Summer Scholar Report

The Role of Leaf-associated Transcription Factors (LTFs) in the Regulation of Vindoline Biosynthesiss

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Introduction

The medicinal plant *Catharanthus roseus* produces the anticancer compounds, vinblastine (0.0002% by weight) (1) and vincristine, from the precursors, vindoline and catharanthine. The pathway for vindoline biosynthesis was recently mapped (2); starting with the compound tabersonine, the pathway consists of 7 steps: T16H2, 16OMT, T3O, T3R, NMT, D4H, and DAT (Figure 1). Unlike catharanthine, the expression of the vindoline pathway genes is found exclusively in young leaves (3) and is not present in the roots (4)(5). This localized vindoline production prevents vinblastine extraction from any other part of the plant other than the leaves. Understanding the regulation of the vindoline pathway and why it preferentially occurs in leaves may allow for transfer of vindoline production to a more industrially efficient culture system, such as cell suspensions or hairy root cultures.

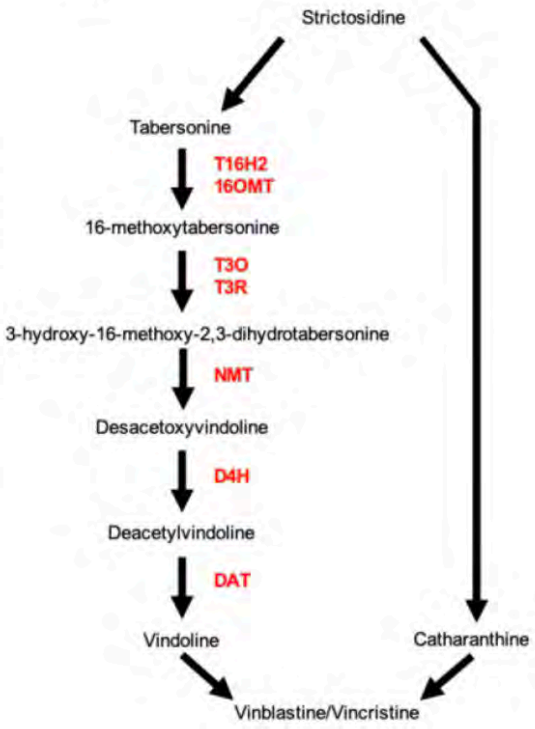

Figure 1: Strictosidine is the precursor of both vindoline and catharanthine biosynthesis. Each of the 7 vindoline pathway genes is highlighted in red. The condensation of vindoline and catharanthine leads to the production of vinblastine and vincristine.

According to a correlation analysis based on *C. roseus* RNAseq data, the expression of a class of transcription factors we dubbed LTFs (Leaf-associated Transcription Factors) was highly correlated to that of the 7 vindoline pathway genes, but not to that of the pathway leading to tabersonine, which is found throughout the plant (3) (6). We hypothesized that LTFs activate the vindoline pathway genes, causing their expression and vindoline production only in the leaves. The goal of this project was to investigate the regulation of the vindoline pathway genes by LTFs through overexpression, silencing, and transactivation experiments.

Methods:

Cloning Overexpression Plasmids

According to the correlation analysis, LTFs 8, 9, 11, and 15 were most closely correlated to the vindoline pathway genes (6). Thus, the coding sequences for each LTF were amplified from *C. roseus* cDNA and transferred to an overexpression plasmid. The expression of the LTF sequences was driven by the constitutive 2x35S promoter. The GUS reporter gene was also overexpressed using the 2x35S promoter as a control; this protein has no effect on the vindoline pathway.

Virus Induced Gene Silencing (VIGS)

VIGS is a method that relies on post-transcriptional gene silencing to decrease the expression of the gene of interest (i.e. LTF8 and LTF9). A plasmid containing the Tobacco Rattle Virus (TRV) machinery (pTRV1) and a plasmid encoding a fragment of the LTF coding sequence (pTRV2) were each transformed into *Agrobacterium tumefaciens* (GV3101). These two plasmids were then co-infiltrated into *C. roseus* to silence each LTF, as adapted from Liscombe & O'Connor (7). This viral system transcribes the LTF sequence into doublestranded DNA, which is recognized by the plant as foreign and diced into short-interfering RNA (siRNA). The siRNA is incorporated into the RNA induced silencing complex (RISC), which degrades mRNA complementary to the siRNA throughout the plant. This mechanism effectively silences the target gene (i.e. LTF8 and LTF9).

Cloning Vindoline Pathway Promoters

Modular Cloning (MoClo) is a plasmid construction system which allows various parts, such as promoters, coding sequences, and terminators, to be combined and rearranged by overlapping fusion sequences. Through the "domestication" process, the T16H2, 16OMT, T3O, T3R, NMT, D4H, and DAT promoters were first amplified in fragments from *C. roseus* genomic DNA (gDNA) to mutate their native Type-II restriction sites since these restriction enzymes are necessary for moving sequences into subsequent higher-level plasmids. The promoter fragments were then assembled in their own "Level 0" (L0) vector. Each promoter L0 vector was combined with other L0 vectors containing the

firefly luciferase coding sequence (F-Luc) and the constitutive *Nos* terminator, to produce a L1 vector. This L1 vector contained a transcription unit (TU), with the pathway promoter driving F-Luc. Each L1 vector was combined with another L1 vector containing a *Renilla* luciferase (R-Luc) driven by a constitutive promoter; the R-Luc acts as a reference reporter in promoter transactivation assays. The result was 7 plasmids containing the pathway promoter driving F-Luc, and the reference reporter on the same plasmid, in opposite directions. These plasmids will be used in promoter transactivation assays. Because each pathway promoter is driving F-Luc, the luciferase enzyme will be expressed if the promoter is activated by an added factor. Protein isolation and administration of substrate d-luciferin allows for luminescence, which is measured and corresponds to the level of promoter activity. Such an assay demonstrates regulation of the pathway promoters.

Transformation of Plasmids into C. roseus via EASI

Efficient *Agrobacterium*-mediated Seedling Infiltration (EASI) is a transient expression method in seedlings developed by the Lee-Parsons Lab (8). *Agrobacteria* were transformed with the plasmids of interest (i.e. GUS control, LTF overexpression (OE), or LTF-OE combined with the vindoline pathway promoter driving firefly luciferase gene); *C. roseus* seedlings were transformed with these *Agrobacteria* strains via vacuum infiltration. Transformed seedlings were planted in Gamborg's B5 full strength media without added sucrose in clear petri dishes and incubated in periods of light and dark to allow for transient expression of the LTFs. Cotyledons were harvested for RNA extraction and qPCR analysis. GUS staining was performed with the GUS-OE seedlings, as this enzyme converts a colorless substrate to a blue-colored compound which can be visualized and serves as an indicator that the plant transformation was successful.

RNA Extraction and qPCR

Three days after infiltration, the transformed cotyledons were flash-frozen in liquid nitrogen and stored at -80°C. Homogenization of the tissue using glass beads was followed by RNA extraction using RNAzol and column purification. RNA was reverse transcribed into cDNA using reverse transcriptase, which was used as template in a quantitative PCR reaction with SYBR green dye and designed primer pairs for each pathway gene (7). qPCR was used to measure the resultant levels of vindoline pathway expression in response to overexpression and silencing of LTFs.

Results:

LTF overexpression resulted in inconsistent effects on vindoline pathway gene expression

LTF8 and LTF9 were transiently overexpressed in *C. roseus* seedings and the effect on the expression of the vindoline pathway genes was monitored. GUS staining of the GUS

transformed seedlings within the same experiment confirmed that the vacuum infiltration was successful. GUS was overexpressed throughout the seedlings in all LTF overexpression experiments.

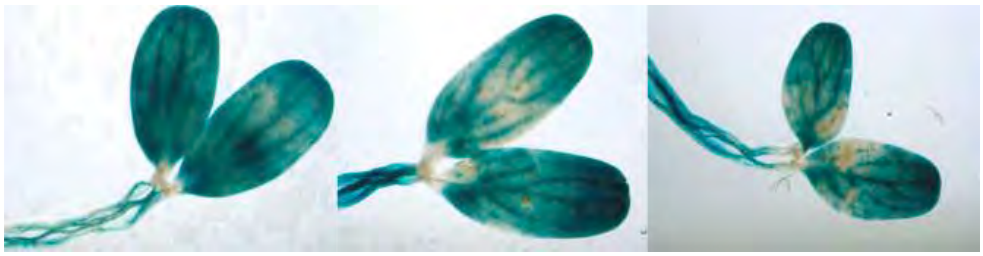

Figure 2: GUS transformed seedlings (shown as blue stained) demonstrate successful transformation.

In the first two overexpression experiments (LTF-OE 1 and 2), LTF8 overexpression was confirmed by qPCR measurement. In comparison with the GUS overexpression control, LTF8 expression was successfully increased up to 30-fold, as seen in Figure 3.

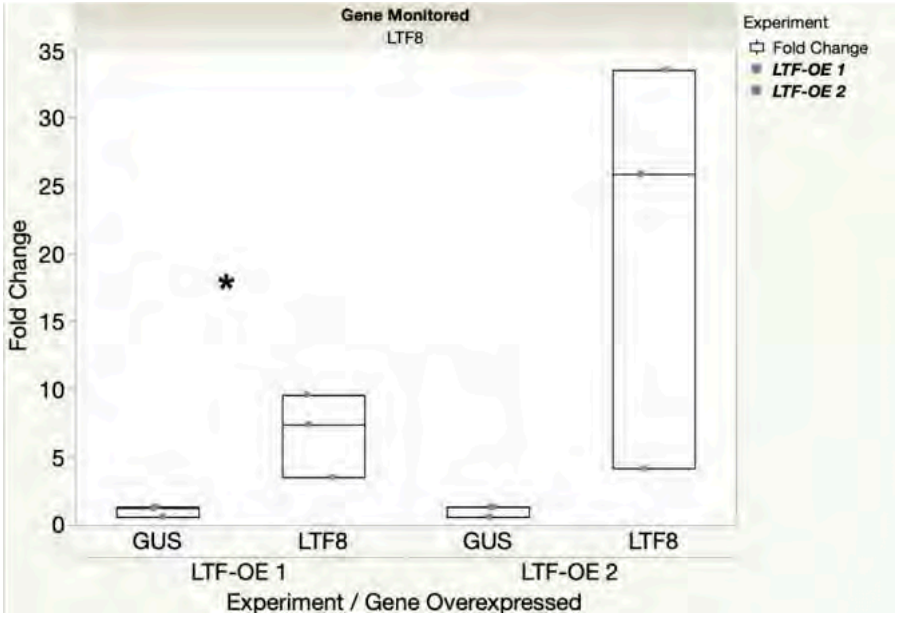

Figure 3: The expression levels of LTF8 in the LTF overexpressed condition versus the GUS control. There is significant overexpression of LTF8 (indicates P=0.040, determined by t-test).*

Given the hypothesized relationship, overexpression of LTFs in *C. roseus* should lead to an increase in transcript levels of the vindoline pathway. In Experiment 1, both D4H and NMT genes were upregulated with LTF8 overexpression (Figure 4). Similar upregulation was observed with other vindoline genes and LTFs (not shown). In Experiment 2, this upregulation was not observed (Figure 4).

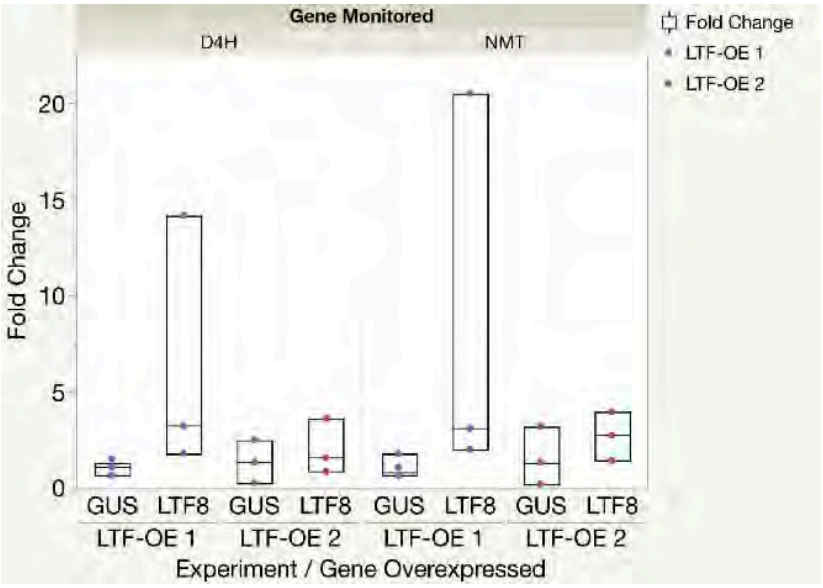

Figure 4: The effect of LTF8 overexpression on the expression of vindoline pathway genes (D4H and NMT). Upregulation of pathway expression is observed in LTF-OE 1.

Jasmonate may be necessary for the activation of the vindoline pathway genes by LTFs

The transformed seedlings were noticeably unhealthier in Experiment 1 than in Experiment 2. Jasmonate is produced in C. roseus during wounding, so we hypothesized that the jasmonate concentration was higher in the unhealthy seedlings of Experiment 1, allowing LTFs to induce the vindoline pathway. To replicate the conditions observed in Experiment 1, seedlings were vacuum-infiltrated with the plasmid-containing *Agrobacteria* followed by methyl jasmonate (100 uM MeJa) treatment. In Experiment 3, LTF and pathway gene expression were measured with and without MeJa. Figure 5 shows that the overexpression of LTF8 was successful.

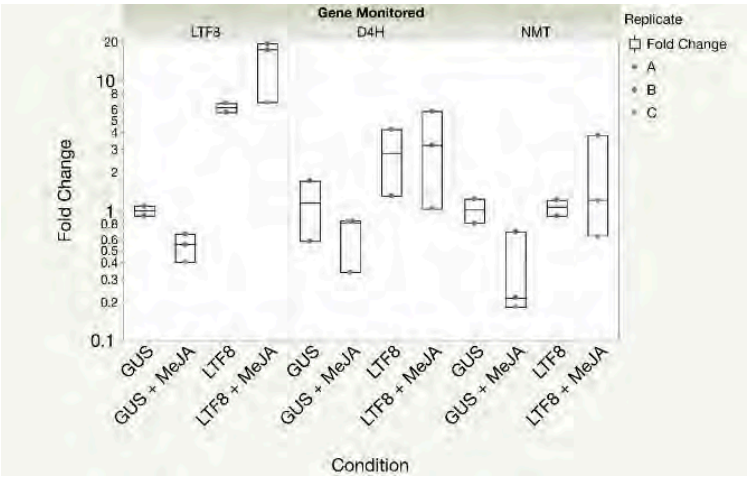

Figure 5: The effect of LTF8 overexpression on the expression of vindoline pathway genes (D4H and NMT) with and without MeJa. All values are normalized to GUS without MeJa.

Similar to Experiment 2, there was no upregulation of D4H and NMT expression with LTF8 overexpression in the absence of MeJa. However, there was an increase in D4H and NMT expression with LTF8 overexpression in the presence of MeJa. For example, D4H increased about 4-fold while NMT increased about 5-fold when LTF8 was overexpressed in the presence of MeJA. The expression of the other pathway genes also increased with LTF in the presence of MeJA (data not shown). These results support the hypothesis that LTFs positively regulate vindoline pathway gene expression. Interestingly, the addition of MeJa decreased LTF and vindoline pathway expression. It is possible that vindoline pathway gene expression decreased in the presence of MeJa due to decreased LTF expression. Overall, MeJa seemed to decrease LTF and vindoline pathway expression, but when LTF was overexpressed, an increase in pathway expression was observed.

Double silencing of LTF8 and LTF9 did not impact vindoline pathway gene expression
Given the hypothesized relationship, silencing of LTF8 and LTF9 expression in C. roseus by VIGS should lead to a decrease in transcript levels of the vindoline pathway. Figure 6 shows significant silencing of LTF8; LTF9 was also significantly silenced (not shown). However, under the LTF silenced condition, NMT and D4H expression showed no downregulation.

Successful cloning of the vindoline pathway promoters will aid in future experiments
The promoter of each pathway gene was amplified in parts from *C. roseus* gDNA, combined, and cloned to drive firefly luciferase (F-Luc) gene expression. Co-infiltration of the LTF overexpression plasmid and the vindoline pathway promoter driving F-Luc plasmid into seedlings would allow for studying potential transactivation of the promoter by the LTF. Luminescence would increase and indicate activation of the pathway promoter by the LTF. Given the hypothesized relationship, the overexpressed LTFs should activate the pathway promoters, either directly by binding the promoters or indirectly by interacting with a secondary factor.

This assay could not be performed within the timeframe, as problems arose in cloning the promoter. The MoClo system used to construct the promoter plasmids requires cutting via BsaI and BpiI restriction enzyme sites. Thus, native sites within the promoters must be removed via mutation and amplification in fragments. For instance, the NMT promoter was divided and amplified in 3 fragments due to 2 BsaI sites. After failed attempts to amplify the T3O promoter, we hypothesized that our genomic sequence (derived from the "Little Bright Eyes" cultivar) differed from the reference genome that was available from which the primers were designed (derived from the "Sunstorm Apricot" cultivar). However, the *C. roseus* reference transcriptome is derived from Little Bright Eyes (3), and although promoter sequences are not part of the transcriptome (as they are not transcribed), the genes immediately downstream of the promoters are. A primer was

designed in this location and this fragment was successfully amplified. Difference in sequences between the two cultivars was confirmed by its sequencing, explaining our earlier failure to amplify the promoter. Using this correct sequence, primers were designed to successfully amplify the promoter. All of the pathway promoters have successfully been cloned into their respective L2 plasmids and are prepared for promoter transactivation assay. These plasmids can be used to detect activation of the pathway promoters by LTFs and other candidate transcription factors, enabling a faster screening process.

Discussion

Controlling the light environment is important when studying the vindoline pathway

The variability in light exposure could potentially affect the expression of the vindoline pathway genes and introduce variations between experiments, such as that observed between Experiment 1 and 2. To reduce these variations, we established a consistent incubation period of dark (48 hours) followed by a consistent period of continuous light (24 hours). Liu et al. (2019) showed that the highest expression of most of the pathway genes occurs after 24 hours of light exposure; thus, harvesting at this time point ensures that light is not limiting expression and that plants have fully recovered from the dark period (10).

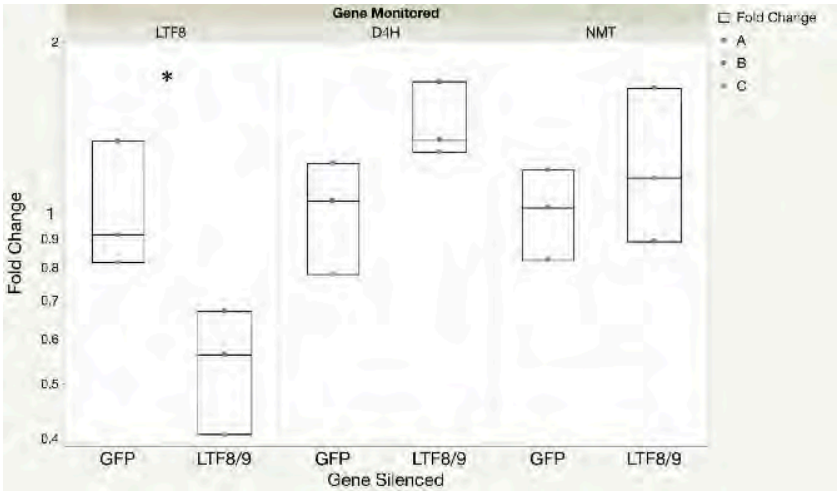

Figure 6: LTF8 was significantly silenced compared to a GFP control (indicates P=0.039, determined by t-test).*

Jasmonate appears to affect the activation of the vindoline pathway by LTFs

In Experiment 1, transformed seedlings appeared unhealthy (wilted and browning) and showed upregulation of the vindoline pathway genes, while in Experiment 2, seedlings were healthy and showed no upregulation. Jasmonate is produced in *C. roseus* during wounding, so we hypothesized that jasmonate was present at higher concentrations in the

unhealthy seedlings of the Experiment 1. The potential effect of jasmonate on the activity of the LTFs is shown in Figure 7. MeJa degrades JAZ so that the co-activating factor (CF) can bind LTFs and therefore activate vindoline pathway expression; this model potentially explains the upregulation of Experiment 1. In Experiment 3, GUS control and LTF-OE conditions with and without MeJa were designed to test this hypothesis.

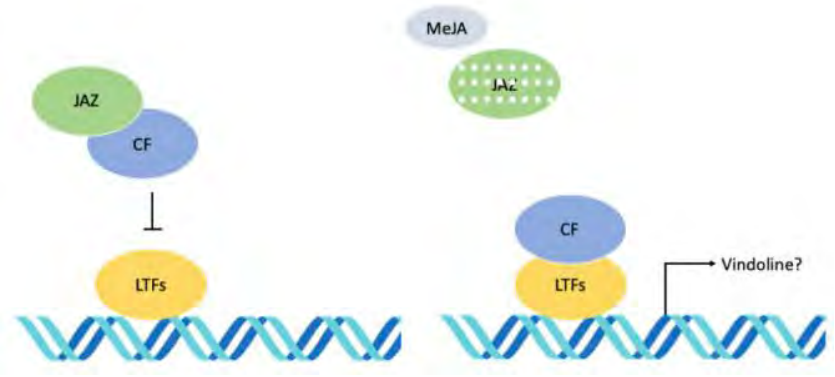

Figure 7: In the absence of MeJa, JAZ is hypothesized to bind and inhibit the CF. In the presence of MeJa, JAZ is degraded and the released CF coactivates LTFs (Produced by Lily Ha).

Based on the model shown in Figure 7, the addition of MeJa in LTF-OE seedlings was expected to induce pathway upregulation. When LTF8 was overexpressed in the presence of MeJA, there was an increase in vindoline pathway gene expression. Interestingly, in the GUS controls, MeJa was shown to decrease LTF and vindoline pathway expression. In Arabidopsis, a decrease in LTF expression was also observed with the addition of MeJa (11). We expected MeJa to increase vindoline pathway expression (12), as explained in Figure 7 resulting from the activation of LTF by the released CF.

LTFs are likely to be redundant

Simultaneous silencing of LTF8 and LTF9 did not lead to a decrease in pathway expression as hypothesized. This lack of decrease may be a result of LTF redundancy. Even though LTF8 and LTF9 were silenced, other LTFs could activate the vindoline pathway genes; no decrease in pathway expression would be observed if other LTFs compensated.

In summary, LTFs are promising candidates in regulating vindoline pathway expression. Additional LTFs will be studied and the experiments conducted and repeated under controlled light and MeJA environments. Promoter transactivation assays will be performed to determine direct regulation of the vindoline pathway by LTFs and other candidates.

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