氏　　名(国籍)	HAMID　RASHID (ハミッド　ラシッド)
学位の種類	博　　士　　(農　　学)
学位記番号	農博第　515　号
学位授与年月日	平成8年3月26日
学位授与の要件	学位規則第4条第1項該当
研究科専攻	東北大学大学院農学研究科 (博士課程)　農学専攻
学位論文題目	Studies on Tissue Culture and Genetic Transformation in *Oryza sativa* ssp. *Indica* and *Moricandia arvensis* (*Oryza sativa* ssp. *Indica* および *Moricandia arvensis* の組織培養と形質転換に関する研究)

論文審査委員(主　査)	教　授	日向康吉
	教　授	江原淑夫
	教　授	亀谷壽昭

論 文 内 容 要 旨

Chapter 1

Introduction: Gene transfer technology, its application on plant breeding with special emphasis on *indica* rice and *Moricandia arvensis*

Genetic transformation is now considered to be a promising technique for plant breeding and has been applied to several crops. However, its applicability is still limited to some crop species. Technical innovation is required for this technique which would be applicable to many other main crops and several experimental plants.

In the present study, efforts were directed to develop a reliable genetic transformation technique of *indica* rice, because there has been no reproducible reports for successful transformation in this crop, while it was successful in *japonica* rice. In order to achieve it, some basic researches on the tissue culture and protoplast culture have also been extended.

Furthermore, a transformation experiment was carried out in *Moricandia arvensis*, which is attractive for the study of photosynthesis and gene regulation, because it has been identified as C3-C4 intermediate with respect to photosynthetic characters. In this connection, a tissue culture study was also carried out to obtain some basic informations for gene transfer in this species.

In this chapter, past investigations on these problems were reviewed.

Abbreviations:
2,4-D= 2,4-dicholorophenoxyacetic acid; NAA= a-naphthaleneacetic acid
BAP= Benzylamino purine; N6 medium: (Chu et al. 1975); MS medium: (Murashige and Skoog 1962); AA medium: (Toriyama and Hinata 1985)
GUS= ß-glucuronidase; HygR= hygromycin-resistance

Chapter 2

An improved method of shoot regeneration from calli in *indica* rice

Three *indica* cultivars, Basmati 370, Basmati 385 and Basmati 6129 were used through the present experiment, because these cultivars are highly evaluated in Pakistan. A 2,4-D concentration at 2mg/l in N6 medium was found to be suitable for callus induction in the former two cultivars, while 3mg/l was in Basmati 6129. When these calli were cultured on MS medium supplemented with 3% sucrose, 3% sorbitol, 2g/l casamino acids, 5mg/l BAP, 1mg/l NAA and 4g/l gelrite, the highest shoot regeneration frequency was attained to be 40 - 56 %. Regeneration frequency was increased 2-7 folds by the addition of sorbitol and gelrite (Table 1) .

Chapter 3

Establishment of embryogenic cell suspensions and plant regeneration from protoplasts of *indica* rice

Cell suspension cultures were initiated from scutella-calli of two rice cultivars, Basmati 370 and Basmati 385. Rapidly growing, embryogenic suspension cultures were established. Use of AA medium, replacement of half suspension medium every 4-6 days (Table 2), and selection of small, spherical and yellowish cells were essential to obtain the suspension cultures.

High yield of protoplasts (1.2×10^7/ml) was obtained from 6-day-old dividing cells by using enzyme solution consisting of Cellulase Onozuka RS, Macerozyme R-10, mannitol and $CaCl_2 \cdot H_2O$.

Nurse culture of rapidly growing cell suspension of Nipponbare was essential for sustaining protoplast division and colony formation in N6 medium (Table 3).

The highest shoot regeneration (30.8%) was attained under a combination of 1mg/l NAA and 5mg/l BAP on MS medium with 3% sucrose, 3% sorbitol, 2g/l casamino acids and 4g/l gelrite. Some regenerated plants were dwarf with low fertility.

Chapter 4

Transgenic plant production mediated by *Agrobacterium* in *indica* rice

A successful *Agrobacterium*-mediated transformaion in *japonica* rice has been reported elsewhere during the tissue culture experiment in the former chapters and this method was considered to be better than the protoplast-electroporation method, if it could be applied to *indica* rice. The present study was, therefore, conducted to know the applicability of *Agrobacterium*-mediated transformaion to the Basmati cultivars, whose characteristics in tissue culture were already studied.

Three-week-old scutella calli were served as the starting material (Fig. 2a). These were infected with an *Agrobacterium tumefaciens* strain EHA101 carrying a plasmid pIG121Hm that contains genes for hygromycin resistance and GUS in the T-DNA region, as well as for kanamycin resistance (Fig. 1a).

Experimental results by using transient GUS assay proved that inclusion of acetosyringone (50μM) in the *Agrobacterium* suspension and co-culture media was indispensable for successful transformation. Hygromycin at the rate of 50 mg/l was a selection

agent for selecting transformed cells.

Referring to these conditions, I obtained 26 plants in Basmati 370 and 4 in Basmati 385, which were hygromycin resistant and GUS positive (Fig. 2 b and c). The transformation efficiency of Basmati 370 was 22%, which was as high as those reported in *japonica* rice and dicots (Table 4) . Plants from 11 independent calli of Basmati 370 and 2 from Basmati 385 were evaluated on their characteristics (Fig. 2d and e). They exhibited normal phenotype and high seed fertility. By Southern blot analysis the GUS DNA was detected as a fragment of the expected length of 3.5 kb in four plants (Fig. 1a and 1b lanes 2,3,5 and 6). One plant showed bands at different positions from 3.5 kb (Fig.1b lane 4). Some bands of unexpected sizes were also observed in all the transgenic plants. These unexpected DNA fragments were probably produced by the rearrangements of DNA upon transformation.

Five transgenic plants from Basmati 370 and one from Basmati 385 were selected, and their selfed progeny were evaluated for hygromycin resistance and GUS expression (Fig. 2f and g). The R1 progeny of plants, N332 and N381, displayed segregation pattern of 3:1, and N382 showed 15:1 for both hygromycin resistance and GUS expression (significant at the 5% level). The R1 progeny of plant N320, N322 and N381 showed unexpected segregation pattern.

Chapter 5
Multiple shoot regeneration from stem internodes and leaf-derived calli in *Moricandia arvensis*

Stem internodes between cotyledon and the first leaf were

excised from aseptically germinated *Moricandia arvensis.* A culture on the MS medium with 0.01 mg/l BAP and 10g/l gelrite resulted in a high frequency of adventitious shoot development; 12 shoots/culture.

Next, abilities of leaf discs (the 1st and 2nd leaves) and the stem internodes were tested to form callus with the subsequent shoot regeneration. Maximum shoot regeneration, as high as 80 %, was achieved for leaf disc-derived calli (Fig. 3a) that was formed on a CIM (callus inducing medium) with 2mg/l NAA and 1mg/l BAP, and that as high as 83% for stem-derived calli on the CIM with 1mg/l NAA and 1mg/l BAP. Multiple shoot regeneration was observed when the calli were transferred to the regeneration medium containing 2mg/l BAP (Table 5).

Chapter 6

Transgenic plant production mediated by *Agrobacterium* in *Moricandia arvensis*

Leaf discs, stem internodes and leaf-derived calli were taken as starting materials. These were infected with *Agrobacterium tumefaciens*, a strain GV3101/pMP90 carrying pIG121Hm. In view of transient GUS expression, leaf discs exhibited a higher rate and stronger expression of genes than the others (Fig. 3b). In a comparison of *Agrobacterium* strains between GV3101/pMP90 and EHA101, the former was better than the latter on the GUS activity. Furthermore, the transient GUS expression was observed only when co-culture medium was supplemented with tobacco feeder cells.

The test of selective agents, kanamycin and hygromycin,

revealed that the callus proliferation was observed only when kanamycin was used (Table 6). A pre-selection period of seven days after co-cultivation was essential (Table 7). Multiple shoot formation was obtained through a process of organogenesis (Fig. 3c). Plants from seven independent transformed calli survived in a glasshouse. They exhibited flowering after 3-4 months (Fig. 3d). One plant is now setting seeds.

In the leaf tissues of the transgenic plants, GUS expression was observed; the 35S promoter being active in epidermis, mesophyll and vascular bundle cells (Fig 3e). No GUS activity was detected in the untransformed control (Fig. 3f). PCR analysis confirmed the presence of GUS DNA in six transgenic plants (Fig. 4). Southern blot analysis showed an expected band of 3.5 kb in transgenic plants M21, M22 and M406. Presence of unexpected band in M29 may be due to rearrangements upon transformation.

Chapter 7

Conclusion

In the present study, *Agrobacterium*-mediated transformation was demonstrated in *Oryza sativa* ssp. *indica* and *Moricandia arvensis*. An effort was also undertaken for cell culture in these plants. It is expected that the genetic transformation technique could be applied to efficient breeding as well as molecular analysis in these plants. The developed genetic transformation technique could also be helpful for transfer of useful genes. The tissue culture techniques developed in this study may also be used according to agricultural and experimental requirements.

Table 1. Effect of osmolarity on shoot regeneration frequency of rice callus. Shoot regeneration medium was supplemented with 3% sorbitol and 4 g/l gelrite. Control was without sorbitol and gelled with 8 g/l agar. Percentage of shoot forming calli was scored after 4-5 weeks of transfer.

Varieties tested	Control medium	Sorbitol & gelrite containing medium
Basmati 370	6.7	51.0
Basmati 385	23.8	43.0
Basmati 6129	21.3	53.3

Table 2. Growth response of cell suspensions to various media; N6, MS,R2 and AA media contained basic salts and vitamins of their respective media and were supplemented with 1 mg/l 2,4-D. N6-CH contained N6 plus 500 mg/l casamino acids, N6-AA had N6 plus the amino acids of AA medium, and N6-Pr had N6 plus 50 mg/l proline.

Tested medium	Basmati 370	Basmati 385	Basmati 6129
N6	++	++	+
N6-CH	++	++	++
N6-AA	-	-	-
N6-Pr	-	-	-
MS	-	-	-
R2	-	-	+
AA	+++	+++	++

- : cells turned brown., + : cells survived with slow growth., ++ : cells grew rapidly producing large cell clumps and +++ : cells grew rapidly producing fine dispersed suspension.

Table 3. Test of two nurse cultures (Nipponbare or Basmati 385) and two media (B5 or N6) for the number of colony formation in a petri plate in which 1×10^6 protoplasts were cultured. Data was recorded after 6 weeks of protoplast isolation.

Nurse culture with	Media	
	B5	N6
Nippon bare	69	124
Basmati 385	5	8
Without nurse culture	0	0

Table 4. Efficiency of transformation by *Agrobacterium* in *indica* rice

Rice	Number of calli or plants					
Cultivars	Co-cultivated calli (A)	Produced HygR calli	Shoot form. calli	Produced HygR plants	Produced HygR and GUS+ plts. (B)	Transform. efficiency % (B/A)
Basmati370	118	102	40	37	26	22.0
Basmati385	84	41	5	4	4	4.8
Basmati6129	53	22	0	0	0	0.0

Table 5. Callus formation and shoot regeneration frequency from leaf discs and stem internodes of *Moricandia arvensis* 40-50 cultures were taken for each treatmment and cultured at 25°C .

Hormone conc.		Leaf discs		Stem internodes	
NAA	BAP	% Callus formation	% Shoot regeneration	% Callus formation	% Shoot regeneration
0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	71.4	0.0	27.0	33.1
0.1	0.1	76.9	12.5	47.3	62.5
0.1	1.0	91.7	28.4	50.2	68.9
1.0	0.0	87.5	41.5	82.5	50.3
1.0	0.1	68.8	51.4	79.2	80.2
1.0	1.0	100.0	63.5	89.9	83.4
2.0	0.0	84.7	32.1	76.2	60.0
2.0	0.1	85.4	59.2	81.2	62.5
2.0	1.0	100.0	80.0	79.3	67.4

Callus inducing medium was consisted of MS medium, 3 % sucrose and 5 g/l gelrite. Shoot regeneration medium consisted of MS medium, 3% sucrose , 2 mg/l BAP and 5 g/l gelrite.

Table 6. Effect of selection agents on transformation efficiency of *Moricandia arvensis* by using *Agrobacterium* strain GV3101/pMP90

Selection agent (mg/l) Km	Hyg	Total explants tested (A)	Selected calli	Shoot forming calli	GUS positive plants (B)	Transform. efficiency % (B/A)
100	0	44	19	7	3	6.8
200	0	58	14	6	6	10.3
0	10	57	5	0	0	0.0
0	20	51	0	0	0	0.0

Km=Kanamycin, Hyg=Hygromycin

Table 7. Effect of pre-selection on transformation efficiency of *Moricandia arvensis* by using *Agrobacterium* strain GV3101/pMP90 and kanamycin as a selection agent.

Pre selection period (days)	Total explants tested (A)	Selected calli	Shoot forming calli	GUS positive plants (B)	Transform. efficiency % (B/A)
0	50	0	0	0	0.0
5	52	4	0	0	0.0
7	56	17	9	7	8.0
10	60	26	14	4	6.7

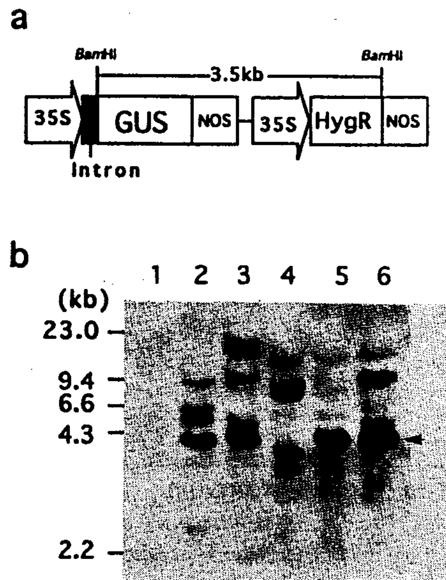

Fig.1. Transformation vector and Southern blot analysis. (a) Schematic diagram of a part of the T-DNA region of transformation vector pIG121Hm. 35S, 35S promoter of cauliflower mosaic virus; intron, the first intron of catalase gene of caster bean; GUS, gene for β-glucuronidase; HPT, gene for hygromycin phosphotransferase; NOS, terminator of nopaline synthase. (b) Southern blot analysis of five transgenic plants of Basmati 370 (lanes 2-6) and an untransformed plant (lane 1). DNA was digested with *Bam* H1 and allowed to hybridize to GUS probe. An arrow indicates the expected band of 3.5 kb. Molecular markers are indicated on the left.

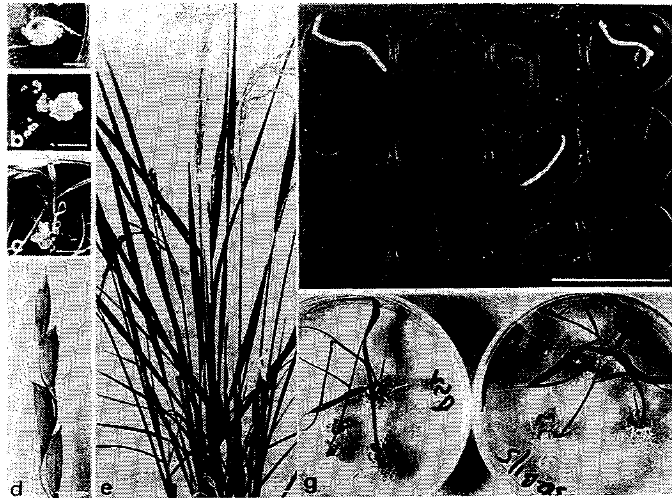

Fig. 2. Production of transgenic plants in *indica* rice cv. Basmati 370 and test of R1 progeny for GUS expression and hygromycin resistance. (a) Three-week-old scutellum derived calli used for co-cultivation with *Agrobacterium.* (b) Hygromycin-resistant colonies of cells proliferated on hygromycin-containing medium 3 weeks after selection. (c) Plant regeneration 4 weeks after transfer. (d) Seeds at maturity stage. (e) A transgenic plant at flowering stage. (f) Test for GUS expression of root segments in R1 progeny. Blue staining indicates GUS expression. Segregation of GUS positive and negative plants were observed. (g) Hygromycin resistance in R1 progeny of transgenic plants (right) and untransformed plants (left). Seedlings were plated on a hygromycin-containing medium. Transgenic plants showed normal growth, whereas untransformed plants were died 7 days later. Bar=1 cm

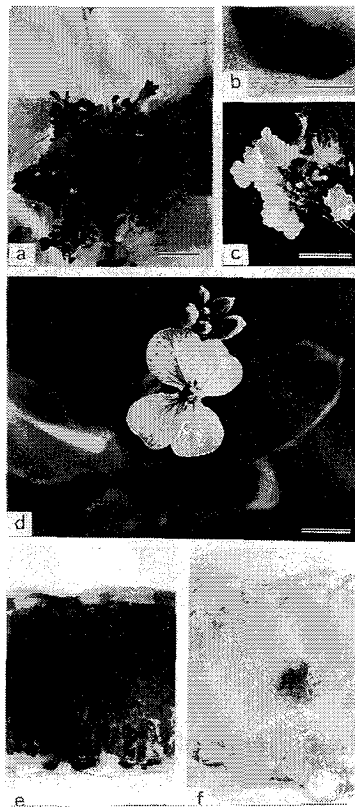

Fig. 3. Shoot regeneration from callus, transgenic plant production and histochemical analysis of GUS expression in *Moricandia arvensis*. (a) Multiple shoot formation from leaf disc-derived callus of untransformed control. Bar=5mm. (b) Transient GUS expression in leaf disc after cocultivation. Bar=1mm. (c) Kanamycin-resistant calli producing shoots after 4 weeks of selection. Bar=1cm. (d) Transgenic plant at flowering stage. Bar=1cm. (e) Section for GUS expression from leaf of transgenic plants. Bar=50μm. (f) Section for GUS expression from leaf of untransformed control plant. Bar=50μm.

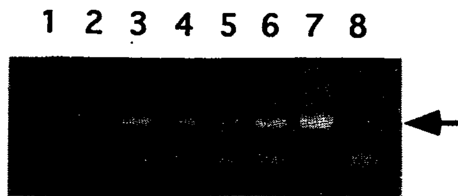

Fig. 4. PCR analysis to detect the presence of GUS gene in transgenic *Moricandia arvensis* plants (lane 1-6), plasmid pIG121Hm (lane 7) and an untransformed plant (lane 8). An arrow indicates a 430bp fragment of GUS gene.

論文審査の要旨

近年遺伝子導入による育種が展望されているが，遺伝子導入の出来る作物はまだ限られており，その技術開発が求められている。本研究は従来遺伝子導入が困難とされていたインド型イネ，およびモリカンデア・アルベンシスへの遺伝子導入方法を確立したものである。

インド型イネのうち，パキスタンで評価の高い，バスマテイ370，バスマテイ385，バスマテイ6129を供試して，これら植物の組織培養における再分化条件を検討し，培地にソルビトールを加え，さらにゲルライトを用いると高い再分化率が得られることを明らかにした。

つぎに，再分化率の高かった前２者を材料として用い，それらからプロトプラストが得られること，そのプロトプラストから再び植物を再分化させるときの条件を明らかにした。

これら培養技術を基礎として，種子胚盤から出来るカルスにアグロバクテリウムを利用した遺伝子導入方法を用いることによって，遺伝子導入したイネを作出することに成功した。さらにそれら遺伝子導入個体の次世代を育成して導入遺伝子が明らかに遺伝することも示した。これらの研究は，アグロバクテリウム法を用いてインド型イネに効率よく遺伝子導入できることを示した世界最初の例である。

さらに，本研究者はＣ３とＣ４光合成の中間型であるモリカンデア・アルベンシスが光合成機能を研究するには良い材料であることに着目し，本種の培養条件および遺伝子導入方法も検討した。

まず，本種の茎切片から直接不定芽が形成される条件，および茎切片と葉切片カルスを経てシュートを得る条件を明らかにした。さらに，本植物において，葉の組織に対してアグロバクテリウム法を用いて遺伝子導入ができること，導入遺伝子が葉内で発現すること，導入遺伝子が次世代に遺伝することなど世界で初めてその実例を示した。

以上，本研究は従来困難とされてきた，インド型イネおよびモリカンデア・アルベンシスへの遺伝子導入技術を世界で初めて開拓し，遺伝子導入育種の技術開発に大きく貢献したものである。よって，審査員一同は本研究者に博士（農学）を授与できるものと判断した。