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# The Strategies to Increase Taxol Production by Using *Taxus mairei* Cells Transformed with TS and DBAT Genes

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**Abstract:** Taxol is an expensive anti-cancer drug to cure ovarian, breast and lung cancers. An increase in taxol yield in *Taxus* cell cultures has been viewed as a great economical potential. Since taxol yield is low in plants and cell cultures, the greater amounts of two taxoids: 10-deactyl baccatin III (DB) and bacctin III (BC) have been used as precursors to synthesize taxol. To increase the taxoid yield in cell cultures, a good cell line and methyl jasnomate (MJ) treatment have been proved to be effective in many studies. In our present study, MJ not only increased taxoid yield, but also increased the kind of taxoids. We presumed that MJ might be a good indicator to select important genes among the ten genes involved in taxol biosynthesis. We selected and cloned genes of taxadiene synthase (TS) and 10-deacetyl baccatin III-10-O-acetyl transferase (DBAT). TS is the first gene of taxol biosynthesis pathway, while DBAT gene controls the reaction of DB to become BC. We found an overexpression of these two genes in both needles and stems of *Taxus mairei* plants, 8 hours after the MJ treatment, indicating that introgression of these two genes in cell cultures might increase taxoid yield. The construction of 35S promoter and sense DBAT, sense TS, and antisens DBAT gene were made and successfully introduced into cell cultures. We presumed that transformed cells would produce taxoids all the time without MJ treatment. Although, the transgenic cells with sense DBAT gene did increase the yield of both BC and taxol, however, MJ treatment was required to enhance the taxoid yield. It suggests that MJ might regulate genes more than what we thought.

**Keywords:** cell cultures; gene transformation; gene expression; taxoids; *Taxus mairei*.

## 1. Introduction

Taxol, mainly extracted from the bark and needles of slow-growing *Taxus* (yew) species, is recognized as a highly effective anticancer drug (Christopher 1993). There have three valuable taxoids: taxol, 10-deacetylbacctin III (DB), and baccatin III (BC). Taxol is very expensive. One kg of taxol costs about 200,000 US$. Usually, taxol concentration in plants is about 0.001 to 0.05%. *T. media* cultivar was the major species to produce taxol. In cell cultures, the concentration of taxol ranged from 0.04 to 0.2%, depending on cell lines. Both DB and BC are precursors of taxol, 1kg of them can be converted into about 0.6 to 0.7 kg taxol (Denis and Greene 1988; Ojima, 1992). The price of DB is about

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25,000 US$. DB is rich in *T. baccata*, while BC is rich in cell cultures. DB level can reach about 0.3% in plants, while BC about 0.4% in cell cultures. There are two sources of taxol production. One from traditional cultivation of *Taxus* plants, and another from cell cultures in bioreactor. Until now, most of taxol in market has been obtained DB, only small part directly extracted from plant. In 1995, Phyton Company announced to have a set up of 75 ton-bioreactor, but could not bring the product in the market. Recently, Korea produced taxol from cell cultures, but it did not have any affect the taxol market. Regardless the difficulty in commercialization, cell cultures are still viewed as great economical potential to replace the traditional cultivation system, since it can produce purer taxoids and in greater quantities, though there are risks involved.

## 2. Cell cultures of *Taxus mairei*

*Taxus mairei*, a giant tree, grows at high mountains of Taiwan about 1000-2000 M in elevation. The needles contain valuable taxoids, especially for taxol and its precursor DB. We found some individual trees contained rich taxoids in their needles (Ho *et al*. 1997). We have established a sizable number of plants *in vitro*, from which hundreds of cell cultures lines have been induced, which were investigated for the potential of taxoid production (Chang and Yang, 1996; Chang et al., 2001, 2004, 2005; Chen et al., 2003, 2004; Ho et al., 1997, 2000). We have identified several good cell lines producing taxol and BC. Quantities of both these components increased significantly after 35 days on addition of methyl jasnomate (MJ) in the cultures. MJ has been proved to be very effective in increasing taxoids as evident from several reports and patents (Yukimuni et al., 1996; Mirjalili and Linden, 1996). On comparing the taxoids levels in cultures with and without MJ treatment, it was observed that the kinds of taxoids in cultures without MJ were much less than the MJ treated ones (Figure 1). It indicates that MJ not only increased taxoid yield, but also increased the kinds of taxoids. The advantage of cell culture lies in its simplicity and purity of extracts. Based on the high quality and yield in cell cultures, if anyone can find a way for mass production of cell cultures in commercial bioreator, it will be a great opportunity to occupy the 20 billion of taxol market a year! That's the reason why so much research has gone into this area and many patents have been applied.

## 3. Taxol biosynthesis pathway

Due to the low concentration of taxol in yew trees, genetic engineering for manipulation of the taxol biosynthetic pathway (Figure 1) has been viewed as an attractive strategy to increase taxol production. The first gene we selected was TS gene, which was the first gene reported to shift MEP pathway into taxol biosynthesis pathway (Wildung and Croteau, 1996). The second gene we selected was DBAT gene, which controlled the reaction from DB into BC (Walker and Croeau, 2000), both are valuable components as described previously. These two genes have been cloned and sequenced. A 2,743-bp TS cDNA clone was identified from *T. mairei* and designated TmTS. (GenBank accession no. AY365032). The coding sequence of TmTS is highly identical (>96%) to TS genes from *T. chinensis* (accession no. AY007207), *T X media* (accession no. AY461450), *T. brevifolia* (U48796) and *T. baccata* (accession no AJ320538). A DBAT cDNA of 1516-bp long was isolated and designated TmDBAT (GenBank accession no. AY 365031). The coding region of TmDBAT shared more than 96% identity to the other DBATs from *T. cuspidata* (AF193765), *T. baccata* (AF456324), and *T X media* (AY452666) (Lung, 2004).

## 4. The response of cell lines to MJ

We have investigated 41 cell lines for more than 3 to 5 years (Table 2). Each cell line has

its own characteristics. Some cell lines grow rapidly but yield no taxoids. Some lines have taxoids in callus, but it is lost in cell suspension. While, some cell lines perform just apposite. Some lines react positively with MJ, while others do not show any response. Finally, we have identified two best lines: Line A is a BC rich line, while Line B is a taxol rich line. These produce high BC and taxol contents when treated with MJ. MJ treatment not only increased taxoid yield, but also increased kind of taxoids as mentioned in Table 1. However, growth rate of cell cultures declined by 20% after MJ treatment.

**Table 1.** Kinds of Taxoids in cell cultures. A: Cultures without MJ treatment (Only one taxoid type seen) B: With MJ treatment (18 taxoids observed).

| | Image | Select | Retention Time (min) | Max. Absorbance (au) | Start Wavelength (nm) | End Wavelength (nm) | Lambda Max. (nm) |
|---|---|---|---|---|---|---|---|
| 1 | | ☐ | 2.933 | 0.02846 | 210.0 | 400.0 | 211.2 |
| 2 | | ☐ | 3.083 | 0.01489 | 210.0 | 400.0 | 248.8 |
| 3 | | ☐ | 3.400 | 0.09422 | 210.0 | 400.0 | 245.3 |
| 4 | | ☐ | 3.733 | 0.01752 | 210.0 | 400.0 | 217.0 |
| 5 | | ☐ | 4.150 | 0.00382 | 210.0 | 400.0 | 234.7 |
| 6 | | ☐ | 4.533 | 0.01489 | 210.0 | 400.0 | 213.5 |
| 7 | | ☐ | 4.683 | 0.02499 | 210.0 | 400.0 | 210.0 |
| 8 | | ☐ | 5.100 | 0.00954 | 210.0 | 400.0 | 214.7 |
| 9 | | ☐ | 5.417 | 0.03988 | 210.0 | 400.0 | 215.8 |
| 10 | | ☐ | 6.317 | 0.00612 | 210.0 | 400.0 | 211.2 |
| 11 | | ☑ | 6.767 | 0.00979 | 210.0 | 400.0 | 225.3 |
| 12 | | ☐ | 7.400 | 0.00652 | 210.0 | 400.0 | 212.3 |
| 13 | | ☐ | 8.083 | 0.00476 | 210.0 | 400.0 | 240.6 |
| 14 | | ☐ | 8.467 | 0.00824 | 210.0 | 400.0 | 244.1 |
| 15 | | ☐ | 9.217 | 0.01310 | 210.0 | 400.0 | 210.0 |
| 16 | | ☐ | 9.633 | 0.00892 | 210.0 | 400.0 | 210.0 |
| 17 | | ☐ | 10.717 | 0.00805 | 210.0 | 400.0 | 257.1 |
| 18 | | ☐ | 11.817 | 0.03007 | 210.0 | 400.0 | 242.9 |
| 19 | | ☐ | 13.033 | 0.03960 | 210.0 | 400.0 | 311.6 |
| 20 | | ☐ | 16.517 | 0.06530 | 210.0 | 400.0 | 214.7 |
| 21 | | ☐ | 23.650 | 0.00734 | 210.0 | 400.0 | 211.2 |
| 22 | | ☐ | 26.050 | 0.04732 | 210.0 | 400.0 | 210.0 |

(A)

| | Image | Select | Retention Time (min) | Max. Absorbance (au) | Start Wavelength (nm) | End Wavelength (nm) | Lambda Max. (nm) |
|---|---|---|---|---|---|---|---|
| 1 | | ☐ | 2.917 | 0.09298 | 210.0 | 400.0 | 212.3 |
| 2 | | ☐ | 3.083 | 0.06164 | 210.0 | 400.0 | 212.3 |
| 3 | | ☑ | 3.350 | 0.17297 | 210.0 | 400.0 | 215.8 |
| 4 | | ☐ | 3.617 | 0.07789 | 210.0 | 400.0 | 210.0 |
| 5 | | ☐ | 3.917 | 0.02116 | 210.0 | 400.0 | 215.8 |
| 6 | | ☑ | 4.150 | 0.06408 | 210.0 | 400.0 | 228.8 |
| 7 | | ☐ | 4.567 | 0.09080 | 210.0 | 400.0 | 219.4 |
| 8 | | ☑ | 4.717 | 0.13825 | 210.0 | 400.0 | 228.8 |
| 9 | | ☐ | 5.100 | 0.01720 | 210.0 | 400.0 | 219.4 |
| 10 | | ☑ | 5.383 | 1.19227 | 210.0 | 400.0 | 232.3 |
| 11 | | ☑ | 6.433 | 0.02520 | 210.0 | 400.0 | 211.2 |
| 12 | | ☑ | 6.817 | 0.17537 | 210.0 | 400.0 | 230.0 |
| 13 | | ☑ | 7.233 | 0.10720 | 210.0 | 400.0 | 230.0 |
| 14 | | ☐ | 8.333 | 0.01570 | 210.0 | 400.0 | 217.0 |
| 15 | | ☐ | 8.900 | 0.04789 | 210.0 | 400.0 | 220.5 |
| 16 | | ☑ | 9.617 | 0.02891 | 210.0 | 400.0 | 230.0 |
| 17 | | ☑ | 10.517 | 0.01407 | 210.0 | 400.0 | 227.6 |
| 18 | | ☑ | 11.100 | 0.01949 | 210.0 | 400.0 | 230.0 |
| 19 | | ☑ | 11.483 | 0.05037 | 210.0 | 400.0 | 232.3 |
| 20 | | ☑ | 12.517 | 0.03702 | 210.0 | 400.0 | 230.0 |
| 21 | | ☑ | 13.817 | 0.00953 | 210.0 | 400.0 | 213.5 |
| 22 | | ☑ | 15.300 | 0.00864 | 210.0 | 400.0 | 210.0 |
| 23 | | ☑ | 18.217 | 0.01660 | 210.0 | 400.0 | 210.0 |
| 24 | | ☑ | 18.983 | 0.00737 | 210.0 | 400.0 | 212.3 |
| 25 | | ☐ | 20.833 | 0.03192 | 210.0 | 400.0 | 213.5 |
| 26 | | ☑ | 23.683 | 0.10962 | 210.0 | 400.0 | 230.0 |
| 27 | | ☑ | 25.250 | 0.05206 | 210.0 | 400.0 | 230.0 |

(B)

## 5. The strategies to increase taxoids by gene cloning

Based on the understanding of MJ effect, we designed a flow chart (Figure 2). First we cloned TS and DBAT genes and used as a probes to investigate the gene expression of plants with or without MJ treatment. If these genes were strongly regulated by MJ, we presumed that these may be important in taxol biosynthesis. Then, we transferred 35s promoter fused with sense/antisense DBAT and sense TS genes into cells. We expected that these transgenic cell cultures may produce the desired products all the time without MJ treatment.

## 6. Expression of TS and DBAT genes in plants

Northern blot hybridization was carried out to determine the expression of the TS and DBAT genes in different organs of *T. mairei* cultivar T1 (Figure 3A). The transcripts of both genes are detectable in all organs tested, with the highest accumulation detected in young leaves, followed by young stems. Expression of both genes decreased as the tissue

aged. Expression was weak in mature leaves, mature stems, old stems and in roots. When T1 plants were treated with MJ, their expression levels prior to MJ induction were consistent with previous experiments and were higher in young leaves than mature leaves. MJ triggered a strong and rapid induction of TS and DBAT genes expression within 8 hours (Figure 3B). Young leaves responded more strongly and quickly than mature leaves (8 hrs vs 1 day). The induction effect was sustained for at least 2 days, but the transcript levels of both genes gradually returned to normal by day 5 (Lung, 2004). These results indicate that both TS and DBAT genes can be regulated by MJ, suggesting that cell cultures transformed with both genes may increase taxoids.

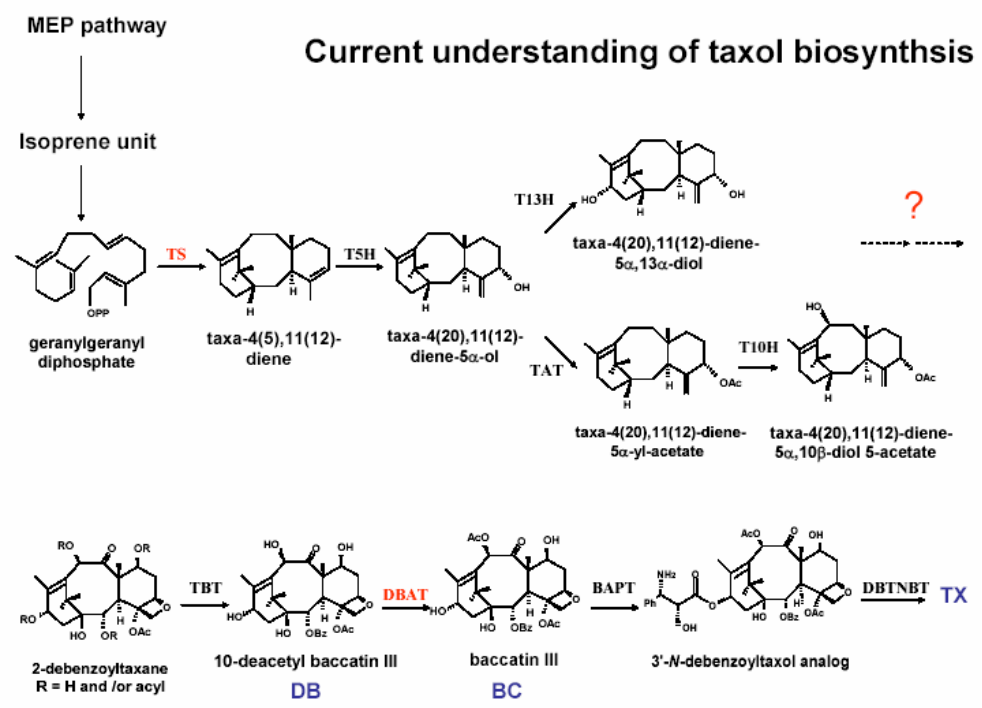


**Figure 1.** Current understanding of taxol biosynthesis

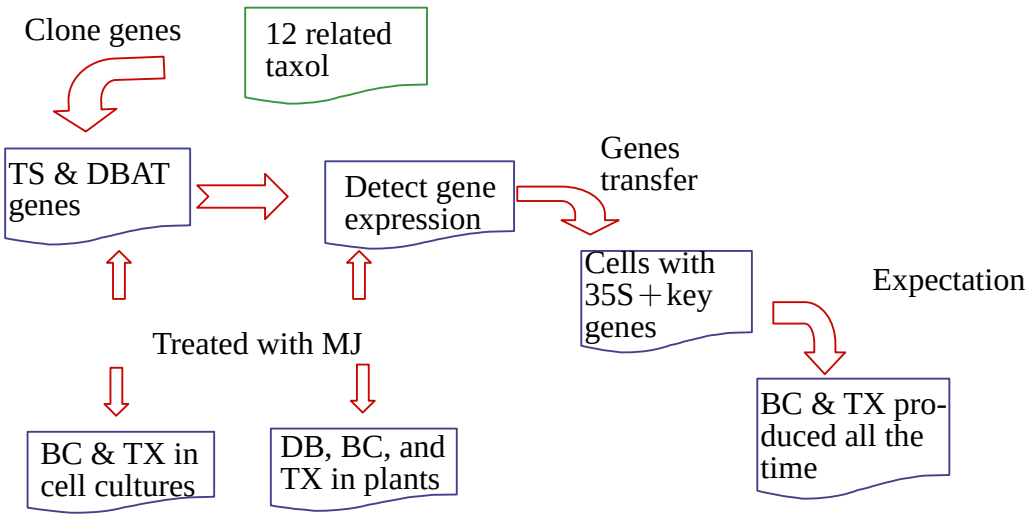


**Figure 2.** The flow chart showing increase in taxoid yield by gene transformation

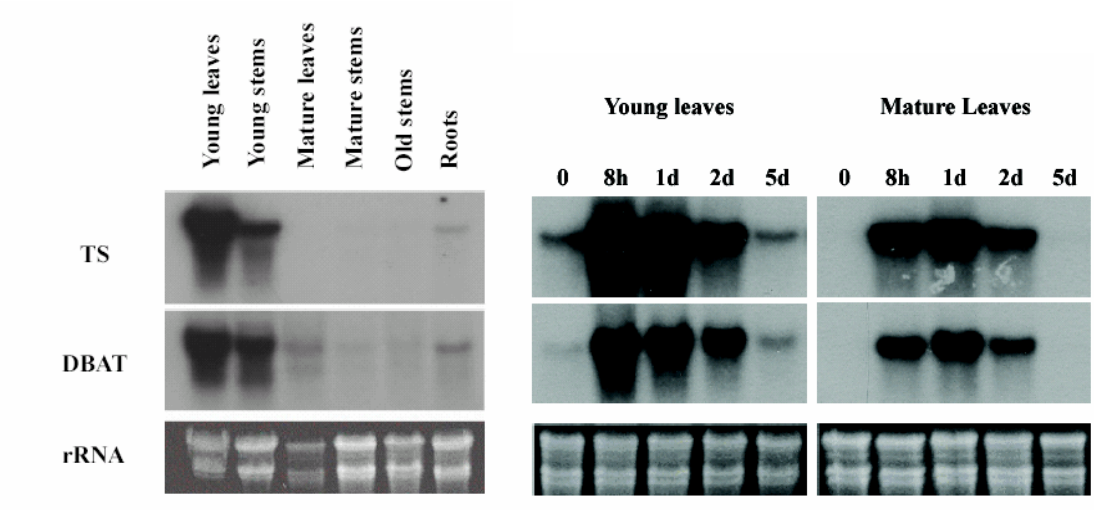


**Figure 3.** The expression levels of the TS and DBAT genes without (A) and with (B) MJ treatment

## 7. Construction of sense/antisense DBAT and TS genes

We constructed sense and antisense DBAT and TS genes as shown in Figure 4. The strong promoter – 35S promotor was used. NPTII gene was used to select transformed lines. At present we have obtained several transformed cell lines (Table 3). Some transgenic cell lines with sense DBAT gene have been cultured in suspension stage, while others in callus stage.

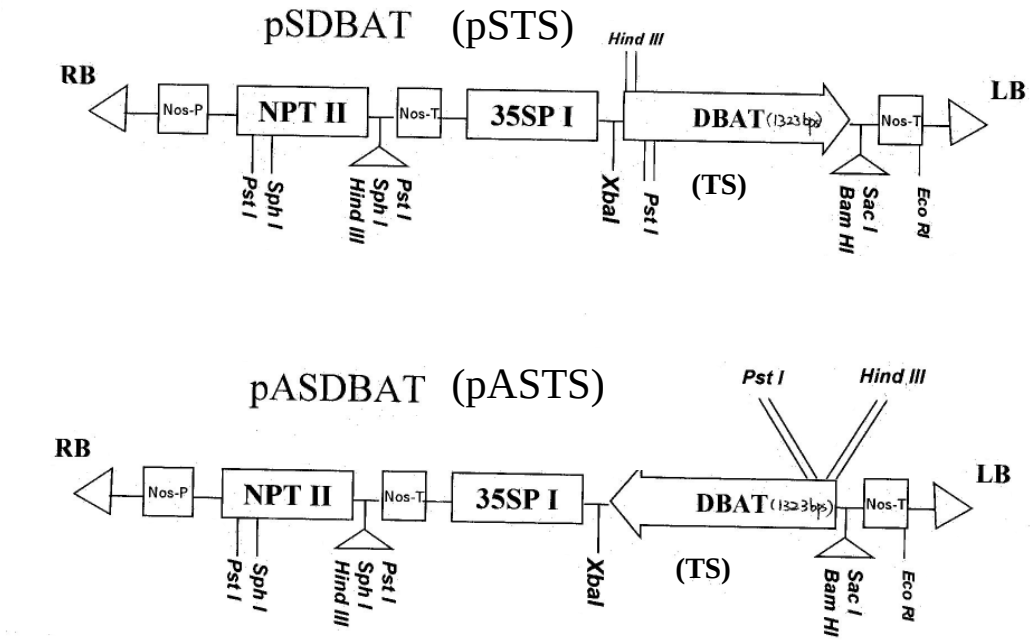


**Figure 4.** Construction of sense and antisense DBAT and TS genes

**Table 2.** The performance of cell lines with/without MJ treatment.

| Cell lines | MJ | Growth % | DB | BC | Taxol |
|---|---|---|---|---|---|
| 41 | - | 15-70 | 0-29 | 0-81 | 0-10 |
| | + | 10-40 | 0-48 | 0-365 | 0-52 |
| Best lines | - | 63 | 10 LineA | 81 | 16 LineB |
| | + | 40 | 48 | 365 | 80 |

**Table 3.** Transformed cell lines and their culture stages.

| Transformed cell lines and cultures | | |
|---|---|---|
| Sense-DBAT | antisense-DBAT | Sense-TS |
| 76 | 41 | 28 |
| Cell cultures | Callus cultures | Callus cultures |

## 8. Performance of transformed cells

We have obtained putative transformed cell lines. Some cell lines transformed with sense DBAT gene were confirmed by using PCR to amplify the 35S/nosT fragments. Though we are interested in the responses of the different transformed cell lines, but presently, only transformed sense-DBAT cell cultures were investigated. First a transformed cell line was induced from E7 clone, which produced only BC. The callus and cell cultures induced from this clone did not produce any taxoids, even after treatment with MJ. Surprisingly, the transformed callus produced BC and taxol. However, in cell cultures the taxoids content became very low. When treated with MJ, both BC and taxol increased significantly. This indicates that for taxoids production an expression of other genes is required, which appears to be regulated by MJ.

The other transformed cell lines was from R5 clone, which contained high concentration of taxoids. Although the callus and cell cultures derived from R5 plants produced few taxoids, taxoid yield increased when treated with MJ. Unlike transformed cells of E7, the transformed cell cultures did not increase taxoids. These required MJ treatment to enhance BC production. BC in transformed cultures with MJ treatment was 2.5 times higher than normal cultures. Thus, this confirmed the previous observation that the greatest yield of taxoids is not controlled by DBAT alone.

**Table 4.** The response of transformed cultures derived from low taxoids lines.

| E7 Clone | DB | | BC | | Taxol | |
|---|---|---|---|---|---|---|
| | Normal | sDBAT | Normal | sDBAT | Normal | sDBAT |
| Seedling (ppm) | 0 | | 460.5 | | 0 | |
| Callus (ppm) | 0 | 0 | 0 | 37.5 | 0 | 36.5 |
| Cell (mg/L) | 0 | 0 | 0 | 3.6 | 0 | 0 |
| Cell +MJ (mg/L) | 0 | 0 | 0 | 75.1 | 0 | 7.8 |

**Table 5.** The response of transformed cultures derived from high taxoids lines.

| R5 Clone | DB | | BC | | Taxol | |
|---|---|---|---|---|---|---|
| | Normal | sDBAT | Normal | sDBAT | Normal | sDBAT |
| Seedling (ppm) | 2570 | | 158 | | 34.2 | |
| Callus (ppm) | 0 | 0 | 1.8 | 0 | 0 | 0 |
| Cell (mg/L) | 0 | 0 | 0 | 0 | 0.6 | 0 |
| Cell +MJ (mg/L) | 0 | 1.4 | 83.2 | 202 | 20.8 | 5.6 |

## 9. Conclusion

In the present study, we found that MJ enhanced the expression of TS and DBAT genes in plants. We expected that cells transformed with sense DBAT gene might increase taxoids yield, but not that transformed cultures would produce taxoids without MJ treatment. The model we propose in this study may be modified further to include more genes for testing. However, the function of genes we cloned are

still waiting for the performance of the cells transformed with sense TS and antisense DBAT genes.

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