F-7 Studies on the gene dispersal from living modified organisms in the natural environment and evaluation of the impact of gene dispersal on biodiversity

Contact person Osami Yagi Professor
Research Center for Water Environment Technology
Graduate School of Engineering, The University of Tokyo
7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
Present address: Advanced Research Institute for the Sciences and Humanities,
Nihon University, 6F Ichigaya Tokyu Building, 2-1 Kudan-kita 4-chome,
Chiyoda-ku, Tokyo, 102-0073 Japan
Tel: +81-3-5275-7951, Fax : +81-3-5275-7852
e-mail: yagi@arish.nihon-u.ac.jp

Total Budget for FY2003-2005 196,150,000Yen (FY2004: 64,121,000Yen)

KeyWords
Gene transfer, Genetically engineered microorganisms, Transgenic fish, Transgenic plant, Microsatellite markers,

1. Introduction and Objectives

During the past decade genetic engineering is being used to develop microorganisms, plants and animals that can perform a variety of commercial tasks in several fields, including agriculture, engineering and medicine. The impacts of these living modified organisms (LMOs) are mainly evaluated on the point of human beings. The potential use of genetically engineered microorganisms (GEMs) in several fields, including agriculture, medicine, and pollution control, is becoming increasingly attractive. There is a need to make the assessment of the risks to prevent environmental damage, before the release of LMO. In 2004, the Law concerning the conservation and sustainable use of biological diversity through regulations on the use of living modified organisms was enforced in Japan. This Law shall have the purpose of ensuring the precise and smooth implementation of the Cartagena protocol on biosafety to the convention on biological diversity. To evaluate the potential risks for the successful deployment of GEMs and genetically modified plants in the environment, it is necessary to get more data of the impact of LMOs on biodiversity. There is not much data on the influence of LMOs on biodiversity. We mainly focused on the gene flow and weediness for plant, and gene flow and population change for microorganisms. We selected and carried out following 10 projects.

2. Results and Discussion

(1) Studies on the gene flow in the environment and evaluation of the impact of genetically modified microorganisms on microbial diversity

① Development of detection methods for genetically engineered micoorganisms

In order to trace the genetically engineered microorganisms (GEMs), it would be highly desirable if we could quantitatively determine the population of the microorganism that has

been intentionally released into the environment by molecular methods. We attempted to insert a marker gene that is distinguishable from background microorganisms/genes in the environment. The targets we set in the experiments were; (i) host genome, (ii) plasmid, and (iii) functional gene on the plasmid/chromosome. We established the method to introduce green fluorescent protein (gfp) gene into a 16S rRNA gene (host genome marking) in Escherichia coli. The plasmid was marked by introducing a red fluorescent protein (dsred) gene (plamid marking). In addition, the functional gene, tomA3, which encodes the alpha subunit of toluene-2-monooxygenase, was selected as a model functional gene, and marked by introducing nucleotide substitutions based on codon degeneracy (functional gene marking). By applying the above mentioned techniques, we have created *Pseudomonas putida* KTTG39: pBBTDTT, a potential bioremediation agent that has the acquired capablility of degrading trichloroethylene. The host microbe itself could be visualized by green fluorescence, and the presence of the plasmid could be detected by the red fluorescence. The marked tomA3 (tomA3m7) can be detected specifically by PCR even in the presence of its wild type genes. Also, we could measure each of three marker sequences by quantitative PCR (QProbe PCR) in the range of 10^2 to 10^6 copies/PCR-tube. By using these three markers, we are able to chase the modified microbes together with its plasmid or functional gene(1),(2).

② Development of rapid method for evaluating the impact of genetically engineered microorganisms on microbial biodiversity

The objectives were to develop a rapid method for evaluating the potential impact of GEMs on microbial biodiversity. In this study, we examined the effect of genetically engineered and wild-type *Pseudomonas putida* strains on indigenous bacteria and its gene in Lake Kasumigaura water.

P. putida PpY101 and PpY101/pSR134 were used as the wild-type reference (WT) and the genetically engineered strain (GE), respectively, in this study. Plasmid pSR134 was encoded the mercury resistance gene as a specific marker. Microcosms consisted of 2000-ml Erlenmeyer flasks containing 1000 ml of Lake Kasumigaura water (Ibaraki prefecture). Microcosms inoculated at a density of 10^7 CFU ml^{-1} of genetically engineered or wild-type *P. putida* strains and the uninoculated control were incubated at 20°C with gentle shaking (60 rpm) in the dark. The number of GEMs and host were counted by the top agar method. On the counting of the population of indigenous bacteria, 1/10 LB agar, R2A agar, 1/10 LB agar with crystal violet, pseudomonas isolation agar, mannitol salt agar and actinomycete isolation agar were used. DNA was extracted from the sample of each microcosm, and the primers GC-350F and 920R were used for the amplification of 16S rRNA gene fragments suitable for analysis by denaturing gradient gel electrophoresis (DGGE). The DCode System for DGGE (Bio-Rad Laboratories Ltd.) was used for DGGE analysis.

In this study, the density of introduced GE and WT was 10^7 CFU ml^{-1}, respectively. In 20 days, the density of GE and WT decreased 10^3 CFU ml^{-1} levels, while the indigenous bacterial density was the same level as the initial period. It seemed that the density of indigenous culturable bacteria was not significantly different between the microcosms inoculated with GE or WT, and the noninoculated microcosms.

In recent years, culture-independent methods have been used in preference to traditional isolation techniques for microbial community analysis. In particular, DGGE is a PCR-based tool that has been applied extensively and effectively for analyzing the biological diversity of bacteria in environments. Banding patterns of DGGE from microcosm samples were compared in detail. To reveal relative changes in the structures of microbial communities in the samples from each microcosm, we analyzed the DGGE profiles by nonmetric multidimensional scaling. In 96 days, the structures of microbial communities in all samples from microcosms were similar. Therefore, it was suggested that genetically engineered *P. putida* had effect neither on the number of indigenous bacteria nor on the total genetic diversity of microbial community.

③ Studies on the behavior of recombinant genes and evaluation of impact of recombinant genes on microbial diversity

Antibiotic resistance genes are generally used as the marker to detect the recombinant microorganisms. However, there is a concern that the antibiotic resistance genes transferred might be transformed into harmful microorganisms and would finally result in the antibiotic resistance. In addition, the behaviors of introduced genes are ambiguous. It is desired that the recombinant DNA is not transferred to other microorganisms.

In this study, the different experimental conditions are examined as follows: in aqueous and in soil environment, the deletion frequency of mercuric reductase gene and the antibiotic resistance gene, existence and frequency of horizontal gene transfer to other microorganisms, effects of environmental factors on the horizontal gene transfer, mechanism and control methods of the horizontal gene transfer.

First, transformation frequencies of Pseudomonad were examined. The uptake of pSR134 by *P. aeruginosa* and *P. alcaligenes* were observed under the high concentration of Mg^{2+} (1mM – 100mM). The plasmid uptake on the filter method showed the high frequency, as the Mg2+ concentration was increasing, and as added plasmid DNA quantity was high. The frequency of plasmid uptake was lower than that of on the membrane filter.

Next, the frequency of plasmid uptake into the environmental microorganisms was studied o evaluate the risk when the recombinant bacteria and the recombinant gene are released into the environment. Bacteria in water samples collected from Shinobazunoike and Lake Kasumigaura were concentrated by centrifugation. Plasmid pSR134 was added into the concentrated bacterial suspensions and plasmid uptake was examined in the natural water. The transformants were detected at the frequency of 10^{-6} – 10^{-7}. The plasmid uptake frequencies did not change, though concentration rates of bacteria or pSR134 dosage were changed. Bacterial species that took pSR34 changed with the concentration rate of bacterial suspensions. Partial sequences of 16S rRNA genes were decided in the isolated 96 transformants and these sequences were distributed to 14 species.

Moreover, 0.1 μg/g soil or 1 μg/g soil of pSR134 or 1.8 x 10^7 cfu/g soil of *P. putida* PpY101/pSR134 were was added into the soil samples. After 1 or 2 days of the plasmid addition, total number of bacteria and transformants were counted. However, uptake or transmission of the plasmid were not detected.

④ Effect of transgenic fish on biodiversity

We aimed to reveal whether a transgene integrated on the fish genome is transferred to bacteria. We heve already established *rpsL*transgenic zebrafish line, in which pML4 plasmid carrying *E. coli KanR* gene (kanamycin-resistant gene) is integrated on the genomic DNA. We have planned to test the possibility of gene-transfer from the fish to bacteria using *rpsL*transgenic zebrafish and pML4 as a model organism and a plasmid, respectively.

In this study, we examined 1) whether a transgene is detected in feces of transgenic fish and 2) whether pML4 gene is transferred to enterobacteria in transgenic fish, 3) whether pML4 is transferred to bacteria in the aquatic environment. We examined whether a typical enterobacteria, *E. coli*, or a soil bacteria, *P. putida*, is transformed to be kanamycin resistant as a result of transfection of pML4 to the bacteria, before testing the possibility of gene-transfer from the fish to bacteria. The possibility of spontaneous transformation of *E. coli* and *P. putida* with pML4 is very low.

As a result of PCR amplification of DNA extracted from feces of *rpsL*transgenic zebrafish, pML4 was detected in feces. This pML4 might be derived from the gut tissue of transgenic fish. We isolated kanamycin-resistant enterobacteria from transgenic zebrafish under the aerobic and anaerobic conditions. However, pML4 was not detected in these isolated enterobacteria.

To confirm the possibility of gene-transfer from transgenic fish to bacteria in the aquatic environment, muscle and gut of transgenic zebrafish were placed in 0.1% artificial sea water or the water of Kasumigaura Lake, and bacteria were cultured. Hundreds of kanamycin-resistant bacterial colonies were isolated form the culture medium, but the pML4-transfermed bacteria were not detected. The transformation rate was estimated to be below $1/10^3$-10^6.

(2) Studies on gene dispersal from genetically modified crops into wild populations and evaluation of the impact of gene dispersal on plant biodiversity

① Development of evaluation method for measurement of gene flow within a natural population and among natural populations: Case study in allogamous plant populations.

This research is aimed to; (1)investigate real gene flow within a population using a typical allogamous plant species common buckwheat population as a model plant of out-crossing GM plant species as like *Brassica napus*, and (2) construct the simulation model of gene introgression that considered the fitness. In detection of a gene flow, the difference of a reproduction system-with self incompatible common buckwheat and self compatible, one paid attention to the influence it has on a gene flow within population. The gene flows between the individuals are restricted very strongly by the self-compatibility. The influence that the difference of the flowering period exerted on the gene flow was observed. It was clarified that the gene exchange between individuals was disturbed only by differing for two weeks the first flowering even if the flowering period came in succession. The simulation model used QTL(Quantitative Trait Loci) information was constructed, and it aimed at the establishment of the technique for evaluating quantitatively the risk that the gene of genetically modified products diffuses to the environment. A basic model was constructed. As a result, it was suggested to remain a lot of introduced genes at the next generation by the linkage drag of the

loci that related to the fitness even if the target gene was neutral when the gene was introduced near the loci related to the fitness. To lower the risk of introgression, these results show that it is effective to modify a reproductive system. It is necessary to clarify the relation to the gene related to the fitness to understand the process that the introduced gene diffuses to a wild population(3)-(6).

② Development of an evaluation method for monitoring potential transgene dispersal into natural populations – 2. Self-pollinating crops

Risk assessment of unintentional transgene dispersal into natural populations from genetically modified self-pollinating crops is less well studied than for out-breeding crops. *Glycine soja* and *Vigna angularis* var. *nipponensis*, the presumed wild ancestors of soybean (*G. max*) and azuki bean (*V. angularis* var. *angularis*), respectively, are distributed across much of Japan. Soybean and azuki bean and their wild relatives are self-pollinating species and sympatric in many areas of Japan. Hence introgression from the crops into these two wild leguminous species needs to be assessed to determine whether there is a potential impact of growing transgenic crops in Japan.

To acquire information of the extent of gene introgression, microsatellite markers that can clearly distinguish between cultivated and wild form of these crops were identified. Soybean and azuki bean and their wild relatives were directly collected from natural populations and analyzed. In azuki bean, many intermediates between cultivated and wild forms were found and the distribution of introgressed individuals in natural populations became clear using microsatellite markers. The results point to the importance of habitat disturbance in relation to gene spread compared to gene dispersal by the rate of natural out-crossing. Introgressed individuals rather than presumed wild plants tend to invade recently abandoned fields and recently vacated land.

In soybean, the potential impact of growing transgenic soybean in Japan needs to be assessed to assist policy makers' deliberations. The large-scale genetic structure of Japanese wild soybean populations and modern cultivated soybean varieties were characterized using microsatellite markers. Genetic admixture analysis implicates persistence of introgressed genes even in morphologically wild individual. In order to clarify the actual situation of introgression from cultivated soybean into wild soybean populations, we identified several intermediates between cultivated and wild soybean in northern and southern Japan. Using microsatellite markers these intermediates were found to be natural hybrid derivatives between wild and cultivated soybean. Thus gene introgression from soybean into wild soybean can occur, but is very rare compared to azuki bean, in natural habitats in Japan. Based on monitoring these habitats for two years, the hybrid derivatives disappeared or tend to disappear and the persistence and movement of soybean alleles from the hybrids was not observed in wild soybean surrounding soybean fields. In natural habitats, pollen dispersal from soybean to wild soybean was not detected in spite of their spatial proximity and overlapping flowering time. On the other hand, pollen dispersal among wild soybeans occurs more frequently than the rare hybridization events from soybean to wild soybean. High out-crossing rate and migration among wild populations by seed dispersal are considered to be important factors for secondary

gene spread.

Natural outcrossing between cultivated soybean and azuki bean and their wild relatives is thought to be rare compared to out-breeding crops. However, hybrid derivatives can persist for several years because of their inbreeding nature. Therefore, knowledge of the growth characteristics and the degree of fitness of hybrids and their derivatives is require to assess possibility of environmental transgene dispersal from self-pollinating crops to their wild relatives(7)-(11).

③ Studies on frequency of transgene introgression from transgenic plants to their wild relatives

To clarify the possibility of transgene introgression from GM plants to their wild relatives and its effect on plant biodiversity, we are trying to obtain hybrids between soybeans and their wild relative, tsurumame, by artificial pollination and to investigate their physiological characteristics including their fitness in the Japanese natural environment. Also, we are investigating the frequency of natural hybridization between soybeans and tsurumame when they are grown side by side in the field.

In 2003, F_1 and F_2 hybrids were obtained by artificial pollination between GM soybeans (*Glycine max* (L.) Merr.) and tsurumame (*Glycine soja* Sieb. and Zucc.). These hybrids showed physiological and morphological characteristics of both of their parents. Herbicide-resistance transgene and phenotype were both inherited from the GM soybeans to the hybrids, according to the Mendel's law.

In 2004, tsurumame and two soybean varieties with different flowering times, were grown at various intervals (1-6 m) in the field of National Institute for Environmental Studies from June, and seeds formed on tsurumame were harvested in November. These seeds were then subjected to the PCR analysis with a SSR marker to detect hybrids. No hybrid was detected in about 2,500 harvested seeds from tsurumame that had been grown at the distance of 1-2 m from soybeans.

In 2005, plants of soybean, tsurumame, and the F_2 hybrids formed between them were grown side by side at intervals of 0.8 or 1 m in the same field. Seeds were harvested from tsurumame plants and subjected to the PCR analysis with a SSR marker as described above. Although all of these plants flowered at almost the same time of the year, no hybrid was detected in 819 harvested seeds from tsurumame. Together with the results obtained in 2004, the frequency of natural hybridization obtained in our experiments is much lower than that reported previously in a study carried at Osaka using a different combination of soybean and tsurumame varieties. The hybridization ratio appears, therefore, to vary greatly depending on factors such as plant varieties and/or environmental conditions under which plants are grown.

④ Study on influence of environmental factors in gene flow among related species

The purpose of this research is to evaluate the influence of several environmental stresses, e.g. lack of nitrogen, restriction of rhizospher and others, on cross compatibility and fitness of progenies between canola (*Brassica napus*) and wild relatives (*B. rapa* and *B.juncea*) in Japan. Although lack of nitrogen did not affect cross compatibility and seeds set, drought stress and

treatment of respiration inhibiter suppressed plants growth. Restriction of rhizosphere affected seed number in a pot and seed set efficiency.

Results of cross pollination (*B. rapa* and *B. napus*, *B. juncea* and *B. napus*) exhibited high cross compatibility. In cross pollination between *B. rapa* and *B. napus*, germinated seeds and non-germinated seeds in pods were confirmed. Flow cytometer analysis detected that 92 % of germinated seeds were hybrid and only 7.3 % of non-germinated seeds was hybrids. Since ratio of germinated seeds was about 5%, production efficiency of hybrid was not higher than expected by cross compatibility. Since the ratio of hybrid is lower in spite of artificial pollination, we speculated that false fertilization might occur in highly frequency. From these results, it seems that progeny of hybrid between *B. rapa* and *B.napus* must not be dominant and will not affect plant biodiversity.

Seed colors of *B. juncea* and *B.napus* in this experiment were yellow and brown, respectively. However seeds color of progeny produced by artificial pollination between *B. juncea* and *B.napus* appeared two types of color which were yellow and brown. Ratio of yellow seeds was 55% and yellow seeds in progeny were revealed *B. juncea* by RAPD analysis. Brown seeds had both PCR fragment derived from *B. juncea* and *B.napus* and were proved hybrid seeds. Since the ratio of brown seeds was too low notwithstanding artificial pollination, we also speculated that this cross combination occurred false fertilization or metaxenia. This result can also be concluded that production efficiency between *B. juncea* and *B.napus* was not high in spite of high cross compatibility.

Morphological and growth traits regarding seed germination, plant height, bolting period and others exhibited intermediate type expect epidermis of leaves. Epidermis of progenies was very similar to waxy epidermis of *B. napus*.

Using ordinal (non-transgenic) plant, *B. rapa*, *B. juncea* and *B.napus*, these results suggest that the cross compatibility between *B. rapa* and *B.napus* or *B. juncea* and *B.napus* are higher than expectation based on the previous knowledge. Moreover fitness of these progenies did not increase and will not give harmful effect on plant biodiversity.

⑤ Evaluation of fitness of hybrids between wild and cultivated soybean and their derivatives

Knowledge of plant growth characteristic in relation to fitness of hybrids between cultivated soybean (*Glycine max*) and wild soybean (*G. soja*) and their derivatives are required to assess the possibility of transgene dispersal from transgenic soybean to wild soybean populations. In the present study, artificial F_1 hybrids of four combinations between two wild soybean accessions and two modern cultivars, having different growth habits and representing northern and southern Japanese accessions, were made. The degree of fitness of the four F_1 hybrids and 2 F_2 populations from two F_1 hybrids were compared with their wild and cultivated parents in three different locations, Akita, Ibaraki and Hiroshima prefectures, in 2004 and 2005.

The flowering period of F_1 hybrids tended to be intermediate between cultivated soybean and wild soybean parent. Due to genetic segregation many F_2 progenies overlapped with that of local wild soybean at the northern location. These data suggest that secondary out-crossing will occur between established hybrids and wild plant even though mean flowering time of

cultivated soybean is different from wild soybean.

The total number of seeds per plant was treated as an indicator of fitness. Generally, stem dry weight and total number of seeds is highly correlated. Total number of seeds of F_1 hybrids was similar or less than the parental wild soybean. However, in the F_2 progenies many individuals produced a similar or greater number of seeds than the parental wild soybean. Although this character was largely affected by environmental conditions, the results suggest that some hybrid derivatives may have more vigorous growth and increase number of seeds (higher fitness) than the parental wild soybean.

The seeds from the wild soybean, soybean, F_1 hybrids and F_2 progenies were sown in the field in the middle of December 2004 and 2005 and compared with their wild and cultivated parents. Seed survival rate during the winter was largely affected by location where seeds were produced rather than the testing location. Most soybean seeds died during winter whereas wild soybeans survived winter conditions. Although seed survival rate from F_1 hybrids were intermediate between cultivated soybean and wild soybean, black and brown seeds from F_2 progenies survived winter conditions like wild soybean. The seed survival rate from F_2 progenies is related to seed color. These data indicate that growth characteristics and survival of seeds in relation to fitness of hybrids and their derivatives are largely affected by genes from cultivated soybean and plant growing conditions. From observed phenotypic data based on limited conditions and number of individuals, hybrid derivatives have the potential to produce a larger number of progenies than native wild soybean, and seeds of some hybrid progenies survive winter conditions. These data will be used for QTL analysis and in simulation studies using outcrossing rate and demographic factors of natural wild soybean populations to assess the probability of transgene dispersal in wild soybean populations.

(3) Development of evaluation methods for the impact of living modified organisms on biodiversity

The evaluation methods for the impact of living modified organisms on biodiversity were proposed. For microorganisms, the gene transfer is influenced by concentrations of donor and recipient cells, plasmid characteristics, donor and host cell characteristics, and environmental conditions such as temperature, pH and nutrient concentration and so on. For plants, the gene transfer is influenced by the self compatibility, plant distance, flowering periods, fitness of hybrids, QTL and environmental conditions. It is very important to evaluate these parameters.

Reference

1. Kitagawa, W., N. Kimura, and Y. Kamagata (2004), A Novel *p*-nitrophenol degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101. J. Bacteriol., 186: 4894-4902 (2004).

2. Lee, T.H., S. Kurata, C.H. Nakatsu, and Y. Kamagata (2004), Molecular analysis of bacterial community based on 16S rDNA and functional genes in activated sludge enriched with 2,4-dichlorophenoxyacetic acid (2,4-D) under different cultural conditions. Microbial Ecol., published online.

3. Kitamoto, N., Ueno, S., Takenaka, A., Tsumura, Y., Washitani, I. and Ohsawa, R. (2006), Effect of flowering phenology on pollen flow distance, and the consequences for spatial genetic

structure within a population of *Primula sieboldii* (Primulaceae). Amer. J. Bot., 93: 226-233.
4. Ueno, S., Kitamoto, N., Ohsawa, R., Tsumura, Y. and Washitani, I. (2005), Nine additional microsatellite markers for *Primula sieboldii* E. Morren, Conservation Genetics Technical Notes, 6: 1063-1064.
5. Iwata, H., Imon, K., Tsumura, Y. and Ohsawa, R. (2005), Genetic diversity among common buckwheat(*Fagopyrum esculentum*) cultivars in Japan as determined from AFLP and SSR markers and quantitative agronomic traits. Genome;48:367-377.
6. Kitamoto, N., Honjo, M., Ueno, S., Takenaka, A., Tsumura, Y., Washitani, I. and Ohsawa, R. (2005), Spatial genetic structure among and within populations of *Primula sieboldii* growing separate streams. Mol. Ecol. 14:149-157.
7. Kuroda, Y., A. Kaga, N. Tomooka, D.A. Vaughan (2006), Population genetic structure of Japanese wild soybean (*Glycine soja*) based on microsatellite variation, Molecular Ecology, 15: 959-974 .
8. Kaga, A., D.A. Vaughan and N. Tomooka.. In H. Lörz and G. Wenzel (eds.) (2004), Molecular markers in plant breeding and crop improvement, Biotechnology in Agriuclture and Forestry, 55: 171-187.
9. Han, O.K. , A. Kaga, T. Isemura, X.W. Wang, N. Tomooka, D.A. Vaughan (2005), A genetic linkage map for azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi],Theor. Appl. Genet., 111: 1278-1287.
10. Wang, X.W., A. Kaga, N. Tomooka and D.A.Vaughan (2004), The development of SSR markers by a new method in plants and their application to gene flow studies in azuki bean *Vigna angularis* (Willd.) Ohwi & Ohashi], Theor. Appl. Genet., 109: 352-360.
11. Zong. X.X. , A. Kaga, N. Tomooka, X.W. Wang, O.K. Han and D. Vaughan (2003), The genetic diversity of the *Vigna angularis* complex in Asia, Genome, 46: 647-658 .