TcJAMYC: A bHLH TRANSCRIPTION FACTOR THAT ACTIVATES PACLITAXEL BIOSYNTHETIC PATHWAY GENES IN YEW
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Running title: A conserved bHLH that affects paclitaxel gene expression

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Abstract:
Paclitaxel (Taxol®) is a highly modified diterpene anti-cancer agent produced by the genus Taxus. Taxus suspension cell cultures have the potential to provide a sustainable source of paclitaxel, but the paclitaxel biosynthetic pathway is not fully characterized, making metabolic engineering efforts difficult. Methyl jasmonate (MJ) is used to elicit paclitaxel production in suspension cultures. Here we show that the promoters of five genes encoding enzymes of the paclitaxel biosynthetic pathway are activated by MJ elicitation. Thus, elicitation of paclitaxel production by MJ is regulated at least in part at the level of transcription. A transcription factor that positively activates the promoters of paclitaxel biosynthetic genes has been cloned. This transcription factor, TcJAMYC, possesses a high degree of similarity to AtMYC2 and JAMYC2, which are known to regulate the expression of jasmonate inducible genes in other systems. TcJAMYC binds to E-boxes found in the promoters of the paclitaxel pathway genes, and these promoters are transiently activated by TcJAMYC overexpression. Addition of MJ attenuates the effect of TcJAMYC on the pathway promoters, suggesting that TcJAMYC could be engineered into Taxus cells to avoid the negative regulation of MJ-induced positive regulation. This strategy could prolong the expression of paclitaxel pathway genes, and increase paclitaxel production.

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paclitaxel occurs much later (three to six days after elicitation), long after steady state transcript levels of the known pathway genes have returned to normal (7). The mechanism(s) underlying the poor correlation between steady state mRNA levels and accumulation of the desired metabolite paclitaxel are not understood. Still, the coordinated expression of the known paclitaxel pathway genes suggests that these genes could be under the control of a single regulatory regime.

Key regulators of specialized metabolite production have been identified in *Catharanthus* and *Arabidopsis thaliana* (8-16). Boter et al. (17) identified a basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factor. This bHLH specifically binds to promoter E-box elements and activates the expression of cell-cycle arrest genes in *Solanum tuberosum* (18). An Arabidopsis homolog has been shown to modulate jasmonate defenses (18). More recently, AtMYC2 was shown to regulate a wide array of MJ-elicitable responses (19), and was implicated in priming of enhanced defenses (20).

Here, the mechanism of MJ elicitation of gene expression in *Taxus* suspension cell cultures was investigated and a regulator of the paclitaxel metabolic pathway was identified. This regulator has been named *Taxus cuspidata* JAMYC (TcJAMYC). There is similarity in sequence and function between TcJAMYC and AtMYC2, suggesting a conserved response to MJ despite the 150 million years of divergence between angiosperm and gymnosperm lineages (21). The results presented here suggest that TcJAMYC is a key candidate gene for engineering increased paclitaxel accumulation in *Taxus* cell cultures.

**Materials and Methods:**

**Cell Culture:**

*Taxus cuspidata* suspension cultures were used for all experiments. Suspensions were sub-cultured every two weeks into Gamborg’s B5 (Sigma, St. Louis, MO) basal salts (3.2 g/L) with 20 g/L sucrose, supplemented with 2.7 μmol/L naphthalene acetic acid (NAA) and 0.01 μmol/L benzyladenine (BA). Ascorbic acid (156 mg/L), citric acid (156 mg/L) and glutamine (906 mg/L) were filter-sterilized (Millipore Millex 0.2 μm syringe filters) and added post-autoclaving. Cultures were maintained in 125 mL Erlenmeyer flasks capped with Belco (Vineland, NJ) foam closures at 24°C and shaking at 125 rpm in the dark. Cells-transfers were accomplished through the addition of 10 mL of 14 day-old suspension cultures into 40 mL of fresh medium. The approximate packed volume of cells transferred was at least 2 mL to maintain optimum culture density.

**Transient Transformation and MJ Elicitation:**

Cells were either mock-elicited by adding 50% EtOH or elicited with MJ (dissolved in 50% EtOH) at a 100 μM final concentration. After three h, both batches of cells (~0.5 g) were transferred onto B5-agar plates, spread out in a circle in the center of the plate with a diameter of about three cm. Cells were then left overnight to induce the expression of the test genes. Luciferase activity was measured using a Turner designs 20/20 luminometer set at 70 % sensitivity using 10 μl of the cell lysate. The luciferase activity was detected using the Applied Biosystems Dual Light® System (Foster City, CA) per the manufacturer’s instructions. GUS assays were performed using a Turner Designs (Sunnyvale, CA) 20/20 luminometer set at 70 % sensitivity using 10 μl of the cell lysate. The luciferase activity was detected using the Applied Biosystems Dual Light® System (Foster City, CA) per the manufacturer’s instructions. GUS assays were performed using a Turner Designs 450 fluorometer with an NB360 excitation filter and a SC430 emission filter. 4-Methyl umbelliferone (Sigma, St. Louis, MO) was used as a calibration standard. GUS assays contained 75 μl protein extract, 50 μl MeOH, and 125 μl 2X MUG buffer, as described (23).
The upstream flanking regions of the biosynthetic pathway genes were cloned using inverse-PCR (25) using the cDNA sequences of T5αH (AY628434), DBAT (AF193765), PAM (AY582743), BAPT (AY082804) and DBTNBT (AF466397). PCR products were cloned into pDESTG221 to form translational fusions to GUS with 5 to 30 N-terminal amino acid residues of the pathway biosynthetic gene prior to the fusion point with GUS.

Vector Construction:
A vector, pDESTG221, was constructed by modifying pPZP221 (24) to contain a Gateway recombination cassette B (Invitrogen, Carlsbad, CA), GUS reporter gene (23) and NOS terminator. The Taxus promoter DNA fragments were re-amplified from I-PCR fragments using primers that contained attL sites 5' to the gene specific nucleotides. PCR products were placed into pDESTG221 using Gateway LR Clonase II (Invitrogen, Carlsbad, CA), creating translational fusions to the GUS reporter gene. The TcJAMYC effector gene was amplified from Taxus cuspidata suspension cell culture first-strand cDNA and ligated in between the CaMV35S promoter and a NOS 3' sequence. The 35S:GUS construct was taken from pBI121:GUS and placed into the multiple cloning site of pPZP221 using EcoRI and BamHI restriction sites. The 35S: LUC construct was ligated into pTZ19u.

Degenerate Primer Amplification of TcJAMYC:
The conserved bHLH domain in the JAMYC proteins from Solanum tuberosum (AJ630505) (17) and Arabidopsis (At1g32640) (26) was used to design degenerate primers with the Consensus-Degenerate Hybrid Oligonucleotide Primers (CODE-HOP) program (27). Five primers were chosen, and two (Forward primer oDEGmyc.1 GAGAAGACCTCTGAATCATGTTGARGCN GARMG; Reverse primer oDEGmyc.3 CAGCTTACATTTCCAGTCTATAAGAAATNGCRTCNCC) produced a PCR product that was then used to screen a cDNA library by hybridization.

cDNA Library Construction and Screening:
Total RNA was extracted from two grams of Taxus cuspidata cell culture line P991, by guanidium isothiocyanate and cesium chloride gradient ultracentrifugation at 104,000 x g for 18 h, followed by phenol-chloroform extraction. Poly-A RNA (5 µg) was obtained from 1 mg total RNA using Poly-A Purist Mag-Kit (Ambion, Austin, TX). cDNA construction and cloning was performed using the ZAP Express cDNA Synthesis Kit (Agilent Technologies, Cedar Creek, TX). Plaques (1x10⁶) from the primary library were screened using the Taxus bHLH fragment obtained by PCR. A 2.5 kb cDNA clone was isolated and sequenced, but the 5'-end was truncated. 5'-Rapid Amplification of cDNA Ends (RACE) was performed using RLM-RACE kit (Ambion, Austin, TX). The full-length cDNA was cloned by PCR using the 5'-sequence obtained by RACE, and this product was cloned and sequenced.

Phylogenetic Analysis:
Each of the bHLH proteins from Arabidopsis and the TcJAMYC was used to create an unrooted phylogram. The amino acid sequence was parsimony informative, so this limited sequence was used to construct the unrooted phylogram. Amino acid sequences were aligned using ClustalX with default settings.

TcJAMYC Protein Purification:
The TcJAMYC cDNA was recombined into pDEST17 (Invitrogen), an E. coli expression vector containing an N-terminal 6X His tag for affinity purification on a Nickel agarose column (Qiagen, Valencia, CA). The Rosetta ™ 2(DE3) pLysS (EMD biosciences, Gibbstown, NJ) strain of E. coli containing this construct was grown to late log phase (OD₆₀₀ = 0.8), induced with 1 mM IPTG, and then incubated with shaking for four more hours at 37°C. Cells were pelleted by centrifugation at 4400 x g, resuspended in 50 mM Tris-Cl pH 6.8, 20 mM β-mercaptoethanol, 2% SDS, 10% glycerol and 10 mM imidazole, and 50 µl DNase1 (10 mg/ml), then incubated on ice for 30 minutes. Debris was removed by centrifugation at 17,000 x g for 20 min, and the TcJAMYC protein was bound to Ni-NTA resin (Qiagen, Valencia, CA). The resin was washed with buffer containing 250 mM NaCl, 50 mM Tris-Cl pH 6.8, 20 mM imidazole, and eluted with buffer containing 250 mM NaCl, 50 mM Tris-Cl pH 6.8, 300 mM imidazole. The eluted protein is Withdrawn at Authors Request.
was further purified using a Centricon YM-3 centrifugal filter device (Millipore, Danvers, MA) and brought to a final protein concentration of 25 ng/µl in 50% glycerol.

**Electrophoretic Mobility Shift Assays (EMSA):**

Oligonucleotide probes (see Table 1) contained a six nucleotide E-box at the center of a 22 bp sequence. A four-nucleotide 5′-overhang was included in each double stranded probe to allow for 32P-labeling. Labeling reactions contained 100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 0.025 % Triton X-100, pH 7.5, 2.2 mM of each dTTP, dATP, and dGTP and 22 µM double stranded oligo in a 23 µl total volume with 5 U of Klenow large fragment DNA polymerase (New England Biolabs (NEB), Ipswich, MA). Unincorporated nucleotides were removed using a 2 ml Sephadex G-25 (Sigma, St. Louis, MO) column. For each EMSA reaction, 175 fmol of double stranded oligonucleotide were used (labeling reaction diluted 1:125). EMSA buffer consisted of 20 mM HEPES-KOH pH 7.9, 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 0.5 mM PMSF, 1 mM DTT, 15 mM MgCl₂, and 5 µg BSA. For standard reactions, 2 µl of TcJAMYC protein was used and the final volume of the reaction was 20 µl. Native polyacrylamide gel electrophoresis was used to separate the DNA probe that was bound by the protein and the free DNA probe. Gel was run at 7°C for 1.5 h at 82 V after being pre-run for 30 minutes.

**Results:**

**Paclitaxel Pathway Gene Promoter Activation:**

Prior work in the Taxus system demonstrated a positive correlation between the steady state mRNA abundance of the paclitaxel biosynthetic pathway genes (hereafter referred to as "pathway genes") and MJ elicitation (7). This suggested that increased transcription of pathway genes was occurring in response to MJ elicitation. To investigate this possibility, promoters of five of the known pathway genes: T5αH (AY628434), DBAT (AF193765), PAM (AY582743), BAPT (AY082804) and DBTNBT (AF466397) were cloned (Figure 2A). The DNA fragments encoding these promoters were placed upstream of the GUS (GUS) reporter gene to form translational fusion constructs containing the pathway promoter, the first few amino acids encoded by the pathway gene (T5αH: five amino acids; DBAT: six; PAM: ten; BAPT: seven; and DBTNBT, thirty), and the reporter gene.

These expression constructs were used to transform *Taxus cuspidata* P991 suspension culture cells by particle bombardment; the cells were either mock-elicited or elicited with 100 µM MJ. To control for variable transformation efficiency, 35S:LUC was co-bombarded in all experiments (Figure 2B). Reporter gene activity is presented as a GUS/LUC ratio to control for transformation efficiency between samples. Measurements of reporter activity were performed 48 h after bombardment. Expression of the 35S:GUS control construct (Figure 2B) was not affected by the addition of MJ (Figure 3). For each pathway gene promoter construct, GUS enzyme activity was at least fourfold higher in MJ treated cells than in the absence of MJ (Figure 3). Thus, we concluded that the promoters of these pathway genes are activated by MJ. This result, in combination with increased amount of steady state mRNA for the endogenous genes after MJ elicitation (7), implies that MJ treatment causes increased transcription of pathway genes.

**Promoter Analysis:**

Since the most of the pathway gene promoters were activated upon MJ elicitation, and the pathway genes all respond in a uniform time course (7), we reasoned that there could be an MJ-responsive *trans*-acting factor that influences these promoters. To test this idea, an in silico analysis of the pathway promoters was performed using PLACE software (http://www.dna.affrc.go.jp/PLACE/) (28). This analysis revealed that there are 36 E-boxes (CANNTG) found in the 6544 nt of the collective pathway gene promoter sequence that we cloned (Figure 2A). A disproportionately large number of potential E-box sites are found in the promoters of the pathway genes, since only 25 E-boxes would be expected to occur at random in a sequence of this length. E-boxes have been found in defense gene promoters in other plants (29). bHLH proteins typically bind to these E-box nucleotide motifs (30). For example, the Arabidopsis bHLH MYC2 protein preferentially binds to the E-box sequence CACGTG. Furthermore, E-boxes are
commonly found on the promoters of genes that respond to MJ (19). The Solanum JAMYC2 binds a related, "G-box" sequence (AAGCTG) on the proteinase inhibitor promoter (17).

**Cloning TcJAMYC:**

The large number of E-box binding sites in pathway promoters suggested that there could be a jasmonate responsive MYC regulator that binds to E-boxes and activates the pathway genes. The well conserved bHLH sequences from Arabidopsis MYC2 (At1g32640) and Solanum JAMYC10 (AJ630506) were used to design degenerate primers with the CODE-HOP program (27). These primers were used to amplify a 172 nt fragment was amplified from Taxus cDNA derived from suspension cultures that had been elicited with MJ (27). The TcJAMYC sequences displayed 94% identity at the amino acid level to the Arabidopsis MYC2 promoter:GUS reporter constructs (see Figure 2A). Mock-elicited cells were bombarded with each pathway gene promoter:GUS construct either alone or in combination with 35S:TcJAMYC. TcJAMYC activated the T5caH, PAM, BAPT, and DBTBNT promoters by at least 2-fold (Figure 5). Only the DBAT promoter was unaffected by co-bombardment with TcJAMYC, although activation of the STN2 promoter was seen (< 2-fold). This corresponds to TcJAMYC promoter:GUS reporter constructs for many pathway genes that encode oxidoreductases at various points along the paclitaxel biosynthetic pathway.

**Activation of Pathway Promoters by TcJAMYC:**

To investigate whether TcJAMYC influences transcription of the paclitaxel pathway genes, co-bombardment experiments were performed using a full-length TcJAMYC cDNA under the control of the CaMV35S promoter in combination with the pathway gene promoter:GUS reporter constructs (see Figure 2A). Mock-elicited cells were bombarded with each pathway gene promoter:GUS construct either alone or in combination with 35S:TcJAMYC. TcJAMYC activated the T5caH, PAM, BAPT, and DBTBNT promoters by at least 2-fold (Figure 5). Only the DBAT promoter was unaffected by co-bombardment with TcJAMYC, although activation of the STN2 promoter was seen (< 2-fold). This corresponds to TcJAMYC promoter:GUS reporter constructs for many pathway genes that encode oxidoreductases at various points along the paclitaxel biosynthetic pathway.

**To clarify the relationship of the Taxus gene to the bHLH genes from Arabidopsis, an unrooted phylogram was generated from the Taxus bHLH sequences using the maximum parsimony analysis in Figure 4B.** Families that were not well-supported evolutionarily are indicated. The Taxus bHLH falls into the same clade as AtMYC2, confirming the similarity of the two genes. Based on the similarity to the other well-characterized JAMYC bHLH transcription factors, we refer to the Taxus gene as TcJAMYC, for Taxus cuspidata jasmonate MYC.

Accumulation of mRNA from the previously characterized JA-MYC genes from tomato and Arabidopsis is positively regulated by MJ (17,19). To determine whether TcJAMYC responds to MJ application, semi-quantitative RT-PCR was performed to compare relative transcript abundance at one hour after MJ elicitation (Figure 4C). This result indicates that there is an increase in TcJAMYC transcripts after MJ elicitation, again emphasizing the resemblance between TcJAMYC and the Arabidopsis JAMYC, AtMYC2.

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tagged TcJAMYC was expressed in *E. coli* and purified on Ni-NTA agarose columns. The expected molecular weight for the tagged TcJAMYC protein is 73kD (Figure 6A). To determine whether TcJAMYC binds to E-box elements, the most abundant E-box (CATGTG) in the pathway promoters was used initially as a probe sequence (Table 1). The TcJAMYC protein bound the CATGTG sequence (Figure 6B). To determine if the binding was specific to TcJAMYC, the GUS protein, expressed in and purified from *E. coli* (Figure 6A), was used in the EMSA. This assay demonstrated that the binding of the CATGTG sequence is specific to the TcJAMYC protein, as GUS did not bind to the DNA probe (Figure 6B). Finally, a mutated DNA probe, the CACGTG E-box, was used in the EMSA (Figure 6C). The TcJAMYC protein did not bind this DNA sequence, demonstrating that the TcJAMYC protein specifically binds to the E-box element CATGTG.

To test the specificity of binding further, a competition assay was performed using the CATGTG probe against increasing amounts of non-specific DNA competitor (the mutated DNA described above). The binding efficiency of the CATGTG probe did not diminish (Figure 6D), again demonstrating that the TcJAMYC protein specifically binds to the CATGTG sequence. Eight additional E-box sequences were found in the pathway promoters (Figure 2A). To determine whether TcJAMYC also binds these E-boxes, oligonucleotides containing these sequences were used in competition assays against the CATGTG probe. As shown in Figure 6E, the only E-box element that competed well against the CATGTG probe was the CACGTG element. This demonstrates that the TcJAMYC protein preferentially binds more strongly to two specific E-box sequences found in the pathway gene promoters: CACGTG and the CATGTG.

To test which of these two sequences, CATGTG or CACGTG, is most efficiently bound by TcJAMYC, competition assays between these two DNA elements were performed. The CATGTG probe competed against itself, as previously shown in Figure 6E, and this was used as reference for binding specificity (Figure 6F). A competition assay was performed using the CATGTG probe against increasing amounts of CACGTG competitor (Figure 6G). The CACGTG fragment competed for binding of the CATGTG probe more effectively than CATGTG self-competitor (compare Figure 6F and 6G). This demonstrates that TcJAMYC preferentially binds to the CACGTG as compared to the CATGTG sequence. To further characterize this binding selectivity, a competition assay was performed with the CACGTG probe against the unlabeled CATGTG competitor (Figure 6H). Increasing amounts of CATGTG competitor did not efficiently compete for binding, confirming the preference for binding of CACGTG. Thus, the TcJAMYC protein specifically binds to CACGTG and secondarily the CATGTG E-box elements.

**Discussion:**

Transcriptional Activation of Pathway Promoters:

MJ has been shown to induce terpene production in conifers (32), glucosinolates in *Arabidopsis* (33,34), alkaloids in *Papaver* (35) and *Catharanthus* (36), as well as proteinase inhibitors in many plant species including important agricultural crops (37). MJ elicitation has also been implicated in the activation of the defense related leucine aminopeptidase promoter in *Nicotiana*, the strictosidine synthase promoter in *Catharanthus*, and a sesquiterpene synthase promoter in *Nicotiana* are activated with MJ elicitation (17,36,40). In this investigation, we have shown that MJ elicits activation of five paclitaxel biosynthetic pathway gene promoters in *Nicotiana* (Figure 5B). This result suggests that the transcriptional response to MJ is conserved across land plant phylogeny from gymnosperms to angiosperms.

Our finding that the promoter of the gene encoding the penultimate step in paclitaxel biosynthesis (BAPT, cf. Figure 5) is strongly activated by MJ is somewhat surprising. The endogenous BAPT transcript was undetectable using RNA gel blot analysis, and up-regulation of mRNA expression from this gene was only detectable using a more sensitive RT-PCR assay (7). This difference in expression between the BAPT reporter construct and the endogenous BAPT gene implies a separate level of regulation of the endogenous gene, perhaps at the post-transcriptional level. Endogenous mRNA from
the terminal gene of the paclitaxel biosynthetic pathway, DBTNBT, does not accumulate to higher levels after MJ elicitation (7). Likewise, compared to the other pathway promoters, the DBTNBT promoter was only weakly affected by MJ (Figure 3). This suggests that manipulating the expression of the DBTNBT gene using a stronger promoter could allow increased enzyme production and increased flux through this point in the pathway.

The 35S promoter, which in this assay drove GUS expression as a control, exhibited a low level of expression compared to the pathway gene promoters, and was not affected by MJ. This may indicate that the CaMV35S promoter is not a strong promoter in Taxus and that other promoters should be investigated for the genetic engineering of Taxus cultures.

A Jasmonate-Responsive bHLH in Taxus:

By leveraging information obtained in model systems (Arabidopsis and Solanum) we have potentially obtained a key regulator of important natural product production. This bHLH transcription factor, TcJAMYC, activated transcription of the genes in the paclitaxel biosynthetic pathway in the gymnosperm genus Taxus. Many of the enzymatic conversions in the pathway are unknown, so obtaining global regulators of the entire pathway may allow for manipulating the pathway in situ without the need to identify all the enzymes involved. The degree of relatedness between TcJAMYC and AtMYC2, compared to all other bHLH proteins in Arabidopsis, suggests that this transcription factor has evolved across a wide span of evolutionary time (Figure 4B).

There is a correlation between the presence of certain binding sites and the activation of specific promoters. TcJAMYC preferentially binds the sequences CACGTG and CATGTG, and at least three copies of these sequences are found in the highly activated promoters (Figure 2A and Figure 5). Among the promoters tested in our studies, only the DBAT promoter contains fewer than three copies of these sequences, and the DBAT promoter is the only one that is not up-regulated when co-bombarded with TcJAMYC into Taxus cells. Since DBAT mRNA expression and DBAT promoter activity do increase following MJ application, there are likely additional regulatory factors that control MJ-induced gene expression in Taxus.

Further work in this system is required for a complete description of the factors involved in MJ-elicited gene expression. A limitation of the Taxus system is that stable transformation is not yet possible for most of the commonly used, paclitaxel-producing cell lines, such as the P991 cell line used in this study. Stable transformation of Taxus suspension cultures with the constructs described here would further define whether TcJAMYC is able to cause increased taxane accumulation and could be used to demonstrate whether there is an in vivo interaction between TcJAMYC and the promoters of pathway genes.

Negative Regulation of Pathway Promoters Following MJ Elicitation:

In the studies presented here, we uncovered evidence for negative regulation of the promoters of the paclitaxel biosynthetic genes. In previous studies, a decrease in steady state mRNA levels of pathway genes was observed six hours after MJ elicitation, remain high for about 24-30 hours, and return to original low levels by about 48 hours (7). This rapid up-then-down-regulation is consistent with negative regulation of the promoters by TcJAMYC. Interestingly, the promoter of T5αH, an early pathway gene, was highly activated when elicited by MJ, but did not demonstrate this negative regulation. Instead, this promoter was highly activated when elicited by both MJ and activated

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TcJAMYC. We hypothesize that, in the bombardment assays reported here, negative regulators are acting to antagonize TcJAMYC regulation of the reporter constructs by 48 hours, thus preventing TcJAMYC’s stimulatory effect on pathway promoter expression. It is both interesting and important that TcJAMYC alone (without MJ) appears not to induce this negative regulatory response (Figure 5). Because of this effect, it may be possible to bypass negative regulation from MJ elicitation by engineering TcJAMYC into Taxus. This strategy may prolong pathway gene expression by allowing positive regulation to occur without the usual MJ-induced negative feedback loop that would usually follow. This could increase metabolic flux through the paclitaxel pathway.

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Figure 1 The biosynthetic pathway leading to paclitaxel.
GGPP is synthesized by geranylgeranyl diphosphate synthase (GGPPS). GGPP is then converted to taxadiene by taxadiene synthase (TASY) (41) and then to taxadien-5α-ol by taxadien-5α-ol hydroxylase (T5αH) (42). Several steps leading from the diol intermediate to functionalized taxanes, including the formation of the oxetane ring, are unknown. Taxane 2α-O-benzoyltransferase (DBBT) (43) produces 10-deacetylbaccatin III (10-DAB). 10-DAB is then converted to baccatin III by 10-deacetylbaccatin III-10-O-acetyltransferase (DBAT) (44). Baccatin III: 3-amino, 3-phenylpropanoyltransferase (BAPT) ligates the side chain (derived from phenylalanine via phenylalanine aminotransferase (PAM) (45)) to produce 3’-N-debenzoyl-2-deoxypaclitaxel (46). An unknown P450-mediated hydroxylation of the side chain forms 3’-N-debenzoyl paclitaxel. 3’-N-debenzoyl-2-deoxypaclitaxel-N-benzoyltransferase (DBTNBT) then produces paclitaxel by benzyolation of the side chain (47).

Figure 2 The pathway promoters cloned using inverse-PCR and the locations of the various E-box elements next to the triangles indicate the frequency of that box on the respective pathway promoter. The US sector 5′-codons after complete functional pathways are included as an N-terminal extension on the GUS protein.

(B) The GUS (GUS), TcJAMYC full-length cDNA, and firefly luciferase (LUC) genes, all driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter, that were used in bombardment assays.

Figure 3 Pathway promoter activation by MJ.
Taxus suspension cultures were plated onto B5 agar plates and bombarded with the promoter:GUS reporter constructs. 35S:GUS was also bombarded as control. 48 h was allowed between bombardment and assay of the GUS activity. Results are presented as a GUS/LUC ratio. N=4 in all samples, error bars are SE.

Figure 4 Cloning of TcJAMYC.
(A) Degenerate primers were designed from the conserved bHLH region in deoxypaclitaxel Taxus DNA fragments to obtain a full-length cDNA. 35S:GUS reporter constructs. 35S:GUS was also bombarded as control. 1 h of MJ elicitation allowed the development of an assay of the GUS activity. Results are presented as a GUS/LUC ratio. N=4 in all samples, error bars are SE.

(B) An unrooted phylogram using the DBD of all bHLH proteins from Arabidopsis and the TcJAMYC protein generated by maximum parsimony analysis. Roman numerals indicate the classes of bHLH transcription factors that were identified by Heim et al. Shading indicates class IIIe. Bootstrap values are calculated from 1000 replicates. Consistency index = 0.3565, retention index = 0.3608, homoplasy index = 0.6435, rescaled consistency index = 0.133.

(C) RT-PCR analysis of TcJAMYC, Taxadiene synthase (TASY, AY424738) rRNA (AF259290), and actin (derived from P. contorta actin; Genbank M36171.1) after 1 h of MJ elicitation. Both rRNA and actin fragments were amplified as internal controls. The control (mock elicited) cells are labeled 1C and the MJ-elicited cells are labeled 1E. The lanes labeled (--) represent the results of amplification of RNA without reverse transcription, as a control for contaminating genomic DNA in our RNA preparations.

Figure 5 Promoter activation by TcJAMYC.
Mock-elicited Taxus cultures were plated onto B5 agar plates and bombarded with the promoter:GUS fusions alone (-MJ, -MYC) or combination with the CaMV35S:TcJAMYC effector plasmid (-MJ, +MYC). Separately, MJ-elicited Taxus cultures were plated onto B5 agar plates and bombarded with the promoter:GUS fusions alone (+MJ, -MYC) or in combination with the CaMV35S:TcJAMYC effector plasmid (+MJ, +MYC). Results are presented as a GUS/LUC ratio. N=4 in all samples, error bars are SE.
Figure 6 Electrophoretic mobility shift binding assays for TcJAMYC.
(A) The TcJAMYC and the GUS proteins on a Coomassie blue-stained SDS page gel after nickel column purification.
(B) EMSA using the TcJAMYC (left to right: ~25ng, 50ng, 75ng, 100ng) and GUS (left to right: ~25ng, 50ng, and 75ng) proteins at increasing amounts using the most common E-box element found on pathway promoters (CATGTG) as probe.
(C) Binding assay using TcJAMYC at increasing amounts (left to right: ~25ng, 50ng, and 75ng) and a mutated probe (Table 1).
(D) Competition assay with the TcJAMYC protein and the CATGTG probe against increasing amounts of mutated cold competitor at 0X, 1X, 2X, 4X, 8X, and 16X excess.
(E) Competition assays using TcJAMYC protein and the CATGTG radio labeled probe against all other E-box elements found in the pathway promoters. The cold competitor is listed to the right of the panels. +0: no competitor, 20X Self: the CATGTG cold competitor at 20X excess, 20X Comp: the cold competitor at 20X excess.
(F) TcJAMYC self-competition assay (to determine binding affinity to CATGTG). The 1-100X range of cold competitor is 1X, 5X, 10X, 20X, 50X, and 100X excess, left to right in each gel.
(G) A competition assay with radio labeled CATGTG and the CACGTG cold competitor. The cold competitor is listed to the right of the panels. +0: no competitor, 20X Self: the CATGTG cold competitor at 20X excess, 20X Comp: the CACGTG cold competitor.
Table 1. Oligonucleotide Probes Used for EMSA

26mer oligonucleotides were used as double stranded DNA in the EMSA. Putative E-box elements are in bold face. The mutated probe does not contain the CANNTG sequence that defines the generic E-box sequence. All 9 E-boxes that are found in the pathway promoters are represented.

| Probe | Oligonucleotide Sequence |
|-------|--------------------------|
| CATGTG: | TAGCCGATCGCATGTGATCGATCGGCTAGCTAGCTAGCATGC |
| CACGTG: | TAGCATCGATCGACGGTACGGATCGCTAGCTAGCATGC |
| CAAGTG: | TAGCGCATCGCATAGGTAGCGATCGCTAGCTAGCATGC |
| CAATTG: | TAGCGCATCGCATATGTAGCGATCGCTAGCTAGCATGC |
| CAACTG: | TAGCGCATCGCATGTGTAGCGATCGCTAGCTAGCATGC |
| CACCTG: | TAGCGCATCGCATGTGTAGCGATCGCTAGCTAGCATGC |
| CATCTG: | TAGCGCATCGCATGTGTAGCGATCGCTAGCTAGCATGC |
| CATTTG: | TAGCGCATCGCATGTGTAGCGATCGCTAGCTAGCATGC |
| CATATG: | TAGCGCATCGCATGTGTAGCGATCGCTAGCTAGCATGC |
| Mutated: | TAGCGCATCGATGAGCCTATCGCTAGCTAGCATGC |

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