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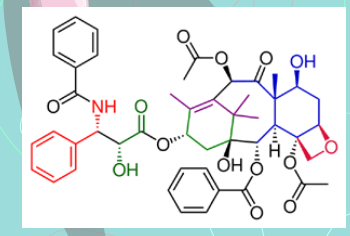

Figure 1. Paclitaxel (Taxol) Structure

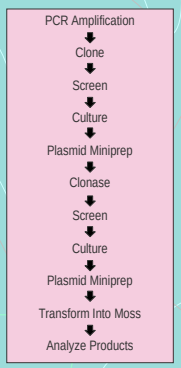

Figure 4. Experiment Flow Chart

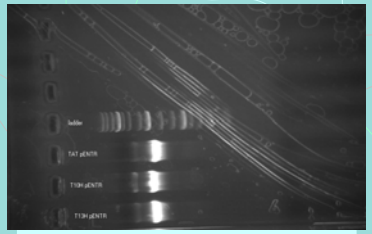

Figure 5. Electrophoresis Gel After PCR Amplification

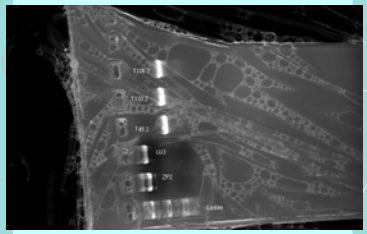

Figure 6. Electrophoresis Gel After Screening and Collection

Figure 3. Moss Prior to Transformation With T10H, T13H, and T5AT

ABSTRACT

Taxol is a cancer fighting drug that was initially isolated from the Pacific Yew. However, the isolation process is not very efficient and the tree is being excessively harvested and faces extinction. To synthetically make Taxol is an inefficient and costly process. If the precursor taxadiene-5 alpha-acetoxy-10 beta-ol can be produced with ease, then the synthetic modification of that precursor would be an efficient way to produce the potent cancer fighting drug. Several genes from the Pacific Yew were isolated and amplified so that they could be inserted into the moss *Physcomitrella patens*. Using competent E. coli cells as entry vectors, the genes were transferred so that the metabolic pathway responsible for taxadiene-5 alpha-acetoxy-10 beta-ol synthesis could be replicated in *Physcomitrella patens*. When the final transfer was made to the moss, a transient expression of the genes resulted in small amounts of product being obtained. After gas chromatography mass spectrometry analysis, the chromatogram plots showed a few more promising peaks representing other Taxol precursors. With a permanent transfer to the moss, a much larger sample could be analyzed and more Taxol precursor could be produced.

INTRODUCTION

Paclitaxel, also known as Taxol, was originally isolated from the bark of *Taxus brevifoli*, which is better known as the Pacific Yew. The compound was discovered during the U.S. Department of Agriculture's random collection program (Lewis 2003). In 1977 it was selected for a more rigorous clinical trial and was found to have cytotoxic properties, and acts by stabilizing microtubules and preventing depolymerization (Lewis 2003). Paclitaxel's first clinical trial cured thirty percent of the participants with late stage ovarian cancer (Success). Taxol has also been shown to help treat breast, lung, head, neck, bladder, esophagus, and cervical cancer as well as Kaposi's sarcoma, which is prevalent among individuals affected by AIDS (Brague 2001). Taxol's cytotoxic and antimitotic activity stems from its ability to prevent the shrinking of microtubules and consequently, the division of a growing cell. Taxol only binds tubulin, which is the main component of microtubules, when it is part of a microtubule. This binding prevents microtubules from breaking down and which then stops chromosome segregation and cell division (Yarris 1995).

Taxol's effective cancer fighting traits have made it a high priority for production and use in the medical community. In fact, Taxol sales reached 1.6 billion dollars in 2000 and became the best selling anti cancer drug (Success). However, there are several problems that arose because of Taxol's popularity. Taxol's presence in the bark of the Pacific Yew is very minor, on the range of 0.01 percent. Therefore, it takes the death of four fully grown Pacific Yew trees to produce one human dose of Taxol. For these reasons, the Pacific Yew is being extensively harvested and faces extinction (Davidson). The synthetic production of paclitaxel requires thirty steps and has a yield of less than 0.05 percent (Azu 1997). Because of its eleven chiral centers, as can be seen in Figure 1, synthetic production of Taxol will not be cost effective or efficient until its production is reduced to twenty five or fewer steps (Azu 1997). By inserting genes into *Physcomitrella patens*, a paclitaxel precursor will be made in the plant that will allow production of the compound to be more time and cost effective.

MATERIALS AND METHODS

Several precursors must be synthesized before the final Taxol product can be produced (Figure 2). *P. patens* has already been altered to produce taxadiene and taxadiene-5alpha-ol which are the first and second precursors respectively (Figure 3). Three plasmids, which contained either T5AT, T10H, or T13H were amplified using polymerase chain reaction. Once a sufficient amount of each sequence was produced, it was sub-cloned into an entry vector, purchased from Invitrogen Labs. This entry vector allowed the plasmids to be transferred to an expression vector, which contained a selection marker. The marker allows *E. coli* containing the plasmid to survive on an antibiotic plate. This allowed us to select only the cells that contained the desired expression vectors. When enough of the expression vectors were created, the plasmids were collected using a plasmid miniprep kit. The plasmids were sequenced to check for quality (Figure 7) and then were shipped to collaborators at the Washington University who transformed *Phscomitrella patens* with the recombinant DNA holding the desired genes. There are two processes for transforming the moss. One process produces permanent transformations while the other produces only transient transformations. The associates at Washington University used the transient process. *P. patens* was then grown in a media with the appropriate antibiotic to ensure transfer. Once the modified *P. patens* matured, it was analyzed in Dr. Anterola's laboratory using gas chromatography and mass spectrometry. An experimental flow chart can be seen in Figure 4.

RESULTS

The initial PCR amplification proved successful, which was determined by the presence of bands for T13H, T10H, and T5AT in an electrophoresis gel (Figure 5). After the plasmids were cloned and screened, colony three was picked for T13H, referred to as T133. Colony six was picked for T10H, referred to as T106. Finally, colony five was picked for T5AT, which is referred to as T45. The electrophoresis gel after the plasmid miniprep showed bands of the expected size for all of the plasmids (Figure 6). The plasmid sequencing confirmed the quality of the amplified plasmids as well(Figure 7). After the transient transformation was performed, *P. patens* colonies were grown in a selective medium.

The products of transformed *P. patens* were analyzed using gas chromatography and mass spectroscopy. Twelve versions of the transformed moss were created, each containing different plasmid combinations (Table 1). The products were analyzed at the molecular weight (MW) of 304 and 330. These weights were used because they represent the products taxadiene-five alpha, thirteen alpha-diol and taxadiene-five alpha-yl acetate respectively. The chromatogram read at the MW of 304 (Figure 8) was analyzed to determine if the T133 and subsequent T10H transformations were successful. The chromatogram read at the MW of 330 (Figure 9) was analyzed to determine if the transformation T45 and T10H was successful. When analyzing the chromatograms, the largest peaks were found in versions five through seven, which contained a single plasmid. These peaks were then compared against the chromatograms of the wild type. If the peaks found in the modified moss' chromatograms were not found in the wild type chromatograms, then there is a better chance that those peaks represent the products of the genes that the mosses were transformed with. Then, the proposed peak retention times are compared to the other chromatograms to find corresponding peaks. The 304 MW chromatogram shows some promising peaks at the fifty minute retention time for 1st , 4th , and 6th versions. Each of these were transformed with T133. Version six shows the greatest peak, followed by version four and finally, version one has the smallest peak. Several peaks, which possibly represent T10H, were seen in versions 1, 3, and 4 at the 49.75 retention time range. Once again, version one had the smallest peak and version four had the largest peak. The 330 MW chromatogram showed promising peaks at the 48.75 minute for versions 1, 2, and 5. The peak in version five was the largest and there were small peaks in versions 1 and 2. There was another promising peak at 50.75 minutes in version five that could represent T45. A peak also appears in version one at the same time.

Version	Vector Added	Protoplast Present
1	T10H, T13H, T5AT	TS6, T5H
2	T13H, T5AT	TS6, T5H
3	T10H, T5AT	TS6, T5H
4	T10H, T13H	TS6, T5H
5	T5AT	TS6, T5H
6	T13H	TS6, T5H
7	T10H	TS6, T5H
8	n/a	TS6, T5H
9	n/a	Wild Type
10	T5AT	Wild Type
11	T13H	Wild Type
12	T10H	Wild Type

Table 1. Moss Versions With Vectors Added and the Protoplasts That Were Already Present

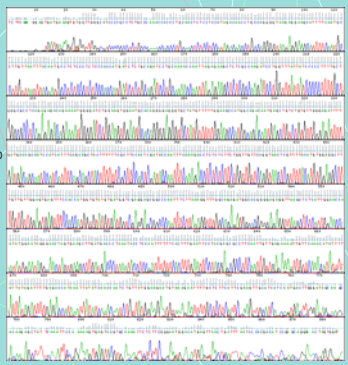

Figure 7. Plasmid Sequences

Chromatogram Plots

Figure 8. GCMS Results For 304 Molecular Weight

Chromatogram Plots

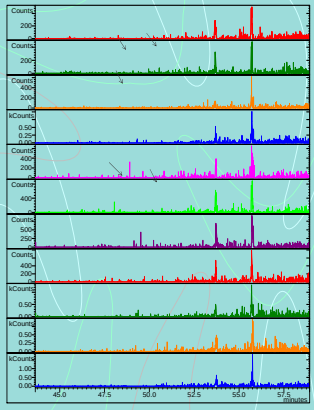

Figure 9. GCMS Results For 330 Molecular Weight

DISCUSSION

Version six was transformed with just T133 and had the largest peak at fifty minutes. Version four was transformed with T133 and T10H. It had a significantly smaller peak at fifty minutes, representing T133, and a larger peak at the 49.75 retention minute, which represents T10H. As can be seen in Figure 2, T10H can only act after T133 or T45 has acted. Therefore, when T10H and T133 are combined, the T133 product is used by T10H to make the final product. Finally, version one was transformed with T10H, T133, and T45. The proposed T133 and T10H peaks were small but present. They could be smaller because their products are being outcompeted by that of T45 whose product is not seen on the 304 MW chromatogram. Based on these results, it is believed that T133 and T10H were both successfully transferred to the moss and expressed. The results from the 330 MW chromatogram showed some promising peaks; however, the peaks were not very large. Based on this, it is believed that T45 was not successfully transferred to the moss. However, with the results from the screens, electrophoresis gels, and sequencing, it is believed that the plasmids isolated were the correct versions and in working order. Therefore, a viable T45 could have been present in the moss, but in so small of a quantity that it did not produce visible results. All of these results were obtained from a transient transformation of moss and a small amount of product being analyzed. With this said, more products need to be analyzed before a stronger conclusion can be made. To create more products, a permanent transformation must be completed. Upon completion, the pathway's viability will be determined and the precursor product can be made.

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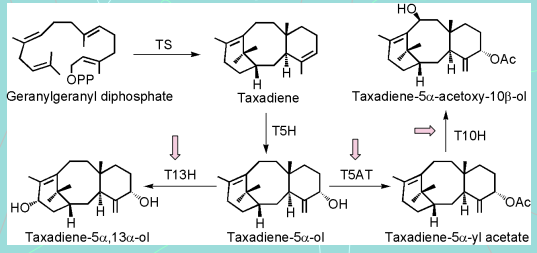

Figure 2. Taxol Precursor Pathway and Target Genes