reverse-transcription quantitative PCR (RT-qPCR). Expression of ORCA5 was induced 9.5-fold by MeJA but remained unaffected by ACC; however, MeJA-induced expression of ORCA5 was attenuated in the presence of ACC, reduced to 7.5-fold. Expression of ORCA4 and ORCA3 was induced 7 and 12 fold, respectively, by MeJA.
and reduced to 0.2-0.3 fold by ACC (Figure 1B). Similar to ORCA5, MeJA-responsive expression of ORCA4 and ORCA3 was reduced to 2.5 and 7-fold, respectively, in the presence of ACC. Expression of STR and TDC, two key targets of ORCAs, and CrMYC2a, were induced 4, 12 and 5.7 fold, respectively, by MeJA treatment. The MeJA-induced expression was reduced to 2-4 fold in the presence of ACC (Fig 1B). In addition, we measured the TIA contents in seedlings treated with MeJA and ACC either alone or in combination. MeJA induced, whereas ACC repressed the accumulation of tabersonine and ajmalicine. Moreover, MeJA-induced accumulation of tabersonine, but not that of ajmalicine, was attenuated by ACC. Accumulation of catharanthine was reduced in MeJA or ACC-treated seedlings (Figure 1C).

**ORCA5 is a nucleus-localized transcriptional activator**

To determine the transactivation activity, ORCA3, ORCA4, or ORCA5, fused to the GAL4-DNA-binding domain (GAL4-DBD), were co-electroporated into tobacco protoplasts with a luciferase reporter driven by a minimal CaMV 35S promoter with GAL4-responsive elements as described previously (Paul et al., 2017). Transactivation activities of ORCA3, ORCA4, and ORCA5 were 6.5, 6.6, and 12-fold, respectively, higher than the reporter-only control (Supplemental Figure S2A). The significant inductions of reporter activity in plant cells suggest that three ORCAs are transcriptional activators. To determine the sub-cellular localization, ORCA5 coding sequence was fused in-frame to the enhanced green fluorescent protein (eGFP) and expressed in tobacco protoplasts. Compared to the protoplasts expressing the eGFP-control, in which GFP was detected throughout the cell, the ORCA5-eGFP fusion protein was localized to the nucleus (Supplemental Figure S2B), consistent with its putative function as a TF.

**ORCA4 and ORCA5 bind to the JRE in the STR promoter**

We have shown that ORCA4 and ORCA5 activate the promoters of key TIA pathway genes, including STR, TDC, and CPR in tobacco cells (Paul et al., 2017). A previous study has shown that ORCA3 binds to the JRE in the STR promoter (Van Der Fits and Memelink, 2001). To determine whether ORCA4 and ORCA5 also bind the same JRE in the STR promoter, we performed electrophoretic mobility shift assay (EMSA). We purified the recombinant, glutathione S-transferase (GST)-tagged ORCA3, ORCA4, ORCA5, (GST-ORCA3/4/5) and CrMYC2a (GST-CrMYC2a) proteins from E. coli using GST affinity chromatography as
described previously (Paul et al., 2017; Patra et al., 2018) (Figure 2A; Supplemental Figure 3A). CrMYC2a, which binds T/G-box motif, was used as a negative control. The purified ORCA3, ORCA4, ORCA5 or CrMYC2a protein was incubated with a 5’ biotin-labeled probe covering the JRE of the STR promoter. Similar to ORCA3, ORCA4 and ORCA5 also bind to the JRE, resulting in a mobility shift (Figure 2B). The binding of GST-tagged ORCA5 to the JRE of the STR promoter was further confirmed by competition using 10X, and 100X excess of the unlabeled (cold) probe. The intensity of the signal decreased gradually with the increase of the concentration of cold probe (Supplemental Figure 3B). As shown in Figure 2B, the unlabeled probe, 1000-fold in excess, out-competed the labeled probe and abolished the signal, suggesting the shifted-band was indeed the ORCA5-JRE complex. We thus used 1000X excess of the cold probe for the competition experiments with ORCA3 and ORCA4, and, similar to ORCA5, the cold probe completely abolished the signals on the gel, indicating the shifted-band was indeed the ORCA3/ORCA4-JRE complex (Figure 2B). We did not detect any signal for CrMYC2a, suggesting that CrMYC2a does not bind to JRE in the STR promoter (Supplemental Figure 3B).

**ORCA TFs differentially activate TIA pathway genes**

The ORCAs are known to regulate a number of genes of the indole pathway and downstream branches (van der Fits and Memelink, 2000; Paul et al., 2017). In this study, we investigated their roles in regulation of additional genes in the TIA pathway. Biosynthesis of secologanin in *C. roseus* (Figure 1A) requires nine enzymes, seven of which involved in the conversion of geranyl diphosphate (GPP) to loganic acid are regulated by BIS1 (Van Moerkercke et al., 2015) and BIS2 (Van Moerkercke et al., 2016). Loganic acid is converted to secologanin by loganic acid methyltransferase (*LAMT*) and secologanin synthase (*SLS*). We used protoplast-based transactivation assay to determine whether *LAMT* and *SLS* are regulated ORCAs. The *LAMT* (1376 bp) or *SLS* (980 bp) promoter, fused to a firefly-luciferase reporter gene, was co-electroporated into tobacco protoplasts with or without the constructs expressing ORCA3, ORCA4, or ORCA5 (Figure 2C). ORCA3, ORCA4, and ORCA5 significantly activated the *LAMT* promoter compared to the control. ORCA5, but not ORCA3 and ORCA4, significantly activated the *SLS* promoter (approximately 2.5-fold) compared to the control (Figure 2C).

**Derepressed CrMYC2a and ORCA5 have synergistic effects on TIA pathway genes**
A recent study has shown that mutation of a conserved aspartic acid to asparagine (D126N) in the JAZ-interaction domain (JID) of CrMYC2a prevents CrMYC2a from interacting with CrJAZ3 and CrJAZ8, thus derepressing CrMYC2a from the inactive complex with the JAZ proteins. In addition, coexpression of the derepressed CrMYC2a (CrMYC2a\textsuperscript{D126N}) with ORCA3 has synergistic effect on expression of several TIA pathway genes (Schweizer et al., 2018). To determine whether the derepressed CrMYC2a acts synergistically with ORCA5, we generated the CrMYC2a\textsuperscript{D126N} mutant by site-directed mutagenesis and evaluated its effect on four key TIA pathway gene promoters, TDC, STR, LAMT and SLS, which are regulated by ORCA5. As shown in Figure 3, CrMYC2a had no additive effect on the STR promoter activity when co-expressed with ORCA5. The TDC, LAMT and SLS promoter activities were slightly higher when CrMYC2a was co-expressed with ORCA5. However, coexpression of CrMYC2a\textsuperscript{D126N} with ORCA5 had synergistic effect on activation of all four promoters (Figure 3).

**ORCA5 overexpression activates TIA pathway genes and boosts TIA accumulation in C. roseus hairy roots**

To further elucidate the regulatory role of ORCA5 in TIA biosynthesis, we generated transgenic C. roseus hairy roots overexpressing ORCA5 (ORCA5-OE). The transgenic status of hairy roots was confirmed by PCR (Supplemental Figure 4A). Two empty vector (EV) control and two overexpression lines (OE-1 and OE-2) were selected for further analysis. Compared to EV control, expression of ORCA5 was 24-40 fold higher in the transgenic lines (Supplemental Figure 4B). Expression of a number of TIA pathway genes, including AS\textalpha, TDC, CPR, G10H, IS, SLS, STR, and SGD, were significantly higher in the ORCA5-overexpression lines compared with the EV control. In addition, expression of the genes encoding C2H2 zinc finger repressors, ZCT1, ZCT2, and ZCT3 were also increased. Interestingly, expression of ORCA3 and ORCA4 were increased significantly in ORCA5-overexpressing hairy roots, suggesting that ORCA5 possibly regulates other members in the ORCA cluster (Figure 4A).

Previous studies have shown that overexpression of ORCA3 in C. roseus hairy roots does not result in increased TIA accumulation (Peebles et al., 2009; Wang et al., 2010; Zhou et al., 2010). In this study, overexpression of ORCA5 significantly increased the transcripts levels of genes in both indole (i.e. AS and TDC) and iridoid branches (i.e. CPR, G10H, IS, and SLS) of the TIA pathway. In addition, expression of the downstream pathway genes, STR and SGD, were also
significantly increased. To determine the metabolic outcomes of ORCA5-overexpression, we measured the alkaloids in the two independent hairy root lines. Accumulation of tabersonine, ajmalicine, and catharanthine increased significantly in ORCA5-OE lines compared to the EV lines (Figure 4B).

In C. roseus, tabersonine, ajmalicine, and catharanthine are detected in roots and aerial parts, while vindoline is only accumulated in aerial parts (van der Heijden et al., 2004). Recent studies have shown that four separate hydrolases (HL1 to HL4) are involved in the conversion of the unstable intermediate derived from O-acetylstemmadenine to tabersonine by HL1, to catharanthine by HL2, and to vincadifformine by HL3/4 (Qu et al., 2018; Qu et al., 2019) (Figure 1A). In roots, the tabersonine is converted to hörhammericine catalyzed by tabersonine 19-hydroxylase (T19H) (Giddings et al., 2011) and minovincinine 19-O-acetyltransferase (MAT) (Laflamme et al., 2001). Recently, a BADH acetyltransferase, tabersonine derivative 19-O-acetyltransferase (TAT), has been characterized in C. roseus. TAT is highly expressed in roots, and has been shown to acetylate 19-hydroxytabersonine derivatives from C. roseus roots at a higher efficiency than MAT (Carqueijeiro et al., 2018). In addition, two conserved cytochrome P450s, tabersonine 6,7-epoxidase isoforms 1 and 2 (TEX1 and TEX2), have been identified in C. roseus. TEX1 is preferentially expressed in roots whereas TEX2 transcripts are present in stem, leaf, and flower (Carqueijeiro et al., 2018). TEX1/2 catalyze the stereo-selective epoxidation of tabersonine to lochnericine which is then converted to hörhammericine by T19H and subsequently acetylated by TAT to form 19-O-acetylhörhammericine. In a parallel branch, a root-specific cytochrome P450, vincadifformine 19-hydroxylase (V19H) catalyzes the conversion of vincadifformine to minovincinine, which is then O-acetylated by MAT to form echitovenine (Williams et al., 2019). We found that similar to other TIA pathway genes, expression of HL2, HL4, T19H, TAT, MAT, and TEX2 was induced by 1.5 to 18 fold in MeJA-treated C. roseus seedlings (Figure 5A). However, we did not observe significant change in the expression of HL1, HL3, V19H and TEX1 in response to MeJA treatment. Next, we measured the expression of these genes in EV and ORCA5-OE hairy root lines and found that expression of MAT and T19H was induced by 20-500 fold ORCA5-OE compared to EV (Figure 4A). Expression of HL3, V19H, TEX1, TEX2, and TAT was also induced by 2-11 fold in the ORCA5-OE lines (Figure 5B), suggesting that these genes are likely regulated by ORCAs. In the ORCA5-OE lines, expression of HL1 and HL4 was slightly repressed whereas HL2 expression did not
change significantly. This is similar to a recent study where transient overexpression of \textit{ORCA3} and/or \textit{MYC2a} in \textit{Catharanthus} flower petal had no effect on \textit{HL1} and \textit{HL2} expression, indicating that additional factors are involved in regulation of TIA pathway (Schweizer et al., 2018).

\textbf{ORCA5 activates the \textit{ZCT3} promoter}

\textit{ZCTs} are negative regulators of TIA pathway (Pauw et al., 2004). In both \textit{ORCA4} (Paul et al., 2017) and \textit{ORCA5} overexpressing hairy root lines (Figure 4A), expression of \textit{ZCTs} were significantly increased. We analyzed the \textit{cis}-elements in the \textit{ZCT} promoters, and found that the \textit{ZCT3} promoter contains putative AP2/ERF binding sites (GC-rich motif). The findings suggest that \textit{ORCA5} regulates \textit{ZCT3} possibly by binding to its promoter, while indirectly regulating \textit{ZCT1} and \textit{ZCT2}. We thus tested the activation of \textit{ZCT3} by \textit{ORCAS}. The \textit{ZCT3} promoter (961 bp) was fused to a firefly-\textit{luciferase} reporter gene and co-electroporated into tobacco protoplasts with or without the constructs expressing \textit{ORCA3}, \textit{ORCA4} or \textit{ORCA5} (Figure 5C). Only \textit{ORCA5} moderately but significantly activated the \textit{ZCT3} promoter compared to the control. To test whether \textit{ORCA5} is regulated by \textit{ZCT3}, \textit{ORCA5} promoter was fused to a firefly-\textit{luciferase} reporter gene and co-electroporated into tobacco protoplasts with or without the construct expressing \textit{ZCT3}. No significant repression of the \textit{ORCA5} promoter was observed (Figure 5D).

To demonstrate that \textit{ORCA5} activates \textit{ZCT3} likely by binding to its promoter, we performed yeast one-hybrid (Y1H) assay. Plasmids expressing GAL4-AD-ORCA5 fusion, controlled by the \textit{ADH} promoter, and the \textit{HIS3} nutritional reporter driven by the \textit{ZCT3} promoter were co-transformed into yeast cells. Transformed yeast cells, harboring the \textit{ZCT3-HIS3} reporter and AD-\textit{ORCA5}, grew on selection medium (-leu-trp-his) with 50 mM of 3-AT, indicating activation of the \textit{ZCT3} promoter by \textit{ORCA5} (Figure 6A).

\textbf{ORCA5 activates the \textit{ORCA4} promoter}

Expression of both \textit{ORCA3} and \textit{ORCA4} were increased significantly in \textit{ORCA5-OE} hairy root lines (Figure 4A), indicating that \textit{ORCA5} possibly regulates the expression of \textit{ORCA3} and \textit{ORCA4}. To test this possibility, \textit{ORCA3} (778 bp), \textit{ORCA4} (883 bp), or \textit{ORCA5} (890 bp) promoters, fused to a firefly-\textit{luciferase} reporter, were co-electroporated into tobacco protoplasts with or without the plasmids expressing \textit{ORCA3}, \textit{ORCA4} or \textit{ORCA5}. None of the \textit{ORCAS} could activate the \textit{ORCA3} promoter, suggesting the \textit{ORCAS} are unable to bind to the promoter despite
the induction of ORCA3 in ORCA5-OE lines (Figure 6B). ORCA3 and ORCA5, but not ORCA4, significantly activated the ORCA4 promoter (Figure 6C). In addition, ORCA5 activated its own promoter compared to the control (Figure 6D). However, ORCA3 or ORCA4 had no effects on transcriptional activity of the ORCA5 promoter (Figure 6D). The activation of ORCA4 by ORCA3 and ORCA5, activation of ORCA3 and ORCA4 by ORCA5, and self-regulation of ORCA5 allude to the possible presence of auto-regulatory and feed-forward loops in the ORCA cluster.

304 ORCA3 and ORCA5 bind to the ORCA4 promoter

We identified a GC-rich motif (AGCCCGCCC) to be a putative AP2/ERF binding site in the ORCA4 promoter and mutated it to AGCAAAACC by site-directed mutagenesis. The mutant promoter, mORCA4-pro was fused to the luciferase reporter to generate a reporter vector. The reporter vectors harboring the wild-type or mutant ORCA4 promoter were co-electroporated into tobacco protoplasts with the plasmid expressing ORCA5. Mutation in the GC-rich motif reduced activation of the ORCA4 promoter by ORCA5 (Figure 6C), suggesting that ORCA5 activates ORCA4 likely by binding to the GC-rich motif in its promoter.

To further verify that ORCA3 and ORCA5 bind the GC-rich element in the ORCA4 promoter, we performed Y1H assay. ORCA3 or ORCA5 fused to the GAL4-AD was co-transformed into yeast cells with the HIS3 reporter driven by the ORCA4 promoter. Yeast cells, harboring the ORCA4-HIS3 reporter and AD-ORCA3 or AD-ORCA5, grew on selection medium (-leu-trp-his) with 50 mM of 3-AT, suggesting that ORCA3 and ORCA5 can activate the ORCA4 promoter (Figure 6A).

We also carried out EMSA to validate the binding of ORCA3 and ORCA5 to the GC-rich motif in the ORCA4 promoter. Recombinant, GST-tagged ORCA3 or ORCA5 protein was purified and incubated with 5’ biotin-labeled probes covering the GC-rich motif of the ORCA4 promoter. Figure 6E shows that ORCA3 and ORCA5 proteins individually interacted with the GC-rich motif, resulting in a mobility shift. The binding of ORCA3 and ORCA5 to the labeled probe was confirmed by a competition experiment using unlabeled (cold) probes. The binding signals of the biotin-labeled probes could be eliminated by excess concentrations (1000x) of cold probe (Figure 6E), suggesting that ORCA3 or ORCA5 binds to the GC-rich motif in the ORCA4 promoter.
GC-rich motifs are present in the promoters of AP2/ERF gene clusters in other plants

AP2/ERFs are known to bind the GC-rich motifs in target gene promoters (Fujimoto et al., 2000; Shoji et al., 2013). Group IX ERFs bind differentially to three GC-rich motifs, P-box (CCGCCCTCCA), CS1-box (TAGACCGCCT) and GCC-box (AGCCGCC) (Shoji et al., 2013). A recent study has identified consensus sequence for GC-rich motifs ([A/C]GC[A/C][T/C][C/T]C) present in the promoters of nicotine biosynthetic genes in tobacco (Kajikawa et al., 2017). In addition, ORCA3 and ORCA5 bind to a GC-rich motif (AGCCCGCC; this study) in the ORCA4 promoter. The question thus arose whether GC-rich elements are also present in the promoters of AP2/ERF gene clusters identified in other plant species. To address this question, we manually searched for similar GC-rich motifs approximately 1kb 5’ of the protein coding regions of NIC2 and GAME9 genes in tobacco, tomato, and potato. Of the ten NIC2 promoters, two GC-rich sequences were found each of ERF168, ERF115 and ERF179. Both tomato GAME9-like 1 (Solyc01g090300) and 2 (Solyc01g090310) contain a single GC-rich sequence, while potato GAME9-like 2, 3, 4, and 7 (Cardenas et al., 2016) contain several in their promoters (Supplemental Figure S5).

Intra-cluster and mutual regulation in AP2/ERF gene clusters

The conserved nature of the GC-rich elements in promoters of the AP2/ERF clusters alludes to the possible intra-cluster regulatory mechanisms that are mutually shared among different plant species. To test this possibility, the C. roseus ORCA4-promoter-luciferase reporter construct was co-electroporated into tobacco protoplasts with or without the plasmids expressing tobacco ERF189 or ERF221. Both tobacco ERF189 and ERF221 significantly activated the ORCA4 promoter compared to the control (Figure 7A). Similarly, tobacco ERF115 (1056 bp) or ERF179 (1070 bp) promoter-luciferase reporter construct was co-electroporated into tobacco protoplasts with or without the construct expressing ERF189 or ORCA5. The activation of the ERF115 and ERF179 promoters by ERF189 or ORCA5 were moderate, but statistically significant (Figure 7B). In addition, we found two potential ERF binding motifs (GGCACCT and GGCCAAGC) in the ERF115 promoter. Mutation of either individual motif did not significantly affect the activation of ERF115-LUC by ERF189; however, mutation of both motifs reduced the activity of ERF115-LUC reporter by 70% compared with the wild-type promoter (Figure 7C). Collectively,
these findings suggest the presence of intra-cluster and mutual regulation in both NIC2 and ORCA clusters.

**C. roseus ORCA ERFs and tobacco NIC2-locus ERFs are likely interchangeable**

*C. roseus* ORCA3/4/5 are homologous to tobacco NIC2 locus AP2/ERFs, ERF189 and ERF221 (a.k.a. ORC1) (Shoji et al., 2010; De Boer et al., 2011). In addition, both ORCAs and NIC2 ERFs are induced by MeJA and recognize GC-rich motifs in target gene promoters in two diverse metabolic pathways (Shoji et al., 2010; De Boer et al., 2011). It is thus intriguing to speculate that *C. roseus* ORCAs and tobacco NIC2-locus ERFs are functionally equivalent and interchangeable. We tested this assumption by co-electroporation of the *putrescine N-methyltransferase* (*PMT*; 1500 bp) or *quinolinate phosphoribosyltransferase* (*QPT*; 1579 bp) promoter-*luciferase* reporter vector into tobacco protoplasts with or without the plasmids expressing *ERF221, ORCA3, ORCA4* or *ORCA5*. As expected, ERF221 significantly activated the *PMT* and *QPT* promoters compared to the control. ORCA3 and ORCA5 also activated the *PMT* and *QPT* promoters although to lower levels compared to the activation by ERF221 (Figure 8A). The *STR* promoter (587 bp) fused to the *luciferase* reporter was co-electroporated into tobacco protoplasts with or without the construct expressing *ORCA3, ERF189*, or *ERF221*. Similar to ORCA3, a known *STR*-activator, both ERF189 and ERF221 significantly activated the *STR* promoter (Figure 8A), suggesting that the tobacco ERF189 and ERF221 are functional equivalents of *C. roseus* ORCAs. To determine the activation specificity of *PMT* and *QPT* by NIC2 ERFs or ORCAs, we cloned a tobacco bZIP TF which is not involved in the regulation of nicotine biosynthesis (Yang et al., 2001) and used it as a negative control. As shown in Supplemental Figure S6, the bZIP TF was unable to activate the *PMT* or *QPT* promoter in tobacco cells. Similarly, CrMYC1, a *C. roseus* bHLH TF not known to regulate the TIA pathway (Chatel et al., 2003), was unable to activate the *STR* promoter in tobacco cells.

To functionally verify the conserved regulatory roles of AP2/ERFs of different clusters, we generated tobacco hairy roots overexpressing *ORCA5*. Transgenic status of the hairy roots was confirmed by PCR (Supplemental Figure S7), and two hairy root lines were used for further analysis. Expression of *PMT* and *QPT* were 2.5-3.0 fold higher in *ORCA5*-expressing hairy roots compared to the empty vector control (Figure 8B). Moreover, nicotine contents in the two *ORCA5*-overexpressing lines were 3-4 fold higher compared to the control lines (Figure 8C), a
result that is consistent with a previous study showing that overexpression of ERF189 in tobacco hairy roots resulted in 2-3 fold increase in PMT and QPT expression and alkaloid accumulation (Shoji et al., 2010). We also generated C. roseus hairy roots overexpressing ERF189, and two transgenic lines were used for further analysis (Supplemental Figure S8). STR expression was approximately 2-fold higher in ERF189-expressing hairy roots compared to the empty vector control (Figure 8D). In addition, the two ERF189-overexpressing lines accumulated 2-7 fold higher ajmalicaine, catharanthine and tabersonine compared to the controls (Figure 8E).

Discussion

Physically linked clusters of non-homologous, structural genes have been identified in numerous plant species, including Arabidopsis, rice, maize, oat, tomato, potato, and opium poppy (Boycheva et al., 2014; Nützmann and Osbourn, 2014; Nützmann et al., 2016). These gene clusters generally encode enzymes that are involved in the biosynthesis of specialized metabolites (Boycheva et al., 2014; Nützmann and Osbourn, 2014; Nützmann et al., 2016). Unlike the structural gene clusters, TF gene clusters comprise homologous genes that likely arose as the results of duplication events. It is unclear whether the duplicated TF genes are functionally redundant and co-regulated by the same transcriptional circuit, or if they have evolved through gene divergence to possess unique functions, including differential responses to hormonal signals and regulation of one another.

We showed that ORCAs and key TIA pathway genes exhibit two distinct expression patterns in response to ET alone, or the combined treatment of ET and MeJA (Figure 1B). CrMYC2a, ORCA5, and TDC were upregulated by MeJA, but not affected by ET. On the other hand, expression of ORCA3, ORCA4, and STR was significantly induced by MeJA and repressed by ET. Moreover, when treated simultaneously, ET antagonizes the MeJA-induced expression of ORCA3, ORCA4, ORCA5, TDC, and STR (Figure 1B). Expression divergence has been observed among the tobacco NIC2 ERFs (Shoji et al., 2010) and tomato JREs (Nakayasu et al., 2018) in response to MeJA and ET. MeJA-induced expression of ERF189/199 is antagonized by ET, whereas expression of other NIC2 ERFs are insensitive to ET treatment (Shoji et al., 2010). Other duplicated regulatory genes in Arabidopsis also exhibit expression divergence (Ganko et al., 2007). The plant genomes sequenced to date have shown whole-genome, tandem, and/or segmental duplications that result in neo-, sub-, or pseudo-functionalization of duplicated genes.
These findings suggest that duplicated regulatory genes, including ORCA and NIC2 ERFs, experience sub-functionalization (Shoji et al., 2010). We demonstrated that ORCA5 has a broader transactivation specificity than ORCA3 and ORCA4 (Figure 2C). Overexpression of ORCA5 in C. roseus hairy roots significantly induced expression of genes in the indole branch and downstream of the iridoid branch, such as SLS, resulting in increased TIA accumulation (Figure 4A). In addition, expression of CrMYC2a was also upregulated in ORCA5-overexpressing hairy roots. In tobacco, not all NIC2 ERFs are equally effective in activating nicotine pathway genes. This functional divergence among the ERFs may be attributed to the sequence differences in the AP2 DNA binding domain and/or the region outside of the AP2 domain (Shoji et al., 2010). MYC2 is known to regulate plant specialized metabolites, including nicotine, TIAs, and SGA. Previously, we have demonstrated that CrMYC2a expression strongly correlates with those of TIA structural and regulatory genes. Similar to MYC2 regulation of nicotine biosynthesis in tobacco (Shoji and Hashimoto, 2011), CrMYC2a co-regulates TIA pathway genes with ORCA3 (Paul et al., 2017). In addition, CrMYC2a expression is induced in response to JA (Figure 1B) and increased in ORCA5-overexpressing hairy roots (Figure 4A). A recent study has shown that transient co-overexpression of a derepressed CrMYC2a (CrMYC2aD126N) with ORCA3 synergistically affects several TIA pathway gene expression (Schweizer et al., 2018). Similar to the previous study, we found that CrMYC2aD126N, when coexpressed with ORCA5, has additive effects on activation of TIA pathway genes. Collectively, these findings suggest that CrMYC2a and ORCAs are part of a regulatory network that modulate TIA biosynthesis in C. roseus. Transient overexpression of CrMYC2a or CrMYC2aD126N alone or in combination with ORCA3 activate a limited number of TIA pathway genes, suggesting that additional but unidentified TFs are likely involved in the TIA gene regulatory network.

Positive and negative regulatory loops are the hallmarks of metabolic pathways in plants. In Arabidopsis JA signaling pathway, MYC2 activates the expression of JAZ repressors, which, in turn, interact with MYC2 to attenuate the intensity of JA signal (Chini et al., 2007; Kazan and Manners, 2013). AtMYBL2, a repressor of anthocyanin biosynthesis in Arabidopsis, is regulated by the bHLH activator, TRANSPARENT TESTA8 (TT8). AtMYBL2 competes with the R2R3 MYBs, PAP1 and PAP2, to form a complex with TT8 that represses anthocyanin accumulation.
(Matsui et al., 2008). Recently, we demonstrated that in *C. roseus*, CrMYC2a and BIS1 activate the expression of the bHLH TF, *RMT1*, which acts as a repressor of CrMYC2a targets (Patra et al., 2018). Similarly, in tomato MYC2 regulates expression of a small group of JA-responsive bHLH TFs, MYC2-targeted bHLH 1 (MTB1), MTB2 and MTB3. MTB proteins inhibit the formation of MYC2-MED25 complex and compete with MYC2 to bind to its targets (Liu et al., 2019). Here, we showed that, similar to ORCA4 (Paul et al., 2017), overexpression of ORCA5 significantly activates *ZCTs* in *C. roseus* hairy roots (Figure 4A). Moreover, we demonstrated that ORCA5 activates *ZCT3* possibly by binding to the GC-rich element in the promoter (Figure 5C, 6A). In *C. roseus* cells, *ZCTs* repress *STR* and *TDC*, the direct targets of ORCAs, by binding to their promoters (Pauw et al., 2004). The up-regulation of *ZCTs* by ORCA4- or ORCA5-overexpression suggests the existence of a negative regulatory loop that is probably involved in the fine-tuning of TIA biosynthesis (Figure 6F).

Individual genes in the tobacco and *C. roseus* ERF gene clusters play overlapping and unique roles in controlling the structural genes in nicotine and TIA biosynthetic pathways, respectively (Shoji et al., 2010; Paul et al., 2017). However, the regulatory relationship among the members within an ERF cluster, or any known plant TF clusters, has not been elucidated prior to this study. Here we demonstrated that an intra-cluster regulatory mechanism exists in both *C. roseus* ORCA cluster and tobacco *NIC2* cluster. The self-regulated ORCA5 activates *ORCA4* by binding to its promoter and *ORCA3*, likely through an uncharacterized TF. ORCA3 also activates *ORCA4* by binding to the GC-rich motif in its promoter (Figure 6). In tobacco, ERF189 activates both the *ERF115* and *ERF179* promoters (Figure 7B). GC-rich sequences are not found within the 1kb promoter regions of *ERF189* and *GAME9*. The fact that GC-rich motifs are present only in the promoters of some ERFs indicates that certain key regulators, such as ERF189, likely play important role in controlling amplification loop in the ERF cluster. The intra-cluster regulation of ERF clusters implies that the individual components within a cluster are not simply redundant duplication of one another. The positive amplification loops help plants to make sufficient precursors required for the spatial-temporal biosynthesis of specialized metabolites. The mechanism is likely conserved in other ERF clusters in plants. It is also reasonable to predict that similar self-regulation mechanisms exist in other TF clusters, such as those formed by group III AP2/ERFs, CBFs (Zhang et al., 2004), and ARFs (Hagen and Guilfoyle, 2002).
The conserved nature of the intra-ERF-cluster regulation prompted us to speculate that ERFs from gene clusters of different plant species are functionally equivalent and interchangeable, despite low sequence similarity (35-41%). In this study, we showed that *C. roseus* ORCA5 can activate both tobacco *ERF115* and *ERF179*, and tobacco ERF189 and ERF221 can activate the *C. roseus* ORCA4 promoter (Figure 7A and B). Furthermore, ORCA3 and ORCA5 can activate the *PMT* and *QPT* promoters (Figure 8A). Similarly, the *STR* promoter in the TIA pathway can be activated by tobacco ERF189 and ERF221 (Figure 8A). Moreover, ORCA5 overexpression in tobacco hairy roots induced expression of *PMT* and *QPT*, resulting in increased nicotine accumulation (Figure 8B and C). Similarly, *ERF189* overexpression in *C. roseus* hairy roots activated the expression of *STR* and induced TIA accumulation (Figure 8D and E). The mutual activations of two distinct metabolic pathways by the ORCA and NIC2 clusters support our hypothesis that the AP2/ERFs are functionally equivalent and are likely interchangeable (Figure 8F). Other *ERF* gene clusters, such as *GAME9* ERFs of tomato and potato (Supplemental Figure S5) also contain the GC-rich elements in their promoters and respond to JA-induction similar to the ORCA and NIC2 clusters. We thus propose that the intra-cluster and mutual regulatory functions are widely conserved among the ERF gene clusters of diverse plant species, although additional experimental verifications are required.

TF gene clusters have been identified in non-plant organisms, including nematodes, *Drosophila*, mouse, and human. The non-plant, homeodomain HOX TF clusters, which play critical roles in invertebrates and vertebrates development, have been characterized (Lappin et al., 2006; Montavon and Duboule, 2013). By comparison, the plant TF clusters are poorly investigated. As more and more TF clusters are being identified, the unique functions and mutual regulatory relationships of the clustered TFs require in-depth examination. Central to the knowledge gaps is the regulatory relationship within a cluster and among different species. Understanding of such relationships will shed light on TF evolution, as well as the functional equivalence and divergence of TFs involved in specialized metabolism. This study demonstrates intra-cluster and mutual regulation of AP2/ERF gene clusters, suggesting that a conserved regulatory mechanism modulates biosynthesis of diverse groups of plant specialized metabolites.

**MATERIALS AND METHODS**

**Plant materials**
Catharanthus roseus (L.) G. Don var. ‘Little Bright Eye’ (NE Seed, USA) was used for cloning, gene expression, and generation of transgenic hairy roots. Nicotiana tabacum var. Xanthi cell line was used for protoplast-based transient expression assays. N. tabacum var. SamsunNN was used for generation of hairy roots.

RNA isolation and cDNA synthesis

C. roseus (L.) G. Don var. ‘Little Bright Eye’ seeds were surface-sterilized using 30% (v/v) commercial bleach for 15 min, washed five times with sterile water and inoculated on half-strength Murashige and Skoog (MS) medium (Caisson Labs, USA). The plates were kept at 28°C in dark for two days and then transferred to a growth room at 28°C, with constant light (Patra et al., 2018). Ten-day-old seedlings were immersed in half-strength MS medium with 100 μM methyl jasmonate (MeJA) and/or 50 μM of ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) for 2h. Mock-treated seedlings were used as control. Total RNA isolated from the seedlings were used for cDNA synthesis as described previously (Suttipanta et al., 2007).

Reverse-transcription quantitative PCR

Reverse-transcription quantitative PCR (RT-qPCR) was performed as described previously (Suttipanta et al., 2011). The primers used in RT-qPCR are listed in Supplemental Table S1. In addition to the C. roseus Elongation Factor 1α (EF1α), 40S Ribosomal Protein S9 (RPS9) gene, was used as a second internal control (Liscombe et al., 2010). All PCRs were performed in triplicate and repeated at least twice.

Total RNA isolated from empty-vector control and ORCA5-overexpressing hairy roots were used for cDNA synthesis and RT-qPCR as previously described (Suttipanta et al., 2011). The comparative cycle threshold (Ct) method (Applied Biosystems, http://www.appliedbiosystems.com) was used to measure transcript levels. In addition to tobacco elongation factor-1α (Shoji et al., 2010) (GenBank accession number D63396), α-tubulin (GenBank accession number AJ421411) was also used as a reference gene.

Sub-cellular localization
For sub-cellular localization, the full-length cDNA of ORCA5 was fused to the N-terminus of the enhanced GFP (eGFP) driven by the CaMV3S promoter and rbcS terminator in a pBS plasmid to generate pORCA5-eGFP. A pBS plasmid containing only eGFP was used as a control. The plasmids containing either eGFP or ORCA5-eGFP were individually electroporated into tobacco protoplasts as described previously (Pattanaik et al., 2010) and visualized after 20h incubation in dark under a fluorescent microscope (Eclipse TE200, Nikon, Japan).

Tobacco protoplast isolation and electroporation

The 5' flanking regions of LAMT (-1375 to -1; relative to the ATG), SLS (-979 to -1), STR (-586 to -1), ZCT3 (-960 to -1), ORCA3 (-777 to -1), ORCA4 (-882 to -1) and ORCA5 (-889 to -1) promoters were PCR-amplified from C. roseus genomic DNA. The PMT (-1499 to -1), QPT (-1578 to -1), ERF115 (-1055 to -3) and ERF179 (-1069 to -3) promoters were amplified from tobacco genomic DNA using gene specific primers. The two GC-rich motifs, TGGCACCT and GGCCAAGC, in ERF115 promoter were mutated to aaaACCT and GaaaAAGC using site-directed mutagenesis. The reporter plasmids for transient protoplast assays were generated by cloning LAMT, SLS, STR, ZCT3, ORCA3/4/5, PMT, ERF115 and ERF179 promoters in a modified pUC vector containing a fire-fly luciferase (LUC) and rbcS terminator. The effector plasmids were constructed by cloning ORCA3/4/5, ERF189/221, and ZCT3 into a modified pBS vector under the control of the CaMV35S promoter and rbcS terminator. The ß-glucuronidase (GUS) driven by the CaMV35S promoter and rbcS terminator was used as an internal control in protoplast assay. For transactivation assay, ORCA3, ORCA4 and ORCA5 were fused to the GAL4 DNA binding domain (GAL-DBD) in a pBS plasmid containing mirabilis mosaic virus (MMV) promoter and rbcS terminator. The reporter plasmid used in the assay contains firefly luciferase driven by minimal CaMV 35S promoter with five tandem repeats of GAL4 Response Elements (5X GALRE), and rbcS terminator fused. Protoplast isolation from tobacco cell suspension cultures and electroporation with supercoiled plasmid DNA were performed using previously described protocols (Pattanaik et al., 2010). The reporter, effector, and internal control plasmids were electroporated into tobacco protoplasts in different combinations; luciferase and GUS activities in transfected protoplasts were measured as described previously (Suttipanta et al., 2007). Each experiment was repeated three times.
Construction of plant expression vector and generation of hairy roots

For plant transformation, ORCA5 and ERF189 were PCR-amplified from C. roseus and tobacco seedlings cDNA, respectively and cloned in pCAMBIA2301 vector containing the CaMV35S promoter and the rbcS terminator (Pattanaik et al., 2010). The pCAMBIA2301 vector alone was used as an empty vector (EV) control. The plasmids were mobilized into Agrobacterium rhizogenes R1000 by freeze-thaw. Transformation of C. roseus seedlings and generation of hairy roots were performed using the protocol described previously (Suttipanta et al., 2011; Paul et al., 2017). Transgenic status of the hairy root lines was verified by PCR amplification of rolB, rolC, virC, nptII and GUS genes. Primers used in this study are listed in Supplemental Table S1. Two independent hairy root lines were selected for further analysis.

Alkaloid extraction and analysis

For extraction of alkaloids, ten day-old seedlings were immersed in half-strength MS medium with 100 μM MeJA and/or 50 μM of ACC for 24h. MeJA and/or ACC-treated seedlings, and transgenic hairy roots were frozen in liquid nitrogen and ground to powder. Samples were extracted in methanol (1:100 w/v) twice for 24 h on a shaker. Pooled extracts were then dried via a rotary evaporator and diluted in methanol 10 μL/mg of the initial sample. The samples were then analyzed using high performance liquid chromatography (HPLC), followed by electrospray-injection (ESI) in a tandem mass spectrometry (MS/MS), as previously described (Suttipanta et al., 2011; Paul et al., 2017). The known alkaloid standards were run to identify elution times and mass fragments.

Yeast one-hybrid assay

The ORCA4 (883 bp)/ZCT3 (961 bp) promoter was cloned in the pHIS2 vector (Clontech), containing the HIS3 reporter gene to generate the reporter plasmid (pORCA4/ZCT3-HIS3). The full-length ORCA3 and ORCA5 cDNAs were cloned into the yeast expression plasmid, pAD-GAL4-2.1 (Stratagene), to generate the effector plasmids (pORCA3/ORCA5-AD). The reporter and effector plasmids were transformed into yeast strain Y187, and transformants were selected on synthetic dropout (SD) medium lacking Leu and Trp (-Leu-Trp). Transformed colonies were then streaked on SD medium lacking His-Leu-Trp (-Leu-Trp-His) with 50 mM 3-AT to check promoter activation.
Recombinant Protein Production and EMSA

The ORCA3, ORCA4 and ORCA5 genes were cloned into the pGEX 4T-1 vector (GE Healthcare Biosciences, Pittsburgh, PA, USA) to generate GST-fusion proteins. The constructs were verified by DNA sequencing and transformed into E. coli BL21 cells containing pRIL (Agilent, Santa Clara, CA, USA). Protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1mM to the cell cultures at A600 ~ 0.8 and induced for 2 h at 37°C. The cells were harvested by centrifugation and lysed using CelLytic B (Sigma, USA) according to the manufacturer’s instructions. The GST fusion proteins were bound to Glutathione Sepharose 4B columns (Amersham) and eluted by using 10mM reduced glutathione in 50mM Tris–HCl (pH 8.0) buffer. Bacterial expression and purification of recombinant CrMYC2a protein were performed as previously described (Patra et al., 2018). For EMSA experiments, biotin-labeled DNA probes were synthesized by Integrated DNA Technologies (IDT) and annealed to produce double-stranded probes. Complementary DNA probes were designed to include the jasmonate-responsive elements (JRE) of STR promoter (\(1^{100}\)ACATCAGCTTGTGACCTTCTTTGAAA GTGATTCCCTTGAGCTT\(^{58}\) relative to transcription start site; TSS) (Van Der Fits and Memelink, 2001) and putative GC rich element of the ORCA4 promoter (\(1^{106}\)CCTTCATAGCCCGCCCAATTGGTAAACGTGCACCAACCTCC\(^{66}\) relative to the translation start, ATG). EMSA experiment was carried out using light shift chemiluminescent EMSA kit (ThermoFisher Scientific). For the binding reactions 40 fmole of DS DNA was incubated with purified protein (500 ng of each protein) for 60 min at room temperature. The protein-DNA binding for ORCA5 was further confirmed by performing competition experiment, where 10X-, 100X- and 1000X-fold excess amount of cold probe (without biotin-label) was added to the binding reactions. For ORCA3 and ORCA4, 1000X-fold excess amount of cold probe was added to the binding reactions. Recombinant CrMYC2a protein was used as a negative control on biotin-label STR probe. The DNA-protein complexes were resolved by electrophoresis on 6% non-denaturing polyacrylamide gels and then transferred to BiodyneB modified membrane (0.45 mm; Pierce). The band shifts were detected by a chemiluminescent nucleic acid detection module (Pierce) and exposed to X-ray films.

Phylogenetic analysis of group IX AP2/ERFs
Protein sequences for tobacco, tomato, and potato were downloaded from Sol Genomics Network database (Fernandez-Pozo et al., 2015), and protein sequences for *C. roseus* were obtained from the medicinal plant genomics resource database (http://medicinalplantgenomics.msu.edu/). The *Arabidopsis* AP2/ERFs sequences were obtained from a previously published report (Nakano et al., 2006). The group IX AP2/ERFs protein sequences from *Arabidopsis* were used as queries using Basic Local Alignment Search Tool (BLAST) (Camacho et al., 2009) to identify the AP2/ERFs from tobacco, tomato, potato, and *C. roseus*. Putative AP2/ERFs sequences were screened using the Pfam database for the AP2/ERFs domain (Finn et al., 2016). The group IX AP2/ERFs protein sequences were aligned using ClustalW with the default settings, and MEGA6.0 was used to construct the phylogenetic tree using Neighbor Joining (NJ) method with bootstrap values set as 1000 replicates. The tree image was generated with the Evolview v2 (He et al., 2016).

**Generation of tobacco hairy roots and measurement of nicotine**

Leaf discs of *in vitro* grown *N. tabacum* var. SamsunNN plantlets were infected with *A. rhizogenes* strain (R1000) harboring the pCAMBIA2301-ORCA5 overexpression construct. After 2 days of co-cultivation, leaf discs were transferred to MS medium supplemented with 400 mg/L cefotaxime and kept at 25°C in the dark. Hairy roots developed from the leaf discs were transferred to MS medium with 400 mg/L cefotaxime and 100 mg/L kanamycin for further proliferation.

Freeze-dried empty-vector control and ORCA5-overexpressing hairy roots were exhaustively extracted for pyridine alkaloids by methyl tert-butyl alcohol (MTBE) and aqueous sodium hydroxide. Alkaloid contents were determined using Gas Chromatograph with Flame Ionization Detectors (GC-FID, PerkinElmer, USA) (Lewis et al., 2008). Nicotine content was reported as percentages on a dry-tobacco-weight basis.

**Statistical analyses**

The data presented here were statistically analyzed by Student’s t-test or one-way analysis of variance (ANOVA), and Tukey’s Honestly Significant Difference (HSD) for multiple comparisons. The significance level (P value) was described in legends to each figure.
Accession numbers: ORCA3 (AJ251249), ORCA4 (KR703577), ORCA5 (KR703578), ERF189 (AB827951) and ERF221 (XM_016622819).

Supplemental Data

Supplemental Figure S1. Phylogenetic analysis of group IX AP2/ERFs in tobacco, tomato, potato and C. roseus.

Supplemental Figure S2. Sub-cellular localization of ORCA5 and transactivation assay of ORCAs in tobacco cells.

Supplemental Figure S3. ORCA5 and CrMYC2a binding to the GC-rich motif in the STR promoter.

Supplemental Figure S4. Molecular analysis of ORCA5-overexpressing C. roseus hairy roots.

Supplemental Figure S5. Positions and sequences of GC-rich motifs in the AP2/ERF promoters of C. roseus, tobacco, tomato, and potato.

Supplemental Figure S6. Activation assays of the QPT, PMT, and STR promoters using tobacco bZIP and CrMYC1.

Supplemental Figure S7. Molecular analysis of ORCA5-overexpressing tobacco hairy roots.

Supplemental Figure S8. Molecular analysis of ERF189-overexpressing C. roseus hairy roots.

Supplemental Table S1. Oligonucleotides used in this study.

Acknowledgements

This work is supported partially by the Harold R. Burton Endowed Professorship to L.Y. and by the National Science Foundation under Cooperative Agreement no. 1355438, to L.Y. We thank Mr. J. May (Department of Civil Engineering and Environmental Research Training Laboratories, University of Kentucky) for assistance on LC-MS and Huihua Ji (KTRDC, University of Kentucky) for assistance on nicotine measurement.

Figure legends

Figure 1. Expression of ORCA3, ORCA4, and ORCA5 in response to JA and ACC. (A) A simplified diagram of the TIA biosynthetic pathway in Catharanthus roseus. TIA pathway genes studied in this work are highlighted in blue, and genes regulated by ORCAs and CrMYC2a (this study; Schweizer et al. 2018; Paul et al. 2017; van der Fits and Memelink, 2000) are indicated by
circle and green triangle, respectively. (B) Ten-day-old C. roseus seedlings were treated with 100 µM MeJA (JA) and/or 50 µM ACC for 2h, and gene expression in whole seedling was measured by RT-qPCR. Mock-treated seedlings were used as controls (CN). (C) Measurement of ajmalicine, catharanthine, and tabersonine in JA-, ACC- and JA+ACC-treated C. roseus seedlings. Alkaloids were extracted and analyzed using LC-MS/MS. The levels of alkaloids were estimated based on peak areas compared to standards. Data represent means ± SDs of three biological samples each with 15-17 seedlings. Different letters denote statistical differences as assessed by one-way ANOVA and Tukey HSD test, p < 0.05. ASα, anthranilate synthase; CPR, cytochrome P450 reductase; G10H, geraniol 10-hydroxylase; HL1/2/3/4, hydrolase 1/2/3/4; IS, iridoid synthase; LAMT, loganic acid methyltransferase; MAT, minovincine 19-O-acetyltransferase; SGD, strictosidine β-glucosidase; SLS, secologanin synthase; STR, strictosidine synthase T19H, tabersonine 19-hydroxylase; TAT, tabersonine derivative 19-O-acetyltransferase; TEX1/TEX2, tabersonine 6,7-epoxidase isoforms 1 and 2; V19H, vincadifformine 19-hydroxylase. ACC, 1-aminocyclopropane-1-carboxylic acid; MeJA, methyl jasmonate; TIA, terpenoid indole alkaloid.

Figure 2. ORCA binding to the GC-rich motif in the STR promoter and differential activation of TIA pathway gene promoters. (A) ORCA3, ORCA4, and ORCA5 were expressed in E. coli and the recombinant proteins were purified to homogeneity as demonstrated by SDS-PAGE. (B) Binding of ORCA3, ORCA4, and ORCA5 to the GC-rich motif in the STR promoter. Nucleotide sequence of GC-rich motif and position of JRE (-100 to -58) relative to the transcription start site (TSS) is shown on the top panel. Autoradiograph shows the DNA-protein complex of biotin-labeled GC-rich motif probe with ORCA3, ORCA4, or ORCA5. The labeled probe was outcompeted by 1000X unlabeled probe (+). (C) Transactivation of the LAMT and SLS promoters, fused to the firefly luciferase (LUC) reporter, by ORCA3, ORCA4, and ORCA5 in tobacco cells. Control (CN) represents reporter plasmid alone. A plasmid containing the β-glucuronidase (GUS) reporter, driven by the CaMV 35S promoter and rbcS terminator, was used as an internal control. LUC and GUS activities were measured 20 h after electroporation. LUC activity was normalized against GUS activity. Data presented here are the means ± SDs of three biological replicates. Statistical significance was calculated using the Student’s t-test, * p <0.05, ** p <0.01. JRE, jasmonate responsive element.
Figure 3. Derepressed CrMYC2a and ORCA5 synergistically effect TIA pathway genes. Activation of the TDC, STR, LAMT, and SLS promoters, fused to the firefly luciferase (LUC) reporter, by ORCA5, CrMYC2a, and CrMYC2aD126N in tobacco cells. A reporter plasmid containing the promoter-LUC cassette was co-electroporated into tobacco protoplasts with effector plasmids harboring TF genes. Control (CN) represents reporter plasmid alone. A plasmid containing the β-glucuronidase (GUS) reporter, driven by the CaMV 35S promoter and rbcS terminator, was used as internal control. LUC and GUS activities were measured 20 h after electroporation. The LUC activity was normalized against the GUS activity. Data presented here are the means ± SDs of three biological replicates. Different letters denote statistical differences as assessed by one-way ANOVA and Tukey HSD test, p < 0.05

Figure 4. Relative expression of key TIA pathway genes and alkaloid accumulation in ORCA5-overexpressing C. roseus hairy roots. (A) Relative expression of the TIA pathway genes and TF genes in two empty vector (EV) controls and two ORCA5-overexpression (OE-1 and OE-2) hairy root lines as measured by RT-qPCR. (B) Measurement of tabersonine, ajmalicine, and catharanthine in EV controls, OE-1, and OE-2. Alkaloids were extracted and analyzed using LC-MS/MS, and the levels of alkaloids were estimated based on peak areas compared to standards. Data presented here are the means ± SDs of three biological replicates. Statistical significance was calculated using the Student’s t-test, * p <0.05, ** p <0.01, *** p <0.001

Figure 5. Relative expression of TIA pathway genes in response to MeJA and in ORCA5-overexpressing hairy roots. (A) Ten-day-old C. roseus seedlings (15-17 seedlings in each replicate) were treated with 100 µM MeJA (JA) for 2h, and expression of HL1 to HL4, V19H, TEX1, TEX2, T19H, TAT, and MAT in seedling was measured by RT-qPCR. Mock-treated seedlings were used as controls (CN). (B) Relative expression of HL1 to HL4, V19H, TAT, TEX1, and TEX2 in empty vector (EV) controls and two ORCA5-overexpression hairy root lines (OE-1 and OE-2) were measured by RT-qPCR. (C) Activation of the ZCT3 promoter, fused to the LUC reporter, by ORCA3, ORCA4, or ORCA5 in tobacco cells. (D) Transactivation of the ORCA5 promoter, fused to the LUC reporter, in tobacco cells. Control (CN) represents reporter plasmid alone. In both C and D, a plasmid containing the GUS reporter, driven by the CaMV 35S promoter and rbcS terminator, was used as an internal control. LUC and GUS activities were
measured 20 h after electroporation. The LUC activity was normalized against the GUS activity.

Data presented here are the means ± SDs of three biological replicates each with 4-5 samples.

Statistical significance was calculated using the Student’s t-test, * p <0.05, ** p <0.01

**Figure 6. Intra-cluster regulatory relationship among the members of ORCA cluster.** (A) Yeast one-hybrid assay demonstrating activation of the ORCA4 promoter by ORCA3 or ORCA5, and the ZCT3 promoter by ORCA5. ORCA3 or ORCA5, fused to the GAL4 activation domain (pAD-ORCA3/ORCA5), was co-transformed into yeast cells with the pORCA4-HIS3 or pZCT3-HIS3 reporter plasmid. The transformants were grown in either the double selection medium (SD-Leu-Trp) or triple selection medium (SD-Leu-Trp-His) with 50 mM 3-amino-1,2,4-triazole (3-AT). Transactivation of the promoters of (B) ORCA3, (C) ORCA4 and mutant-ORCA4 (m-ORCA4), and (D) ORCA5 by ORCA3, ORCA4, or ORCA5 in tobacco cells. Data presented here are the means ± SDs of three biological replicates each with 4-5 samples. Statistical significance was calculated using the Student’s t-test, * p <0.05, ** p <0.01 (E) Binding of ORCA3 and ORCA5 to the GC-rich motif in the ORCA4 promoter. Nucleotide sequence and position of the GC-rich motif relative to the translation start site (TSS) is shown on the top panel. Autoradiograph shows the DNA-protein complex of the biotin-labeled probe covering the GC-rich motif with ORCA3 or ORCA5. The binding of the labeled probe was outcompeted by 1000X unlabeled probe (+). (F) A model summarizing the intra-cluster regulation among the ORCAs and co-regulation of ORCAs and ZCTs of the TIA pathway. The ORCA genes are activated by JA but repressed by ET. ORCA3 and ORCA5 regulate ORCA4. ORCA5 regulates its own expression. ORCA5 activate ZCT3 whereas ORCAs indirectly regulate ZCT1 and ZCT2. Solid blue arrows indicate activation by JA; solid yellow T-bars represent repression by ET. Solid black arrows represent direct activation, whereas broken arrows represent indirect or undetermined activation. ORCA5 activates whereas ZCT represses several genes in the indole and iridoid branches of the TIA pathway.

**Figure 7. Mutual regulatory relationship among C. roseus ORCA and tobacco NIC2 AP2/ERFs.** (A) Transactivation of the ORCA4 promoter by NIC2 ERF, ERF189 or ERF221, and (B) the tobacco ERF115 and ERF179 promoters by ERF189 or ORCA5. Data represent means ± SDs of three biological samples. Different letters denote statistical differences as assessed by one-way ANOVA and Tukey HSD test, p < 0.05. (C) Transactivation of the mutant ERF115
promoter by ERF189 in tobacco protoplast-based transactivation assay. A plasmid containing the GUS reporter, driven by the CaMV 35S promoter and rbcS terminator, was used as an internal control. Control represents reporter plasmid alone. The LUC and GUS activities were measured 20 h after electroporation. The LUC activity was normalized against the GUS activity. Data presented here are the means ± SDs of three biological replicates each with 4-5 samples. Statistical significance was calculated using the Student’s t-test, * p <0.05.

Figure 8. C. roseus ORCAs and tobacco NIC2 ERFs are likely interchangeable (A) Transactivation of C. roseus STR promoter by ORCA3, ERF189, or ERF221 and tobacco PMT and QPT promoters by ERF221, ORCA3, ORCA4, or ORCA5 in the tobacco protoplast assay. A plasmid containing the GUS reporter, driven by the CaMV 35S promoter and rbcS terminator, was used as internal control. Control (CN) represents reporter plasmid alone. The LUC and GUS activities were measured 20 h after electroporation. The LUC activity was normalized against the GUS activity. Data represent mean ± SDs of three biological replicates each with 4-5 samples. Different letters denote statistical differences as assessed by one-way ANOVA and Tukey HSD test, p < 0.05 (B) Relative expression of PMT and QPT in two empty vector (EV1 and EV2) control and two ORCA5-overexpressing (OE-1, OE-2) tobacco hairy root lines, as measured by RT-qPCR. The tobacco elongation factor 1 α (EF1α) was used as an internal control. (C) Nicotine contents in two empty vector-control (EV1 and EV2) and two ORCA5-overexpression (OE-1 and OE-2) tobacco hairy root lines. Nicotine concentrations are presented as percentage dry weight (%DW). (D) Relative expression of STR in EV1 and EV2 (control) and two ERF189-overexpressing (189OE-1, 189OE-2) Catharanthus hairy root lines, as measured by RT-qPCR. The Catharanthus EF1α was used as an internal control. (E) Measurement of ajmalicine, catharanthine, and tabersonine in EV1 and EV2 controls, and OE-1, and OE-2. Alkaloids were extracted and analyzed using LC-MS/MS, and the levels of alkaloids were estimated based on peak areas compared to standards. Data presented here are the means ± SDs of three biological replicates. Statistical significance was calculated using the Student’s t-test, * p <0.05, ** p <0.01, *** p <0.001. (F) A model depicting the mutual regulatory relationship among and between the ORCA and NIC2 locus AP2/ERFs. The thin solid arrows represent direct activation and broken arrows represent indirect activation within a cluster. The thick arrows indicate the inter-species mutual regulation of the ERFs.
Literature Cited

Boycheva S, Daviet L, Wolfender JL, Fitzpatrick TB (2014) The rise of operon-like gene clusters in plants. Trends Plant Sci 19: 447-459

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+: architecture and applications. BMC Bioinformatics 10: 421

Cardenas PD, Sonawane PD, Pollier J, Vanden Bossche R, Dewangan V, Weithorn E, Tal L, Meir S, Rogachev I, Malitsky S, Giri AP, Goossens A, Burdman S, Aharoni A (2016) GAME9 regulates the biosynthesis of steroidal alkaloids and upstream isoprenoids in the plant mevalonate pathway. Nat Commun 7: 10654

Carqueijeiro I, Brown S, Chung K, Dang TT, Walia M, Besseau S, Duge de Bernonville T, Oudin A, Lanoue A, Billet K, Munsch T, Koudounas K, Melin C, Godon C, Razafimandimby B, de Craene JO, Glevarec G, Marc J, Giglioli-Guivarc'h N, Clastre M, St-Pierre B, Papon N, Andrade RB, O'Connor SE, Courdavault V (2018) Two Tabersonine 6,7-Epoxidases Initiate Lochnericine-Derived Alkaloid Biosynthesis in Catharanthus roseus. Plant Physiol 177: 1473-1486

Chae L, Kim T, Nilo-Poyanco R, Rhee SY (2014) Genomic signatures of specialized metabolism in plants. Science 344: 510-513

Chatel G, Montiel G, Pre M, Memelink J, Thiersault M, Saint-Pierre B, Doireau P, Gantet P (2003) CrMYC1, a Catharanthus roseus elicitor- and jasmonate-responsive bHLH transcription factor that binds the G-box element of the strictosidine synthase gene promoter. J Exp Bot 54: 2587-2588

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448: 666-671

Colinas M, Goossens A (2018) Combinatorial Transcriptional Control of Plant Specialized Metabolism. Trends Plant Sci 23: 324-336

De Boer K, Tillemans P, Pauwels L, Vanden Bossche R, De Sutter V, Vanderhaeghen R, Helson P, Hamill JD, Goossens A (2011) APETALA2/ETHYLENE RESPONSE FACTOR and basic helix–loop–helix tobacco transcription factors cooperatively mediate jasmonate-elicited nicotine biosynthesis. Plant J 66: 1053-1065

Fernandez-Pozo N, Menda N, Edwards JD, Saha S, Tecle IV, Strickler SR, Bombarely A, Fisher-York T, Pujar A, Foerster H, Yan A, Mueller LA (2015) The Sol Genomics Network (SGN)--from genotype to phenotype to breeding. Nucleic Acids Res 43: D1036-1041

Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, Salazar GA, Tate J, Bateman A (2016) The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res 44: D279-285
Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression. Plant Cell 12: 393-404

Ganko EW, Meyers BC, Vision TJ (2007) Divergence in Expression between Duplicated Genes in Arabidopsis. Mol Biol and Evol 24: 2298-2309

Giddings L-A, Liscombe DK, Hamilton JP, Childs KL, DellaPenna D, Buell CR, O’Connor SE (2011) A stereoselective hydroxylation step of alkaloid biosynthesis by a unique cytochrome P450 in Catharanthus roseus. J Biol Chem 286: 16751-16757

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J 16: 433-442

Hagen G, Guilfoyle T (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol 49: 373-385

He Z, Zhang H, Gao S, Lercher MJ, Chen WH, Hu S (2016) Evolview v2: an online visualization and management tool for customized and annotated phylogenetic trees. Nucleic Acids Res 44: W236-241

Kajikawa M, Sierro N, Kawaguchi H, Bakaher N, Ivanov NV, Hashimoto T, Shoji T (2017) Genomic Insights into the Evolution of the Nicotine Biosynthesis Pathway in Tobacco. Plant Physiol 174: 999-1011

Kazan K, Manners JM (2013) MYC2: The Master in Action. Molecular Plant 6: 686-703

Kellner F, Kim J, Clavijo BJ, Hamilton JP, Childs KL, Vaillancourt B, Cepela J, Habermann M, Steuernagel B, Clissold L, McLay K, Buell CR, O’Connor SE (2015) Genome-guided investigation of plant natural product biosynthesis. Plant J 82: 680-692

Laflyamme P, St-Pierre B, De Luca V (2001) Molecular and biochemical analysis of a Madagascar periwinkle root-specific minovincinine-19-hydroxy-O-acetyltransferase. Plant Physiol 125: 189-198

Lappin TR, Grier DG, Thompson A, Halliday HL (2006) HOX genes: seductive science, mysterious mechanisms. Ulster Med J 75: 23-31

Lewis RS, Jack AM, Morris JW, Robert VJ, Gavilano LB, Siminszky B, Bush LP, Hayes AJ, Dewey RE (2008) RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves. Plant Biotechnol J 6: 346-354

Li J, Zhang K, Meng Y, Hu J, Ding M, Bian J, Yan M, Han J, Zhou M (2018) Jasmonic acid/Ethylene signaling coordinates hydroxycinnamic acid amides biosynthesis through ORA59 transcription factor. Plant J 95: 444-457

Liscombe DK, Usera AR, O’Connor SE (2010) Homolog of tocopherol C methyltransferases catalyzes N methylation in anticancer alkaloid biosynthesis. Proc Natl Acad Sci USA 107: 18793-18798

Liu Y, Du M, Deng L, Shen J, Fang M, Chen Q, Lu Y, Wang Q, Li C, Zhai Q (2019) MYC2 Regulates the Termination of Jasmonate Signaling via an Autoregulatory Negative Feedback Loop. Plant Cell 31: 106-127

Lu X, Zhang L, Zhang F, Jiang W, Shen Q, Zhang L, Lv Z, Wang G, Tang K (2013) AaORA, a trichome-specific AP2/ERF transcription factor of Artemisia annua, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to Botrytis cinerea. New Phytol 198: 1191-1202
Matsui K, Umemura Y, Ohme-Takagi M (2008) AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant J 55: 954-967

Mirjalili N, Linden JC (1996) Methyl jasmonate induced production of taxol in suspension cultures of Taxus cuspidata: ethylene interaction and induction models. Biotech Progress 12: 110-118

Montavon T, Duboule D (2013) Chromatin organization and global regulation of Hox gene clusters. Philos Trans R Soc Lond B Biol Sci 368: 20120367

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol 140: 411-432

Nakayasu M, Shioya N, Shikata M, Thagun C, Abdelkareem A, Okabe Y, Ariizumi T, Arimura GI, Mizutani M, Ezura H, Hashimoto T, Shoji T (2018) JRE4 is a master transcriptional regulator of defense-related steroidal glycoalkaloids in tomato. Plant J 94:975-990

Nützmann H-W, Osbourn A (2014) Gene clustering in plant specialized metabolism. Curr Opin Biotech 26: 91-99

Nützmann HW, Huang A, Osbourn A (2016) Plant metabolic clusters–from genetics to genomics. New Phytol 211: 771-789

Patra B, Pattanaik S, Schluttenhofer C, Yuan L (2018) A network of jasmonate-responsive bHLH factors modulate monoterpenoid indole alkaloid biosynthesis in Catharanthus roseus. New Phytol 217: 1566-1581

Pattanaik S, Kong Q, Zaitlin D, Werkman J, Xie C, Patra B, Yuan L (2010) Isolation and functional characterization of a floral tissue-specific R2R3 MYB regulator from tobacco. Planta 231: 1061-1076

Pattanaik S, Werkman JR, Kong Q, Yuan L (2010) Site-Directed Mutagenesis and Saturation Mutagenesis for the Functional Study of Transcription Factors Involved in Plant Secondary Metabolite Biosynthesis. In AG Fett-Neto, ed, Plant Secondary Metabolism Engineering: Methods and Applications. Humana Press, Totowa, NJ, pp 47-57

Paul P, Singh SK, Patra B, Sui X, Pattanaik S, Yuan L (2017) A differentially regulated AP2/ERF transcription factor gene cluster acts downstream of a MAP kinase cascade to modulate terpenoid indole alkaloid biosynthesis in Catharanthus roseus. New Phytol 213: 1107-1123

Pauw B, Hilliou FAO, Martin VS, Chatel G, de Wolf CJF, Champion A, Pré M, van Duijn B, Kijne JW, van der Fits L, Memelink J (2004) Zinc Finger Proteins Act as Transcriptional Repressors of Alkaloid Biosynthesis Genes in Catharanthus roseus. J Biol Chem 279: 52940-52948

Peebles CA, Hughes EH, Shanks JV, San KY (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

Qu Y, Easson M, Simionescu R, Hajicek J, Thamm AMK, Salim V, De Luca V (2018) Solution of the multistep pathway for assembly of corynanthean, strychnos, iboga, and aspidosperma monoterpenoid indole alkaloids from 19E-geissoschizine. Proc Natl Acad Sci U S A 115: 3180-3185

Qu Y, Safonova O, De Luca V (2019) Completion of the canonical pathway for assembly of anticancer drugs vincristine/vinblastine in Catharanthus roseus. Plant J 97: 257-266
Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T, Perroud P-F, Lindquist EA, Kamisugi Y (2008) The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science 319: 64-69

Sanchez-Perez R, Pavan S, Mazzeo R, Moldovan C, Aiese Ciglione R, Del Cueto J, Ricciardi F, Lotti C, Ricciardi L, Dicenta F, Lopez-Marques RL, Moller BL (2019) Mutation of a bHLH transcription factor allowed almond domestication. Science 364: 1095-1098

Schweizer F, Colinas M, Poller J, Van Moerkercke A, Vanden Bossche R, de Clercq R, Goossens A (2018) An engineered combinatorial module of transcription factors boosts production of monoterpenoid indole alkaloids in Catharanthus roseus. Metab Eng 48: 150-162

Shen Q, Lu X, Yan T, Fu X, Lv Z, Zhang F, Pan Q, Wang G, Sun X, Tang K (2016) The jasmonate-responsive AaMYC2 transcription factor positively regulates artemisinin biosynthesis in Artemisia annua. New Phytol 210: 1269-1281

Shoji T, Hashimoto T (2011) Tobacco MYC2 regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NIC2-locus ERF genes. Plant Cell Physiol 52: 1117-1130

Shoji T, Kajikawa M, Hashimoto T (2010) Clustered transcription factor genes regulate nicotine biosynthesis in tobacco. Plant Cell 22: 3390-3409

Shoji T, Mishima M, Hashimoto T (2013) Divergent DNA-binding specificities of a group of ETHYLENE RESPONSE FACTOR transcription factors involved in plant defense. Plant Physiol 162: 977-990

Shoji T, Nakajima K, Hashimoto T (2000) Ethylene Suppresses Jasmonate-Induced Gene Expression in Nicotine Biosynthesis. Plant and Cell Physiology 41: 1072-1076

Suttipanta N, Pattanaik S, Gunjan S, Xie CH, Littleton J, Yuan L (2007) Promoter analysis of the Catharanthus roseus geraniol 10-hydroxylase gene involved in terpenoid indole alkaloid biosynthesis. Biochim Biophys Acta 1769: 139-148

Suttipanta N, Pattanaik S, Kulshrestha M, Patra B, Singh SK, Yuan L (2011) The Transcription Factor CrWRKY1 Positively Regulates the Terpenoid Indole Alkaloid Biosynthesis in Catharanthus roseus. Plant Physiol 157: 2081-2093

Thagun C, Imanishi S, Kudo T, Nakabayashi R, Ohyama K, Mori T, Kawamoto K, Nakamura Y, Katayama M, Nonaka S, Matsukura C, Yano K, Ezura H, Saito K, Hashimoto T, Shoji T (2016) Jasmonate-Responsive ERF Transcription Factors Regulate Steroidal Glycoalkaloid Biosynthesis in Tomato. Plant Cell Physiol 57: 961-975

Udomsom N, Rai A, Suzuki H, Okuyama J, Imai R, Mori T, Nakabayashi R, Saito K, Yamazaki M (2016) Function of AP2/ERF Transcription Factors Involved in the Regulation of Specialized Metabolism in Ophiorrhiza pumila Revealed by Transcriptomics and Metabolomics. Front Plant Sci 7: 1861

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 der Heijden R, Schripsema J, Verpoorte R (2004) The Catharanthus alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11: 607-628
Van Moerkercke A, Steensma P, Gariboldi I, Espoz J, Purnama PC, Schweizer F, Miettinen K, Vanden Bossche R, De Clercq R, Memelink J, Goossens A (2016) The basic helix-loop-helix transcription factor BIS2 is essential for monoterpenoid indole alkaloid production in the medicinal plant Catharanthus roseus. Plant J 88: 3-12
Van Moerkercke A, Steensma P, Schweizer F, Pollier J, Gariboldi I, Payne R, Vanden Bossche R, Miettinen K, Espoz J, Purnama PC, Kellner F, Seppanen-Laakso T, O’Connor SE, Rischer H, Memelink J, Goossens A (2015) The bHLH transcription factor BIS1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in Catharanthus roseus. Proc Natl Acad Sci U S A 112: 8130-8135
Wang C-T, Liu H, Gao X-S, Zhang H-X (2010) Overexpression of G10H and ORCA3 in the hairy roots of Catharanthus roseus improves catharanthine production. Plant Cell Rep 29: 887-894
Williams D, Qu Y, Simionescu R, De Luca V (2019) The assembly of (+)-vincadifformine- and (-)-tabersonine-derived monoterpenoid indole alkaloids in Catharanthus roseus involves separate branch pathways. Plant J 99: 626-636
Winz RA, Baldwin IT (2001) Molecular Interactions between the Specialist Herbivore Manduca sexta (Lepidoptera, Sphingidae) and Its Natural Host Nicotiana attenuata. IV. Insect-Induced Ethylene Reduces Jasmonate-Induced Nicotine Accumulation by Regulating Putrescine N-Methyltransferase Transcripts. Plant Physiol 125: 2189-2202
Yang SH, Berberich T, Sano H, Kusano T (2001) Specific association of transcripts of tbzF and tbz17, tobacco genes encoding basic region leucine zipper-type transcriptional activators, with guard cells of senescing leaves and/or flowers. Plant Physiol 127: 23-32
Yu Z-X, Li J-X, Yang C-Q, Hu W-L, Wang L-J, Chen X-Y (2012) The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in Artemisia annua L. Mol Plant 5: 353-365
Zhang H, Koes R, Shang H, Fu Z, Wang L, Dong X, Zhang J, Passeri V, Li Y, Jiang H, Gao J, Li Y, Wang H, Quattrocchio FM (2019) Identification and functional analysis of three new anthocyanin R2R3-MYB genes in Petunia. Plant Direct 3: e00114
Zhang P, Chopra S, Peterson T (2000) A segmental gene duplication generated differentially expressed myb-homologous genes in maize. Plant Cell 12: 2311-2322
Zhang X, Fowler SG, Cheng H, Lou Y, Rhee SY, Stockinger EJ, Thomashow MF (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant Arabidopsis. Plant J 39: 905-919
Zhao J, Zheng SH, Fujita K, Sakai K (2004) Jasmonate and ethylene signalling and their interaction are integral parts of the elicitor signalling pathway leading to β-thujaplicin biosynthesis in Cupressus lusitanica cell cultures. J Exp Bot 55: 1003-1012
Zhou M-L, Zhu X-M, Shao J-R, Wu Y-M, Tang Y-X (2010) Transcriptional response of the catharanthine biosynthesis pathway to methyl jasmonate/nitric oxide elicitation in Catharanthus roseus hairy root culture. Appl Microbiol Biotechnol 88: 737-750
Boycheva S, Daviet L, Wolfender JL, Fitzpatrick TB (2014) The rise of operon-like gene clusters in plants. Trends Plant Sci 19: 447-459
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+: architecture and applications. BMC Bioinformatics 10: 421
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Cardenas PD, Sonawane PD, Pollier J, Vanden Bossche R, Dewangan V, Weithorn E, Tal L, Meir S, Rogachev I, Malitsky S, Giri AP, Goossens A, Burdman S, Aharoni A (2016) GAME9 regulates the biosynthesis of steroidal alkaloids and upstream isoprenoids in the plant mevalonate pathway. Nat Commun 7: 10654
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Carqueijeiro I, Brown S, Chung K, Dang TT, Walia M, Besseau S, Duge de Bermonville T, Oudin A, Lanoue A, Billet K, Munsch T, Koudounakas N, Melin C, Godon C, Razafirondimby B, de Craene JO, Glevarec G, Marc J, Giglioli-Guivarc’h N, Claстрre M, St-Pierre B, Papon N, Andrade RB, O’Connor SE, Coudavault V (2018) Two Tabersonine 6,7-Epoxidases Initiate Lochnericine-Derived Alkaloid Biosynthesis in Catharanthus roseus. Plant Physiol 177: 1473-1486
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Carqueijeiro I, Duge de Bermonville T, Lanoue A, Dang TT, Teijaro CN, Paetz C, Billet K, Mosquera A, Oudin A, Besseau S, Papon N, Glevarec G, Aethorlau T, Classtrre M, Giglioli-Guivarc’h N, Schneider B, St-Pierre B, Andrade RB, O’Connor SE, Coudavault V (2018) A BAHD acyltransferase catalyzing 19-O-acetylation of tabersonine derivatives in roots of Catharanthus roseus enables combinatorial synthesis of monoterpenoid indole alkaloids. Plant J 94: 469-484
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chae L, Kim T, Nilo-Poyanco R, Rhee SY (2014) Genomic signatures of specialized metabolism in plants. Science 344: 510-513
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chatel G, Montiel G, Pre M, Memelink J, Thiersault M, Saint-Pierre B, Doireau P, Gantet P (2003) CrMYC1, a Catharanthus roseus elicitor- and jasmonate-responsive bHLH transcription factor that binds the G-box element of the strictosidine synthase gene promoter. J Exp Bot 54: 2587-2588
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448: 666-671
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Colinas M, Goossens A (2018) Combinatorial Transcriptional Control of Plant Specialized Metabolism. Trends Plant Sci 23: 324-336
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

De Boer K, Tilleman S, Pauwels L, Vanden Bossche R, De Sutter V, Vanderhaeghen R, Hilson P, Hamill JD, Goossens A (2011) APETALA2/ETHYLENE RESPONSE FACTOR and basic helix–loop–helix tobacco transcription factors cooperatively mediate jasmonate-elicited nicotine biosynthesis. Plant J 66: 1053-1065
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Fernandez-Pozo N, Menda N, Edwards JD, Saha S, Tedc IY, Strickler SR, Bombarely A, Fisher-York T, Pujar A, Foerster H, Yan A, Mueller LA (2015) The Sol Genomics Network (SGN)—from genotype to phenotype to breeding. Nucleic Acids Res 43: D1036-1041
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Finn RD, Coggill P, Eberhardt RY, Eddy SR, Punta M, Qureshi M, Sangrador-Vegas A, Salazar GA, Tate J, Bateman A (2016) The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res 44: D279-285
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohsako-Takagi M (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression. Plant Cell 12: 393-404
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ganko EW, Meyers BC, Vision TJ (2007) Divergence in Expression between Duplicated Genes in Arabidopsis. Mol Biol and Evol 24: 2298-2309
Giddings L-A, Liscombe DK, Hamilton JP, Childs KL, DellaPenna D, Buell CR, O'Connor SE (2011) A stereoselective hydroxylation step of alkaloid biosynthesis by a unique cytochrome P450 in Catharanthus roseus. J Biol Chem 286: 16751-16757

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J 16: 433-442

Hagen G, Guilfoyle T (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol 49: 373-385

He Z, Zhang H, Gao S, Lercher MJ, Chen WH, Hu S (2016) Evolview v2: an online visualization and management tool for customized and annotated phylogenetic trees. Nucleic Acids Res 44: W236-241

Kajikawa M, Sierro N, Kawaguchi H, Bakaher N, Ivanov NV, Hashimoto T, Shoji T (2017) Genomic Insights into the Evolution of the Nicotine Biosynthesis Pathway in Tobacco. Plant Physiol 174: 999-1011

Kazan K, Manns JM (2013) MYC2: The Master in Action. Molecular Plant 6: 686-703

Kellner F, Kim J, Clavijo BJ, Hamilton JP, Childs KL, Vaillancourt B, Cepela J, Habermann M, Steuernagel B, Clissold L, McClay K, Buell CR, O'Connor SE (2015) Genome-guided investigation of plant natural product biosynthesis. Plant J 82: 680-692

Lafframme P, St-Pierre B, De Luca V (2001) Molecular and biochemical analysis of a Madagascar periwinkle root-specific minovincinine-19-hydroxy-O-acetyltransferase. Plant Physiol 125: 189-198

Lappin TR, Grier DG, Thompson A, Halliday HL (2006) HOX genes: seductive science, mysterious mechanisms. Ulster Med J 75: 23-31

Lewis RS, Jack AM, Morris JW, Robert VJ, Gavilano LB, Siminszky B, Bush LP, Hayes AJ, Dewey RE (2008) RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves. Plant Biotechnol J 6: 346-354

Li J, Zhang K, Meng Y, Hu J, Ding M, Bian J, Yan M, Han J, Zhou M (2018) Jasmone/ethylene signaling coordinates hydroxycinnamic acid amides biosynthesis through ORA59 transcription factor. Plant J 95:444-457

Liscombe DK, Usera AR, O'Connor SE (2010) Homolog of tocopherol C methyltransferases catalyzes N methylation in anticancer alkaloid biosynthesis. Proc Natl Acad Sci USA 107: 18793-18798

Liu Y, Du M, Deng L, Shen J, Fang M, Chen Q, Lu Y, Wang Q, Li C, Zhai Q (2019) MYC2 Regulates the Termination of Jasmonate Signaling via an Autoregulatory Negative Feedback Loop. Plant Cell 31:106-127

Liu X, Zhang L, Zhang F, Jiang W, Shen Q, Zhang L, Lv Z, Wang G, Tang K (2013) AeORA, a trichome-specific AP2/ERF transcription factor of Artemisia annua, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to Botrytis cinerea. New Phytol 198: 1191-1202

Matsui K, Umemura Y, Ohme-Takagi M (2008) A MYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant J 55: 954-967
Mirjalili N, Linden JC (1996) Methyl jasmonate induced production of taxol in suspension cultures of Taxus cuspidata: ethylene interaction and induction models. Biotech Progress 12: 110-118

Montavon T, Duboule D (2013) Chromatin organization and global regulation of Hox gene clusters. Philos Trans R Soc Lond B Biol Sci 368: 20120367

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol 140: 411-432

Nakayasu M, Shiyoa N, Shikata M, Thagun C, Abdelkareem A, Okabe Y, Ariizumi T, Arimura GI, Mizutani M, Ezura H, Hashimoto T, Shoji T (2018) JRE4 is a master transcriptional regulator of defense-related steroidal glycoalkaloids in tomato. Plant J 94:975-990

Nützmann H-W, Osbourn A (2014) Gene clustering in plant specialized metabolism. Curr Opin Biotech 26: 91-99

Nützmann HW, Huang A, Osbourn A (2016) Plant metabolic clusters—from genetics to genomics. New Phytol 211: 771-789

Pattanaik S, Kong Q, Zaitlin D, Werkman J, Xie C, Patra B, Yuan L (2010) Isolation and functional characterization of a floral tissue-specific R2R3 MYB regulator from tobacco. Planta 231: 1061-1076

Patra B, Pattanaik S, Schluttenhofer C, Yuan L (2018) A network of jasmonate-responsive bHLH factors modulate monoterpenoid indole alkaloid biosynthesis in Catharanthus roseus. New Phytol 217: 1566-1581

Pattanaik S, Werkman JR, Kong Q, Yuan L (2010) Site-Directed Mutagenesis and Saturation Mutagenesis for the Functional Study of Transcription Factors Involved in Plant Secondary Metabolite Biosynthesis. In AG Fett-Neto, ed, Plant Secondary Metabolism Engineering: Methods and Applications. Humana Press, Totowa, NJ, pp 47-57

Paul P, Singh SK, Patra B, Sui X, Pattanaik S, Yuan L (2017) A differentially regulated AP2/ERF transcription factor gene cluster acts downstream of a MAP kinase cascade to modulate terpenoid indole alkaloid biosynthesis in Catharanthus roseus. New Phytol 213: 1107-1123

Pauw B, Hilliou FA O, Martin VS, Chatel G, de Wolf CJF, Champin A, Pré M, van Duijn B, Kijne JW, van der Fits L, Memelink J (2004) Zinc Finger Proteins Act as Transcriptional Repressors of Alkaloid Biosynthesis Genes in Catharanthus roseus. J Biol Chem 279: 52940-52948

Peebles CA, Hughes EH, Shanks JV, San KY (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

Qu Y, Easson M, Simionescu R, Hajicek J, Thamm AMK, Salim V, De Luca V (2018) Solution of the multistep pathway for assembly of corynanthean, strychnos, iboga, and aspidosperma monoterpenoid indole alkaloids from 19E-geissoschizine. Proc Natl Acad Sci U S A 115: 3180-3185

Qu Y, Safonova O, De Luca V (2019) Completion of the canonical pathway for assembly of anticancer drugs vincristine/vinblastine in Catharanthus roseus. Plant J 97: 257-266

Rensing SA, Lang D, Zimmer AD, Troup S, Salamov A, Shapiro H, Nishiyama T, Peyrout P, Lindquist EA, Kamisugi Y (2008)
Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science 319: 64-69

Sanchez-Perez R, Pavan S, Mazzeo R, Moldovan C, Aiese Cigliano R, Del Cueto J, Ricciardi F, Lotti C, Ricciardi L, Dicenta F, Lopez-Marques RL, Moller BL (2019) Mutation of a bHLH transcription factor allowed almond domestication. Science 364: 1095-1098

Schweizer F, Colinas M, Pollier J, Van Moerkercke A, Vandens Bossche R, de Clercq R, Goossens A (2018) An engineered combinatorial module of transcription factors boosts production of monoterpenoid indole alkaloids in Catharanthus roseus. Metab Eng 48: 150-162

Shen Q, Lu X, Yan T, Fu X, Lv Z, Zhang F, Pan Q, Wang G, Sun X, Tang K (2016) The jasmonate-responsive AaMYC2 transcription factor positively regulates artemisinin biosynthesis in Artemisia annua. New Phytol 210: 1269-1281

Shoji T, Hashimoto T (2011) Tobacco MYC2 regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NIC2-locus ERF genes. Plant Cell Physiol 52: 1117-1130

Shoji T, Kajikawa M, Hashimoto T (2010) Clustered transcription factor genes regulate nicotine biosynthesis in tobacco. Plant Cell 22: 3390-3409

Shoji T, Mishima M, Hashimoto T (2013) Divergent DNA-binding specificities of a group of ETHYLENE RESPONSE FACTOR transcription factors involved in plant defense. Plant Physiol 162: 977-990

Shoji T, Nakajima K, Hashimoto T (2000) Ethylene Suppresses Jasmonate-Induced Gene Expression in Nicotine Biosynthesis. Plant and Cell Physiology 41: 1072-1076

Suttipanta N, Pattanaik S, Gunjan S, Xie CH, Littleton J, Yuan L (2007) Promoter analysis of the Catharanthus roseus geraniol 10-hydroxylase gene involved in terpenoid indole alkaloid biosynthesis. Biochim Biophys Acta 1769: 139-148

Suttipanta N, Pattanaik S, Kulshrestha M, Patra B, Singh SK, Yuan L (2011) The Transcription Factor CrWRKY1 Positively Regulates the Terpenoid Indole Alkaloid Biosynthesis in Catharanthus roseus. Plant Physiol 157: 2081-2093

Thagun C, Imamishi S, Kudo T, Nakabayashi R, Ohyama K, Mori T, Kamamoto K, Nakamura Y, Katayama M, Nonaka S, Matsukura C, Yano K, Ezura H, Saito K, Hashimoto T, Shoji T (2016) Jasmonate-Responsive ERF Transcription Factors Regulate Steroidal Glycoalkaloid Biosynthesis in Tomato. Plant Cell Physiol 57: 961-975

Udomsom N, Rai A, Suzuki H, Okuyama J, Imai R, Mori T, Nakabayashi R, Saito K, Yamazaki M (2016) Function of AP2/ERF Transcription Factors Involved in the Regulation of Specialized Metabolism in Ophiirrhiza pumila Revealed by Transcriptomics and Metabolomics. Front Plant Sci 7: 1861

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 der Heijden R, Schripsema J, Verpoorte R (2004) The Catharanthus alkaloids: pharmacoognosy and biotechnology. Curr Med Chem 11: 607-628
Van Moerkercke A, Steensma P, Gariboldi I, Espoz J, Purnama PC, Schweizer F, Miettinen K, Vanden Bossche R, De Clercq R, Memelink J, Goossens A (2016) The basic helix-loop-helix transcription factor BIS2 is essential for monoterpenoid indole alkaloid production in the medicinal plant Catharanthus roseus. Plant J 88: 3-12

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Van Moerkercke A, Steensma P, Schweizer F, Pollier J, Gariboldi I, Payne R, Vanden Bossche R, Miettinen K, Espoz J, Purnama PC, Kellner F, Seppanen-Laakso T, O’Connor SE, Rischer H, Memelink J, Goossens A (2015) The bHLH transcription factor BIS1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in Catharanthus roseus. Proc Natl Acad Sci U S A 112: 8130-8135

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Wang C-T, Liu H, Gao X-S, Zhang H-X (2010) Overexpression of G10H and ORCA3 in the hairy roots of Catharanthus roseus improves catharanthine production. Plant Cell Rep 29: 887-894

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Wang C-T, Liu H, Gao X-S, Zhang H-X (2010) Overexpression of G10H and ORCA3 in the hairy roots of Catharanthus roseus improves catharanthine production. Plant Cell Rep 29: 887-894

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Yu Z-X, Li J-X, Yang C-Q, Hu W-L, Wang L-J, Chen X-Y (2012) The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in Artemisia annua L. Mol Plant 5: 353-365

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Zhang H, Koes R, Shang H, Fu Z, Wang L, Dong X, Zhang J, Passeri V, Li Y, Jiang H, Gao J, Li Y, Wang H, Quattrocchio FM (2019) Identification and functional analysis of three new anthocyanin R2R3-MYB genes in Petunia. Plant Direct 3: e00114

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Zhang H, Koes R, Shang H, Fu Z, Wang L, Dong X, Zhang J, Passeri V, Li Y, Jiang H, Gao J, Li Y, Wang H, Quattrocchio FM (2019) Identification and functional analysis of three new anthocyanin R2R3-MYB genes in Petunia. Plant Direct 3: e00114

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Zhao J, Zheng SH, Fujita K, Sakai K (2004) Jasmonate and ethylene signalling and their interaction are integral parts of the elicitor signalling pathway leading to β-thujaplicin biosynthesis in Cupressus lusitanica cell cultures. J Exp Bot 55: 1003-1012

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title

Zhou M-L, Zhu X-M, Shao J-R, Wu Y-M, Tang Y-X (2010) Transcriptional response of the catharanthine biosynthesis pathway to methyl jasmonate/nitric oxide elicitation in Catharanthus roseus hairy root culture. Appl Microbiol Biotechnol 88: 737-750

Pubmed: Author and Title
Google Scholar: Author Only, Title Only, Author and Title