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Full Length Research Paper

Optimized integration of T-DNA in the taxol-producing fungus *Pestalotiopsis malicola*

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We previously reported a taxol-producing fungus *Pestalotiopsis malicola*. There, we described the transformation of the fungus mediated by *Agrobacterium tumefaciens*. T-DNA carrying the selection marker was transferred into the fungus and randomly integrated into the genome as shown by Southern blotting. Approximately 66% of the transformants obtained a single copy of T-DNA. Conditions of transformation were optimized. The method can be used for taxol-related genes searching and manipulation in *P. malicola*. This is the first report of T-DNA-mediated transformation in taxol-producing fungi.

Key words: T-DNA, random insertional mutagenesis, taxol, *Pestalotiopsis.*

INTRODUCTION

Paclitaxel (Taxol®), a natural anti-tumor drug, was originally isolated from the bark of pacific yew, *Taxus brevifolia* (Wani et al., 1971), and were later found in a number of endophytic fungi (Strobel et al., 1996; Zhou et al., 2010). By estimate, more than two dozen taxol-producing fungi have been documented from all over the world (Zhou et al., 2010). Since the raw material of yew trees is limited, large-scale fungal fermentation is regarded as a promising solution for the short supply of the drug in the market. As many believe, it may make taxol affordable for more patients (Fu et al., 2009). Currently, problems need to be solved before its industrial application. For instance, the current yield of paclitaxel in fungi is still low. And in some cases, instability of the metabolite was also observed (Miller et al., 2008; Bi et al., 2011). Illustration of taxol biosynthesis, particularly, at the molecular level, is significant for addressing these issues. However, little progress has been made towards this goal. A surprising fact is that transformation of foreign DNA into taxol-producing fungi has not been well established. This is perhaps due to the lack of sophisticated molecular tools in these non-model taxol-producing fungi.

We previously isolated a taxol fungus, *Pestalotiopsis malicola* Nk101 (Bi et al., 2011). In this report, we briefly describe a method for high frequency transformation of the fungus in conjugation with *Agrobacterium tumefaciens*. *A. tumefaciens*-mediated transformation (ATMT) has been successfully applied in numerous fungi (Michielse et al., 2005). However, ATMT has shown fungal strain specificity and particular marker need to be developed accordingly. In the optimized conditions, a large number of transformants was obtained. We constructed a hygromycin B resistance cassette and showed it worked properly in *P. malicola*. Therefore, we used it as the selection marker that was ligated to the T-DNA of *A. tumefaciens*. We designed a protocol for a high frequency transformation via ATMT. In the optimized conditions, a large number of transformants was obtained. Southern blot showed that the T-DNA was integrated into the chromosomes in a random way. This method may be useful for gene complementation and targeting and for large-scale random mutagenesis in *P. malicola*.

MATERIALS AND METHODS

For transformation of NK101, a proper selectable marker was first chosen and its expression cassette was constructed. NK101 showed a high sensitivity to hygromycin B (100 µg/ml and above,

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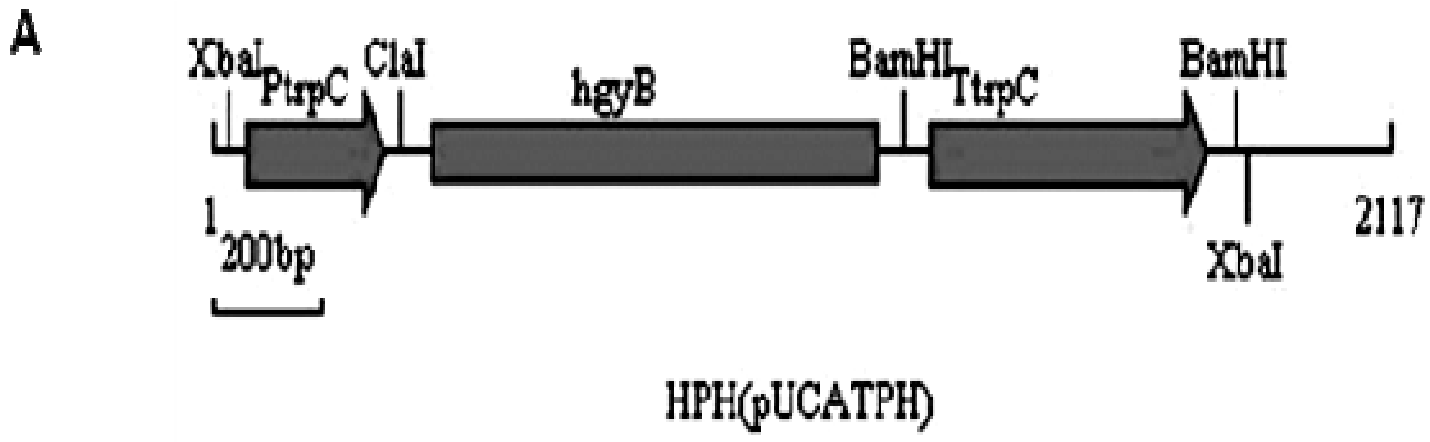

Figure 1 A. Diagrammatic structure of the hygromycin B resistant cassette. The cassette was on a 2.1-kb *Xba* I fragment derived from pUCATPH (Lu et al., 1994). The cassette was then ligated to pBI121 at *Xba* I site to form a new binary vector pBI121-HPH for fungal selection. The plasmid pBI121-HPH was introduced into *A. tumefaciens* LBA4404 grown at 28°C overnight in 5 ml LB that contained antibiotics: 100 mg/L streptomycin and 25 mg/L rifampicin. The cells were collected at 5,000 rpm for 5 min at 4°C, washed with ice-cooled $CaCl_2$ (20 mM) twice, and resuspended in 500 µl 20 mM $CaCl_2$. Aliquots (100 µl) of the cells were incubated with approximately 2 µg pBI121-HPH DNA on ice for 30 min, and in liquid nitrogen for 2 min, before incubated in a water bath for 5 min at 37°C. After centrifugation at 12,000 rpm for 30 s, the mixture was grown in 500 µl LB medium at 28°C for 3.5 h and aliquots of 200 µl were plated on LB agar containing both streptomycin (100 mg/L) and rifampicin (25 mg/L) at 28°C.

data not shown). As a test, we used a hygromycin B resistant gene, *hph*, as the marker in the transformation. The *hph* ORF was under the control of the *trpC* promoter (P-trp) and *trpC* terminator (T-trp) from *Aspergillus nidulans*. This *hph* cassette has been used successfully in numerous fungi (Cullen et al., 1987; Lu et al., 1994). The *hph* cassette, a 2.1-kb *Xba* I fragment, on the plasmid pUCATPH was inserted into T-DNA at the *Xba* I site on the binary vector pBI121 (GenBank accession No. AF485783.1) to generate a new vector pBI121-HPH (Figure 1A). By $CaCl_2$-mediated transformation, pBI121-HPH was then introduced into *A. tumefaciens* LBA4404 which carried the T-DNA transfer machinery (Bundock et al., 1995; Michielse et al., 2004). The plasmid pBI121-HPH extracted from LBA4404 was conventionally verified by multiple restrictions and sequencing. Fortunately, this P-trp-*hph*-T-trp functioned properly in *P. malicola*.

RESULTS AND DISCUSSION

NK101 conidia were co-cultivated with the LBA4404 cells carrying pBI121-HPH in the presence of acetosyringone (AS) to induce the transfer of T-DNA. Fungal hygromycin resistant colonies grew up in two days after the filter carrying both donors and recipients was transferred to the selection medium (Figure 1B, the left panel). A negative control was set by incubation of fungal conidia with LBA4404 cells carrying only pBI121 without *hph* and did not give rise to any resistant colonies (Figure 1B, the central panel). This result demonstrates that LBA4404 successfully transformed the T-DNA-HPH to NK101. Transformation efficiency varied from up to 180 transformants per plate (10^7 conidia) by estimate (Figure 1B, the left panel). Transformants were incubated at 22°C, 12 to 14 days, under lights, for sporulation. Transformants were purified by individual spore isolation and individual spores were allowed to grow up on hygromycin B (100 µg/ml) plates (Figure1B, the right panel).

We further determined the optimal transformation condition. The time for co-cultivation of the conidia with bacterial cells was one of the critical factors affecting the transformation efficiency (Figure 1C). When co-cultivation time was less than 12 h, few transformants were obtained. With longer incubation, more transformants were gained until 60 h.

The efficiency of transformation reached a maximal value after that time point (approximately 180 to 200 transformants/10^7 conidia). The presence of AS (200 µM) in the medium prior to co-cultivation was also an important factor on the efficiency (Figure 1C). Approximately 2-fold transformants were obtained in the presence of AS. Furthermore, transformation efficiency was also dependent either on the number of bacterial cells or the conidia used in the experiment. As tested, 10^8 bacterial cells (CFU) and 10^7 conidia were optimal for transformation (Assays 1 and 3 in Table 1). When the number of *A. tumefaciens* cells was over 10^9 or NK101 conidia over 10^8, the transformation efficiency fell sharply instead (data not shown). Additionally, the ratio of *A. tumefaciens* cells to the fungal conidia was determined to be 10:1 (bacterium to conidia). The highest number of transformants in the assays was achieved at this ratio (Assays 1 and 2 in Table 1).

Acquisition of hygromycin B resistant transformants suggests that ATMT was achieved in *P. malicola*. The P-trp-*hph*-T-trp cassette worked properly in this fungus and can be used as a selection marker. The best number of *A. tumefaciens* cells and NK101 conidia were 10^8 and 10^7, respectively. The best ratio between bacterium and

B

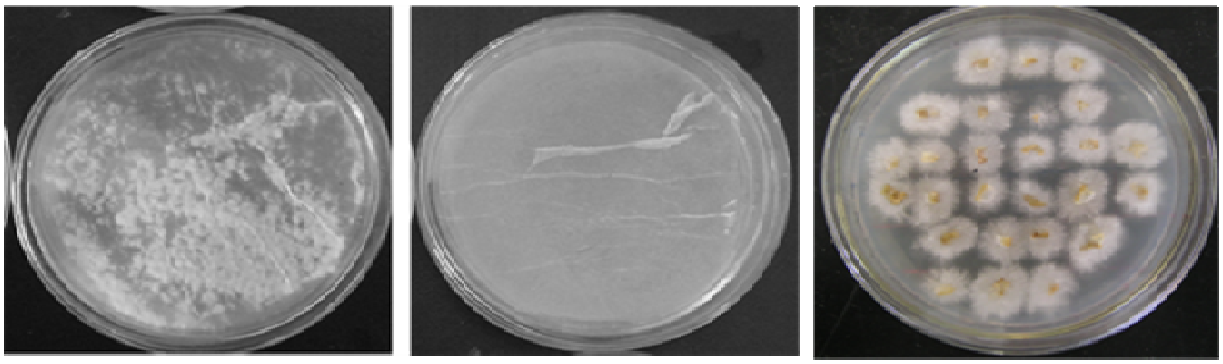

Figure 1 B. Transformation of *P. malicola* NK101. The left panel, a large number of hygromycin resistant Nk101 transformants grew up on hygromycin (100 µg/ml) PDA plate after a cocultivation with LBA4404 carrying pBI121-HPH, at 24°C for 48 h. As a control, no positive colonies grew from a co-incubation with the LBA4404 cells containing the plasmid pBI121 without *hph* gene (the central panel). The right panel shows purified transformants via single spore isolation on hygromycin-containing PDA plate. The medium PDA (20 potato, 2 dextrose and 2% agar) was used for fungal growth. For sporulation, NK101 was incubated at 22°C, 12 to14 days, under continuous illumination. For T-DNA transfer, 200 µM acetosyringone (AS, Sigma, St. Louis, USA) was added to Induction medium (IM) (Michielse et al., 2004). Fungal transformants were selected on PDA supplemented with 100 µg/ml hygromycin B and 200 µg/ml cefotaxime that killed LBA4404. For conjugation, fungal conidia were stripped off the agar with sterile H_2O and spun down. LBA4404 cells were grown in LB at 28°C for 18 h and diluted to an OD_{600} reading 0.15 in induction medium (IM), with or without 200 µM AS, shaking at 200 rpm, 28°C to OD_{600} of 0.5 to 0.8. Approximately 100 µl of the bacterium culture (10^8 CFU) was mixed with an equal volume of NK101 conidia (10^7) and spread on a nitrocellulose filter (45 mm in diameter). The membrane was placed on co-cultivation plates in the presence of 200 µM AS. After incubation at 24°C for 48 h, the filter was transferred to PDA supplemented with hygromycin B (100 µg/ml) and cefotaxime (200 µg/ml), at 24°C for 48 h. The filter was then removed and the plate was incubated at 22°C for two weeks for sporulation.

C

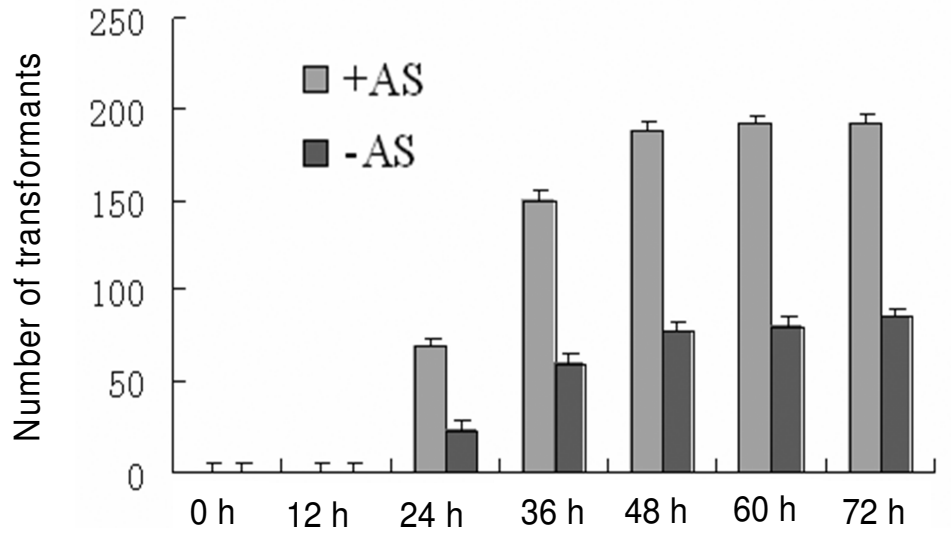

Figure 1 C. Effects of co-cultivation time and AS on transformation efficiency. The number of transformants approximately doubled in the presence of AS. When the time of conjugation was shorter than 12 h, few transformants were obtained. After 24 h, the number of transformants increased with longer incubation till 60 h. Each test was conducted in triplicate. The error bars represents the standard deviation.

Table 1. The effect of the ratio of LBA4404 cells to NK101 conidia on transformation frequency. Each co-cultivation was carried out in triplicate. Transformants were counted and errors were calculated as standard deviation.

Assay	Number of LBA4404	Number of conidia	*A. tumefaciens* : *Pestalotiopsis* sp. ratio	Number of transformant
	1×10^8	1×10^7	10:1	200 ± 11
1	1×10^8	1×10^6	100:1	81 ± 5
	1×10^8	1×10^5	1000:1	3 ± 1
	1×10^7	1×10^7	1:1	25 ± 3
2	1×10^6	1×10^7	1:10	3 ± 2
	1×10^5	1×10^7	1:100	0
	1×10^8	1×10^7	10:1	180 ± 13
3	1×10^7	1×10^6	10:1	7 ± 2
	1×10^6	1×10^5	10:1	0

fungal conidia was 10:1. AS in the culture of *A. tumefaciens* LBA4404 significantly increased the transformation frequency. The co-cultivation period should be no shorter than 48 h for an efficient transfer of T-DNA. Under this condition, the frequency can reach approximately 200 distinguishable transformants on a single plate. Notably, we tried another transformation method for *P. malicola*, that is, using the conventional protoplast-PEG protocol. Due to the production of extracellular polysaccharides by the fungus, the making of protoplasts became a challenge in this fungus. Transformation has not been successful by this method so far (data not shown).

T-DNA destination in transformants and taxol-deficient candidates

We analyzed the destination of T-DNA in the transformants by Southern blotting. Genomic DNA was prepared for 18 randomly selected transformants. In one gel for undigested genomic DNA blotting, all positive *hph* bands were detected at the level corresponding to the genomic DNA bands, suggesting that T-DNA was integrated in the genome (only one gel with 10 wells was shown). In the lane containing the wild type DNA (lane W), no *hph* band was detected (Figure 2A). In another blotting (Figure 2B), *Hind* III, which did not cut the *hph* cassette, was used to digest the genomic DNA, seven out of the nine transformants, except lanes 8 and 9, showed different band patterns, suggesting that the insertion occurred largely in a random way. Additionally, transformants 7, 8 and 9 had two positive bands, suggesting that two copies of T-DNA existed in these transformants (34% of 9 transformants); the other 66% of the 9 transformants harbored only a single copy of T-DNA (Figure 2B).

Interestingly, in a PEG/protoplast-mediated transformation of another taxol-producing fungus, *P. microspora*, the transformed foreign plasmid DNA remained as independent extrachromosomal molecules with telomeric repeats (Long et al., 1998). Southern blot in that report showed that all the eight randomly picked transformants contained multiple plasmid bands ranging from 6 to 30 kb in size. The data indicates that the double-stranded plasmid DNAs in the protoplast/PEG transformation is likely a substrate for the telomeric modification. In this respect, the single-stranded T-DNA transfer by *A. tumefaciens* in our case was likely immuned from a similar modification, and thus promoted the integration of T-DNA into the chromosomes.

We designed an optimized T-DNA integration procedure in the taxol-producing fungus, *P. malicola* (Bi et al. 2011). Random integration of T-DNA is of practical benefit for further study in several aspects, for instance, to make tagged mutants and for targeted gene disruption, as ATMT has been successfully applied in numerous fungi (Michielse et al., 2005). With ATMT, we constructed an insertional mutant library. Variants in phenotype have been observed. In fact, after screening more than 800 transformants, four exhibited a taxol-deficient phenotype as demonstrated by LC-mass screening procedure (Figure 3). The characteristic peaks of taxol at 854 or 876 m/z disappeared in these four transformants. Interestingly, the yield of an unknown secondary products increased dramatically in another transformant, B19 (data not shown). The analysis for these mutants with molecular approaches is now under the way. Unfortunately, so far, we have not obtained any transformants with improved yield of taxol. Thus, further screening is necessary for a mutant with a higher taxol yield.

It is noteworthy that *Pestalotiopsis* spp. is actually one of the most encountered fungi in nature (Ge et al., 2009). They are frequently associated with plants as a pathogen or endophytes (Ge et al., 2009). Based on the information we collected, at least eight different species from the genus *Pestalotiopsis* have been documented so far to produce taxol, indicating that taxol biosynthesis is likely conserved in this genus. Investigation on *Pestalotiopsis* fungi may shed light on the origination and evolution of the biosynthesis of taxol. This work presents a tool for the purpose.

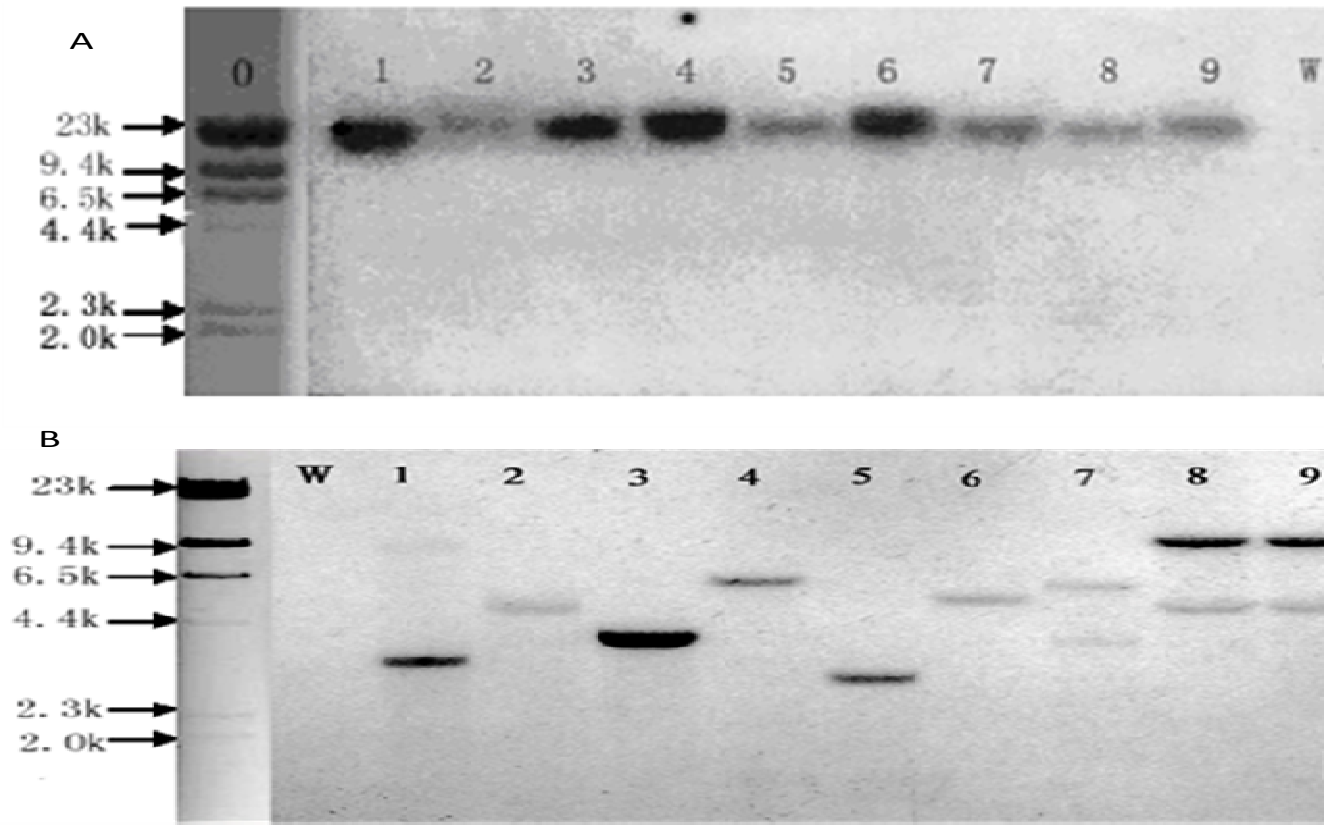

Figure 2. Integration of T-DNA-HPH in NK101 transformants. (**A**) Southern blot for undigested genomic DNA of nine randomly picked transformants (lane 1 to 9). The membrane was probed with the 2.1-kb *Xba* I hygromycin resistant cassette. Positive bands were located at the genomic DNA level. Lane 0 was the marker of λ DNA-*Hin*d III; Lane W contained the wild type NK101 DNA, as control. (**B**) Southern blot for *Hin*d III digested genomic DNA of the nine transformants, probed with the same fragment. *Hind* III did not cut T-DNA-HPH. Six out of nine transformants have one copy of T-DNA inserted in the genome. DNA preparation was described by Raeder and Broda (1985). The fungal DNA undigested or digested with *Hin*d III was separated by electrophoresis with 0.8% agarose gel in 1× TAE (40 mM Tris-acetate, pH 8.5 and 2 mM EDTA). The gel then was treated with 0.25 M HCl before blotting onto a charged nylon transfer membrane (Hybond-N^+, Osmonics, U.K.) with 0.4 M NaOH as transfer buffer. The blotting procedure was carried out by following the membrane manufacturer's instruction. The 2.1-kb *Xba* I fragment of *hph* cassette was labeled with ^{32}P-dCTP.

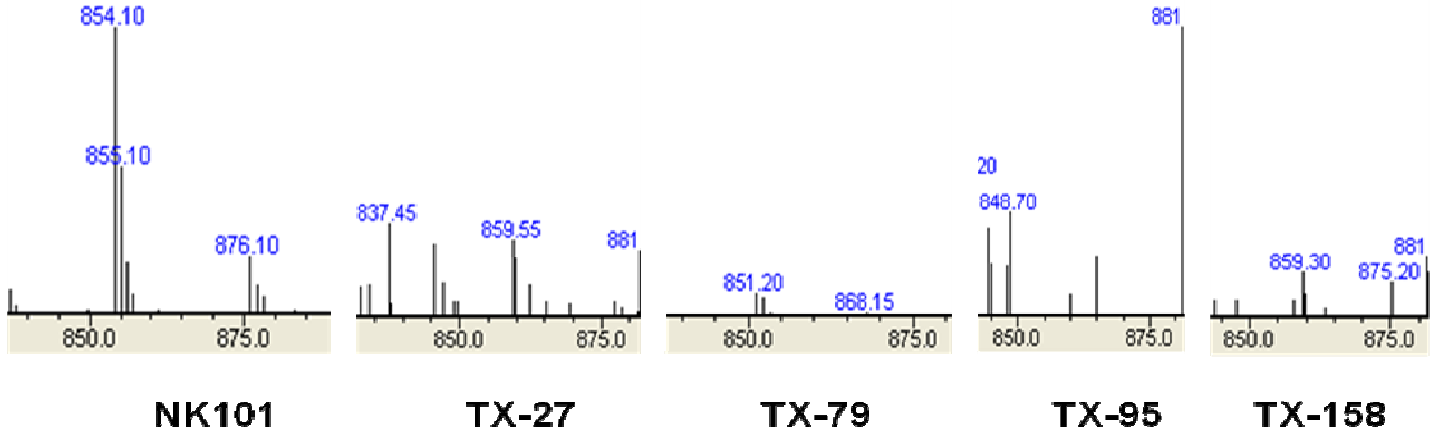

Figure 3. Electrospray mass spectrum (MS) for taxol-deficient candidates from the transformant library by ATMT. The mutants(TX-27, TX-79, TX-95, and TX-158） and the wild type strain were inoculated in 200 ml PDA and incubated for 6 days at 28℃. The fermentation broth was treated as described by Bi et al. (2011). The extracts were subjected to liquid chromatograph-mass spectrometry. The characteristic peaks of taxol at 854 $(M+H)^+$, 876 $(M+Na)^+$ were observed in the sample of wild type. In contrast, no equivalent peaks were detected in the samples in the mutant candidates.

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