Transformation of the Endophyte *Neotyphodium* with the *iaaM* Gene

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Symbiotic associations of grasses with *Neotyphodium* endophytes often provide the grass hosts with major fitness enhancements⁸⁻¹¹. These associations are considered to be mutualistic, with the plants providing nutrients for the fungi and the fungi synthesizing alkaloids that protect the plants from mammalian and insect herbivores, plant pathogens and nematodes, as well as increased drought tolerance and competitiveness^{1,2,6}.

The *Neotyphodium* endophytes of grasses are found predominantly in the intercellular spaces of the leaf sheath and are usually seed-transmitted. In nature, they are not known to cause disease or to produce external structures^{2,5}.

To use endophytes to endow novel functions in plants, we have attempted to introduce *iaaM* gene from *P. syringae* pv. *savastanoi* into *Neotyphodium*. *Pseudomonas syringae* pv. *savastanoi*, 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^{3,13}. 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 hydrolase⁹. Production of IAA 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 *Neotyphodium* can be transformed and that the marker genes introduced were stably maintained⁹, opens the way for using these fungi as a vehicle for introducing the *iaaM* gene into grasses. Since some extent of IAM accumulation is presumed to be automatically converted to IAA, we did not attempt to clone the *iaaH* gene in *Neotyphodium*. The ability to transform *Neotyphodium* endophyte and the development of efficient methods for introducing the fungus

back into the plant⁴, offers an alternative surrogate method to transform grasses. Once the endophyte association with host plant is established in the grass, the introduced *iaaM* gene will be maternally transmitted as a consequence of fungal invasion by fungal hyphae of the ovule of the developing seed.

Neotyphodium protoplasts were prepared using a modification of the method as described¹⁴. The concentration of protoplasts obtained in these experiments was 1.8×10^7 /ml. Transformation by polyethylene glycol (PEG) was done using a modification of the procedure as described¹². Twenty μ l of a 40% PEG solution (40% PEG 4000, 50 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 M sorbitol, 50 mM Tris-HCl pH 8.0, 2 μ l spermidine (50 mM), 5 μ l heparin (5 mg/ml) and 10 μ g of DNA (2 μ g/ μ l in H_2O) was added into 80 μ l of protoplasts in STC buffer (1M Sorbitol, 50 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mM Tris-HCl pH 8.0). The solution was mixed gently and incubated on ice for 30 min, then 900 μ l of 40% PEG was added and incubated at room temperature for 15-20 min. One hundred μ l of this mixture was put into 5 ml of 0.8% regeneration medium (at 50°C) to overlay to a 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 μ g/ml). The plate was inverted and incubated in the dark at 22°C for 4 to 7 days. Transformation of *Neotyphodium* protoplasts of strain Ti 91093 with 5 μ g of pAY2 (Fig. 1C) gave 107 transformants. Genomic DNA was prepared from fresh mycelium (60 mg) of each strain by the mini-preparation procedure as described^{7,15}, except the mycelium was first ground to a powder in liquid nitrogen. To determine the integration of the *hph* (hygromycin resistance gene) and *iaaM* genes into the genome of *Neotyphodium*, the DNA fragment of *hph* and *iaaM* gene was amplified by PCR with appropriate primers. The result of the PCR showed that the transformant had a signal band of 560 bp, corresponding to the size of the *hph* gene, and PCR

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for amplifying *iaaM* fragment showed a signal band of 1.7 kb corresponding to a fragment of the *iaaM* gene (Fig. 2A), indicating that *hph* and *iaaM* gene had been integrated into the genome of the transformant.

Introduction of the *iaaM* gene into *Neotyphodium* protoplast by electroporation was also attempted. That is, protoplasts were washed free of media by centrifugation at $750 \times g$ for 3 min, then resuspended in 100 μ l sterile distilled water and pAY2 (5 μ g) was added. Before electroporation, this mixture was incubated on ice for 5 min, and electroporation was performed with Gene Pulser (Bio-Rad). To develop the electroporation

Fig. 2. PCR products of *hph* and *iaaM* gene in transformants. Genomic DNA of non-transformants (lane 1) and transformants (lane 2 and 3) of strain Ti 91093 by PEG (A), strain Ti 91093 by electroporation (B), strain TF 91006 by PEG co-transformation (C), and strain TF 91006 co-transformant by electroporation (D) were used for PCR amplification of *iaaM* gene (lane 1 and 2) and *hph* gene (lane 3). Bands at 1.7 kb and 560 bp indicate *iaaM* gene and *hph* gene, respectively.

Fig. 1. Map of the plasmid pAN7-1, pAY1 and pAY2. A: Plasmid pAN7-1. Restriction sites for *EcoRI*, *BamHI*, and *HindIII* are shown, as are the genes for ampicillin resistance (*amp*), hygromycin B resistance (*hph*). The *hph* gene is fused to the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter fragment (*PgpD*) and an *A. nidulans* *trpC* terminator (*TtrpC*). B: Plasmid vector pAY1. *NcoI* DNA fragment of *iaaM* gene is fused to the *PgpD* and the *TtrpC* at *NcoI* site upstream from the phleomycin in pAN8-1. C: Plasmid vector pAY2. DNA fragment of *hph* gene and its promoter (*PptrpC*) and terminator (*TtrpC*) are inserted into *XbaI* site downstream from the *TtrpC* in pAY1. *PptrpC* indicates *A. nidulans* *trpC* gene promoter. Restriction sites for *NcoI* and *XbaI* are shown.

procedure, we investigated the best conditions for electroporation with the varied capacitance and voltage. The optimal condition for *Neotyphodium* protoplasts was, resistance 400 Ω , capacitance 25 μFD and voltage 2

Table 1. The effect of capacitance and voltage on number of transformants after electroporation

Capacitor (μF)	Voltage (V)	Transformant*		
		I	II	III
1	1500	0	0	0
	2000	0	0	0
	2500	0	0	1
3	1500	1	1	0
	2000	1	2	1
	2500	1	2	2
25	1500	3	3	4
	2000	5	6	5
	2500	2	2	1

*I: experiment 1; II: experiment 2; III: experiment 3.

kV/cm. Under this condition, the highest number of transformants could regenerate on the selective medium (Table 1). After electroporation, aliquots were put into 5 ml of molten 0.8% regeneration medium and overlaid onto regeneration medium. The plate was incubated at 25°C overnight and then overlaid with 0.8% agar containing hygromycin B (HmB; 200 $\mu\text{g/ml}$). The plate was further incubated at 22°C for 4 to 7 days.

Transformation by electroporation with pAY2 (5 μg) of strain Ti 91093 gave three transformants. A low transformation efficiency was presumed with low protoplast regeneration frequencies. These results might indicate that transformation by PEG is more effective than that by electroporation. The development of a high efficiency transformation system for the *Neotyphodium* endophyte of perennial ryegrass using hygromycin resistance as a dominant selectable marker has been reported⁵⁾. The high efficiency transformation for filamentous fungi using a PEG-based transformation system is presumably the result of using a medium that gives good regeneration of protoplasts, as well as the

Fig. 3. Analysis of the accumulation of IAA and IAM by reverse-phase HPLC. Details are described in the text. HPLC profiles of a transformant (A) and non-transformant (B) of strain TF 91006 were shown. Mobile phase; a: Methanol, b: 1% acetic acid, 1% 1-heptance sulfonic acid sodium salt in water, a : b = 1 : 1. Flow rate: 1 ml/min. Detector: photodiode array monitor at 280 nm (L745H: Hitachi).

inclusion of sorbitol as an osmoticum, not only during the preparation of the protoplast but also in the 40% PEG solution used in the transformation step¹⁴). Integration of *hph* and *iaaM* gene into the genome of *Neotyphodium* was confirmed by PCR as well as the transformation of strain Ti 91093 by PEG (Fig. 2B).

Co-transformation of *Neotyphodium* protoplast with 2 μ g pAN7-1 which shows resistance to hygromycin B (Fig. 1A) and 10 μ g pAY1 (Fig. 1B) into strain TF 91006 gave six transformants by the PEG method, while co-transformation by electroporation gave four transformants. Integration of *hph* and *iaaM* gene into the genome of *Neotyphodium* was confirmed by PCR as described above (Fig. 2C, D), and normal nucleotide sequences of *iaaM* integrated into the genome of *Neotyphodium* were analysed by DNA sequencer (data not shown).

To determine expression of the *iaaM* gene, the amount of IAM and IAA in the culture medium was examined by HPLC after addition of 0.2 mM L-tryptophan. The culture was centrifuged at $10,000\times g$ for 15 min at 4°C to remove the cells. After centrifugation, 1 N HCl was added to the supernatant to adjust the pH to 3.0, and 100 ml of ethyl acetate was added to the supernatant to extract the indole compounds. Seventy-five ml of the ethyl acetate extract was evaporated to dryness and redissolved in ethanol. For HPLC analysis, samples in ethanol solution were applied to the column Inertsil ODS3 4.6 $\times$ 250 (GL Science).

Accumulation of both IAM and IAA from the culture filtrate of transformant strain TF 91006 and strain Ti 91093 could be detected by HPLC. Detection by HPLC showed an accumulation of IAM and IAA in the transformant of strain TF 91006 of 56.97 μ M/g cells and 30.26 μ M/g cells, respectively (Fig. 3A), while accumulation of IAM and IAA in the transformant of strain Ti 91093 was 36.42 μ M/g cells and 21.38 μ M/g cells, respectively. On the other hand, the accumulation of IAA in the non-transformant of strain TF 91006 was 23.12 μ M/g cells (Fig. 3B) and of strain Ti 91093 was 18.24 μ M/g cells. No accumulation of IAM in the culture filtrate of non transformants was detected by HPLC analysis, indicating that transformants significantly accumulated IAM. However, not all transformants showed high expression by accumulating IAM as we expected. We are currently examining the copy number and the integration of *iaaM* gene in the transformants. These result showed that the promoter of *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*Pgpd*) was enough to promote the expression of *iaaM* gene. A non-transformant of *Neotyphodium* also produced a small amount of IAA, but not IAM, although the pathway of IAA biosynthesis is still unclear. Most microorganisms carry the IAA biosynthetic pathway from tryptophan (Trp) through indole-3-pyruvic acid (IPA) and indole-3-acetaldehyde (IAAld) as intermediates¹³). The production of IAA is widespread in the plant kingdom, including bacteria and fungi, although the amount of IAA produc-

tion and its biosynthetic pathway vary from one organism to the other¹³).

Our study has indicated that introducing a foreign gene into grasses by using this fungus as a vector is possible, since the development of efficient methods for introducing the fungus back into the grasses has been established⁴). It will be interesting to examine whether IAA biosynthesis through the IAM pathway in these transformants could facilitate a better interaction with the host. If so, the IAM pathway may have a role in the interaction with host, or the beneficial effect of IAA production by these transformants may depend on the IAA biosynthesis pathway. Inoculation of grasses with these fungi and examination of the effects of the expression of *iaaM* gene in *Neotyphodium* to the association with host plants is underway.

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和 文 摘 要

Ahmad YUNUS・川又伸治・島貫忠幸・村上泰弘・一瀬勇規・白石友紀・山田哲治：ネオティフォディウム・エンドファイトへの *iaaM* 遺伝子形質転換体の作出

オリーブこぶ病細菌の *iaaM* 遺伝子（トリプトファンモノオキシゲナーゼ遺伝子）をネオティフォディウム・エンドファイト菌に導入し形質転換を試みた。その結果、ポリエチレングリコール（PEG）を用いる方法がエレクトロポレーション法に比べて高い形質転換効率を示した。ネオティフォディウム TF91006 株と Ti91093 株の形質転換体における *iaaM* 遺伝子と薬剤耐性マーカーのハイグロマイシン耐性遺伝子の染色体 DNA への組み込みは、PCR 法によって予想される 560 bp の *iaaM* 遺伝子と 1.7 kb のハイグロマイシン耐性遺伝子の増幅によって確認した。これらの形質転換体における *iaaM* 遺伝子の発現を培養液中に蓄積されたインドールアセトアミド（IAM）量を HPLC によって定量することによって調べたところ、多くの形質転換体では有意な IAM の蓄積が検出された。

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