The transient effect of jasmonic acid feeding along with ORCA3 overexpression in *Catharanthus roseus* hairy roots

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Introduction

Jasmonic acid is an important signaling molecule in plants that regulates a wide variety of cellular responses including the responses to abiotic and biotic stresses. The external application of jasmonic acid has been shown to increase the accumulation of the monomeric terpenoid indole alkaloids, TIAs, in *Catharanthus roseus* seedlings and hairy root cultures (1-3). Jasmonic acid regulates the expression of the ORCA3 (Octadecanoid-Responsive *Catharanthus* AP2/ERF-domain) transcription factor (4). The overexpression of ORCA3 in *C. roseus* cell suspension cultures resulted in an increase of the mRNA levels of the following TIA biosynthetic genes: anthranilate synthase, ASα, tryptophan decarboxylase, TDC, 1-deoxy-D-xylulose synthase, DXS, NADPH:cytochrome P-450 reductase, CPR, secologanin synthase, SLS, strictosidine synthase, STR, strictosidine beta-glucosidase, SGD, and desacetoxyvindoline 4-hydroxylase, D4H (5). The accumulation of mRNA transcripts did not result in an increase in TIA compounds which could be explained by the lack of an observed increase in the transcripts of geraniol 10 hydroxylase, G10H, and deacetyl vindoline 4-O-acetyl transferase, DAT (5).

The TIA pathway leads to the production of vinblastine and vincristine; two important anti-cancer drugs that are produced in small quantities within *C. roseus*. This study explores the transient effects of overexpressing ORCA3 under the control of the glucocorticoid inducible promoter in *C. roseus* hairy roots along with simultaneous feeding of jasmonic acid. Our goal is to gain a better understanding of the effects of jasmonic acid and ORCA3 expression in differentiated cell types such as hairy roots that will lead us to an intergrated metabolic engineering approach to increase the production of the TIAs within *C. roseus*.

Methods

Induction and Elicitation

Catharanthus roseus hairy roots transgenic for the expression of ORCA3 under the control of the glucocorticoid inducible promoter were fed with 3 μM dexamethasone (induced), 50 mg/L jasmonic acid, 3 μM dexamethasone and 50 mg/L jasmonic acid, or ethanol (uninduced) during late exponential growth phase. Tissue was harvested at 0, 6, 12, 24, 48 and 72 hours after feeding. 300 mg of fresh weight tissue was ground in liquid nitrogen and stored at -80 °C for further mRNA analysis. The rest of the tissue was harvested for alkaloid extraction.

Alkaloid Extraction

The harvested tissue was frozen at -80 °C. The tissue was then lyophilized for 36 hrs. Approximately 50 mg dry weight of the ground tissue was extracted in 25 mL methanol by

ultrasonication for 10 min. The extracts were clarified by centrifugation at 1,300g for 15 min at 4 °C. The supernatants were concentrated to 2 mL using a vortex evaporator, and passed through a 0.22 µm nylon filter (13 mm). 10 µL of each samples was analyzed for indole alkaloids ajmalicine, serpentine, catharanthine, hörhammericine, lochnericine, and tabersonine on the HPLC as previously described (6).

cDNA Synthesis and Q PCR Amplification

mRNA was extracted from frozen tissue by using the Trizol protocol from Invitrogen. cDNA was generated using the Reverse Transcription System (Promega). The Q PCR reaction was carried out using the SYBR Green Master Mix (Applied Biosystems) on a 96 well plate as previously described (7). We used the comparative C_T (threshold cycle) method for relative quantification of gene expression. The 40S ribosomal protein S9 (rsp9) was used as our control gene. We examined the following mRNA transcripts: ASα, TDC, DXS, G10H, CPR, SLS, STR, SGD, and ORCA3 and used the uninduced hairy root sample at 0 hrs to normalize the data.

Results and Discussion

Jasmonic Acid Feeding

The greatest positive effects on TIA accumulation and TIA gene expression occurred during the jasmonic acid feeding alone. mRNA transcripts in general increased to a max between 12 and 24 hrs and returned close to the levels of the uninduced control by 72 hrs. Specifically the terpenoid genes reached their max at 12 hrs with DXS and G10H increasing approximately 12 and 60 fold, respectively, over the uninduced control at 0 hrs. At 24 hrs a maximum increase was observed of approximately 8 fold for ASα, 7 fold for TDC, 6.5 fold for SLS, 25 fold for STR, 5 fold for SGD, and 5 fold for ORCA3 when compared to the 0 hr control.

These increases in TIA biosynthetic transcripts correlated with an increase in alkaloid accumulation. Serpentine, ajmalicine, catharanthine and tabersonine reach their max between 24 and 48 hours followed by a decrease in the concentration of these compounds. Jasmonic acid feeding significantly ($p>0.05$) increases serpentine by 40%, ajmalicine by 35% and tabersonine by 74% at 24 hours over the uninduced line. Catharanthine was increased by 62% at 48 hrs. Hörhammericine levels reached their max at 24 hours (170% increase) and leveled out where as lochnericine levels continued to increase throughout the entire 72 hrs by 128% over the uninduced line.

The observed increases in mRNA transcripts and TIA compounds upon jasmonic acid treatment is consistent with other studies that showed increases in TIA compounds in *C. roseus* seedlings, hairy roots, and cell suspension cultures (1-3, 5) and increases in mRNA transcripts in cell cultures (5).

ORCA3 overexpression

While we observed a 90 fold increase in ORCA3 expression in the induced sample over the uninduced control, ORCA3 overexpression had little effect on the levels of mRNA transcripts observed in the hairy root lines. ORCA3 overexpression had a negative impact on the levels of tabersonine, lochnericine and hörhammericine which decreased significantly ($p<0.05$) by 81%, 42%, and 78%, respectively, at 72 hrs.

These results are contrary to what we expected to see since ORCA3 overexpression in *C. roseus* cell suspension cultures resulted in an increase in a number of TIA genes transcripts (5). This may point to other mechanisms of regulation of the TIA pathway within differentiated tissues.

Jasmonic acid feeding with ORCA3 overexpression

The results for the combined effects of ORCA3 overexpression with jasmonic acid elicitation lie somewhere in between the individual cases of induction and elicitation. As with ORCA3 overexpression, we see a similar drop in tabersonine, lochnericine, and hörhammericine levels but it takes longer for lochnericine levels to drop and ajmalicine increases significantly by 59% at 48 hrs. Catharanthine reaches a max at 24 hrs (43% increase) after which it declines to the uninduced levels by 72 hrs.

ORCA3 mRNA levels increase 170 fold times over the uniduced culture by 72 hrs. Other increases in mRNA transcripts occur by 24 hrs for ASα (5 fold), STR (15.1 fold), and SLS (6.5 fold).

Conclusion

These results show that a coordinate overexpression of many enzymes may be necessary to increase the production of the terpenoid indole alkaloids and point us towards a multigene strategy to engineer *C. roseus* hairy roots for increased production of TIAs. These results also suggest that ORCA3 overexpression with or without jasmonic acid feeding is not an effective means of coordinately overexpressing many enzymes within *C. roseus* hairy roots.

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