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Transformation of Mutualistic Fungal *Acremonium* Endophyte

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Conditions have been developed for transforming protoplasts of the *Acremonium* endophyte by PEG 4000 and electroporation. Transformation by PEG exhibited a higher number of transformants than by electroporation. Integration of *iaaM* gene into the genome was examined by PCR and Southern blot hybridization analysis. PCR product showed that transformants banded at around 1.7 kb corresponding to the size of *iaaM* gene. Hybridization of the digests of genomic DNA with *iaaM* gene as DNA probe showed that the number of hybridized band signals was different between transformant and non-transformant. These results might indicate that PEG is an effective method for the transformation of *Acremonium* endophyte and that there are repeated copies of the *iaaM* homologous sequences in the genome of *Acremonium*.

Key words : *Acremonium* sp., endophyte, transformation, iaaM gene, hph gene

Introduction

Acremonium endophytes are asexual filamentous fungi which form symbiotic associations with many cool season grass species[10]. They are strictly mutualistic, conferring considerable benefits upon their grass hosts. These include protection from drought, plant pathogens, and insect and mammalian herbivores, as well as increased production of seeds and biomass[2,7]. The endophytes are found in the reproductive and vegetative tissues of the host grasses. In natural associations they are not known to cause disease or produce external structures, and are transmitted within the seed of their hosts[2].

The *Acremonium* endophytes can be regarded as, in some sense, analogous to the cellular organelles of many eukaryotes. Because of their maternal line transmission in seed, the endophytes are heritable components of the grass-fungus symbiota[12]. However, unlike mitochondria and plastids, they are not required for cellular functions of the plant. The endophytes contribute biosynthetic capacity manifested, in part, by the production of several classes of protective metabolites[11]. The effects of these metabolites and of other benefits e.g., drought tolerance[1] that are attributable to the endophytes can result in ecological interdependence. Endophytes are required for the ecological fitness of the grass hosts and provide a dramatic enhancement of competitiveness over plants that have had the endophyte removed[2].

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Abbreviations

DNA, deoxyribonucleic acid; IAA, indole-3-acetic acid; IPA, indole-3-pyruvic acid; IAM, indole-3-acetamide; IAAld, indole-3-acetaldehyde; Trp, L-tryptophan; *iaaM*, gene encodes tryptophan 2-monooxygenase; PEG, polyethylene glycol; HmB, hygromycin B

The endophytes are thought to have evolved from *Epichloe* spp. (*Ascomycetes*), the causative agents of 'choke' disease in grasses, and share many similarities with them[9,11]. *Epichloe* spp. normally grow within the plant as an endophyte but upon flowering inflorescence choke often occurs. Choke is the result of a mycelial growth on host leaf sheaths and inflorescences which prevents seed production on affected tillers. The sexual cycle of *Epichloe* is completed upon the stromata, at which stage ascospores are ejected from mature perithecia and these are thought to reinfect susceptible grasses[10].

A wide variety of microorganisms that live in close association with plants either as parasites or as symbionts have developed the capacity to alter host physiological processes to their own advantage. One example is that microbial pathogens use phytohormone to alter physiological conditions in the host. Other examples are the production of indoleacetic acid (IAA) and cytokinin by *Pseudomonas syringae* pv. *savastanoi*, *Agrobacterium tumefaciens*, and *Agrobacterium rizhogenes* that cause plant neoplastic diseases[3,15,16].

The production of IAA itself is widespread in the plant kingdom, including bacteria and fungi, although the amount of IAA production and its biosynthetic pathway vary from one organism to another. Most microorganisms carry the IAA biosynthetic pathway from tryptophan (Trp) through indole-3-pyruvic acid (IPA) and indole-3-acetaldehyde (IAAld) as intermediates[17].

Pseudomonas syringae pv. *savastanoi*, a causal agent of olive and oleander knot, produces indole-3-acetic acid (IAA) from L-tryptophan (Trp) via indole-3-acetamide (IAM) as an intermediate[3,15]. The so-called olive knot tumor, is thought to result from abnormal plant cell multiplication at the infection site as a host response to the large amount of IAA produced by this pathogen. The genes involved in IAA biosynthesis in this pathogen are *iaaM*, which encodes tryptophan monooxygenase, and *iaaH*, which encodes indoleacetamide hydrolase[3]. *iaaM* and *iaaH* form an operon, with *iaaM* promoter proximal. These genes are located on the plasmid, pIAA, in oleander isolates of *P. syringae* pv. *savastanoi*, but on the chromosome or the megaplasmid in olive isolates[4,15]. These observations suggest that the genes for enzymes of the IAM pathway in plant-associated bacteria have a common origin and have been widely distributed by the horizontal transfer of *iaaM* and *iaaH* genes. It has been speculated that IAA production in these plant-associated microorganisms might facilitate interaction with the host. This study was designed to develop this concept in fungi, too. The demonstration that *Acremonium* can be transformed and that the marker genes introduced were stably maintained[6], opens the way for using these fungi as a vehicle for introducing *iaaM* gene into grasses. The ability to transform *Acremonium* endophyte and the development of efficient methods for introducing the fungus back into the plant[5], offers an alternative surrogate method to transform grasses. Once the endophyte is established in the grass, the *iaaM* gene introduced will be maternally transmitted as a consequence of invasion by fungal hyphae of the ovule of the developing seed. Here we present the methods of transformation of *Acremonium* to introduce *iaaM* gene into the *Acremonium* genome.

Materials and Methods

Fungal strains

Acremonium strain TF 91006 and TF 91019 are an isolate from Tall Fescue, Ti 91093 and Ti 92093 are an isolate from Timothi, strain no. 2 is an isolate from *Poa trivialis* L.

Plasmids

pAN8-1, pAN7-1, pDH25 and pTET40. These plasmids were transformed into *Escherichia coli* strain XLI Blue by standard procedures. Plasmid DNA was isolated by the standard method as

described[8] and purified by equilibrium centrifugation in CsCl-ethidium bromide gradient.

Growth of bacteria and fungi

Acremonium sp. was maintained on 2.4 % potato dextrose agar (PDA) containing 20 μg/ml chloramphenicol. Cultures were grown at 22 °C in either liquid PD medium, or on solid PD medium. *E. coli* cultures were grown in LB medium supplemented where necessary with ampicillin to 50 μg/ml.

Enzymes

Restriction enzymes, alkaline phosphatase, DNA ligation kit and DNA labelling kit were obtained from Takara (Japan). Novozyme 234 was obtained from Novo Nordisk (Denmark). Hybridization membranes were obtained from Amersham (UK). Primers for amplifying *iaaM* fragment and *hph* fragment were obtained from Sawady (Japan).

Preparation of *Acremonium* Protoplast

Protoplasts were prepared using a modification of the method as described[18]. Fungal cultures were grown in PD broth at 22 °C with gentle shaking for 5-6 days, using ground fungal mycelium from a plate culture as inoculum. The mycelium was harvested by filtering through sterile sintered glass funnel and washed with sterile distilled water. Mycelium from a 100 ml culture was resuspended in 15 ml of filter sterilized osmotic medium containing 10 mg/ml of Novozyme 234. This mixture was shaken at 80 rpm for 6-7 hours at 30 °C until the culture cleared and then transferred to centrifuge tubes and overlaid with ST buffer (1 M Sorbitol; 100 mM Tris-HCl). Protoplasts were banded at the interface of the two solutions by centrifugation at 750 × g for 5 minutes. Protoplast layers were carefully removed to a new tube. The protoplasts were washed once with 5 ml of STC buffer (1 M Sorbitol; 50 mM Tris-HCl; 50 mM $CaCl_2$. $2H_2O$) by shaking gently and centrifuged at 100 × g for 5 minutes to remove hyphal debris. Supernatant was collected and centrifuged at 750 × g for 5 minutes or until protoplasts should form a pellet in the bottom. Supernatant was discarded and then the protoplasts were resuspended in 5 ml of STC buffer and centrifuged at 750 × g for 5 minutes. The protoplasts were resuspended in 0.5 ml of STC buffer. The concentration of protoplasts obtained in these experiments was 1.8 × 10^7/ml.

Subcloning of *iaaM* gene and *hph* gene into the plasmid pAN8-1

To direct the expression of *P. savastanoi-iaaM* gene in *Acremonium* endophyte, a DNA fragment of *iaaM* was inserted into the *Nco*I site upstream from phleomycin sequences in pAN8-1, a fungal expression plasmid vector. This plasmid was designated pAY1 as shown in Fig. 1. Furthermore, the hygromycin B phosphotransferase gene (*hph*) under the control of an *Aspergillus nidulans trp*C gene promoter and *trp*C gene terminator from pDH25 was inserted into the *Xba*I site downstream from *trp*C terminator in pAY1. This plasmid was designated pAY2 as shown in Fig. 2.

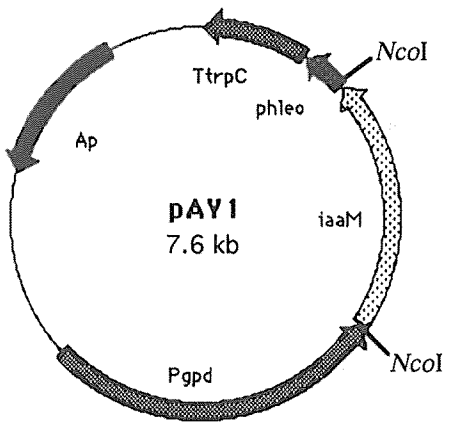

Fig. 1 Map of plasmid vector pAY1.
Restriction site for NcoI is shown. *iaaM* gene is fused to the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehidrogenase promoter fragment (Pgpd) and an *A. nidulans trpC* terminator (TtrpC). *iaaM* gene was subcloned at NcoI site upstream from the phleomycin in pAN8-1. Ap indicates ampicillin resistance gene and phleo indicates phleomycin resistance gene.

Transformation of *Acremonium* protoplast by 40 % PEG 4000

Transformation of *Acremonium* protoplast was prepared using a modification of the procedure as described[14]. 20 μl of a 40 % polyethylene glycol (PEG 4000) in a solution (50 mM $CaCl_2$, 1 M sorbitol, 50 mM Tris-HCl pH8), 2 μl spermidine (50 mM), 5 μl heparin (5 mg/ml) and 10 μg of DNA (2 μg/μl in H_2O) was added into 80 μl of protoplasts in STC buffer. In the case of co-transformation, pAY1 (20 μg) and pAN7-1 (2 μg) were used. The solution was mixed gently and incubated on ice for 30 minutes, then 900 μl of 40 % PEG was added and incubated at room temperature for 15-20 minutes. 100 μl aliquots of this mixture were put into 5 ml of molten 0.8 % regeneration medium (at 50 °C) and overlaid onto regeneration medium plate. The plate was incubated at 25 °C overnight, then overlaid with molten 0.8 % agar containing an appropriate amount of Hygromycin (200 μg/ml). Plate was inverted and incubated in the dark at 22 °C for 4-7 days.

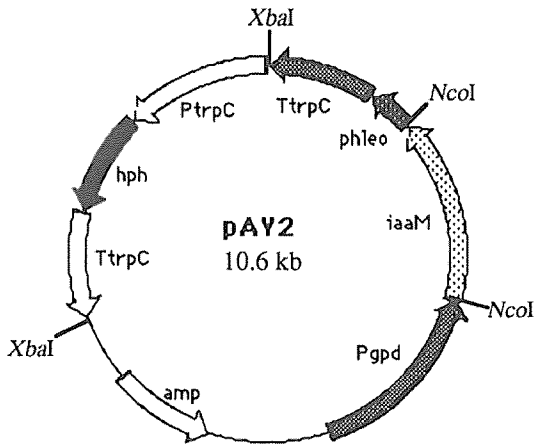

Fig. 2 Map of plasmid vector PAY2.
Restriction sites for NcoI and XbaI are shown. *hph* gene is fused to the *Aspergillus nidulans trpC* gene promoter (PtrpC) and *trpC* terminator (TtrpC). *hph* gene and its promoter and terminator were subcloned at XbaI site downstream from the TtrpC. *hph* indicates Hygromycin B resistance gene and amp indicates ampicillin resistance gene.

Transformation of *Acremonium* protoplast by electroporation

We prepared *Acremonium* protoplasts using a modification of the method as described[18]. Protoplasts were washed free of media by centrifugation at 750 × g for 3 minutes, then resuspended in 100 μl sterile distilled water. Into the solution, 10 μg pAY1 with 2 μg pAN7-1 were added, and another plasmid, pAY2 (5 μg) was also used in the separate electroporation. Before electroporation, this mixture was incubated on ice for 5 minutes. The electroporation machine used was a Bio-Rad Gene Pulser with a 5 Ω resistor in series with the sample chamber. 100 μl of protoplasts were mixed with these plasmids and then electroporated once at voltage 2.0 kV, capacitance 25 μFD and resistance 400 Ω. After electroporation, all of the aliquots were put into 5 ml of molten 0.8 % regeneration medium and overlaid onto regeneration medium plate. The plate was incubated at 25 °C overnight and then overlaid with 0.8 % agar containing HmB (200 μg/ml). Plate was incubated at 22 °C for 4-7 days.

Preparation of *Acremonium* genomic DNA and Southern blot hybridization

Genomic DNA was prepared from freeze-dried mycellium (60 mg) of each strain by the mini-preparation procedure as described[19], except the mycelium was first ground to a powder in liquid nitrogen. Ground lyophilized mycellium (40-60 mg dry) was put into a 1.5 ml microcentrifuge tube to one-third up the conical portion. To the microcentrifuge tube 700 μl of lysis buffer (50 mM Tris-HCl pH 7.2; 50 mM EDTA; 3 % SDS; and 1 % 2-mercaptoethanol) was added and vortexed so the mixture was homogenous; the mixture was incubated at 65 °C for 1 hour. The mixture was centrifuged at 10,000 rpm for 15 minutes at room temperature. 500 to 600 μl of aqueous phase containing the DNA was removed to a new tube and 700 μl of chloroform: TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA)-saturated phenol (1:1, v:v) was added, and vortexed

briefly. The mixture was centrifuged at 10,000 rpm for 15 minutes at room temperature. Aqueous phase containing the DNA was removed to a new tube and 50 μl of 3 M sodium acetate was added, followed by 2x volumes of isopropanol, and vortexed. After the centrifugation of the mixture at 10,000 rpm for 15 minutes at room temperature, the supernatant was discarded, and the pellet was rinsed once with 70 % ethanol. Tubes were inverted to dry them and placed in a vacuum oven at 37 °C for 10 minutes or until dry. Pellet was then resuspended in 100 μl of TE. To avoid the presence of excess polysaccharide in the preparations, very young cultures of the fungus were used for DNA preparations.

Genomic DNA (15 μg) was digested overnight with *EcoR*1 restriction enzyme (5-10 U per μg of DNA). The DNA fragments were separated by electrophoresis on 0.8 % agarose gel. After electrophoresis, the agarose gel was depurinated in 0.25 M HCl with gentle shaking until the dyes changed colour. Agarose gel was rinsed with distilled water and soaked in denaturation buffer (1.5 M NaOH; 0.5 M NaCl) so to completely cover the gel, and left for 20 minutes at room temperature with shaking. Gel was rinsed with distilled water and placed on top of the wet Hybond N^+ membrane, the wet 3MM papers then placed on top of the gel. Buffer (0.4 N NaOH) was drawn from a reservoir and passed through the gel into a stack of paper towels. DNA was transferred from the gel by the moving stream of buffer, and was deposited on a nylon membrane. After 4 hours, the 3MM papers above the gel were removed, then the membrane was rinsed with distilled water and placed on a paper towel to dry for 10 minutes at room temperature. The membrane was covered with kitchen wrap, then put in a vacuum oven at 80 °C for 30 minutes to 1 hour. Pre-hybridization (2-4 h) and hybridization (16-20 h) with ^{32}P-labelled *iaaM* gene probe were carried out in the aqueous hybridization buffer according to the manufacturer's instructions.

Results and Discussion

Transformation of *Acremonium* endophyte

To examine the plasmid vector which could be integrated into the *Acremonium* genome, transformation was performed using pAN7-1, pAN8-1 and pDH25. Transformation of *Acremonium* protoplasts strain no. 2 with pDH25 and pAN7-1 gave 100-190 and 90-190 transformants, respectively, while pAN8-1 gave 40-60 transformants after 2 weeks incubation (Table 1). To determine the integration of these plasmids into the genome of transformant, hygromycin B resistance gene (*hph*) from genomic DNA of transformant was amplified by PCR with appropriate primers. The result of the PCR showed that transformants have a signal of about 560 bp, corresponding to the size of the *hph* gene, indicating that these plasmids (pAN7-1 and pDH25) had integrated into the genome of these transformants (Fig. 3).

Transformation of Acremonium protoplast by 40 % PEG 4000

Transformation of *Acremonium* protoplast of strain TF 91006 and TF 91019 with pAY1 (10 μg) gave 4 and 200 transformants, respectively, while strain Ti 91093 and no. 2 gave no transformant on the selection medium containing phleomycin. For co-transformation with pAY1 (20 μg) and pAN7-1 (2 μg), strain TF 91006 and TF 91019 gave 6 and 200 transformants, respectively. However, strain Ti 91093 and no. 2 gave no transfor-

Table 1 Plasmid transformation of *Acremonium* protoplast

Transformant			
	Plasmids		
Plate	pDH25	pAN7-1	pAN8-1
1	100	190	50
2	180	90	60
3	190	—	45

—:plate was contaminated.
Transformation by PEG 4000

mant on the selection medium (Table 2). No transformant was observed on plates for strain Ti 91093 and strain no. 2, probably due to inadequate conditions of protoplasting. On the other hand, transformation of strain Ti 91093, Ti 92093 and TF 91006 with pAY2 (5 μg) gave 107, 12 and 96 transformants, respectively (Table 3).

Transformation of *Acremonium* protoplast by electroporation

To establish electroporation procedure, we investigated the best conditions for capacitance, resistance and electric field strength. The optimal condition of electroporation for *Acremonium* protoplast was as follows; resistance 400 Ω, capacitance 25 μFD and electric field strength 2 kV/cm. For this condition, about 60 % of *Acremonium* protoplast could regenerate on the regeneration medium after subjection to electroporation. Different types of plasmids such as pAY1, pAY2 and pAN7-1 were electroporated into *Acremonium*. Electroporation with pAY2 (5 μg) into strain TF 91006, Ti 91093 and Ti 92093 gave 37, 3 and 3 transformants, respectively (Table 3). Co-transformation by eletroporation of *Acremonium* protoplast with pAY1 (10 μg) and pAN7-1 (2 μg) into strain TF 91006, Ti 91093 and no. 2 gave 6-8, 20-23 and 0-2 transformants, respectively, whereas strain TF 91019 gave 46 and 200 transformants (Table 4).

To determine the integration of *iaaM* gene into the genome, the DNA fragment of *iaaM* gene was amplified by PCR. The size of PCR products of most transformants was around 1.7 kb, indicating that the *iaaM* gene had integrated into the *Acremonium* genome (Fig. 4). On the other hand, non-transformant also has a band at around

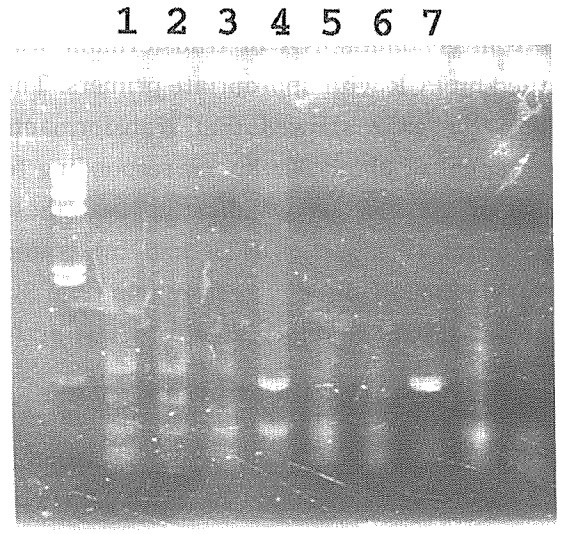

Fig. 3 Amplification of *hph* gene from the *Acremonium* genome. Non transformant (lanse 1-3 and 5-6) showed no band at 560 bp, while transformants (lane 4 and 7) generated a single band at 560 bp, the size of *hph* gene when amplified by appropriate primers.

Table 2 Transformation of *Acremonium* by PEG 4000

	Transformant	
Strain	Transformation	co-transformation
TF 91006	4	6
TF 91019	200	200
Ti 91093	0	0
Strain no. 2	0	0

Transformation: using (only) one plasmid (pAY1)
co-transformation: using two plasmids (pAY1 and pAN 7-1)

Table 3 Transformation of *Acremonium* protoplast using pAY2

	Transformant	
Strain	PEG 4000	Electroporation
TF 91006	96	37
TF 91019	107	3
Ti 91093	12	3

Table 4 Co-transformation of *Acremonium* by electroporation

	Transformant	
Strain	Electroporation I	Electroporation II
TF 91006	6	8
TF 91019	46	200
Ti 91093	20	23
Strain no. 2	2	0

Two electroporations were done for each of the strain of *Acremonium*, protoplasts were plated onto regeneration medium over night and then overlaid with molten 0.8 % containing 200 μg/ml HmB.

1.7 kb. Non-transformant of *Acremonium* might naturally contain *iaaM* gene.

Molecular analysis of transformant

To confirm the integration of *iaaM* gene into the genome and to determine the copy number of integrated *iaaM* gene in the transformants, DNA from 8 representative transformants were digested with *EcoR*I, an enzyme that does not cut *iaaM* sequence. Then Southern blot hybridization analysis was performed with a probe of ^{32}P-labelled *iaaM*. Hybridizing signals concomitant with both the size and number of fragments among these transformants were observed, but there were some differences between non-transformants and transformants. The results for the *EcoR*I digests are shown in Fig. 5. Transformant of a strain TF 91006 lane 2 (transformation by PEG), lane 3 (co-transformation by PEG) and lane 4 (co-transformation by electroporation) showed three hybridization signals with equal intensity corresponding to fragments of about 4 kb, 6 kb and 9 kb in size, indicating that integration of *iaaM* gene had occurred into at least three sites in the genome, or they might be intrinsically hybridizing bands. Non-transformant of a strain TF 91006 showed four hybridization signals of about 4 kb, 5 kb, 9 kb and 21 kb; it is possible that repeated copies of *iaaM* gene were found in the genome. Transformant of a strain TF 91019 (lane 6 and 7, transformation by PEG) and lane 8 (transformation by electroporation) showed four hybridizing signals of about 4 kb, 6 kb, 9 kb and 22 kb, suggesting that *iaaM* gene has integrated at four sites in this transformant, whereas non-transformant of a strain TF 91019 had two hybridization signals corresponding to fragments of 22 kb and 7 kb in size, indicating the presence of *iaaM* gene at two sites in the genome. The results here indicate that the *iaaM* gene which has integrated in the transformants also derived from these transformations.

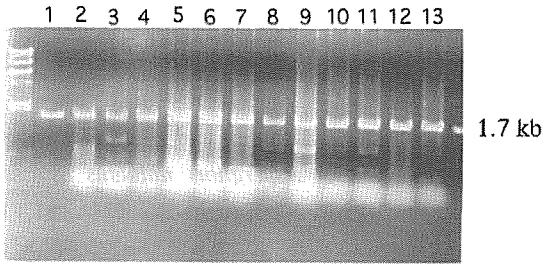

Fig. 4 Amplification of *iaaM* gene from the *Acremonium* genome. Most transformants generated a band at around 1.7 kb, the size of *iaaM* gene. Lane 1 is *iaaM* gene, lane 2 shows ampification of *iaaM* gene from the genome of non-transformant, lane 3–13 show amplifications of *iaaM* gene from the genomes of transformants.

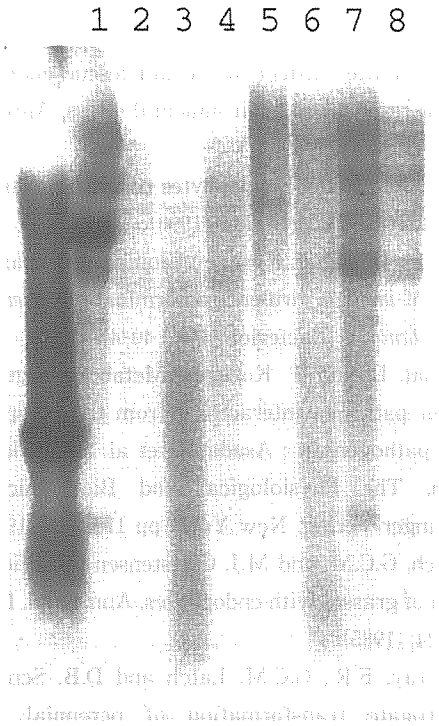

Fig. 5 Hybridization of *iaaM* to genomic DNA digests of transformants and non-transformants. Autoradiograph of a Southern blot of *EcoRI* digests of genomic DNA hybridized with ^{32}P-labelled *iaaM* gene. Lane 1, non-transformant of a strain TF 91006; lane 2, transformant of a strain TF 91006 (transformation by PEG 4000); lane 5, transformant of strain TF 91006 (co-transformation by PEG 4000); lane 4, transformant of strain TF 91006 (co-transformation by electroporation); lane 6, non-transformant of a strain TF 91019; lanes 7 and 8, transformant of a strain TF 91019 (transformation by PEG 4000); lane 8, transformant of strain TF 91019 (co-transformation by electroporation).

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共生真菌 アクレモニウム エンドファイトの形質転換

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アクレモニウムエンドファイトの形質転換の条件を検討した．アクレモニウムエンドファイトからプロトプラストを調製し、PEG 4000とエレクトロポレーションを用いて形質転換を試みた．その結果，PEG で形質転換すると，エレクトロポレーションによる場合よりも多くの形質転換体が得られた．PCR 解析によって *iaaM* 遺伝子のゲノムへの導入を確認したところ，形質転換体の PCR 産物は *iaaM* 遺伝子のサイズに相当する約1.7 kb のバンドを持っていた．一方，*iaaM* 遺伝子をプローブとしたサザンブロット解析においては，形質転換体と非形質転換体の間にはハイブリダイズした断片数に違いがあることが明らかとなった．以上の結果は，アクレモニウムエンドファイトの形質転換には PEG 法が有効であること，アクレモニウムエンドファイトのゲノム中には *iaaM* 遺伝子様配列の反復コピーが存在することを示唆している．