Metabolomics analysis reveals both plant variety and choice of hormone treatment modulate vinca alkaloid production in *Catharanthus roseus*.

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ABSTRACT.

The medicinal plant *Catharanthus roseus* produces numerous secondary metabolites of interest for the treatment of many diseases—most notably for the terpene indole alkaloid (TIA) vinblastine, which is used in the treatment of leukemia and Hodgkin's lymphoma. Historically, methyl jasmonate (MeJA) has been used to induce TIA production, but in the past, this has only been investigated in either whole seedlings, cell culture, or hairy root culture. In this work, we investigate the induction capabilities of MeJA and ethylene, a different phytohormone, in both the shoots and roots of two varieties of *C. roseus*. Using LCMS and RT-qPCR, we demonstrate the importance of variety selection, as we observe markedly different induction patterns of important TIA precursor compounds. Additionally, both phytohormone choice and concentration have significant effects on TIA biosynthesis. Finally, our study suggests that several early-induction pathway steps as well as pathway-specific genes are likely to be transcriptionally regulated. Our findings highlight the need for a complete set of 'omics resources in commonly used *C. roseus* varieties.

Many plant-derived secondary metabolites have chemical properties that give them therapeutic value for the treatment of cancers, hypertension, and other illnesses[1]. In the medicinal plant *Catharanthus roseus* (L.) G. Don, the terpene indole alkaloid (TIA) family of natural products, which include the clinically used antineoplastic agents vinblastine and vincristine, and the antihypertensive agent ajmalicine. Vinblastine and vincristine, used in the treatment of lymphoblastic leukemia[2, 3] are naturally produced at low levels in the leaves of the plant, which makes the chemical extraction of the two alkaloids difficult and time consuming[4]. Pharmaceutical scientists generally extract the more abundant precursor compounds from the leaf and perform an *in vitro* coupling to increase the yield of vinblastine and vincristine, which is then isolated[5, 6]; this process, however, can be cost prohibitive. The plant signaling hormone methyl jasmonate (MeJA) is typically used to induce higher levels of the alkaloid products in the laboratory through gaseous or hydroponic application after being dissolved in DMSO[7, 8], but recent studies have found that ethylene also induces alkaloid production[9, 10]. While MeJA is too expensive for practical use in a large-scale agricultural production, ethephon (a commercially available ethylene derivative) is a viable and cost-effective option for increasing alkaloid yields prior to chemical extraction.

Over the last 50 years, vinca alkaloid production has been induced *in planta* with MeJA via root-uptake from growth medium or through exposure to vapor in an enclosed system[7-9]. Ethylene has only recently been identified as an induction agent[10, 11]; when used, it is generally applied via addition to growth medium or foliar application[9, 10]. Foliar application of ethephon (ETPN), a compound that is quickly converted to ethylene upon uptake into the cell, does not require any special equipment and is a method that can be straightforwardly transferred from a laboratory setting into a greenhouse setting for agricultural-scale production of these desirable

compounds. If large scale biopharmaceutical production is the ultimate goal, foliar ETPN treatment is ideal since it is inexpensive and does not need to be reapplied to obtain the desired result.

Hairy root culture is a commonly studied system with strong potential for *C. roseus* for alkaloid production and extraction; however, it is a technically challenging system[12]. In particular, this and similar culture systems require special equipment and impeccable sterile technique to prevent contamination. Additionally, not all precursor alkaloids of interest in the TIA pathway can be found in the roots at levels that would make extraction viable (e.g. vindoline) in the absence of further genetic engineering developments in this system[13-15], and those that are present are regulated differently than in seedlings[16]. Alternatively, *C. roseus* seeds are easy to germinate and are relatively fast-growing in soil. Gently uprooting seedlings from the soil and thoroughly washing in deionized water allows collection of all parts of the plant in a relatively short amount of time and with minimal concern regarding contamination. These considerations make plants a good system not only for biological studies but also provides potential for greenhouse-level scale-up of alkaloid precursor production.

Many different cultivars of *C. roseus* have been developed for ornamental uses and, of those, some have also been evaluated for their utility in alkaloid production. Of this genetic diversity, however, only a few have been selected for genomic resource development[17-20]. “Little Bright Eye” (LBE) is a variety that was commonly used for plant pathology research and was used in the initial efforts to identify the TIA biosynthetic genes. More recently other varieties have been investigated as part of transcriptional and metabolomic studies[17-20]. “SunStorm Apricot” (SSA) was developed for horticultural use and recently was subjected to genome sequencing[18]. Given that different varieties of *C. roseus* have been used by different groups in transcriptomic,

genomic, metabolomic studies of the TIA pathway (Table S1), we sought to investigate the inter-varietal differences in alkaloid production and response to stimuli. With this in mind, we designed a study of the alkaloid induction patterns of ethylene and MeJA in these two varieties of *C. roseus* (LBE vs. SSA). As shoots and roots are very different from one another, we chose to separate the plant into two parts, which has not been done in previous *C. roseus* work. Testing hormonal induction in LBE has allowed us to compare to previous studies, while including SSA provides an opportunity for future investigation into the regulation of induction on a genome-wide scale.

In this work, the natural differences in alkaloid levels between the two varieties were investigated. Additionally, the *in planta* effects of foliar MeJA or ETPN treatments on the metabolomic profiles in both roots and shoots of both varieties were revealed. Finally, this work examines the transcriptional effect of the phytohormones on the expression of genes involved in the terpene indole alkaloid biosynthetic pathway and its relation to the metabolomics profile. We show that unexpectedly, not only do varietal differences play a major role not only in hormonal induction influence on alkaloid production, but also genetic variation between SSA and LBE is substantial enough to affect wildtype levels of alkaloids in both roots and shoots.

RESULTS AND DISCUSSION

In order to investigate the effect that different phytohormone treatments have on the concentrations of key precursor alkaloids in SSA and LBE, we set up the randomized treatment experiment displayed in Figure 1. Seedlings from each variety were treated with control treatment (vehicle only) or methyl jasmonate (100 μM) or ethephon (100 μM or 1 mM) for 24 hours, and then were subsequently divided at the hypocotyl. After harvest, we extracted total

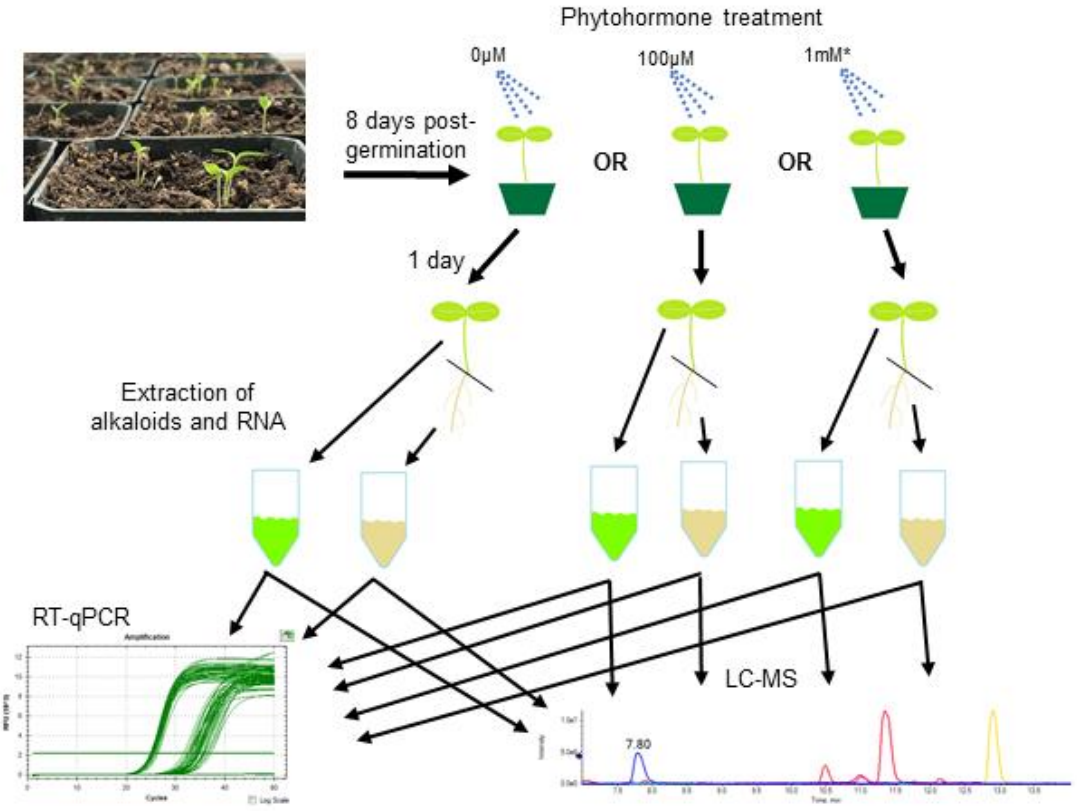

Figure 1 Experimental design of this study. Seeds of two Catharanthus roseus varieties (LBE and SSA) were sown and grown to 8 days post-germination. At that time, the plants were treated with various concentrations of either ethephon or MeJA. The asterisk on the 1mM denotes that the concentration was only used for ethephon. Seedlings were harvested 24 hours after treatment, divided into roots and shoots, and flash frozen. Total alkaloid content and RNA were extracted from the frozen tissues, which were then used for LCMS and RT-qPCR analyses.

RNA and total alkaloids from each treatment for use in LCMS and RT-qPCR analyses of selected components of the terpene indole alkaloid (TIA) biosynthesis pathway, as well as two upstream pathways (Figure 2). These experiments revealed that not only does ethephon induce the production of important precursor alkaloids at concentrations typically used in agricultural systems, but also that there are baseline differences between the two varieties. This is the first study comparing two research-relevant *C. roseus* varieties and measuring outcomes in both treated and untreated plants.

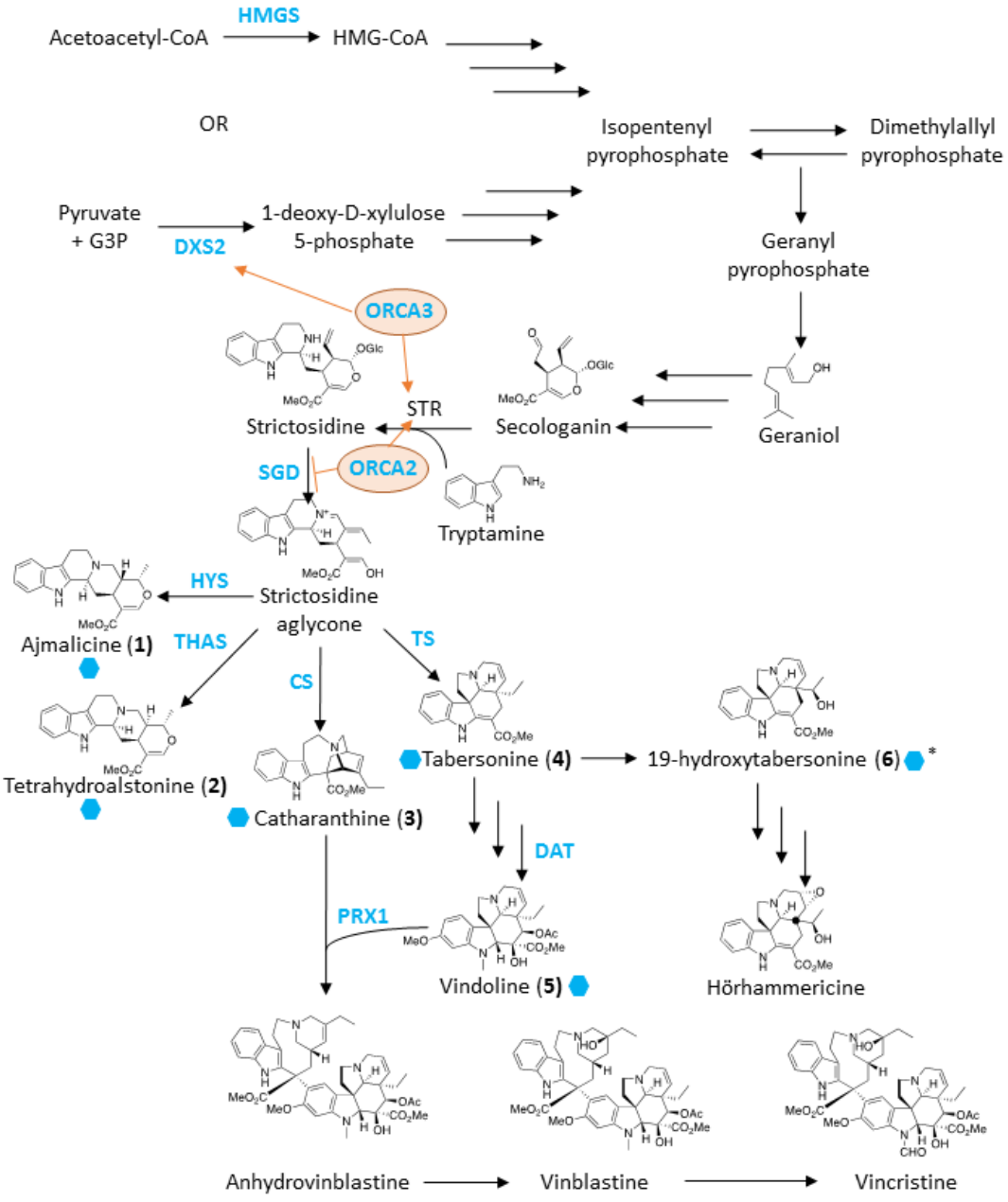

Figure 2 Pathway diagram from MVA and MEP to TIA. Three arrows symbolizes multiple enzymatic steps and intermediates. Abbreviations in blue are the genes selected for RT-qPCR. Orange ovals represent the TFs selected for RT-qPCR. Compounds marked with a hexagon were quantified on LCMS. An asterisk over the hexagon denotes our hypothesis for the identity of the uncharacterized compound.

Alkaloid levels substantially differ between varieties. Previous studies have investigated the genetic and metabolomics between many different *C. roseus* varieties[21, 22]. While many of the compared varieties are used mainly for ornamental purposes, others are historically used medicinally. None of the studies, however, included LBE and SSA which would allow for the utilization of available genomic and transcriptomic resources to inform future bioengineering efforts. We selected these two varieties for this exact reason. Prior to beginning these studies, we validated our extraction techniques to ensure that technical and biotic influences were minimized using *C. roseus* SSA. To test our technical reproducibility, we pulverized up 10-20 shoots in liquid nitrogen and then, after thorough mixing of the resulting powder, the powder was divided into three approximately equal portions of plant material. The replicate plant material was extracted and analyzed via LCMS (in technical duplicate) as described in the Experimental section. The vindoline concentration was determined to be 12.2 ± 2.7 μg/mg wet weight. To test for biological variability, 20 plants were grown under identical conditions and randomly allocated to three samples. The samples were pulverized in liquid nitrogen, extracted with methanol and analyzed by LCMS (in technical duplicate) as described in the Experimental section. In these samples the vindoline concentration was determined to be 10.92 ± 3.0 μg/mg wet weight. This demonstrated that our extraction protocol was reproducible and accurate.

A comparison of the spectrometric results of the untreated control plants highlights notable differences between the plant varieties themselves. As these control plants were only sprayed with deionized water and the pots were arranged randomly to avoid positional effects, changes observed are attributable to variety. Because some of the precursor alkaloids are restricted to certain plant parts[14, 15], we opted to divide our plants at the base of the hypocotyl (see Figure 1 above). In the shoots, untreated SSA plants have markedly higher levels catharanthine and

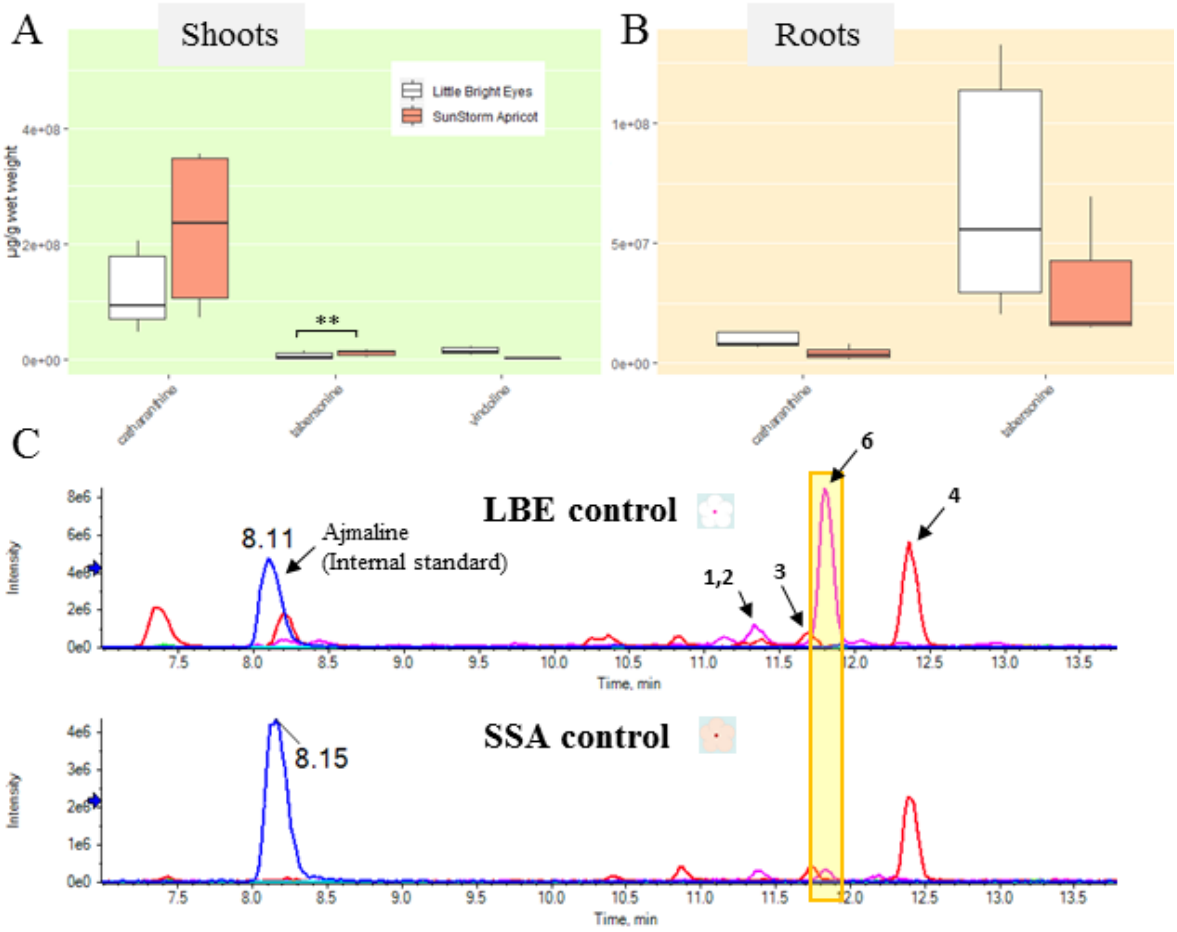

Figure 3 Alkaloid concentrations differ greatly between untreated plants of the two varieties. ** denotes a p-value ≤ 0.01 (A) In shoots, SunStorm Apricot has a higher concentration of catharanthine and tabersonine, while Little Bright Eye has a much greater concentration of vindoline. (B) In roots, Little Bright Eye has higher concentrations of catharanthine, tabersonine, and ajmalicine/tetrahydroalstonine. (C) Representative LCMS traces from the 0 µM ethephon treatment group; even in the control treatment, there are obvious differences between the varieties. Labeled peaks are as follows: 1 = ajmalicine; 2 = tetrahydroalstonine; 3 = catharanthine; 4 = tabersonine; 6 = 19-hydroxytabersonine (putative assignment).

tabersonine (Welch's t-test, $p \leq 0.01$), while LBE has a higher concentration of vindoline (Figure 3A). The difference in concentration of vindoline is nearly significant but falls just short due to LBE having much more intra-varietal variation (Welch's t-test, $p = 0.1033$). In roots, however, untreated LBE has markedly higher concentrations of catharanthine and tabersonine. (Figure 3B). An alkaloid with an m/z = 353 is present at high levels in the roots of untreated LBE and at lower levels in the roots of untreated SSA (Figure 3C). Due to this alkaloid's m/z and its retention time with respect to identified peaks, we suspect that this compound is a hydroxylated

tabersonine. We endeavored to confirm our hypothesis about our uncharacterized alkaloid's identity using commercially available standards for alkaloids with the appropriate molecular weight (including 11-hydroxytabersonine, yohimbine, and lochnericine), however none of the commercially available standards had an identical retention time as this unknown peak. We therefore posit that this compound is 19-hydroxytabersonine for which we were unable to identify a commercial supplier. If this is 19-hydroxytabersonine, a significant increase is not necessarily a desirable result as it channels tabersonine to hörhammericine, echitovenine, or minovincine—none of which are clinically used[23]. Overall, we observe that important differences arise in alkaloid concentration between varieties and between the tissues of these varieties, even without the application of an induction agent.

Induction of alkaloid levels differs markedly based on which phytohormone is used. As seen in other studies, treatment with these phytohormones induced alkaloid production[7, 9-11, 24]. In both shoots and roots, both methyl jasmonate (MeJA) and ethephon (ETPN) either caused an increase in alkaloid level or had no effect—there was no evidence of a significant decrease in any of the alkaloids examined.

For three of the alkaloids we investigated (catharanthine and ajmalicine/tetrahydroalstonine), treatment with MeJA increased the concentration in the shoots of both varieties but not significantly (Figure 4, S1). For the other two alkaloids, MeJA only increased the concentration in one of the varieties—in LBE for vindoline and significantly in SSA for tabersonine (Figure 4; Welch's t-test, $p \leq 0.01$). In LBE shoots, at least one ETPN treatment significantly increases the concentration of all five alkaloids investigated (Welch's t-test, $p \leq 0.01$). In SSA shoots, ETPN only significantly increases the levels of tabersonine (Welch's t-test, $p \leq 0.01$; it less significantly

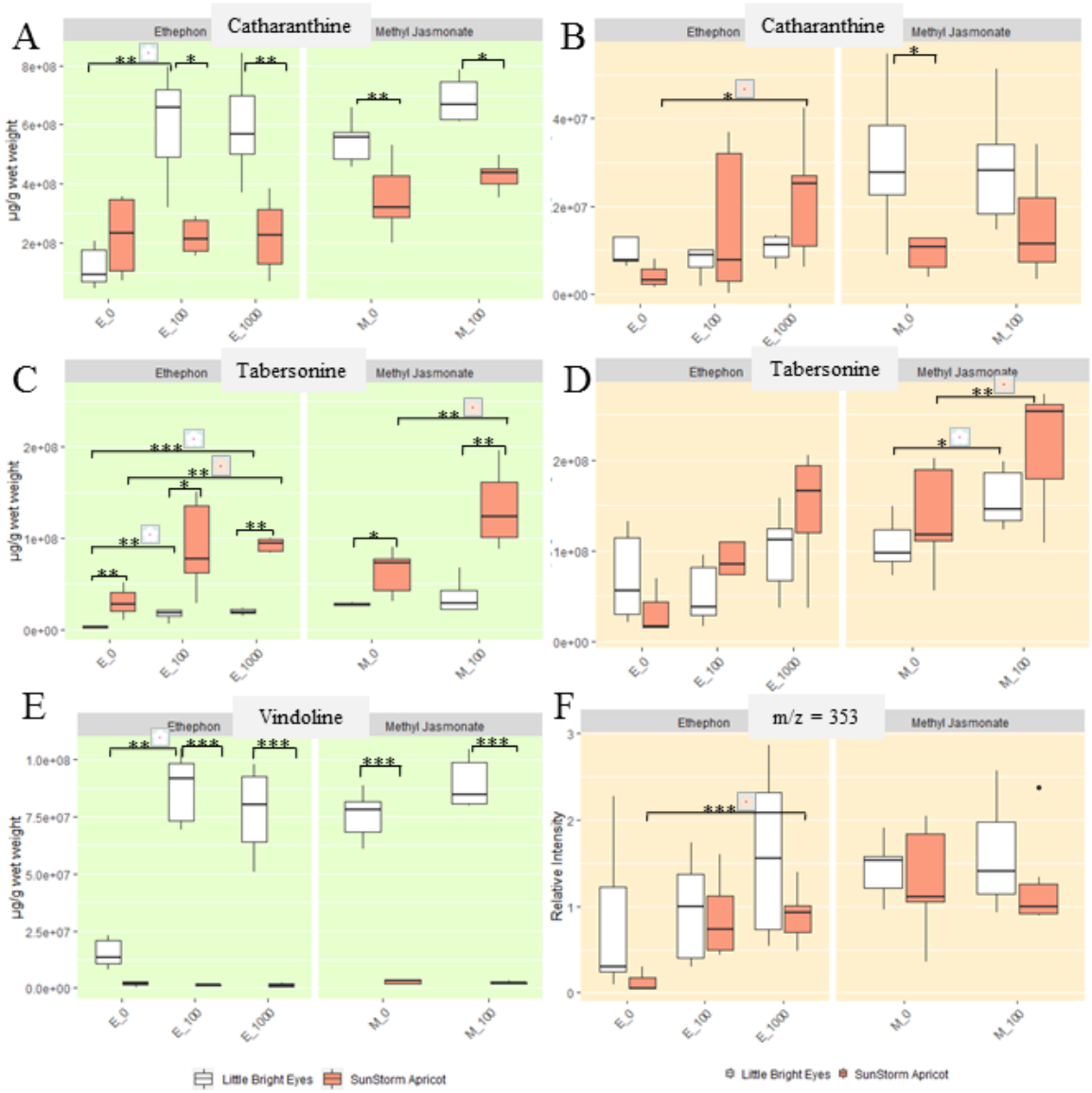

Figure 4 Alkaloid concentrations differ between varieties and treatment. * denotes a p-value ≤ 0.05; ** denotes a p-value ≤ 0.01; *** denotes a p-value ≤ 0.001; all represented statistics are from Welch's t-test post-hoc analyses. Significance markers with a white flower represent treatment differences in LBE, while those with a peach flower represent treatment differences in SSA. (A) Catharanthine concentrations increased in LBE shoots after treatment with both hormones, but only with methyl jasmonate in SSA shoots. (B) In roots, catharanthine increased markedly in SSA after treatment with ethephon. (C) In shoots, tabersonine levels increase greatly in SSA upon treatment with either phytohormone, but only after treatment with ethephon in LBE. (D) Tabersonine levels increase significantly in the roots of both varieties after treatment with either phytohormone. (E) Vindoline concentration increases significantly in LBE shoots after treatment with ethephon. (F) The amount of the unidentified alkaloid present relative to the internal standard increased significantly in the roots of SSA after treatment with ethephon.

increases vindoline and ajmalicine/tetrahydroalstonine. Despite multiple attempts, we were unable to separate ajmalicine and tetrahydroalstonine as they have the same mass, the same fragmentation pattern, and identical retention times. Therefore, we report ajmalicine/tetrahydroalstonine here as a single combined value relative to the internal standard.

In the roots, MeJA increases levels of three alkaloids (tabersonine in both varieties; ajmalicine/tetrahydroalstonine in SSA only), but not significantly (Figure 4, S1; Welch's t-test, $p \geq 0.05$). ETPN significantly increased all five of the alkaloids investigated in SSA (Welch's t-test, $p \leq 0.05$). In the roots of LBE, only tabersonine and ajmalicine/tetrahydroalstonine are increased by treatment with ETPN, but neither increase is particularly significant.

Overall, ethephon significantly increased the levels of a larger number of alkaloids than MeJA. All of the alkaloids mentioned in this study are valuable precursors in the TIA pathway. Additionally, the interaction between treatment and variety was significant for some of the alkaloids. In shoots, catharanthine and vindoline were significantly increased by the interaction of ETPN and variety while the interaction of MeJA and variety significantly affected tabersonine. In the roots, however, none of the alkaloids had significant interaction effects.

Master regulators are upregulated by hormonal induction. The ORCA family of transcription factors have been documented as central regulators of early stage TIA intermediate production in *C. roseus*. In the shoots of both varieties, the higher concentration of ETPN induces an increase in the number of ORCA2 transcripts (Fig 5A; ANOVA, $p \leq 0.001$). ORCA2 transcripts in roots, however, respond oppositely in the two varieties: increasing with ETPN treatment in SSA while decreasing in LBE and decreasing with MeJA treatment in SSA

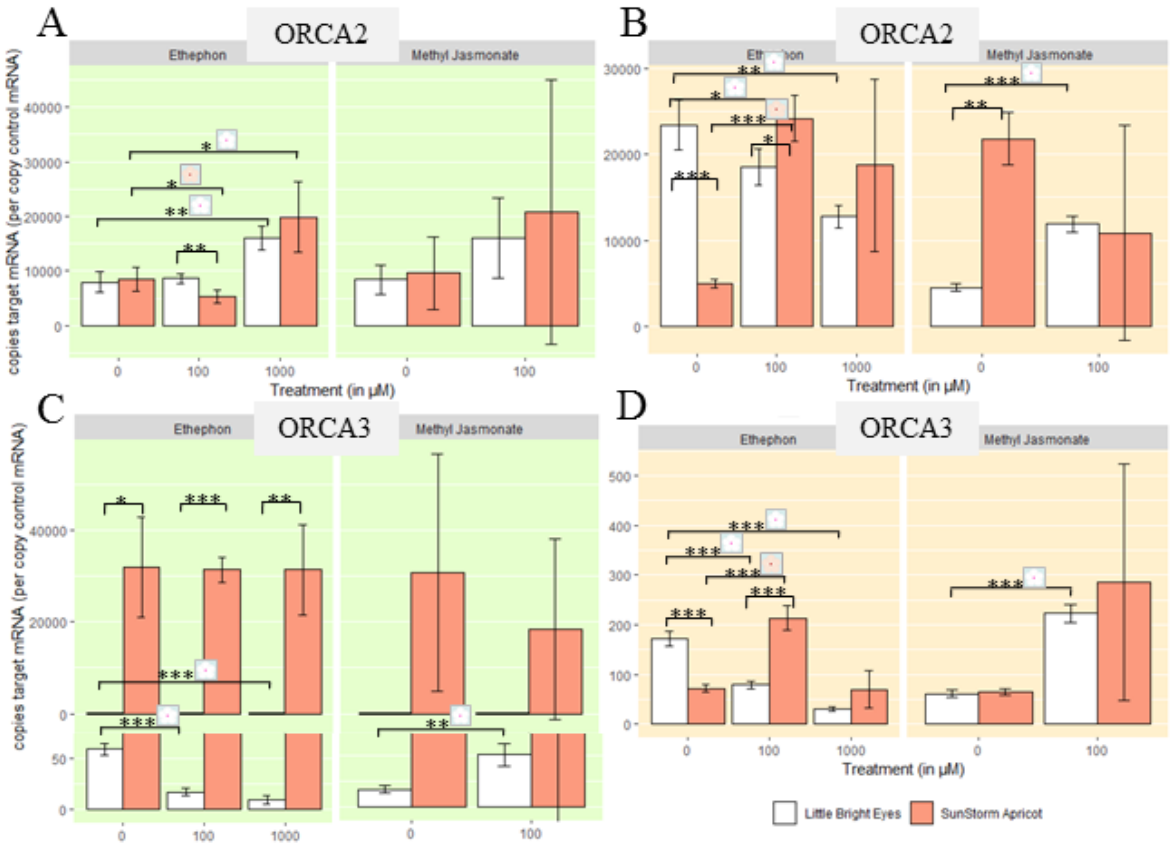

Figure 5 Expression of key regulatory genes are transcriptionally regulated upon phytohormone treatment. * denotes a p-value ≤ 0.05; ** denotes a p-value ≤ 0.01; *** denotes a p-value ≤ 0.001; all represented statistics are from Welch's t-test post-hoc analyses. Significance markers with a white flower represent treatment differences in LBE, while those with a peach flower represent treatment differences in SSA. (A) ORCA2 transcripts in shoots (B) ORCA2 transcripts in roots (C) ORCA3 transcripts in shoots (+ zoomed in panel) (D) ORCA3 transcripts in roots.

increasing in LBE (Fig 5B; ANOVA, $p \leq 0.001$). Previous studies in hairy root culture have shown that the overexpression of ORCA2 significantly increases concentrations of catharanthine and vindoline levels[25] while decreasing tabersonine levels[26], but our results do not appear to have the same correlations. In untreated plants, ORCA3 transcripts are present at significantly higher levels in the shoots of SSA than in the same tissue in LBE (Fig 5C; Welch's t-test, $p \leq 0.001$). These levels in SSA, however, do not respond to treatment with ETPN—unlike in LBE, where they are significantly decreased (ANOVA, $p \leq 0.01$). In roots, treatment with MeJA increases transcripts in both varieties; however, we see opposing effects depending on variety in shoots (Fig 5D). Previous studies have found that overexpression of ORCA3 in cell culture or plants

significantly altered the levels of vindoline, catharanthine, ajmalicine, and tabersonine[27, 28]. The changes in ORCA3 in SSA do mirror the changes seen in catharanthine and ajmalicine in roots, though this is not true of ORCA3 in LBE. These results underscore the need for broader testing in different varieties and the care needed prior to extrapolating results from one variety of *C. roseus* to inform results or pathway engineering of another variety.

As ORCA3 positively regulates two key genes in the TIA pathway[27], the significant difference between its expression in SSA and LBE makes its promoter an interesting target for further investigation and future bioengineering efforts. ORCA2 would also make a good candidate for engineering, as its transcript levels increased upon treatment. As with any master regulator, though, there is the possibility of activation of potential repressors, so a careful investigation in to genes controlled by these two TFs regulate would be advisable.

Early-induction pathway steps are likely transcriptionally regulated. We first wanted to investigate whether genes that are fundamental to TIA biosynthesis early on in the process show major transcriptional changes on hormone induction in our experimental system. We selected two biosynthetic genes that play roles upstream of the TIA pathway which have also been the focus of other studies[19, 29]: hydroxymethylglutaryl-CoA synthase (HMGS) in the mevalonate (MVA) pathway and 1-deoxy-D-xylulose-5-phosphate synthase (DXS2) in the pathway methylerythritol phosphate (MEP). We found that DXS2 transcript level increased in the roots of our young plants treated with MeJA (Figure S2C); meanwhile, HMGS transcript abundance decreased in the shoots of the MeJA-treated plants (Fig S2A). Previous work has demonstrated that ethylene induces the MVA pathway while jasmonate induces the MEP pathway in older seedlings[19, 29]. It is interesting to note that our results in roots and shoots treated through foliar application of the phytohormones show different induction patterns than were observed in those

previous studies, where treatments were applied to entire seedlings via hydroponic supplementation. A different physiological outcome in roots and shoots may be expected given that the immediate uptake happens through a different tissue; in some ways, however, our results are not directly comparable to that of past work since outcome specific to the individual plant parts has not been investigated. Regardless, both of these genes encode key enzymes in the formation of the indole component of terpene indole alkaloids, so a change in transcript abundance due to phytohormone treatment could have downstream effects on concentration of each alkaloid.

TIA-pathway specific genes are transcriptionally regulated. We selected seven biosynthetic genes that encode key enzymes directly related to the biosynthesis of terpene indole alkaloids. Of these enzymatic genes, five perform important reactions in the path toward vinblastine; the remaining two genes are involved in reactions that branch off from the vinblastine biosynthesis pathway but catalyze the formation of other medicinally relevant alkaloids. We felt it was necessary to investigate the expression of these genes in separate plant parts, as this information will be useful for engineering alkaloid production in biopharmaceutical settings.

Catharanthine synthase (CS) and tabersonine synthase (TS) produce catharanthine and tabersonine, respectively, and were recently determined to be two of the four missing enzymes in the TIA pathway[30]. We were particularly interested in discovering how the various phytohormone treatments effected their transcription, since relatively little research has been done with the genes since their discovery. In shoots, the CS transcript levels increase in all groups except for ETPN treated SSA, which is consistent with the trends in catharanthine production (Fig 6A; ANOVA, $p \leq 0.001$). While catharanthine appears to be transcriptionally

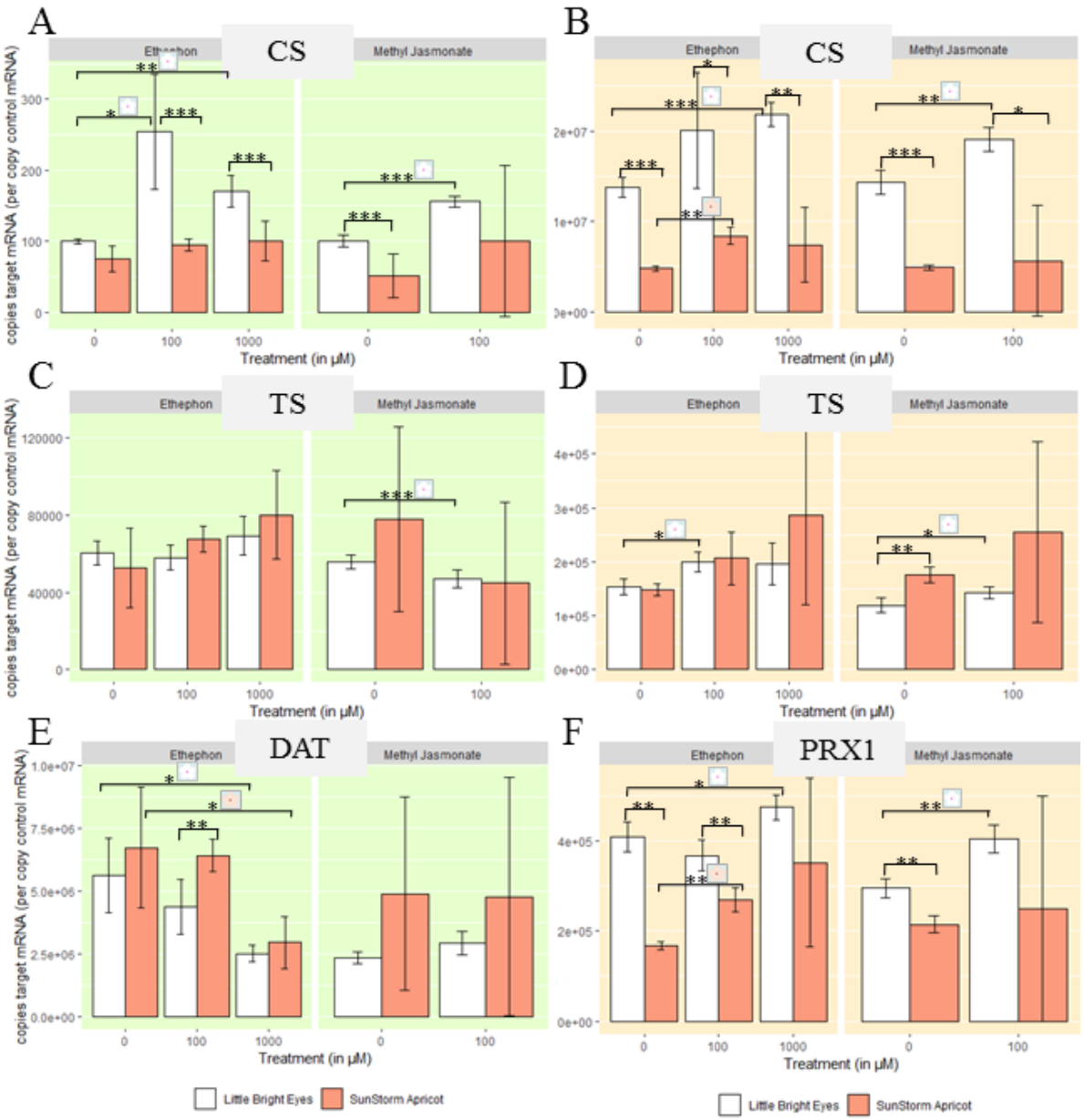

Figure 6 Expression of some key enzymes in the TIA pathway are transcriptionally regulated upon phytohormone treatment. * denotes a p-value ≤ 0.05; ** denotes a p-value ≤ 0.01; *** denotes a p-value ≤ 0.001; all represented statistics are from Welch's t-test post-hoc analyses. Significance markers with a white flower represent treatment differences in LBE, while those with a peach flower represent treatment differences in SSA. (A) CS shoots (B) CS roots. (C) TS shoots (D) TS roots (E) DAT (F) PRX1 transcript levels in roots increase after treatment with ethephon in both varieties and decrease after treatment with MeJA, but only in LBE.

regulated in LBE roots by both ETPN and MeJA, CS levels in SSA roots are only increased by ETPN (Fig 6B: ANOVA, $p \leq 0.001$). In the shoots of both varieties, tabersonine concentrations do not appear to be transcriptionally regulated, as TS transcript levels increase after treatment

with ETPN and decrease after treatment with MeJA, which is not at all consistent with the trends in alkaloid concentration (Fig 6C). This could be the due to an enzyme directly upstream acting as a bottleneck in the pathway, while the amount of TS present remains consistent because it is expressed at a level that is sufficient to handle an increased amount of substrate. In roots, on the other hand, tabersonine does appear to be transcriptionally regulated in both varieties, as a significant induction of TS transcripts after treatment with ETPN is observed which are correlated with observed changes in the tabersonine concentrations (Fig 6D).

Interestingly, although vindoline concentrations increase significantly in LBE shoots upon treatment with ETPN, there is no associated increase in deacetylvindoline *O*-acetyltransferase (DAT) mRNA levels; in fact, we observe a significant decrease in both varieties (Fig 6E; ANOVA, $p \leq 0.01$). Shoots treated with MeJA, however, do have similar increases in DAT mRNA and vindoline (Welch's t-test, $p \leq 0.1$), which is consistent with the changes in vindoline concentration observed in previous studies of *C. roseus* plants over-expressing DAT[31].

In roots and shoots of both of the varieties, ETPN treatment caused a statistically significant increase in the transcription of PRX1 (Fig 6F, S2; ANOVA, $p \leq 0.001$). Meanwhile, MeJA decreased transcription levels in all tissues of SSA and, significantly, in the roots of LBE (Fig 6F, S2; Welch's t-test, $p \leq 0.01$). Although α-3',4'-anhydrovinblastine and vinblastine levels were below the detection limit of our mass spectrometer, they are still key alkaloids, which is why we chose to examine this gene. Previous work in cell culture demonstrated a correlation between the over-expression of PRX1, an increase in the number of SGD transcripts, and an increase in ajmalicine accumulation[32]. The change in ajmalicine/tetrahydroalstonine is consistent with the patterns that we observed in SSA roots and the increase that we saw in SGD transcripts is seen in all SSA tissues (Figure S3; ANOVA, $p \leq 0.001$).

Overall, production of many key TIAs appear to be transcriptionally regulated in at least one tissue. ETPN and MeJA induce approximately equal numbers of the biosynthetic genes that code for key enzymes in the TIA pathway. They also induce genes upstream of the TIA pathway, which may be useful information for future bioengineering attempts. When taken in conjunction with the changes observed in alkaloid concentrations and with consideration of the cost of large-scale application, ETPN is a great option for alkaloid production in a biopharmaceutical setting.

Summary. In summary, our work demonstrates that choice of variety of *C. roseus*, phytohormone type, and concentration of treatment all have an impact on the levels of key alkaloids in each plant part. Either a genomic or transcriptomic resource is available for the two varieties investigated here, but neither variety has both. The differing baseline metabolic profile as well as the differing responses to phytohormone treatment emphasize the importance of choosing an appropriate variety for one's desired outcomes. Additionally, optimization of treatments is crucial; timing of phytohormone application and harvest, as well as the concentration applied, can have significant effects on both the health of the plants and the induced changes in alkaloid concentrations. Finally, this study shows that ethephon is a viable and agriculturally-relevant induction agent for key alkaloids in a large-scale biopharmaceutical production setting.

EXPERIMENTAL SECTION

General Experimental Procedures. Quantitation of metabolites was obtained using a Shimadzu Prominence HPLC (consisting of a degasser, two LC-10AD HPLC pumps, an autosampler, a photodiode array, and system controller) upstream of a 3200 QTrap mass spectrometer (AbSciex) operated using the Analyst software package. Data was analyzed offline using

Peakview version 2.2 software. Quantitation and purity analysis of total extracted RNA was obtained using a Thermo-Fisher Nanodrop and Agilent BioAnalyzer 2100 in the Center for Genomics and Biocomputing Core Facilities at Oregon State University. Quantitation of selected transcripts was obtained on a BioRad C1000 Touch thermocycler with a BioRad CFX96 detection unit. Transcript data was extracted using BioRad CFX Manager software. Primer efficiencies were calculated using LinRegPCR[33].

LCMS grade H_2O, acetonitrile, methanol and were purchased from MilliporeSigma. RNEasy Mini Kit, RNase-free DNase Set, and QIAgen SYBR PCR kit were purchased from QIAgen and were used according to the manufacturer's instructions. SuperScript RT kit was purchased from Invitrogen for reverse transcription and used according to the manufacturer's instructions. LCMS grade formic acid was purchased from Fisher Chemicals. Vindoline, vinblastine sulfate, vincristine sulfate, and catharanthine were obtained from Cayman Chemicals. Ajmaline and tetrahydroalstonine were obtained from Extrasynthese, while ajmalicine was obtained from Millipore-Sigma. Lochnericine and 16-hydroxytabersonine (aka 11-hydroxytabersonine) were obtained from MuseChem. All other chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise specified. Oligonucleotides were designed with the PrimerQuest tool from Integrated DNA Technologies, purchased from Sigma-Aldrich with standard desalting and used without further purification.

Plant Material and Growth. Two *Catharanthus roseus* varieties were selected for these experiments: "SunStorm Apricot" (obtained from www.expressseed.com) and "Little Bright Eyes" (obtained from www.neseeds.com). 10-12 seeds of a single variety were planted in 4-inch plastic pots filled to 1 cm below the top with MetroMix potting mix (35%-45% Sphagnum moss, bark, pumice, dolomite limestone). Pots were arranged on labeled trays, which were covered

with plastic domes to increase humidity until seedlings emerged through the soil. The plants were grown in an environmentally controlled growth room under a 12-hour light/12-hour dark photo-cycle with a 22°C ambient temperature.

Phytohormone Treatments and Sample Collection. We optimized our ethephon concentrations and treatment timing via a series of tests in a single variety (SSA). For the treatment timing optimization experiment, only SunStorm Apricot seedlings were grown to either 2 or 8 days post-germination and then were sprayed with 5mL of either water (control) or 100uM ethephon (treatment). After treatment, plants were sealed inside 2-gallon zip-top bags and returned to the growth chamber for 24 hours. On the next day, the plants were carefully uprooted, washed with DI water, separated at the hypocotyl into roots and shoots with a surgical blade, and flash-frozen in liquid nitrogen. Samples were stored at -80 °C until they could be processed, a minimum of 24 hours.

In the concentration optimization experiment, SSA seedlings were grown to 8 days post-germination and were then sprayed with 5mL of a control or 100uM, 500uM, or 1mM ethephon. Ethephon was mixed in DI water alone for the treatments, while the control treatment consisted of DI water. The plants were then handled as described above. Each sample from both of these experiments consisted of all the plants from a single pot; there were 12 pots for each variety. At higher concentrations, the plants began showing signs of senescence, becoming yellow and wilted.

For the larger comparison experiment, both SSA and LBE seedlings were grown to 8 days after germination. 6 pots of each variety were selected at random from the trays, sprayed with a combined volume of 5mL of DI water (ethephon control), DI water + 0.02% DMSO (methyl

jasmonate control), 100uM ethephon, 1mM ethephon, or 100uM methyl jasmonate + 0.02% DMSO. After treatment, the plants were handled as described above. We processed 6 replicates for each treatment. Each sample contained all of the plants from a single pot (~8 plants).

Alkaloid Extraction and Mass Spectrometry. Shoots were ground in liquid nitrogen with a mortar and pestle; roots were macerated by hand with a metal spatula directly in the methanol solution to prevent sample loss during the grind and transfer process due to the small amount of tissue. Shoot extractions were performed using 1 mL methanol containing 10 µM ajmaline (internal standard) per 100 mg tissue. Root extractions were performed using 1 mL of methanol containing 1 µM ajmaline (internal standard) per 10 mg tissue. The extracts were then allowed to stand at room temperature (~22 °C) for 20 minutes and then the cellular debris was pelleted by centrifugation (3,220 *x g,* 22 °C, 20 min). The cleared extracts were then filtered through a 0.22 µm nylon syringe filter to remove remaining particulate. The shoot alkaloid extracts were diluted 1:10 in methanol and 20 µl was transferred to HPLC vials containing glass sample inserts. The root extracts were used undiluted. The filled HPLC vials were stored at -80°C until they could be analyzed via LC-MS (described below). Unfortunately, some samples were lost during processing. In the end, the final replications for each treatment were as follows: LBE E0-S = 6; LBE E0-R = 5; LBE E1 = 6 and 6; LBE E4 = 6 and 6; LBE M0 = 6 and 6; LBE M1 = 6 and 6; SSA E0-S = 6; SSA E0-R = 3; SSA E1-S = 6; SSA E1-R = 5; SSA E4 = 6 and 6; SSA M0-S = 6; SSA M0-R = 5; SSA M1 = 6 and 6.

LCMS quantitation of *C. roseus* alkaloids. LCMS analysis was achieved using a Shimadzu Prominence HPLC upstream of a 3200 QTrap AbSciex mass spectrometer. Separation was achieved using Luna C18(2) column (150 x 2.00 mm, 3 µm) at a flow rate of 0.2 ml/ min and the

following gradient. Line A was water with 0.1% (v/v) formic acid and line B was acetonitrile with 0.1% (v/v) formic acid. The column was pre-equilibrated with 85% A/15% B. Upon injection (2 μL of prepared HPLC sample) the mobile phase composition was maintained for 1 minutes followed by changing the mobile phase to 60% A/40% B over 14 minutes using a linear gradient. The mobile phase was then changed to 0% A/100% B over the next 1 minute and held at this ratio for 8 minutes. The mobile phase was changed to 85% A/15% B over 1 minute and the column was equilibrated at 85% A/15% B for 7 minutes prior to the next injection. The mass spectrometer settings were as follows: MS (EMS positive mode, 50-1500 m/z), Curtain gas, 40.0; Collision gas, Medium; IonSpray voltage, 4500.0; Temperature, 400.0; Ion Source Gas 1, 35.0; Ion Source Gas 2, 35.0; Interface heater, ON; Declustering potential, 45.0; Entrance potential, 4.0; Collision energy, 5.0, number of scans to sum, 2; scan rate, 4000 Da/sec. MS/MS (MRM mode) For catharanthine (Q1, 337.3; Q3, 144.2; time 40 msec, CE (volts) 20.0); for tabersonine (Q1, 337.3; Q3, 305.3; time 40 msec, CE (volts) 20.0); for vinblastine (Q1, 406.2; Q3, 271.9; time 40 msec, CE (volts) 30.0); for vincristine (Q1, 413.2; Q3, 353.4; time 40 msec, CE (volts) 30.0). Curtain gas, 40.0; Collision gas, Medium; IonSpray voltage, 4500.0; Temperature, 400.0; Ion Source Gas 1, 35.0; Ion Source Gas 2, 35.0; Interface heater, ON; Declustering potential, 45.0; Entrance potential, 10.0; Collision cell exit potential, 3.0. Data was acquired using the Analyst software package.

Standard curves were generated by analyzing commercial standards at known concentrations using the identical LCMS settings.

RNA Extraction and qRT-PCR. Stored tissues were ground with mortars and pestles that had been treated with RNase Zap to prevent sample degradation. The ground tissues were divided into two 2mL microfuge tubes, which were used immediately to extract total RNA using the

RNeasy Mini Kit (QIAgen) in conjunction with their RNase-Free DNase Set (QIAgen) as directed. The total RNA for each sample was quantified on a Nanodrop (Thermo Scientific) and integrity was confirmed on a Bioanalyzer 2100 (Agilent). Only samples with RINs ≥ 8.0 were used for two-step qRT-PCR. Each biological replicate was used for two technical replicates, bringing the total to four reps for each sample. 300ng of input RNA from each sample was reverse transcribed using the SuperScript RT kit (Invitrogen). qPCR and melt curve analyses were performed using the QIAgen SYBR PCR kit on a CFX96 detection system (BioRad). qPCR data was extracted using CFX Manager software (BioRad). Primers not sourced from literature were designed using PrimerQuest tool (Integrated DNA Technologies).

Data Analyses. Relative intensities for each were determined from LCMS data by calculating the area under the peak (AUC) using Peakview version 2.2 (AbSciex) and then dividing that value by the AUC of our internal standard, ajmaline. Absolute concentrations were calculated from the AUC and a standard curve for each alkaloid; each quantity was then normalized using the original wet weight of the sample. We performed Welch's t-tests to determine the significance of differences in alkaloid concentrations between varieties and two-way ANOVA followed by Tukey pairwise comparison post-hoc analyses to determine the significance of treatments. For qPCR data analysis, LinRegPCR [33] was used to determine primer for efficiencies. Absolute copy numbers of transcripts were determined ($\bar{X}_{0_s} = \Delta T * \hat{E}_s^{\left[\bar{b}_a * \log_{\hat{E}_s}(\bar{E}_a) - \bar{C}_{q_s}\right]}$) and then normalized to the absolute copy number of 40S ribosomal protein S9 (RPS9), our control gene, from the same sample. The resulting data was analyzed using ANOVA and Welch's t-tests. All statistical analyses were performed in R (version 3.4.3).

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge:

Supplementary Information.pdf contains supplementary figures (S1-S3) and tables (S1-S7) referenced in the manuscript.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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