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GENETIC TRANSFORMATION OF *TRICHODERMA VIRENS* WITH *ASPERGILLUS NIDULANS amdS* GENE AS POSITIVE SELECTABLE MARKER

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ABSTRACT: *Trichoderma virens protoplast was transformed with the acetamidase (amdS) gene from Aspergillus nidulans. Transformation was done using the pamdS plasmid harboring Aspergillus nidulans 3.8 kb amdS fragment that code for an acetamidase. The transformants were able to grow on acetamide as sole source of nitrogen as vigorously growing colonies against weak background growth for wild type. Two types of transformant colonies can be distinguished. Type I show continuous vigorous growth and regenerated on (protoplast regeneration selective medium) PRSM at frequencies of 7 transformants per µg plasmid DNA. Type II gave small colonies that were appeared at frequency of 22 transformants per µg plasmid DNA. Ten colonies out of the thirty-five type I transformants were able to give spores. However, the remaining transformants were sterile. All colonies of type II transformants may be considerd as abortive transformants. Five colonies out of the ten type I transformants were mitotically stable. Southern blot analysis was performed to verify the presence of A. nidulans amdS gene. The amdS gene was integrated in T. virens genome at random fashion. The copy number of amdS integrated gene and the sites of integration appear to be differ with each transformants. Extracellular protein was subjected to SDS-polyacrylamide gel electrophoresis to examine the amdS gene expression of the mitotically stable transformants compared to mitotically unstable transformants. The protein-banding pattern of the transformants revealed high level of 55 - 60 KD significant band that was not present in non-transformant recipient isolate of T. virens. This band was consistent with the size of the A. nidulans acetamidase polypeptide.*

Key Words: acetamide, pamdS plasmid, Protoplast, SDS-PAGE, Southern blot analysis, Transformation, Trichoderma virens.

INTRODUCTION

At the end of 20th century, the public attitude toward the use of chemical pesticides was changed. *Trichoderma* species have been investigated as biological control agents for over 70 years (Elad *et. al.*, 1981; Papavizas 1985; Harman and Kubicek 1998 and Hjeljord and Tronsmo 1998). Nowadays, such species have become commercially available. *Trichoderma virens* is one of the most important biological control agents against plant pathogenic fungi

(Miller *et. al.*, 1975). This fungus was formerly classified morphologically as *Gliocladium virens*. Recently, Rehner and Samuels (1994), Rehner and Samuels (1995) and Samuels (1996) found that the *G. virens* is very similar to the anamorph of *Hypocrea gelatinosa*. Moreover, the direct sequence of its internal transcribed spacer (ITS) has classified *T. virens* among other *Trichoderma* species and close in proximity to *T. harzianum* (Lieckfeldt *et. al.*, 1998). *T. virens* grows poorly on acetamide as nitrogen source and lacks sequences detectable homologous to *amdS* gene encoding the acetamidase. This observation led to develop a transformation system for *Trichoderma* species using *amdS* gene as a dominant heterologous marker for selecting transformants based on acetamide utilization. Transformation system allowed detecting and measuring the population dynamics of commercially available strains in soil and opened up the possibilities of DNA-mediated manipulation of *Trichoderma* species. Transformation systems have been previously described (Lorito 1993, Ossanna and Mischke 1990, Herrera-Estrella *et. al.*, 1990, Goldman *et. al.*, 1990, Thomas and Kenerley 1989, Penttila *et. al.*, 1987). The present study was carried out to: 1. transform the protoplast of an Egyptian *T. virens* isolate using *Aspergillus nidulans amdS* gene and 2. examine the *amd*S gene presence and expression upon transformation.

MATERIALS AND METHODS

Strains and plasmid: As recipient for the transformation the *T. virens* isolate, Tv101 was used. The *pamdS* plasmid that derivatives of p3SR2 plasmid 3.8-kb SalI/XbaI fragment comprising the *Aspergillus nidulans amdS* in pUC19, was kindly supplied by Prof. Dr. Robert Mach (Microbial Biochemistry & Gene Technology Group, Institute of Chemical Engineering, Vienna University). Plasmid was propagated in *E. coli* K12 strain JM 109.

Media: Minimal medium (MM) containing, per liter, 20 g glucose, 5 g $(NH_4)SO_4$, 1.5 g KH_2PO_4, 0.6 g $MgSO_4$, 0.6 g $CaCl_2$, 5 mg $FeSO_4.7H_2O$, 6 mg $MnSO_4.H_2O$, 4 mg $ZnSO_4.H_2O$, 2 mg $CoCl_2$ and 2 % agar. The pH was adjusted to 5.5. The selective medium (SM) for *amdS*$^+$ transformants was MM supplemented with 10 mM acetamide, as sole source of nitrogen, instead of $(NH_4)SO_4$ and 12.5 mM CsCl. 1 M Sorbitol was added to MM or SM as an osmotic stabilizer made protoplast regeneration minimal medium (PRMM) and protoplast regeneration selective medium (PRSM), respectively. Mycelium for protoplast preparation and DNA extraction was grown in Potato Dextrose Broth (PDB). For extracellular protein preparation, mycelia were grown in liquid SM or MM. Potato dextrose agar (PDA) was used as a maintenance medium.

Protoplast preparation and Transformation: Preparation of protoplasts was carried out according to Penttila *et al.*, (1987) method with some modifications. Young spores from *T. virens*, Tv101, isolate were grown with shaking for 20 h in PDB medium containing 100 µg ampiciline / ml. The

mycelium was harvested, washed with cold 1.2 M $MgSO_4$ solution, and suspended at concentration of 100 mg wet weight/ml in lytic solution (1.2 M $MgSO_4$, 10 mM sodium phosphate buffer, pH 5.8 + 7.5 mg / ml of Novozyme 234) and incubated for 3 h at 28 °C with gentle agitation. Protoplasts were separated from undigested mycelia debris by filtering through cheesecloth. Equal volume of 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.0 solution was added. Protoplasts were collected by centrifugation at 3000 rpm for 15min at 4 °C and washed twice in 1 M sorbitol, 10 mM Tris-HCl, pH 7.5 solution. Protoplasts were resuspended in STC buffer (1 M sorbitol, 10 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.5) at a density of approximately 5×10^8 / ml and kept on ice until used.

The transformation was carried out as described by Ballance *et. al.*, (1983). 200 µl of 5×10^8 protoplast /ml were mixed with 5 µg *pamdS* plasmid in 20 µl. Then 50 µl of PEG solution (25 % w/v polyethylene glycol 8000, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) was added and the mixture was incubated on ice for 20 min. The incubation was continued at room temperature for 5 min after addition of 2 ml of PEG solution. Then 4 ml of STC buffer was added. Aliquots of 500 µl were plated on agar overlay onto PRSM. Plates were kept at 28° C. Viable numbers of protoplasts, before and after addition of PEG, that were able to give rise to colonies on PRMM were counted to determine regeneration frequencies. For determination of Transformants mitotic stability, the arising single colonies transferred to SM plates and subcultured on MM for three successive growth cycles. Subsequently, the transformants were re-plated on SM.

DNA extraction: DNA was extracted according to Al-Samarrai and Schmid (2000) with minor modifications as follows; each isolate was cultured in 100 ml of PDB at 28 °C for 3 days. Mycelium was harvested and washed, then freezed immediately by immersion in liquid nitrogen. Thirty mg of ground mycelium was suspended and lysed in 500 µl of lysis buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, 1 % w/v SDS pH 7.8), then 20 µg/ml of RNAse A was added. 5 min later at 37 °C, 165 µl of 5 M NaCl solution was added. The suspension was centrifuged at 13000 rpm for 20 min at 4 °C, then supernatant was immediately transferred to fresh tube containing 400 µl of chloroform and 400 µl of phenol. After centrifugation for 20 min the equeous phase was transferred and extracted with an equal volume of chloroform. DNA was precipitated by 95 % ethanol. To free DNA from polysaccharide the precipitate was resuspended in 500 µl of lysis buffer followed by addition of 165 µl of 5 M NaCl solution. The suspension was then chloroform-extracted. The DNA in equeous phase was precipitated again and washed three times with 70 % ice-cold ethanol and resuspended in 50 µl sterile deionized distilled water.

DNA manipulations: 20 µg DNA was digested with *Xba*I restriction endonuclease according to the manufacturer instructions. DNA was

fractionated on 0.6 % agarose gel and transferred from the gel onto Hybond N^+ nylon membrane according to Southern (1975). The nonradioactive AlkPhos Direct kit from Amersham Pharmacia Biotech was used for Southern blot hybridization, according to the manufacturer instructions with hybridization temperature 55 °C. A 100 ng probe (*SalI-XbaI* fragment) was labeled and hybridized to the membrane. For the chemiluminescent detection of alkaline phosphatase, CDP-Star detection reagent was used, according to the manufacture's instructions.

Extracellular protein isolation and SDS- polyacrylamide gel electrophoresis: Preparation of extracellular protein was carried out as follows. A disk of transformants and recipient isolate (4mm diameter) were incubated at 30 °C for one week in 100 ml of MM. Approximately 300 mg young mycelium were incubated at 30 °C for 48h in 100 ml of MM or SM supplemented with 25 mM NaCOOH. Extracellular protein was recovered from 5 ml culture supernatant after addition of an equal volume of methanol and tenth volume of chloroform. Centrifugation at 5000 rpm for 20 min at 4°C was made. Upper phase was discarded and the protein was precipitated by addition 1 ml of methanol to the lower phase. Then protein was pelleted and resuspended, after air-drying, in Laemmli sample buffer (64 % of 0.15 M Tris-HCl buffer, pH 6.8. 20 % glycerol, 6 % SDS, 10 % 2-6-mercaptoethanol and 0.1% bromophenol blue). Fifty-microliter samples were subjected to electrophoresis in 10 % polyacrylamide prepared in 0.1 % SDS with a 5% polyacrylamide stacking gel. Electrophoresis was conducted at 10 °C for about 4 hr at 15 mA (Laemmli, 1970). Electrophoresis was performed in a vertical slab mold (80 × 100 × 1.5 mm). Gels were stained with coomassie blue.

RESULTS AND DISCUSSION

T. virens transformation with A. nidulans amdS gene: The efficient production of protoplasts isolated from 20-h-old mycelium of *T. virens* isolate, Tv101, was approximately 7.4×10^5 protoplasts / 100 mg wet weight of mycelia under the experimental condition. Regeneration frequency of protoplasts was 6.8×10^5 colonies per 10^6 protoplasts on PRMM. However, the regeneration frequency was dropped to 5.1×10^5 colonies per 10^6 protoplasts on PRMM after the addition of PEG solution.

Protoplast transformation experiment was conducted in order to obtain transformants able to grow on acetamide as sole of source nitrogen ($amdS^+$). The *pamdS* plasmid harboring *A. nidulans* 3.8 kb *amdS* fragment that code for an acetamidase was used to transform *T. virens* protoplasts, Tv101 by PEG-mediate. The transformants appeared after a 15 to 20 days of incubation on PRSM as vigorously growing colonies against weak background growth for wild type. Two types of transformant colonies can be distinguished as it shown in (Fig. 1). Type I show continuous vigorous growth and regenerated

on PRSM at frequencies of 7 transformants per µg plasmid DNA. Type II gave small colonies that were appeared at frequency of 22 transformants per µg plasmid DNA as presented in tabel (1). Similar finding was reported by Pe'er *et. al.*, (1991) who developed an efficient method to detect, monitor or recover *T. harzianum* that released into soil by *amd*S transformation. On contrary, Penttila *et. al.*, (1987) obtained large *AmdS*$^{+}$ colonies of *T. reesei* with a frequency of 50-100/µg of plasmid DNA and the smaller ones with a frequency of 100-400/µg. This discrepancy may be due to the differences between the *Trichoderma* species or to DNA vector used.

Appearing colonies on PRSM were transferred to SM. Ten colonies out of the thirty-five type I transformants were able to give spores on SM as shown in tabel (1). However, the remaining transformants were sterile and this was obvious as it appeared in (Fig. 2). All colonies of type II transformants may be consider to be abortive transformants. As they initially grew to a limited extent on PRSM but cannot be rescued into large colonies after transferring the colonies to MM, followed by replating on SM (Kelly and Hynes 1985). Only one of type II transformant can be rescued but failed to give spores.

Five colonies out of the ten type I transformants were mitotically stable. This facet was shown by repeated transfer of the spores for two growth cycles on to MM and subsequent reculturing on SM.

Presence of amdS gene: To detect the presence of integrated amdS gene into genomic DNA, Southern blot analysis was performed. The genomic DNA XbaI digests of the *T. virens* recipient isolate and ten of type I transformants were applied to agarose gel electrophoresis and submitted to analysis by Southern blot technique to verify that the *A. nidulans amdS* gene was integrated into the genome of the transformants. The blot was probed with SalI/XbaI DNA fragment (*A. nidulans amdS* gene) that excised from *pamdS* plasmid (Fig. 3). Transformants appeared to be different regarding copy number of the *amdS* integrated gene. Fig (3) clearly shows that isolates 3, 5, 6, 8 and 9 harboring single copy of amdS gene, as it appeared as single band. Furthermore, two integrated copies were found in transformant number 2 and 10, also three integrated copies were found in transformant number 1 and 4 transformants (Fig. 3). In addition, one transfomant showed four bands reflecting the presence of four amdS copies. The copy number of *amdS* integrated gene and the sites of integration appear to be differ with each transformants. The results showed that acetamide utilization transformant phenotype (*amds*$^{+}$) never detected in control transformation experiment in which protoplasts were incubated without plasmid DNA. This observation indicates that the *amdS* gene is not present in the *T. virens* genome. Moreover, DNA analysis confirmed this finding and showed that the *T. virens* genome lacks sequences detectable homologous to *amdS* gene (Fig. 3). Consequently, *amdS* gene was integrated in *T. virens* genome at random fashion.

Table 1: Classification of *Trichoderma virens* isolate, Tv101, transformed with *amdS* gene using *pamdS* plasmid that comprising 3.8-kb SalI/XbaI fragment of the *Aspergillus nidulans amdS*.

Transformant type	Transformants number / 5µg						
		Total		True		Fertile	
	Total	Abortive	True	Steril	Fertile	Stable	Unstable
Type I	35	0	35	25	10	5	5
Type II	112	111	1	1	0	-	-

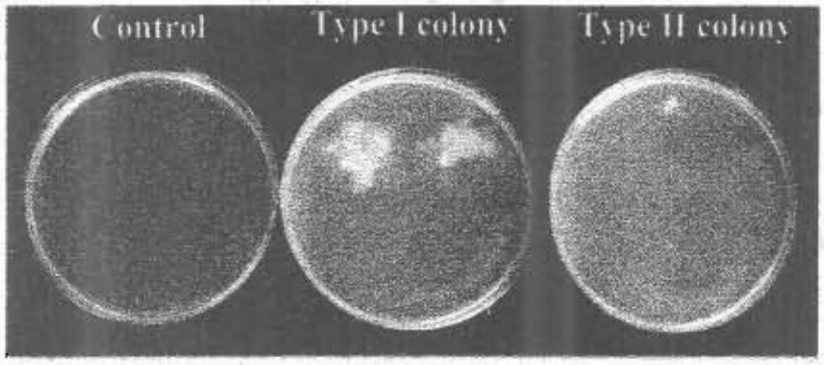

Fig. 1: Two typies of *Trichoderma virens* transformants arised after 15 - 20 days on protoplast regeneration acetamide-CsCl medium, Type I (large) and type II (small) coloneis after protoplast transformation using *pamdS* plasmid harboring *A. nidulans* 3.8 kb *amdS* fragment that code for an acetamidase.

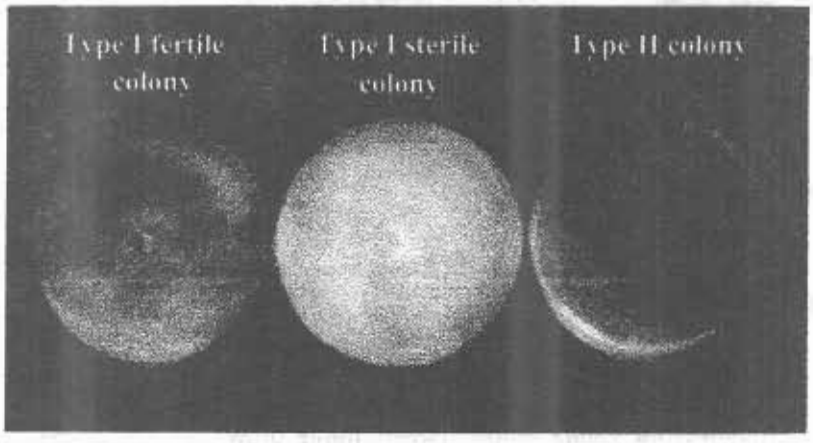

Fig. 2: Appearing colonies of *Trichoderma virens* transformants on protoplast regeneration acetamide-CsCl medium that transferred to acetamide-CsCl medium showing three catogaries of transformant colonies, type I fertile transformants (able to give spores), type I sterile transformants failed to give spores and type II abortive transformants that cannot be rescued into large colonies after transferring.

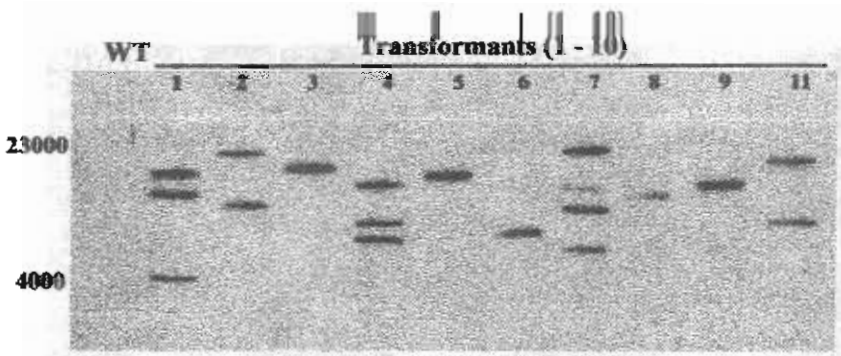

Fig. 3: Southern blot analysis of genomic DNA XbaI digests of the *Trichoderma virens* wild type isolate, Tv101, and the transformants probed with *SalI-XbaI* fragment from *pamdS* plasmid comprising the *Aspergillus* nidulans acetamidase gene.

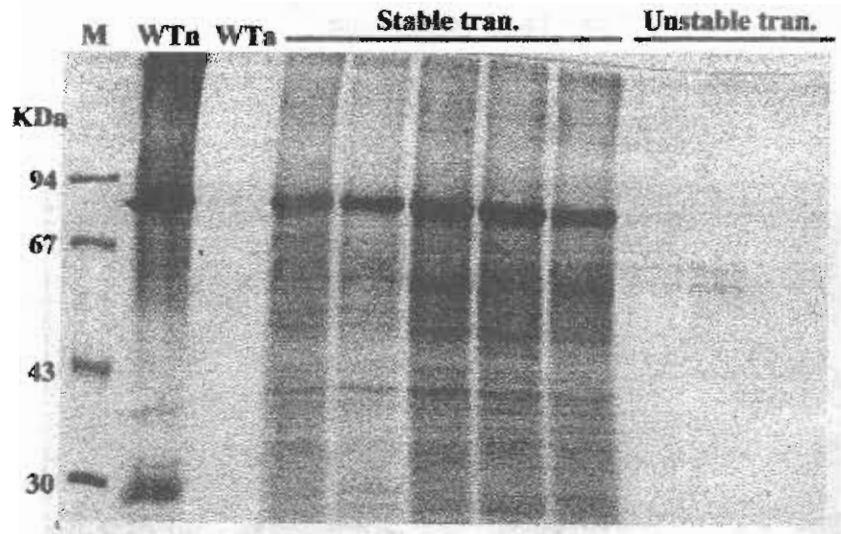

Fig. 4: SDS-PAGE analysis of extracellular protein of the *Trichoderma virens amdS* transformants grown on acetamide-CsCl medium. The protein-banding pattern of the transformants revealed high levels of 55 - 60 KDa significant band that was not present in unstable transformants, non-transformant recipient isolate grown on acetamide-CsCl (TWa) and non-transformant recipient isolate grown on $(NH_4)SO_4$ as nitrogen source (TWn).

Expression of amdS gene in the transformants: The *amd*S gene expression of the mitotically stable transformants was examined. The extracellular protein was recovered from culture supernatant of transformants grown on MM, SM and subjected to SDS-PAGE for the detection of protein bands. The protein-banding pattern of the transformants revealed high levels of 55 - 60 KDa significant band that was not present in non-transformant recipient isolate of *T. virens*. This band was consistent with the size of the *A. nidulans* acetamidase polypeptide that obtained by Hynes *et. al.*, 1983 and Kelly and Hynes 1985. This result suggested that the integrated *amdS* gene of *A. nidulans* could be expressed in *T. virens*.

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التحول الوراثي للتريكودرما فيرنس باستخدام الجين المشفر للأسيتاميداز المعزول من الأسبرجلس نيديولنس كمرقم انتخابي موجب

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الملخص العربي

أجـري هـذا البحث للحصول علي عزلات محولة وراثيا وثابتة يمكنها النمو علي الأسيتاميد كمصـدر وحـيد للنتروجين. ومن أجل هذا تم عزل برتوبلاست الفطر وعمل تحول وراثي له باستخدام بلازمـيد تحمـل جيـن يشـفر لإنـزيم الأسيتامیداز والمعزول من فطر الأسبرجلس نيديولنس. وكانت المسـتعمرات المحولـة وراثـيا ذات نمو كثيف بالمقارنة بالنمو الضعيف للطراز البري. وأمكن تمييز نوعيـن من المستعمرات المحولة، النوع الأول أظهر نمو مستمر وكثيف وظهرت بمعدل ٧ مستعمرات لكل ميكروجرام بلازميد، بينما النوع الثاني أظهر نمو محدود وكثيف وظهرت بمعدل ٢٢ مستعمره لكل ميكروجرام بلازميد. وهذه المستعمرات تم نقلها علي بيئة حدية مضاف إليها الأسيتاميد كبيئة انتخابية فوجـد أن ١٠ مسـتعمرات من بين ٣٥ من النوع الأول أمكنها النمو و إعطاء عزلات لها القدرة علي الـتجرثم، ولكـن باقـي المسـتعمرات أعطت عزلات عقيمة. بينما مستعمرات النوع الثاني ذات النمو المحـدود عـند نقلها علي البيئة الانتخابية لم يمكنها النمو و إعطاء عزلات ذات نمو مستمر حتى بعد نقلهـا علي بيئة بدون أسيتاميد ثم إرجاعها للبيئة الانتخابية، باستثناء مستعمرة واحدة. أظهرت خمسة عزلات من النوع الأول ثباتا وراثيا. كما انه تم التأكد من وجود الجين المستخدم مدمجا ضمن المحتوي الوراثي للفطر بطريقة عشوائية وذلك باستخدام تقنية **Southern blot**. والنتائج أظهرت أيضا أن هـذا الجيـن والـذي يشـفر لإنزيم الأسيتاميداز لا يوجد ضمن المحتوي الوراثي للعزلة البرية والتي كمستقبل في تجارب التحول الوراثي. وفي محاولة لدراسة التعبير الجيني لهذا الجين الغريب في العزلات المحولة وراثيا، تم دراسة نمط حزم البروتينات الناتجة في الوسط عند إنماء هذه العزلات المحولة علي بيئة انتخابية سائلة تحتوي علي الأسيتاميد كمصدر وحيد للنتروجين. فأظهرت النتائج وجود حزمة من البروتيـن ذات وزن جزيئـي يتراوح من ٥٥ إلى ٦٠ كيلو دالتون وذلك في الخمس عزلات المحولة والثابتة وراثيا التي درست، و الوزن الجزيئي لهذه الحزمة يتوافق مع وزن الأسيتاميداز والمعزول من فطر الأسبرجلس نيديولنس. بينما لم تظهر هذه الحزمة في الطراز البري الذى لا يوجد به الجين المشفر لـه ، وكذلك في ثلاث عزلات محولة وراثيا غير ثابتة.