Annual Report

2006

NIAS
National Institute of Agrobiological Sciences
Annual Report 2006
(In the fiscal year 2005)
The National Institute of Agrobiological Sciences (NIAS), which is the largest agricultural research institute of basic life science in Japan, was established on 1 April 2001 as an independent administrative institution of the Ministry of Agriculture, Forestry, and Fisheries and a center for basic studies to develop innovative agricultural biotechnologies and new bioindustries.

The Second Five-year Plan started on 1 April 2006. During the next five years, the Institute will advance the technologies developed in the First Five-year Plan. These include sequencing of the entire rice genome; sequencing of the silkworm genome and recombination of livestock and silkworm genes; and bioscience research that will help improve human nutrition and promote the creation of new bioindustries.

In the Second Five-year Plan, the Institute will focus on: (1) improvement, diversity and utility of agrobiological resources - we will utilize resources that we have built up in the course of research on the genes of rice, silkworms, and pigs and will secure other genetic resources; (2) research on, and development of, innovative agricultural technologies using biological and genome information - we will study organisms with a view to their ability to adapt to their environments, differentiate, and interact with other organisms; and (3) research and development aimed at creating new biotechnology-based industries - we will develop biotechnologies to produce useful materials, such as silk-based products that can be used in everyday items and in medical practice.

There is an urgent need to develop technologies in these three subject areas, because each of them is important and is expected to contribute greatly to society. The Institute will make further efforts to maximize the benefits of biotechnology.

One of the major biotechnologies in which the Institute is involved is the recombination of genes in crops, insects (silkworms), and animals. This technology is based on fundamental principles related to the building blocks of life in which DNA provides the templates for building amino acids into proteins. The principles are essentially the same as those on which conventional breeding technologies and successes are based.

Gene recombination and other biotechnologies are developing rapidly; however, their high level of sophistication may make them difficult for the general public to understand. In addition, the media is now reporting on the potential risks of using biotechnology. Since the development of basic gene recombination technologies in the 1970s, these technologies have been used to develop a number of drugs to help patients with diseases such as diabetes, and to improve human health. Agricultural plants developed using these new technologies are now widely cultivated in many countries and are consumed in Japan and elsewhere in foods for humans and in animal feeds. Because due consideration has been given to securing safety in both the research and development of gene-recombination technology and its application, few concerns have arisen in regard to its influence on the environment and the safety of its use in food production.

Biotechnologies have great potential, and there is fierce global competition to lead in these fields. Gene-recombination technology is spreading widely because it provides opportunities to make use of scientific discoveries in many areas of biology. Advanced biotechnologies are indispensable in helping to solve problems in the areas of global food supply, the environment, and medicine. We believe that biotechnology research will contribute to the well-being of the human race. Thus there is a need for the public to gain an understanding of the importance of the wise use of biotechnology for the future good of humankind.

As part of the Second Five-year Plan, all the staff at NIAS are determined to make a concerted effort to achieve more great results like the sequencing of the entire rice genome achieved under the First Five-year Plan. We thank all of those involved for their cooperation, and we look forward to your continued support, understanding, and collaboration.

Teruo Ishige, President
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Organization

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Animal Cell Biology Laboratory
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Mutation Breeding Laboratory

The color on respective divisions is the same color on pages of “Topics of Research in This Year” and “Research Activities” for each division in Annual Report 2006.
Bombyx mori densovirus (BmDNV) multiplies in the columnar cell nuclei of the midgut epithelia of the silkworm, Bombyx mori. It is classified into two species, DNV-1 and DNV-2 based on their symptoms, serological characters, genome structure and sequences. Some silkworm strains were identified as resistant against DNV-1 and/or DNV-2. In such strains the response reflects non-susceptibility rather than resistance because even high dose of inoculum does not affect their survival rate. So far four non-susceptibility genes have been reported, namely; nsd-1 (L21-8.3 cM), Nid-1 (L17-31.1 cM), nsd-2 (L17-24.5 cM) and nsd-Z (L15-50.7 and 30.0 cM from apical and distal ends, respectively). However, none of them have yet been isolated as responsible genes. Studies on these genes are useful in understanding the mechanism of the viral invasion and multiplication, and introducing those genes into the silkworms or cultured cells will lead us to understand the functions of the genes. We have identified four EST markers closely linked with nsd-2. By taking advantage of Bombyx genome information, positional cloning, BAC-contig construction and linkage analysis of the virus-selected BC1 (backcross 1) progenies (Fig. 1) enabled to find the resistant strain-specific deletion within the candidate region of 500 kb (Fig. 2). We have succeeded in identifying the candidate gene for nsd-2 on the deletion region (Fig. 3). The gene was expressed only in the midgut throughout the larval stage. RT-PCR revealed that mutant strains examined showed a common shorter transcript compared with that of susceptible strains, which was caused by the common deletion in the gene among resistant strains (Fig. 4). Sequence analysis of the cDNA tells that nsd-2 encodes a transmembrane protein (Fig. 5). Mutation of this gene endows the resistance against the virus. Identification of nsd-2 gene strongly accelerates the virus research through establishing transgenic cultured cell lines hypersensitive to the virus, in addition to the direct marker-assisted breeding of Bombyx strains resistant to DNV-2.

Fig. 1
Segregation of BC1 with DNV-2 infection.
Azuki bean (*Vigna angularis* var. *angularis*) belongs to the Asian *Vigna* that consists of 21 species among them 10 are cultivated. Until now none of the Asian *Vigna* had a genetic linkage map that resolved the 11 linkage groups representing the haploid chromosome number. In order to develop a saturated linkage map of azuki bean we first constructed an SSR (microsatellite) library using a new method in plants. The library construction involved an oligo-primed second-strand synthesis enrichment procedure of the single strand genomic library. We found in the azuki bean genome (AG) motifs are a rich source of markers and thus constructed an (AG)-SSR enriched library. The enriched library enabled primer pairs to be designed and these primers had a high percentage (98%) of successful single locus amplification. A total of 401 SSR primer pairs were developed for use in azuki genome.
To construct a fully saturated genetic linkage map for azuki bean, three mapping populations consisting of a total 592 individuals were developed using azuki bean and three closely related taxa (V. angularis var. nipponensis, V. nepalensis and V. riukiuensis). The linkage maps based on the newly developed SSR markers with RFLP and AFLP markers covered 11 linkage groups and the marker order was highly conserved among the three maps. To integrate the marker information into a single map a consensus linkage map was synthesized based on common SSR markers among three maps as a bridge. The consensus linkage map consists of a total 896 markers and has an overall length of 854 cM with an average distance of 3.1 cM between SSR markers (Fig. 1).

The significance of the SSR primers

Fig. 1
A genetic linkage map for azuki bean
developed for azuki bean is that it can be used not only for genetic and QTL mapping in azuki bean but also for many other cultivated Asian Vigna. We have confirmed that approximately 70% of the SSR primer pairs amplify a certain DNA fragment even in Asian Vigna not closely related to azuki bean. Thus using the SSR primers genome maps for other important crops, like mungbean, can now be developed.

The significance of this azuki bean genetic linkage map is that it can act as a standard for comparative mapping in the Asian Vigna. In addition, since azuki bean belongs to the temperate group of legumes, that includes common bean (Phaseolus vulgaris) and soybean (Glycine max), this map will be helpful for comparative genome research across the world's most important legume crops.

**Allele diversity and evolution of GBSS 1 gene generated by multiple transposable elements in foxtail millet**

*Makoto KAWASE*

*Genebank*

Foxtail millet, *Setaria italica* P. Beauv. ssp. *italica* is one of the world's oldest cultivated crops. It is most likely to have been domesticated in Eurasia and genetically differentiated into various landrace groups. The naturally occurring waxy and low-amylose variants of this millet and other cereals were developed in East Asia and Southeast Asia under human selection for sticky foods. Mutations in the *GBSS 1* gene for granule-bound starch synthase 1 are known to be associated with these traits.

Twelve different alleles were found on *GBSS 1* gene in foxtail millet based on the analysis of 871 landraces collected from various regions mainly in Europe and Asia, which are conserved in NIAS Genebank. Twelve *GBSS 1* alleles were identified by Long PCR: types I to X, IVa and IVb (Fig. 1). Types I and II produce non-waxy endosperm starch containing over 22% amylose, types III, VI and IX forms low amylose endosperm (less than 22% amylose), and types IV, IVa, IVb, V, VII, VIII and X cause waxy endosperm (almost no amylose). The differentiation of those alleles has been mediated by eleven transposable elements' insertions, TSI-I to TSI-11, studied in the present study.

Type I is distributed through Asia and Europe, while others have their own distribution particularly in East Asia and Southeast Asia (Fig. 2). All of the twelve accessions of ssp. viridis, the presumed wild ancestor of ssp. *italica*, have the type I allele. Type II similarly produces non-waxy starch, although it has an insertion in an intron, TSI-1.

![Fig. 1](image)

Allele diversity of *GBSS 1* locus in foxtail millet (Kawase et al., 2005)
Types III, IV, IVa, IVb and IX have insertions of different length at the same site of the Intron 1, and types VI and VIII have insertions of different length at the same site of intron 12. TSI-3 of type IX is homologous with TSI-2 of types IV, IVa and IVb but have a deleted portion. Type III has an additional insertion (TSI-6) on TSI-3 of type IX. Type V has a retroposon (TSI-7) inserted in intron 3. TSI-8 of type X is its Solo-LTR variant. Intergenic recombina of types II and VI were rarely found.

Fig. 2
Geographical distribution of GBSS 1 alleles of foxtail millet (Kawase et al., 2005)

Fig. 3
Mode of evolution of GBSS 1 alleles of foxtail millet (Kawase et al., 2005)
An evolutionary sequence was suggested most likely from type I to type IV, from type IV to type IX, and from type IX to type III (Fig. 3). Types IVa and IVb were independently formed from type IV by different short insertions. Type VI was also evolved from type I, and type VIII from type VI.

Five landraces of type V (TSI-7) and one of type X (TSI-8) have an completely identical LTR sequence. The LTRs of TSI-9 were identical among six landraces of type VII. These findings indicate that the insertions occurred quite recently from a paleontological viewpoint.

Allele types I to X can be identified by Long PCR using four sets of primers, and additional two sets can distinguish types IVa and IVb from type IV.

Publication

Development of practical method to discriminate Nagoya breed from other chicken breeds

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Nagoya breed (Fig. 1) is a famous native chicken in Aichi Prefecture of Japan, a dual purpose breed for eggs and meat. The purebred (Nagoya breed × Nagoya breed) has been commercialized, so the commercial chickens are produced by intra-strain mating. Nagoya breed has four strains (NG1, NG2, NG3 and NG4) established at Aichi-ken Agricultural Research Center. They are maintained at Aichi Livestock and Poultry Breeding Center, which supplies parent stocks to the hatcheries using the four strains. Therefore, all commercial Nagoya breed chicken are derived from four strains. Since the taste, palatability and texture of Nagoya breed meat are well recognized in Japan, the market price of Nagoya breed meat is much higher than that of broiler meat. However, cut-up meat of Nagoya breed and broiler cannot be easily distinguished by appearance. So, a technology to discriminate between Nagoya breed and broiler is vital for preventing false sales and guaranteeing the quality of meat. The objective of the present study is to develop a method to discriminate between Nagoya breed and all other chicken on the market using microsatellite DNA markers. Four strains of Nagoya breed established at the Aichi-ken Agricultural Research Center were analyzed using twenty-five microsatellite markers. In these strains, five of the markers

Fig. 1 Nagoya breed
(ABR0015, ABR0237, ABR0417, ABR0495 and ADL0262) had a single allele. Other 448 chicken samples of various breeds and hybrids were analyzed using the same five markers. None of these chicken samples had the same allele combination as the Nagoya breed. These five microsatellite markers provide a practical method to accurately discriminate the Nagoya breed from other chicken and can contribute to checking the validity of labeling Nagoya breed.

Intracellularly expressed antibodies (intrabodies) have been considered useful for not only clinical applications such as viral neutralization and cancer therapy but also functional analysis of proteins inside the cell. A variety of intrabody formats have been designed. Single-chain variable fragments (scFvs) consist of one heavy chain variable region (VH) linked through a flexible peptide spacer, a repeated motif of 3 × GGGGS, to one light chain variable (VL). They are able to fold and retain the antigen-binding specificity and affinity of the parental antibody. However, technical problems, such as stability and functional expression of intrabodies in the cytosol remain to be overcome.

In this study, we constructed the scFv intrabodies derived from hybridoma cells producing the monoclonal antibodies (Fig. 1A) that specifically bind to an Ena/VASP homology 1 (EVH1) domain of Wiskott-Aldrich syndrome protein (WASP). WASP resides in the cytosol as a multifunctional adaptor molecule and mediates actin polymerization

**Intrabody technology: a novel method for domain-specific knockdown of cytosolic protein in mice**

Mitsuru SATO

**Molecular Biology and Immunology Department**

Intracellularly expressed antibodies (intrabodies) have been considered useful for not only clinical applications such as viral neutralization and cancer therapy but also functional analysis of proteins inside the cell. A variety of intrabody formats have been designed. Single-chain variable fragments (scFvs) consist of one heavy chain variable region (VH) linked through a flexible peptide spacer, a repeated motif of 3 × GGGGS, to one light chain variable (VL). They are able to fold and retain the antigen-binding specificity and affinity of the parental antibody. However, technical problems, such as stability and functional expression of intrabodies in the cytosol remain to be overcome.

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**Fig. 1**

Antibody structure

(A) The basic structure of a conventional antibody consists of four polypeptide, two identical heavy (H) chains and two identical light (L) chains, held together by disulfide bonds. Each chain has both a variable (V) and a constant (C) region.

(B) Schematic representation of the two scFv DNA constructions for microinjection. Shown are the leader signal sequence, VH region, polypeptide linker (Gly4Ser)3, VL region, light chain constant (CL) region and Myc tag sequence. The scFv DNA fragments were cloned into the pCAGGS-MCS expression vector.
and interleukin (IL)-2 synthesis in the T-cell receptor (TCR) signaling pathway. The anti-WASP-EVH1 scFvs were constructed with a V\text{H} leader signal sequence at the N-terminus and with a light chain constant (C\text{L}) region behind the V\text{L} region (Fig. 1B), and injected into fertilized mouse eggs. In T cells from transgenic mice overexpressing the anti-WASP-EVH1 scFvs, binding of anti-WASP-EVH1 scFvs to native WASP was confirmed by immunoprecipitation. Furthermore, IL-2 production induced by TCR stimulation was strongly inhibited in the scFv transgenic mice T cells (Fig. 2A). Similar T-cell defects were observed in WASP-EVH1 domain overexpressing transgenic mice (Fig. 2A). So, the WASP-EVH1 scFv intrabodies inhibit only the function of EVH1 domain that regulates IL-2 synthesis signaling, but do not affect the rest of the domain functions of WASP (Fig. 2B). The novel procedure presented here can be applicable to functional analysis of other cytosolic proteins.

![Fig. 2](image)

**Fig. 2**

IL-2 production induced by TCR stimulation was inhibited by anti-WASP-EVH1 intrabodies

(A) Splenic T cells from anti-WASP scFv 21SHL and 21SHL-CL Tg mice, WASP-EVH1 Tg and wild-type mice were cultured in medium alone or in the presence of anti-CD3ε Ab. Each cell culture supernatant was collected at 24 h. IL-2 in the supernatant was quantified by ELISA.

(B) Schematic representation of mode of intrabody action against WASP-EVH1 domain in T cells. WASP contains multiple domains that enable it to interact with different proteins. Shown are the EVH1 domain, GTPase-binding domain (GBD), proline rich region (PRR), verprolin homology (VH) and cofilin homology (CH) domain. Anti-WASP scFvs can specifically bind to the EVH1 domain of WASP in the cytosol of T cells, blocking the collect interaction between WASP and signaling molecules involved in IL-2 synthesis.
Mulberry latex defense mulberry trees from herbivorous insects

Kotaro KONNO
Insect Genetics and Evolution Department

Latex is widely found among plant species; 12,000-35,000 species have been reported to exude it and many kinds of chemicals and proteins have been reported from plant latex. The biological functions of plant latex and its ingredients, however, remained obscure. Recently, we found that several latex-producing plants with no reported toxicities are strongly toxic to insects due to the ingredients of latex. For example, papaya (Carica papaya, Moraceae) and fig (Ficus virgaia, Moraceae) leaves are strongly toxic to insects because of the cysteine proteases in their latex. Our recent findings indicated a need to investigate latex ingredients and their biological functions.

Mulberry trees (Morus spp. Moraceae) grow in Asia and their leaves are used for rearing an economically very important insect, the silkworm, Bombyx mori, for thousands of years. Moraceae plants are characterized by the presence of latex, and mulberry trees also exude latex (Fig. 1, lower left photo) when their leaves are damaged by caterpillars. In wild condition, mulberry leaves are not often damaged by herbivorous insects, although the leaves are soft and contain a lot of nutrients.

Fig. 1
Defense activity of mulberry latex and three sugar-mimic alkaloids, the active compounds contained in the latex in very high concentrations.
Upper left photo, left part: the cabbage moth larvae fed intact (excised) mulberry leaves; Upper left photo, right part: the cabbage moth larvae fed mulberry leaves from which latex was washed off by cutting into narrow leaf strips and washing with water; Lower left photo: latex exuded from mulberry leaves (arrows). Right: three sugar-mimic alkaloids contained in mulberry latex in high concentrations, which are glycosidase inhibitors and were reported to have anti-diabetic activities.
such as proteins. However, there have been no detailed studies about the defense activities of mulberry trees against insect herbivory or the involvement of mulberry latex in the plant defense. In this study, we addressed these subjects.

We found that mulberry leaves are highly toxic to lepidopteran larvae other than the silkworm, *B. mori* (such as the Eri silkworm, *Samia ricini* and notorious pest species, the cabbage moth larvae, *Mamestra brassicae*), due to the ingredients of the latex. When Eri silkworm and the cabbage moth larvae were fed mulberry leaves, they bit in the leaves but they didn’t grow, and died at last (Fig. 1, upper left photo, left part). The toxicity of mulberry leaves was lost when latex was washed off (Fig. 1, upper left photo, right part). Also, latex-added artificial diets showed toxicity and growth inhibitory effects to the Eri silkworm (Fig. 2A). Mulberry (*M. australis*) latex contained very high concentrations of alkaloidal sugar-mimic glycosidase inhibitors (sugar-mimic alkaloids) reported to have anti-diabetic activities, such as 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1), 1-deoxy nojirimycin (DNJ), and 1,4-dideoxy-1,4-imino-D-ribitol (Fig 1, right). Their concentrations, altogether, in latex reached 1.5-2.5% (8-18% to dry weight) in several mulberry varieties (Table 1), which were 100 times the concentrations previously reported from whole mulberry leaves. These sugar-mimicking alkaloids showed toxicities to caterpillars (Fig. 2 B,C), but not to the silkworm, *B. mori* (Fig. 2 D). Our results suggest that mulberry latex and sugar-mimicking alkaloids in it play key roles in defense of mulberry against insect herbivory, and also suggest the existence of some adaptive mechanisms in the silkworm, *B. mori*.

This study shows, for the first time in long history of sericulture, the existence of strong latex-borne defense mechanisms in mulberry, and gives practical answer to mulberry-silkworm interactions. This study, together with our previous studies on latex-borne defense mechanisms of papaya and fig trees carried out by cysteine proteases in latex, shows that medically applicable chemicals (i.e. anti-diabetic sugar-mimic alkaloids) exist in mulberry latex in surprisingly high concentrations (18% of dried latex), and they are easily purified from latex, and shows the possibility of mulberry latex as a source of interesting and applicable chemicals in medical and agricultural field. Also, this study will contribute to researches on pest resistance of mulberry varieties.

Reference

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<th>Table 1 Concentrations of sugar-mimic alkaloids in mulberry latex</th>
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<td><strong>Species, populations, and cultivars</strong></td>
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<tr>
<td><em>M. australis</em> (or <em>M. bombycis</em>)</td>
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<tr>
<td>Wild, Ishigaki, Okinawa, Japan</td>
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<tr>
<td>Wild, Tsukuba, Honshu, Japan</td>
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<tr>
<td>Cultivar “Yukishirazu”</td>
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<td>Cultivar “Ichibei”</td>
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<td><em>M. alba</em></td>
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<td>Cultivar “Shinichinose”</td>
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Values indicate means ± SD (n = 4-5 for wild populations, n = 2-3 for cultivars)
Fig. 2
Growth inhibitory effects of mulberry latex and sugar mimic alkaloids on the Eri silkworm, Samia ricini. Mulberry latex (A) and two sugar-mimic alkaloids, D-AB1 (B) and DNJ (C), show remarkable growth inhibitory effects on the Eri silkworm, Samia ricini. The larvae of the silkworm, Bombyx mori, are not at all affected by either of sugar-mimic alkaloids even at the high concentrations, suggesting that this species has developed some adaptive mechanisms to these compounds (D). Both sugar-mimic alkaloids and unidentified high molecular weight components contribute to the defense activities of mulberry latex (E). Percentages in graphs indicate mortalities of larvae (A-D).
Both Sf9 cell line derived from *Spodoptera frugiperda* and High 5 cell line derive from *Trichoplusia ni*, are utilized actually as a gene expression system by baculovirus in vitro. However, there is not *Bombyx mori* cell line that is suitable for serum-free culture and high level gene expression. This time, we established *Bombyx mori* cell line that is almost equal to *Spodoptera frugiperda* cell line and leaded to develop a new in vitro gene expression system that can actualize a large scale production of recombinant protein.

NIAS-Bm-Ke1 cell line (Ke1 cells) that adjusted to KBM700 of serum-free media was established. Ke1 cells showed high cell proliferation and high susceptibility to BmPTLNPV, a recombinant BmNPV expressing the luciferase gene (Figs. 1, 2).

The heat-treated silkworm hemolymph showed a role of promoter of susceptibility of recombinant BmNPV when added to Ke1 cells cultured in serum-free medium. In comparison of luciferase gene expression of Ke1 cells and Sf9 cells, Ke1 cells cultured in KBM700 serum-free medium containing heat-treated silkworm hemolymph, showed higher luciferase activity than Sf9 cells cultured in SF900 II serum-free medium containing 10% of FBS. A large scale luciferase expression in spinner culture was possible when Ke1 cells were cultured in KBM700 serum-free medium containing heat-treated silkworm hemolymph, because Ke1 cells were floatage (Fig. 3). Heat-treated silkworm hemolymph was eliminated from KBM700 serum-free medium at 24 hours after the virus inoculation and cultured in fresh KBM700 serum-free medium. As a conclusion, it was shown that NIAS-Bm-Ke1 cell line was able to be used as baculovirus gene expression system.
We have isolated phytochrome A (phyA), phytochrome B (phyB) and phytochrome C (phyC) mutants from rice (Oryza sativa) and have produced all combinations of double mutants. Seedlings of phyB and phyBphyC mutants exhibited a partial loss of sensitivity to continuous red light (Rc) but still showed significant de-etiolation responses. The responses to Rc were completely canceled in phyAphyB double mutants. These results indicate that phyA and phyB act in a highly redundant manner to control de-etiolation under Rc. Under continuous far-red light (FRc), phyA mutants showed partially impaired de-etiolation and phyAphyC double mutants showed no significant residual phytochrome responses, indicating that not only phyA but also phyC is involved in the photoperception of FRc in rice. Interestingly, the phyBphyC double mutant displayed clear R/FR reversibility in the pulse-irradiation experiments, indicating that both phyA and phyB can mediate the low-fluence response for gene expression (Fig. 1). Rice is a short-day plant and we found that mutation in either phyB or phyC caused moderate early flowering under the long-day photoperiod, while monogenic phyA mutation had little effect on the flowering time. The phyA mutation, however, in combination with phyB or phyC mutation caused dramatic early flowering (Fig. 2).

Functional analysis of phytochromes in rice

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We have isolated phytochrome A (phyA), phytochrome B (phyB) and phytochrome C (phyC) mutants from rice (Oryza sativa) and have produced all combinations of double mutants. Seedlings of phyB and phyBphyC mutants exhibited a partial loss of sensitivity to continuous red light (Rc) but still showed significant de-etiolation responses. The responses to Rc were completely canceled in phyAphyB double mutants. These results indicate that phyA and phyB act in a highly redundant manner to control de-etiolation under Rc. Under continuous far-red light (FRc), phyA mutants showed partially impaired de-etiolation and phyAphyC double mutants showed no significant residual phytochrome responses, indicating that not only phyA but also phyC is involved in the photoperception of FRc in rice. Interestingly, the phyBphyC double mutant displayed clear R/FR reversibility in the pulse-irradiation experiments, indicating that both phyA and phyB can mediate the low-fluence response for gene expression (Fig. 1). Rice is a short-day plant and we found that mutation in either phyB or phyC caused moderate early flowering under the long-day photoperiod, while monogenic phyA mutation had little effect on the flowering time. The phyA mutation, however, in combination with phyB or phyC mutation caused dramatic early flowering (Fig. 2).
Immunotherapy using allergen-specific T cell epitopes is a safe and effective treatment for the control of IgE-mediated allergic diseases, such as Japanese cedar polinosis. We developed transgenic rice plants expressing T cell epitopes of Cry j I and Cry j II allergens of Japanese cedar pollen as a fusion protein with the soybean glycinin (Fig. 1). Under the control of the rice seed storage protein glutelin GluB-1 promoter, the fusion protein was specifically expressed and accumulated in seeds at a level of 0.5 % of the total seed protein.

**Fig. 1**
A schematic illustration of the transformation plasmid
Next, we examined the effect of transgenic rice seeds on allergic immune responses. Mice were orally fed with rice seeds, and then systemically challenged with pollen allergens (Fig. 2). Compared to the control group of mice, the development of allergen-specific IgE and CD4+ T cell proliferative responses was inhibited in the group of mice fed with transgenic seeds (Fig. 3). The levels of allergy-associated cytokine production of IL-4, IL-5, IL-13, and histamine release in serum were also significantly suppressed. In addition, the development of pollen-induced clinical symptoms was inhibited in our experimental sneezing mouse model (Fig. 4). These results provide a positive first step toward developing rice seed-based edible vaccines for the prevention and treatment of allergic diseases.
The National Institute of Agrobiological Sciences (NIAS) is composed of three divisions: (1) the Genome and Biodiversity Research Division, (2) the Insect and Animal Sciences Division, and (3) the Plant Science Division. The research activities of the Genome and Biodiversity Division are interconnected with the Insect and Animal Science Division and Plant Science Division. Therefore, research data and biological resources are shared among these three divisions to maximize research efficiency of the institute.

The Genome and Biodiversity Research Division aims to elucidate major biological phenomena involved in agricultural production. It consists of three departments: (1) the Genome Research Department, (2) the Genetic Diversity Department and (3) the Genebank. The overall research output of these three departments is transmitted to the basic and applied research community. As a result, the Genome and Biodiversity Research Division contributes to science, agriculture and social life by distributing useful bio-resources and scientific results to the local as well as international research community.

The Genome Research Department focuses on genome analyses of three major organisms, which serve as the backbone of Japanese agriculture, namely, rice, pig and silkworm. Large-scale analysis of the genome by genetic mapping, physical mapping and genome sequencing is now considered as the most efficient strategy for understanding the structure and function of many genes controlling the expression of economically important traits. The genome information from these model organisms could be very useful for analysis of related species by comparative genomic approaches.

Genetic erosion caused by various environmental, socio-economical and ecological changes necessitates research on genetic resources to maintain the genetic diversity of the biological world. The Genetic Diversity Department focuses on basic research on plant, microbe and animal diversity. Extensive studies on various germplasm have contributed to the development of rational and efficient methods for classification, characterization, preservation and discovery of new biological resources.

To accelerate research on genetic resources, the NIAS is also the center of the MAFF Genebank System for agricultural genetic resources in Japan. The Genebank has the responsibility for conservation and maintenance of plant, microorganism and animal genetic resources and their DNA. A close linkage between NIAS and other sub-genebanks across Japan facilitates efficient collection, management and distribution of a wide range of genetic resources. All genetic resources available through the Genebank can be used for research and breeding purposes in accordance with the regulations of the Convention on Biological Diversity and International Treaty. Information related to genetic resources and DNA materials available at Genebank can be accessed at [http://www.gene.affrc.go.jp/](http://www.gene.affrc.go.jp/) and [http://www.dna.affrc.go.jp](http://www.dna.affrc.go.jp). The research activities of the Genome Research Department, the Genetic Diversity Department and Genebank are described below.
Introduction

Extensive analysis of the genomes are being undertaken through the three genome research projects of the institute, namely, the Rice Genome Research Program (RGP), Animal Genome Research Program (AGP) and Silkworm Genome Research Program (SGP). In the case of rice, the RGP in collaboration with the International Rice Genome Sequencing Project (IRGSP) has successfully completed the sequencing of the entire genome in 2004. The accurate map-based sequence of the rice genome is now considered as the most important resource for cereal genomics and provides the link to several important projects on rice functional genomics, comparative genomics and applied genomics. Immediately after completion of the sequence, the Rice Annotation Project (RAP) was initiated with the aim of manually curating the annotation of the genome on a regular basis. The first jamboree-style annotation meeting (RAP1) paved the way for the establishment of the Rice Annotation Project Database (RAP-DB), a comprehensive database of manually curated rice genes. Analysis of the genome structure of pig through the Animal Genome Research Program (AGP) led to the development of DNA markers for QTL analysis, construction of a catalog of porcine genes and facilitating traceability in the Japanese pork market. The Silkworm Genome Research Program (SGP), which aims to characterize the silkworm genome, has also made significant progress in EST analysis, genetic and physical mapping, whole genome shotgun sequencing and expression profiling. Informatics support for these projects facilitate large-scale analysis of genome sequence data, development of analysis tools, construction of genome databases and release of information to the public domain. Access to genome information and biological materials generated from these genome projects, as well as other genome-related projects, is provided by the DNA Bank and the Rice Genome Resource Center (RGRC). The following reports summarize the major research topics and achievements of the six laboratories of this department.

Assignment of 205 genes localized in HSA17 to a swine RH (IMpRH) map to generate a dense comparative map between swine and human/mouse

Bi/uni-directional chromosomal painting (Zoo-FISH) and gene mapping have earlier indicated the correspondence of swine chromosome (SSC) 12 harboring economically important quantitative trait loci (QTLs) to human chromosome (HSA) 17. In the present study, we have attempted to assign the genes localized in HSA17 to SSC12 to generate a comprehensive and comparative map between HSA17 and swine chromosomes. A total of 255 primer-pairs were designed using porcine sequences indicated to be orthologous to human genes. Of the 255 primer pairs, 208 (81.6%) were found to be mappable to a swine chromosome using the INRA-Minnesota 7000-rad porcine × Chinese hamster whole genome radiation hybrid (IMpRH) panel. The mapping results revealed the following: 205 genes were integrated into an SSC12 RH linkage map with logarithm of odds (lod) scores greater than 6. One gene (G1T1) was suggested to localize on SSC12, while the remaining two (HT008 and RPL26) were not linked to any markers/expressed sequence tags (ESTs)/genes registered, including those in the present study. A comparison of the gene orders among SSC12, HSA17, and mouse chromosome (MMU) 11 corresponding to HSA17 indicated that intrachromosomal rearrangements occurred frequently in HSA17 of human ancestral species after its speciation.
Analysis of Diversity in the Genus *Oryza* by Comparative Genomics Approach

The high-quality sequence of the rice genome based on the japonica cultivar Nipponbare was revealed in 2004. Since then, the information derived from the genome sequence has been widely used in understanding the biology of rice including the identification and functional characterization of novel genes for agricultural traits. As a model cereal crop, the rice genome sequence could also be used in clarifying how the genus *Oryza* originated from the ancestral cereal genome, as well as how it evolved and diversified. This would not only elucidate the lineage between cultivated rice varieties from their wild relatives but would also be useful in searching for new beneficial alleles of the genes in the wild rice species that had been lost during the establishment of modern varieties. A comparative genomics approach is becoming more and more important because the wild rice has been recognized as one of the major sources for novel gene alleles. Many agriculturally important genes such as disease resistance genes have been found in wild rice and introduced into the modern rice varieties.

We investigated the nucleotide sequence variation among wild rice species using 900 ESTs mapped at intervals of approximately 500 kb throughout the genome. Primers were designed mainly from the 3'-UTR region of the EST sequences and used for PCR amplification of DNA from 45 accessions belonging to 21 *Oryza* species from the germplasm collection of NIAS and the National Institute of Genetics (NIG). The overall efficiency of amplification for each species is shown in Fig. 1. Most of the regions were amplified in the AA genome (*O. sativa* complex, 94% in average for 15 accessions), suggesting that the genome sequence is conserved. On the other hand, the *O. officinalis* complex, which includes BB, BBCC, CC, CCDD and EE genomes, has 50-70% amplification efficiency. The other remote genomes (FF, GG and HHJJ) showed only 20-30% amplification. We have clearly shown here that the rice genome sequence has gradually changed in the course of evolution from the wild species. These results also indicate the utility of the standard genome sequence from the japonica cultivar Nipponbare for analysis of genome diversity as well as the evolutionary relationships between modern rice varieties and wild rice species. Moreover, the conserved regions across species could be good markers for comparative genomics among *Oryza* species.

![Amplification of *O. sativa* EST markers in wild rice species](image)

**Fig. 1**
Amplification of *O. sativa* ssp. *japonica* cv. Nipponbare EST markers in wild rice accessions
The height of the bars represents the efficiency (%) of marker amplification.
The Rice Annotation Project Database (RAP-DB) and Comparative Genome Analysis

Completion of the rice genome sequencing by the International Rice Genome Sequencing Project (IRGSP) enabled us to annotate all the rice genes. Automated annotations of the gene functions were manually curated in a jamboree-style annotation meeting of the Rice Annotation Project (RAP). The annotation data is available in the RAP Database (RAP-DB, http://rapdb.lab.nig.ac.jp/) which was constructed in collaboration with the DNA Data Bank of Japan (DDBJ), National Institute of Genetics (Fig. 2). The RAP-DB provides two types of viewers (GBrowse and G-integra), BLAST/BLAT similarity searches, and keyword searches. Information about TIGR and NCBI annotations on the IRGSP genome assembly is also available. Additionally, the RAP genes are linked to other rice genomics data, such as full-length cDNAs and T-DNA insertion mutant lines. We expect that the RAP-DB will serve as a hub for rice genomics.

The RAP annotation data was envisaged to elucidate the evolutionary process of flowering plants. We compared the genomes of two representative plant species, rice and *Arabidopsis thaliana*. First, our analysis revealed that the number and average length of exons in rice were quite similar to those in *A. thaliana*, while the lengths of introns and intergenic regions varied between the two species. This may be because transposable elements had been inserted extensively in non-exonic regions of rice. Second, we found that 22% of the rice genes had no homologs in the *Arabidopsis* gene set. Although this observation is consistent with the previous view that the rice genome possesses a number of unique genes, the majority of the rice-specific genes might be due to gene loss in the *Arabidopsis* lineage or mispredictions of the protein coding regions. Lastly, despite independent gene duplication events after the speciation between the monocot and dicot plant species, the distribution of the paralogs and protein functions had been retained in a similar manner in both of rice and *A. thaliana*. These data suggest that the rice and *Arabidopsis* gene sets have shared a common feature for over 100 million years. The phenotypic difference between the two species may have been derived from changes of a small number of genes or the divergence of gene regulation.
Construction of a silkworm SNP linkage map based on BAC end-sequences

The silkworm, *Bombyx mori*, is an agriculturally important insect that has been domesticated for an estimated 5,000 years and used extensively for silk production. In addition, it is a key model of the Lepidoptera, the second largest group of holometabolous insects, which include many beneficial insects as well as the most destructive agricultural pests. Due to

![SNP linkage map](image)

**Fig. 3**

*A Bombyx mori* SNP linkage map

The map is based on 534 SNP markers segregated into 28 linkage groups, represented by vertical lines. The BAC clones corresponding to the mapped SNP markers are shown on the right and the corresponding recombination distances between the markers are indicated on the left.
industrial and agricultural concerns, genome analysis has become an urgent necessity for a comprehensive characterization of silkworm. So far, we have been carrying out silkworm whole-genome shotgun sequencing, large-scale EST collection, BAC-contig construction by fingerprinting methods, etc. The integration of those results based on BAC clones will provide a useful tool for post-genome investigations of silkworm and other Lepidopteran species. We have developed a linkage map for silkworm based on single nucleotide polymorphisms (SNPs) initially found on regions corresponding to the end sequences of bacterial artificial chromosome (BAC) clones. Using 190 segregants from a backcross of a p50T female × an F1 (p50 T × C108T) male, we analyzed the segregation patterns of 534 SNPs between p50T and C108T detected among 3,840 PCR amplicons, each associated with a p50T BAC end-sequence. This enabled us to construct a linkage map composed of 534 SNP markers spanning 1,305 cM in total length distributed over the expected 28 linkage groups (Fig. 3). Among 534 BACs with end sequences harboring the SNPs used to construct the linkage map, 89 were associated with 107 different ESTs. Each of the SNP markers is directly linked to a specific genomic BAC clone and to whole genome sequence data. In addition, some are also linked to the EST data. Therefore, the SNP linkage map will be a powerful tool for investigating silkworm genome properties, mutation mapping, and map-based cloning of genes of industrial and agricultural interest.

**Single nucleotide polymorphisms in Toll-like receptor (TLR) genes revealed in pigs**

Opportunistic infections have become major problems for modern pork production. Diseases like pneumonia and diarrhea, which result from intensive breeding of swine, lead to extensive economic losses. The causative pathogens are resident organisms in pig farms and are difficult to eradicate. Hence, the genetic improvement of swine, based on resistance to pathogens may prove to be an effective control measure against these infections. Toll-like receptors (TLRs) play a crucial role in the recognition of pathogen-associated molecular patterns (PAMPs) derived from the pathogenic microbes. Polymorphisms in TLRs may influence their recognition of pathogen-derived molecules; swine TLRs are predicted to be associated with responses to infectious diseases such as pneumonia. We cloned and determined the complete sequence of porcine TLR genes (TLR1, TLR2, TLR4, TLR5 and TLR6) that are expressed on the cell surface and closely related to the microbial infection. We searched for single nucleotide polymorphisms (SNPs) in the coding sequences of porcine TLR1, TLR2, TLR4, TLR5, and TLR6 genes in 96 pigs from 11 breeds and elucidated 21, 11, 7, 13, and 11 SNPs, respectively, which caused amino acid substitutions in the respective TLRs (Fig. 4).

![Fig. 4](image_url)

**Heterozygosity of the SNPs in porcine TLR1 and TLR6 genes investigated in 96 individuals**

Synonymous and non-synonymous SNPs are indicated by closed and open circles, respectively. The green area in each gene corresponds to LRRs, which are important for pattern recognition of molecules, and the yellow area corresponds to TIR (Toll/IL-1 receptor) domains, which are necessary for intracellular signaling. Heterozygosity of the SNPs is calculated using the following formula:

$$\text{Heterozygosity} = 1 - (p_1^2 + p_2^2)$$

where $p_1$ and $p_2$ are the observed frequencies of the first and second alleles at each SNP locus, respectively. Biases in the heterozygosity between non-synonymous and synonymous SNPs are indicated by red lines and were calculated as the differences of the sums of non-synonymous SNPs ($S_{n}$) and synonymous SNPs ($S_{s}$) within every 100 bp window sliding 1 bp.
The distribution of these non-synonymous SNPs was biased; many were located in the leucine-rich repeats (LRRs), particularly in TLR 1. These data demonstrated that the heterogeneity of TLR genes has been preserved in various porcine breeds despite intensive breeding that was carried out for livestock improvement. This suggests that the heterogeneity in TLR genes is advantageous in increasing the possibility of survival in porcine populations.

**Optimization of expression profiling output from microarray analysis**

Much of the researches in the post-genome era will depend on high-throughput technologies. The Rice Genome Resource Center (RGRC, http://www.rgrc.dna.affrc.go.jp/) is providing access to microarray technology to allow a large number of investigators to apply global expression profiling into their specific research programs on rice. A rice oligonucleotide microarray with 22,000 non-redundant oligonucleotide probes based on the full-length cDNA sequence information was constructed in collaboration with Agilent Technologies (http://www.agilent.co.jp). This rice microarray system enables researchers to simultaneously characterize thousands of rice genes associated with biological functions, growth processes, and biotic and abiotic stress response. The open laboratory for microarray analysis is equipped with facilities for hybridization, scanning and data analysis. A 2-day protocol for microarray analysis was developed to allow researchers to conduct expression analysis of samples from hybridization to data analysis. Prior to hybridization, the RNA samples are checked for quality using the BioAnalyzer and concentration using the NanoDrop in order to assure that only high-quality RNA samples are used for linear amplification and labeling. Sample labeling using Cy3 and Cy5, and subsequent hybridization can be performed on the first day. Hybridization is carried out for 17 hours at 60°C. Subsequently, washing and data spot analysis using the Agilent Feature Extraction and Genespring analysis software can be performed on the second day. It is a highly reliable protocol eliminating most of the limiting factors that affect microarray expression analysis such as sensitivity of the quantity of RNA and background intensity. This suggests that hybridization of target genes can be performed with high reproducibility and enhanced sensitivity.

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**Fig. 5**

Optimization of conditions for microarray analysis facilitates analysis of very small amount of RNA derived from various tissues or cells therefore expanding the utility of gene expression profiling in functional genomics.
One of the main problems involving expression profiling is the high level of variability in microarray data. Although some of this variability is relevant because it corresponds to the differential expression of genes, a large portion of variability usually results from undesirable biases introduced during the many technical steps of the experimental procedure. The so-called experimental noise has been addressed to normalize data according to the related effects. Color swaps are now routinely included in microarray experimental designs to correct for labeling biases. The amount of samples for successful hybridization has also been investigated. Although it is normally suggested that about 400 ng of total RNA should be used for labelling, the amount of sample could be reduced to 10 ng of total RNA with almost similar expression intensity for highly and medium expressed genes. Reducing the amount of samples to 2 ng also gave a clear expression pattern. This indicates that the microarray could be used for analysis of small samples of RNA derived from tissues or organs, specific tissues isolated by laser dissection or transient assay in cell culture (Fig. 5).

The microarray open laboratory has been used by almost 247 research groups from different institutes and universities all over Japan since it became operational in August 1, 2003. Currently, an average of two users/groups per week use the facilities. In addition to the rice microarray, the RGRC open laboratory also supports microarray analysis in *Arabidopsis*, silkworm and cow.

**Genome Diversity Department**

The objectives of the Genetic Diversity Department are to conduct basic research into plant, animal and microorganism diversity from the molecular to the population level. Such research contributes to the development of new and improved methods for classification, characterization and preservation of germplasm and discovery of new biological resources for use in agriculture and other industries.

Current plant research includes molecular and biological characterization and evaluation of the genera Hordeum, Oryza, Saccharum, Triticum, Glycine and Vigna. Mechanisms in plants that confer cold hardness are also being investigated. Microbiological research includes functional genomics, biosystematics and proteomics of such organisms as fungi, yeasts and bacteria. In particular, plant pathogens have been focused on. Animal research includes the development of the methods to utilize various types of germplasm and to improve the genetic performance of domestic animals. Major research outputs of the department except for ‘Topics of Research’ during fiscal 2005 are described below.

**Very close relationship of the chloroplast genomes among Saccharum species**

We recently determined the complete sequence of the sugarcane chloroplast genome. Here, we have used the information for a comprehensive phylogenetic analysis of the genus *Saccharum*, using all six species (13 accessions). The polymorphisms between sugarcane and maize in 26 chloroplast genome regions were used for the analysis. In 18 of the 26 regions (a total of 5,381 bp), we found 41 mutations involving 17 substitutions, three inversions, six insertion/deletion mutations, and 15 simple sequence repeat length polymorphisms. Based on these results, we calculated a phylogenetic tree of the genus *Saccharum*, in which all six species are clearly separated (Fig. 1). By the analysis, (1) *S. sinense* and *S. barberi*, which have identical sequences, belong to the same clade, whereas the other four species, *S.*
officinarum, S. robustum, S. edule, and S. spontaneum, form an independent clade; (2) S. spontaneum has a paraphyletic relationship with the other five species; and (3) no or very low intraspecific variation was observed in S. officinarum, S. robustum, S. sinense, S. barberi, and S. edule, whereas higher intraspecific variation was observed in S. spontaneum.

Based on the number of nucleotide substitutions, the divergence time between S. officinarum and S. spontaneum, and between S. officinarum and maize were calculated to be about 730-780 thousand years ago and about 5.9 million years ago, respectively. These results suggest that the cytoplasm of Saccharum species are very closely related.

**Analysis of a large seeded mutant in black gram (Vigna mungo)**

The Asian Vigna consists of domesticated species among them is black gram. A mutant of this species has a seed size about double that of traditional cultivated black gram and six times the size of wild black gram (Fig. 2a). This mutant is also larger in other organs such as leaves (Fig. 2b). In order to determine the location of this mutation(s) on the black gram genome, a genome map was developed based on a cross between the mutant black gram and wild black gram. In a BC₁F₁ population of 180 individuals from this cross 148 marker loci could be assigned to the 11 linkage groups that corresponds to the haploid chromosome number of black gram. This is the first genetic linkage map for black gram and it was compared with that of a close relative, azuki bean. Inversions, insertions, deletions / duplications and a translocation were detected between the black gram and azuki bean linkage maps. Domestication trait QTL, including seed size, locations have been identified on the genome map of black gram.

**Identification and mapping of QTL for rachis internode length associated with cleistogamy in barley**

General knowledge of the closed flowering trait, or cleistogamy, of barley, Hordeum vulgare L., is still limited. We studied the relationship between cleistogamy and characters of spike morphology and detected a linkage of...
cleistogamy genes with a highly significant quantitative trait locus (QTL) for rachis internode length on the long arm of chromosome 2H (Fig. 3). The mapping populations consisted of 129 doubled haploid lines of ‘Mikamo Golden’ x ‘Harrington’ and 150 F2 plants of ‘Misato Golden’ x ‘Satsuki Nijo’. The genetic distance between markers is given in cM. Arrows represent positions; shaded bars represent 90% confidence interval of QTL 2H.

The peaks of the QTL coincided with the positions of the cleistogamy gene loci.

**Development of GCP domain model**

The Generation Challenge Programme (GCP) aims to assist farmers in the developing world by using advances in molecular biology to harness the rich global heritage of plant genetic resources and create a new generation of crops that meet the needs of resource-poor people. Crop research within the GCP has many interrelated data types, focused on germplasm. The scope of the GCP scientific domain model is to cover this range of data types spanning germplasm, phenotype, genotype, mapping, functional genomics and location environment data. NIAS has a role to develop the functional genomics sub-domain model. Software implementations of the GCP domain model will form the middleware of the GCP platform and network that will link and user tools and interfaces to local and remote (web service connected) data source (Fig. 4). The model is documented in Unified Modelling Language (UML). Computable versions of the UML model are published in the “Demeter” package of the GCP Middleware project in CropForge (http://cropforge.irri.org/projects/gcpmiddleware/). The alpha release of the domain model is also published to http://www.generationcp.org/model.

![Fig. 3](image1.png)  
Mapping of QTL for rachis internode length (Qtl2H) on the long arm of chromosome 2H of barley  
A: Genetic map of 126 DH lines of ‘Mikamo Golden’ x ‘Harrington’; B: Genetic map of 150 F2 plants of ‘Misato Golden’ x ‘Satsuki Nijo’. The genetic distance between markers is given in cM. Arrows represent positions; shaded bars represent 90% confidence interval of Qtl2H.

![Fig. 4](image2.png)  
Relationship of GCP domain model to platform
Taxonomic analysis of *Fusarium* species, as the causal pathogens of soybean sudden death syndrome and dry bean root-rot

The etiological agents of soybean sudden death syndrome (SDS) have been reported as *F. solani* or its forma specialis, f. sp. *glycines*. On the other hand, the causal pathogens of dry bean or mung bean root-rot have been known as *F. solani* f. sp. *phaseoli*. Soybean SDS pathogens isolated from the US, Argentina and Brazil, dry bean root-rot pathogens from the US and Japan, and mung bean root-rot pathogen from Canada were investigated. Detailed phenotypic comparisons of macro- and microscopic features, and phylogenetic analyses of multilocus DNA sequence data indicated that the soybean SDS and dry bean root-rot pathogens comprised six morphologically and phylogenetically distinct species (Fig. 5). Soybean SDS in North and South America (US, Argentina and Brazil) was found to be caused by four species: *Fusarium virguliforme*, *F. tucumaniae*, *F. brasiiliense* and an undescribed species of *Fusarium*. By way of contrast, dry or mung bean root-rot in North America (US and Canada) and Japan is caused by two closely related species, *F. phaseoli* and *F. cuneirostrum*. The SDS species do not form an exclusive group within the molecular phylogeny, indicating they may not have a monophyletic origin. Artificial inoculation tests on a susceptible variety of soybean, using the isolates from soybeans, dry beans and mung beans, revealed that all six species can induce typical SDS symptoms on the inoculated soybean plants.

Genome-wide gene expression analysis of *Xanthomonas oryzae* pv. *oryzae*

To survey genes regulated by HrpG or HrpX, we constructed a DNA macroarray system consisting of 2,384 of genomic DNA fragments of strain T7174 (MAFF311018) of *Xoo*. It comprised about 95.5% of the whole genome DNA. Using this macroarray system, it was confirmed that the *hrg* gene cluster (about 87k to 120k region of the Xoo genome) was up regulated in wild type strain under *hrg*-inducing medium, while it was not up regulated in the ∆*hrgX* and ∆*hrgG* mutants (Fig. 6). Moreover, it was revealed that thirteen genomic regions were regulated by HrpG or HrpX. To check that expression of genes within these regions were really controlled by the HrpG or HrpX, a real-time quantitative RT-PCR system was used. Finally, six genes (XOO0037, XOO0078, XOO1388, XOO2263, XOO4042 and XOO4134) were newly identified. In addition,
two genes, XOO2263 and XOO4134 contained a plant-inducible promoter (PIP) box, which was a consensus sequence of HrpX regulons, upstream region of putative initiation codon. These obtained results showed that this macroarray system was useful tool for genome-wide gene expression analysis in Xoo.

**Kluyveromycetes lactis** killer protein entry to sensitive yeast cell mediated by plasma membrane sphingolipid

Although a number of microorganisms surround us, very few succeed in causing infection. Recently, innate immune system in eukaryotes, through the action of antimicrobial peptides and proteins, is recognized as playing an important role in survival and defense. A killer strain of the yeast *Kluyveromycetes lactis* secretes a protein toxin (zymocin) that prevents the proliferation of *Saccharomyces cerevisiae* (baker yeast) cells. We analyzed one of the mutants, kti6, which resists exogenous zymocin but is sensitive to intracellularly expressed toxic subunit of zymocin (Fig.7). KTI6 is allelic to IPT1, and codes for mannosyl-diinositolphospho-ceramide [M(IP)2C] synthase, the enzyme which catalyzes the reaction to produce M(IP)2C, the major plasma membrane sphingolipid. Mutants defective in the synthesis of M(IP)2C intermediates were also found resistant to zymocin. In addition, kti6/ipt1 cells prevented the import of toxic subunit of zymocin. In summary, our results indicates that M(IP)2C plays a role in the accumulation of zymocin in target cells. Interestingly, actions of several antifungal proteins, isolated from plants, are also reported as mediated by sphingolipid. Thus the structure of sphingolipid in the target cell is a key of proteinaceous toxin spectrum.

**Sex chromosome inactivation in male FKBP6 deficient animals**

FK506 binding protein 6 (FKBP6) is a component of the synaptonemal complex (SC). In the male mice and rats, lack of this protein defects chromosome pairing, resulting in meiotic arrest in spermatogenesis. The female deficient animals, however, enable to complete meiosis and shows normal fertility, although the protein has been detected in the SC. The discrepancy brought us an idea that FKBP6 might have a role in inactivation of XY chromosomes at pachytene stage, which is a male specific meiotic event. Therefore we examined co-localization of phosphorylated histone H2AX (γH2AX) with paired sex chromosomes, as a marker of inactivation of XY.

Germ cells obtained from *Fkbp6* +/-(phenotypic normal) and -/- male (meiotic arrest) mice and rats were processed by surface spread method. Nuclei spread on slide glass were reacted with anti SCP3 (for chromosome staining) and γH2AX antibodies, followed with fluorescent labeled 2nd antibodies, then examined under the razor scanning microscope (Fig. 8). In *Fkbp6* +/− mice, most

![Fig. 7](image)

Response to zymocin of heterozygous diploids (kti6/ EUROSCARF gene disruptants) kti6Δipt1 was resistant to zymocin.

![Fig. 8](image)

Pachytene nuclei of Fkbp6 +/- (upper panels) and +/- (lower panels) mice SCP3 (red) and γH2AX (green) staining. Nucleus of +/- showed localization of γH2AX in the restricted region surrounding XY pair. In the +/- nucleus γH2AX diffusively localized to the region surrounding abnormally paired chromosomes including XY (arrows).
pachytene nuclei (96.3%) displayed γH2AX co-localizing to XY pair. In Fkhbp6 -/- mice, 71.9% of pachytene nuclei showed restricted staining of surrounding paired XY. Other nuclei showed diffused γH2AX staining possibly caused by non-homologous pairing of XY. The Fkhbp6 deficient rats showed similar tendency to mice. The results evidenced that sex chromosome inactivation could occur, unless XY paired normally, in FKBP6 deficiency.

Effects of data structures on the solving of animal model equations by preconditioned conjugate gradient iteration

To predict breeding values in dairy cattle, preconditioned conjugate gradient (PCG) algorithm has become of interest lately as a means of solving large sets of mixed model equations (MME). Thereby, the effects of data structure on the solving of MME by PCG algorithms were investigated by using data in a computer simulation. A large number of animals did not cause slow convergence if the data structure (e.g. number of generations and mating ratio) was fixed. The numbers of breeding animals and their progeny affected the structure of the A-inverse matrix (A: additive genetic relationship matrix), but selection methods did not. An increasing number of iterations seemed to be due to an imbalance of nonzero elements in the A-inverse matrix, which resulted in the imbalance of nonzero elements in the MME. The number of iterations until convergence was roughly constant for two-trait animal model when the heritability of a trait was constant regardless of the heritability of another trait, and it was the smallest when the heritabilities of both traits were about 0.5 to 0.6 (Fig. 9). Increasing absolute values of genetic and environmental correlations resulted in slow convergence. In particular, genetic correlation affected convergence strongly.

![Fig. 9](image)

Number of iterations until convergence for different heritabilities (a) and different genetic and environmental correlations (b)
Genebank

Genebank Project

The NIAS Genebank Project consisting of plant, microorganism, animal, and DNA sections has been implemented in national and international collaboration with a large number of organizations in public and private sectors. The Genebank, NIAS coordinates the Project as a center of the project activities including the exploration, collection, characterization, evaluation, preservation, multiplication, and information management of genetic resources (GRs).

In the fiscal year 2005, international collaborative field studies were carried out in cooperation with relevant research institutes in the partner countries. The Project dispatched scientists to explore tartary buckwheat GRs in Sakhalin, wild rice and vegetables GRs in Myanmar, and yam GRs in Vietnam, to do feasibility study on apples, pears, and stone fruits GRs in Xinjiang Uygur Autonomous Region of China, to survey wild rice and wild and cultivated legumes GRs in Papua New Guinea, to collect Exobasidium GRs in Taiwan and Malaysia. The Perilla GRs collected in the collaborative field study program in Korea were studied using AFLP analysis for possible and effective conservation on farm. Five exploration missions were organized by the Project to explore different GRs inside Japan: pear GRs in Aomori, Akita, and Iwate prefectures, plum and pear GRs in Yamagata prefecture, Vaccinium GRs in Hokkaido prefecture and Yakushima Island of Kagoshima prefecture, Cruciferous vegetables in Kagoshima and Miyazaki prefectures, and tea GRs in Shimane prefecture.

Additionally, several sub-banks did explorations by their own efforts, from which collected materials are also registered in the NIAS Genebank system. The total numbers of GRs preserved at the NIAS Genebank and partner institutions are 235,946 accessions of plant GRs, 22,231 accessions of microorganism GRs, and 878 accessions of animal GRs as of December 31, 2005. In FY2005, 4524 accessions of plant GRs, 799 accessions of microorganism GRs and 28 accessions of animal GRs were distributed to internal and external users for basic and applied researches. Passport and characteristics data of freely distributable accessions are uploaded on the website (http://www.gene.affrc.go.jp/).

International Genetic Resources Workshop was held on August 22nd and 23rd, 2005 at Tsukuba on two topics, “Rice Genome: Challenges and Opportunities” and “Global and Regional Strategies for Conservation and Sustainable Use of Plant Genetic Resources”. It was held jointly by the SABRAO and the NIAS as a part of the 10th International Congress of the Society of Breeding Researches in Asia and Oceania (SABRAO).

Discovery of a Chromosomal Region Associated with Root Structure Using a Set of Representative Cultivars Derived from Rice Germplasm Collection

Asian cultivated rice (Oryza sativa L.) holds diversified root systems depending on different water-stress conditions such as rainfed-lowland and upland. Although root traits should play a key role in drought avoidance, limited analyses of QTL loci on a few traits such as length and thickness of root have been reported. We did microscopic observation and comparative analysis of root structures in the Rice Diversity Research Set (RDRS) of rice germplasm developed in the NIAS Genebank. Nodal roots of the main stem were collected from rice plants grown in upland condition for six weeks of summer season, 2004 at Tsukuba. We took
digital images of stained cross-sections using digital microscope and measured root area, stele area, area and number of xylem vessel. Association between alleles at 147 RFLP loci and phenotypic data obtained from 61 varieties (47 of Indica type and 14 of Japonica type) were calculated. We discovered one region on chromosome 4 associated with the stele/root area ratio.

Microscopic observation showed the similarity of frequency distribution for root area and number of xylem vessels between Indica type and Japonica type cultivars. Japonica rice was more variable than Indica rice in root traits such as areas of stele and xylem vessels, xylem vessel area/no. ratio, and stele/root area ratio, and 14 Japonica varieties were divided into two groups corresponding to small (n=9) and large (n=5) stele/root area ratios (Fig. 1). We analyzed genotype frequency at RFLP loci between two groups divided based on variation in stele/root area ratio and found one marker (orange box) associated with stele/root area ratio in long arm of chromosome 4 (Fig. 2). This region is suggested to harbor a gene or genes responsible for size factors of root organs.

**Development of Cryopreservation Protocol for Long-term Storage of Plant Germplasm**

Cryopreservation is becoming a very important tool for long-term storage of plant germplasm, especially vegetatively propagated crops and recalcitrant seed species, with a minimum of space and maintenance. Cryopreservation should be considered as a backup to the collections to insure against loss. In this, we mean the priority of collections to be cryopreserved should be given to the “at risk” crops that have an increased chance of being lost from a collection. In our genebank, the *Morus* germplasm collection at Tsukuba, which include about 1,400 accessions with several species, are processed for long-term preservation. To date, about 750 accessions of them have been cryopreserved in liquid nitrogen tanks.
using dormant winter buds. Also, we started three cryopreservation projects such as practical storage of \textit{in vitro}-grown potato shoots, development of cryopreservation protocol of mat rush and sugarcane. In these, practical cryopreservation method of \textit{in vitro}-grown potato shoot tips by encapsulation-vitrification was improved. Mass propagation system of \textit{in vitro}-grown mat rush shoots was established using induction of multiple shoots.

\textbf{Morphological, molecular and phytopathological variations of \textit{Plectosporium tabacinum}}

\textit{Plectosporium tabacinum} (van Beyma) M.E. Palm, W. Gams & Nirenberg, which was validly described in 1995 (Palm et al. 1995), had been known as a common soil fungus at rhizospheres, and was isolated from lake sediment for the first time in Japan (Tubaki and Ito, 1975). We identified several fungal isolates pathogenic to plants, \textit{i.e.} pumpkin, garden ranunculus, anemone, sweet pepper, lotus ginger and Japanese radish (Fig. 3), as \textit{P. tabacinum}, and found that it had been differentiated morphologically, molecularly and phytopathologically. The isolates used are shown in Table 1. Their morphological characters are as follows (Fig. 4). Colonies in culture on potato dextrose agar (PDA) at 20°C in the dark are flat with little aerial mycelium, smooth and cream to salmon or pale brown in color. Conidiophores are unbranched or occasionally branched, with conidiogenous cells often arising at right angles from vegetative hyphae. Conidiogenous cells are monophialides or rarely polyphialides formed at the apices, or as short (adelophialides) or long branches from vegetative hyphae, hyaline, smooth, cylindrical to obclavate, sometimes crooked or sinuous at the tips, often with single conidiogenous apertures, and occasionally with second apertures. Conidia are produced in colorless slime masses at the tips of the phialides, hyaline, smooth, oblong-ellipsoidal, usually asymmetrical to slightly curved, multiguttulate and most are 1-septate and with a few aseptate.

Growth speed of mycelia and appearance rates of aseptate conidia on PDA at 20°C in the dark varied from 2.7-4.7 mm/day and 0-28.6%, respectively, in 6-8 isolates. Sizes of septate and aseptate conidia that had formed on synthetic low nutrient agar (SNA) at 20°C in the dark ranged from 4.0-12.0×1.0-5.5 µm and 2.5-8.5×1.0-3.5 µm, respectively, depending on the isolates. The 10 isolates were classified into 2 groups based on their sequence data of rDNA ITS.
regions, though their homologies were highest (>94%) with those of *P. tabacinum* registered in the DNA Data Bank of Japan (Fig. 5). When pumpkin, garden ranunculus and lotus ginger were inoculated with the isolates, the respective isolates were virulent only to the original host plants, whereas the others caused no disease in the plants tested (Table 1). There is the possibility that *P. tabacinum* containing various strains morphologically and molecularly has been also differentiating strains with high host-specificity.

Fig. 5
Grouping of *P. tabacinum* isolates based on neighbor-joining analysis for sequence of rDNA ITS regions ITS1F (5'-CTTGGTCATTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used as primers for PCR. Numbers above branches are bootstrap values (%).

Table 1 Isolates used and their pathogenicity to pumpkin, garden ranunculus and lotus ginger.

<table>
<thead>
<tr>
<th>Collection locality</th>
<th>Isolation source</th>
<th>Fig. 1</th>
<th>Pathogenicity to pumpkin</th>
<th>garden ranunculus</th>
<th>lotus ginger</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kagoshima, Japan</td>
<td>pumpkin [Cucurbita sp.]</td>
<td>A</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ibaraki, Japan</td>
<td>pumpkin [Cucurbita sp.]</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Kagawa, Japan</td>
<td>garden ranunculus [Ranunculus asiaticus]</td>
<td>B</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Chiba, Japan</td>
<td>garden ranunculus [Ranunculus asiaticus]</td>
<td>C</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Chiba, Japan</td>
<td>anemone [Anemone coronaria]</td>
<td>D</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ehime, Japan</td>
<td>sweet pepper [Capsicum annuum var. grossum]</td>
<td>E</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tokyo, Japan</td>
<td>lotus ginger [Crucuma alismatfolia]</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tokyo, Japan</td>
<td>lotus ginger [Crucuma alismatfolia]</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tokyo, Japan</td>
<td>lotus ginger [Crucuma alismatfolia]</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Chiba, Japan</td>
<td>lotus ginger [Crucuma alismatfolia]</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Miyazaki, Japan</td>
<td>radish [Raphanus sativus]</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ezypt, Japan</td>
<td>sweet violet [Viola odorata]</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hyogo, Japan</td>
<td>lake sediment</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

+: Virulent. −: Virulence was not detected.
The Insect and Animal Sciences Division consists of the following six departments.

The Developmental Biology Department is conducting research to elucidate the physiological functions of germ cell lines and the hormonal mechanism underlying growth regulation, and to develop embryonic technologies as well. These researches focus on several invertebrates and vertebrates.

The Molecular Biology and Immunology Department is concerned with the molecular events involved in the signaling for antibacterial peptide synthesis in insects and signaling of cytokines and T cell receptors in mammals, the functional analysis of macrophage/microglia, hematopoietic stem cells and T cell subsets, the genetic mechanisms underlying complex disease traits, and the development and improvement of transgenic animal technology. Major research on cytokine signaling in the mammalian immune system, innate immune system in insect, the transformation of spermatogenic cells in vivo and genetic analysis of multi-factorial are being conducted.

The Physiology and Genetic Regulation Department aims to elucidate the mechanisms of unique physiological phenomena observed in insects and animals. The studies include analyses of neural functions including brain, metabolic regulation, and physiological bases for adaptation to environment, perception and function of behavior regulating chemicals, cell differentiation and the genes regulating them, in both invertebrates and vertebrates.

The research activities of the Insect Genetics and Evolution Department are mainly focused on molecular and conventional genetics of insects including silkworm, biochemical analyses of insect-plant interaction, characterization and breeding of natural enemies, and basic and applied studies on insect-associated microbes including pathogens and symbiotes.

The Insect Biomaterial and Technology Department is carrying out research which aims to develop the biomimetic techniques of insects and their utilization such as the sugar reception of the flesh fly, and to clarify the characteristics of insect-born biomaterials for the development of new technologies.

The major targets of the Insect Biotechnology and Sericology Department are the development of functional insect cell lines and transgenic insects, large-scale production of useful substances using the transgenic insect systems, and the development of new silk materials which are good for healthful and wealthy human life by developing new sericultural technologies including breeding of characteristic new varieties of silkworms.

The major research topics for 2005 from these six departments in the Insect and Animal Sciences Division are as follows:
Expression of a nanos homologue in Bombyx eggs

The silkworm Bombyx mori has no recognizable germ plasm, a morphologically distinguishable egg cytoplasm that contains germ cell determinant(s), and its germ cells appear first on the ventral side of the embryo, not on the posterior pole as in Drosophila melanogaster. During the search of germ cell specific genes in Bombyx, we found that transcripts of a nanos homologue were dispersed along the ventral midline of the egg, the region where germ cell formation occurs in the future (Fig. 1a). In later stages of embryogenesis, the transcript was observed specifically in the germ cells (Fig. 1b). The localization of the nanos transcripts may be interpreted as the presence of Bombyx germ plasm in the ventral midline of the egg. However, its composition and localization mechanisms seem to be different from those of Drosophila, suggesting that Bombyx has developed a specific mechanism(s) of germ cell formation during the evolution.

Application of the tetracyclin controlled binary gene expression system (tTA/TRE system) to the sawfly, Athalia rosae

One of the powerful tools to analyze gene function in non-model organisms is the technique to regulate gene expression. We demonstrated that a transgenesis-based binary gene expression system worked in Athalia rosae (Fig. 2). Embryos bearing both tetracycline-controlled transactivator (tTA) and a marker EGFP gene driven by the tTA response element (TRE-EGFP) expressed EGFP, while the embryos bearing either the tTA or the TRE-EGFP did not express EGFP. The EGFP expression in embryos bearing both tTA and TRE-EGFP was suppressed when tetracycline was orally applied to the parents at a concentration of 100 µg/ml.

![Fig. 1](image1.png)
Expression pattern of a Bombyx nanos homologue during the early development
(a) An egg at cleavage stage. Ventral view. Anterior is to the left. (b) An embryo after blastokinesis. The nanos transcript is specifically observed in the gonad (arrow).

![Fig. 2](image2.png)
The tTA/TRE binary expression system works in the sawfly, Athalia rosae
The embryo bearing tTA/TRE -EGFP expresses the marker EGFP (left panel, middle embryo). The expression was suppressed by application of tetracycline (right panel, middle embryo).
Expression patterns of two novel genes isolated from regenerating individuals of an oligochaete annelid, *Enchytraeus japonensis*

The spatiotemporal expression profiles of two novel genes, *Ejrup1* and *Ejrup3*, which were isolated from regenerating *Enchytraeus japonensis* were examined by RT-PCR and whole mount *in situ* hybridization (WISH). For both genes, the expression was scarcely detectable in intact worms, but was dramatically activated following amputation. The expression levels were highest at the blastema formation stage (around 12-24 hours after amputation). The results suggest that these genes play important roles in annelid regeneration (Fig. 3).

Ppet genes have a function on differentiation / proliferation of ES cells and also early development in the mouse

We have previously reported that mouse ES cells were divided into three subpopulations according to the expression levels of platelet endothelial cell adhesion molecule 1 (PECAM-1) and stage-specific embryonic antigen (SSEA)-1. Quantitative RT-PCR and chimera formation revealed that the expression level of PECAM-1 and SSEA-1 were positively correlated with pluripotency of ES cell subpopulations. In order to identify novel regulatory factors in ES cell differentiation, we have performed comparison of gene expression profiles by oligo-DNA array analysis between three subpopulations. In this fiscal year, we focused on uncharacterized 23 genes (Ppet: PECAM-1 Positive ES cell-derived Transcripts) and attempt to elucidate the functions of them in ES cells and early embryos by small interfering RNA (siRNA) mediated gene knockdown.

When fluorescein-labeled Ppet siRNAs were transfected into ES cells, plating efficiency of these ES cells showed no significant difference between fluorescein negative and positive cells in all groups. Alkaline phosphatase (AL-P, a marker for undifferentiated ES cells) positive colonies were decreased by knockdown of Ppet 002, 005, 010, 015, 021 and 023 gene as well as Oct3/4 (Fig. 4). In contrast, AL-P positive colonies were increased by knockdown of Ppet 001, 003, 004 and 019 genes. Next, we performed knockdown of Ppet gene in early mouse embryos. The embryos that injected Ppet015, 019 and 021 siRNA exhibited developmental retardation and degradation. In cases of Ppet 019 and 021 gene knockdown, total cell numbers of blastocysts were reduced without morphological abnormality. Conversely, Ppet 010, 022 and 023 gene knockdown caused developmental facilitation. In cases of Ppet 022
and 023 gene total cell numbers of blastocyst were increased without morphological abnormality. This study showed that some Ppet genes probably play an important role in the specific mechanism of differentiation / proliferation of ES cells and early embryo.

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

**Fusion of primordial germ cell with embryonic blood cell in chickens**

Manipulation of primordial germ cells (PGCs) provides a useful method for nuclear transfer in chickens. The system for producing germline chimaeric chickens by the transfer of PGCs has already been established. If a nucleus of PGC can be replaced by a somatic cell nucleus, the nuclear transferred PGC could give rise to viable offspring via germine chimaeric chicken after being transferred to recipient embryo. Enucleation of PGCs could be done by UV irradiation or some other methods, and somatic cell nuclear transfer could be achieved by fusing the enucleated PGC with a somatic cell. Through these manipulations, it is expected that the nuclear transferred PGCs are produced and the manipulated PGCs migrate to the germinal ridges after being transferred to the bloodstream of recipient embryos and successfully differentiate into germ cells in the gonads of the chimaeric chickens. By mating these chimaeric chickens, somatic cell-derived offspring are expected to be produced. In order to apply this strategy for nuclear transfer in chickens, the techniques for each step involved in the nuclear transfer in PGCs should be developed. The present study was conducted to produce nuclear transferred PGCs, and here we report the preliminary results of fusing PGC with embryonic blood cell (EBC) using inactivated Sendai virus (Hemagglutinating Virus of Japan; HVJ) or electrical stimulation. Fused cells of PGC and EBC were produced using inactivated HVJ as shown in Fig. 5 (A-I). PGC-EBC fused cells were identified by their morphology and the difference in size of the two nuclei after staining with Hoechst 33342. PGC has a large nucleus while EBC has a relatively small nucleus. The fused cell has thus both large and small nuclei. The fused cells were PGC-EBC, PGC-PGC, and EBC-EBC. More than three cells were occasionally fused. The
mean fusion rate of PGC with EBC using inactivated HVJ was 0.96%. When cell fusion was carried out using electrical stimulation, pearl chains were formed within 20 seconds by exposing cells to the AC field. After applying DC pulses, adjacent cells in a pearl chain were fused in some places. Fused cells of more than three cells were also occasionally observed. The fused cells of PGC and EBC were shown in Fig. 5 (J and K). The mean fusion rate of PGC with EBC using electrical stimulation was 5.2%. The system for producing viable offspring derived from nuclear transferred PGCs makes it possible to manipulate the germline of chickens through somatic cells.

**Lack of αGal epitope in transgenic cloned pigs by expression of EndoGalC**

Because organs of pigs are biologically and anatomically similar to human organs, porcine organs are expected to use as replacement for human organs, which are chronically in short supply. The major problem with xenotransplantation of porcine organs into humans is the hyperacute rejection caused by the reactions involving natural human antibodies and the complement system. It is considered that natural antibodies against αGal epitope on pig cells are the main cause of hyperacute rejection. Recently, the overseas companies succeeded to produce α1,3GT knocked out pigs by somatic cell cloning for elimination of αGal epitope.

The endo-β-galactosidase C (EndoGalC) secreted from *Clostridium perfringens* cleaved αGal epitope from pig cells under physiological pH conditions. In addition, transfection with EndoGalC gene effectively reduced αGal expression in cultured porcine aortic endothelial cells. Thus, the cleavage of αGal epitope by EndoGalC would be the alternative to knock out α1,3GT gene for elimination of αGal epitope in whole pigs.

The EndoGalC expression vector was transfected in Meishan fetal fibroblasts by electroporation. After selection under G418 for 10-14 days, the cells lacking αGal epitope were separated and collected by FACS (fluorescent-activated cell sorting). The separated cells were cultured and expanded sufficient for nuclear transfer to produce live piglets. 1144 of nuclear transferred embryos at 2-8 cell stage were transferred to 7 synchronized recipient pigs. Two recipients were pregnant but one...
recipient aborted two fetuses on day 61 and the other recipient delivered one live piglet (Fig. 6). Genomic PCR of DNA extracted from the piglet and the aborted fetuses confirmed the presence of EndoGalC gene. The FACS analysis showed that the levels of elimination of αGal epitope in the fibroblasts collected from the piglet and the aborted two fetuses were 98.3%, 99.0% and 99.2% respectively. The cloned piglet had no physical abnormalities and reached normal maturity.

These results clearly showed that the combination of FACS with nuclear transfer has much superiority to produce transgenic pigs like our previous success of cloned pig with highly expression of human decay accelerating factor (hDAF). Moreover, we are planning to produce transgenic pigs with both hDAF and EndoGalC expression by mating with each transgenic cloned pig for xenotransplantation.

Regulation of Silkworm Larval Development

The regulatory mechanisms on insect development, such as ecdysis and metamorphosis are being studied on the silkworm at Insect Growth Regulation Laboratory. Recent progresses are as follows: (1) A protein of cultured cells derived from silkworm ovary, which was phosphorylated by methoprene, a juvenile homone (JH) analog, was MAP kinase, and it was also observed that the MAP kinase

from follicle cells of the silkworm at late pupal state with high reactivity with JH was phosphohylated by incubation with methoprene for 5 min. (2) New prothoracicostatic factors, Bombyx FMRFamide (BRFa) were identified in Bombyx mori. BRFa is predominantly expressed in neurosecretory cells of thoracic ganglia, and the neurons in prothoracic ganglia innervate the prothoracic glands to supply the peptides to the gland surface (Fig. 7). These results suggest that BRFa is controlling the activity of prothoracic gland by direct innervation. (3) We analyzed transactivation abilities of three isoforms of a metamorphosis-specific transcriptional factor Broad-complex (BR-C) isolated from Bombyx mori by luciferase gene assay using a Bombyx cell line. BR-C Z2 isoform that processes zinc finger DNA binding motives induced luciferase expression via either of three metamorphosis-specific gene promoters, but BR-C NZ1 and NZ4 isoforms without zinc finger motif did not, suggesting that BR-C isoforms with zinc finger motives play an important role for induction of multiple metamorphosis-specific genes. (4) We cultured pieces of larval integument in MGM-450 medium with 10 % fetal bovine serum (FBS). Reduced form glutathione was added in the medium to inhibit melanization of medium and integument. The pieces of integument were put on the supports of polyester membrane in order to expose air on surface of cuticle. At 48 hours after initiation of the culture we observed malformed cells at surround of the wound, but recognized only a few melanizing or damaged

Fig. 6
Cloned transgenic pig expressing EndoGalC for elimination of αGal epitope

Fig. 7
BRFa-immunoreactive axons on the surface of prothoracic glands
cells. The epidermal cells maintained apparently cell growth activity on the culture condition for the immunofluorescent staining of incorporated bromodeoxyuridine (BrdU). These observations suggested that the integument culture was available method for study of growth and development on epidermal cells.

**Identification of new molecules in bovine placenta: Prolactin-related proteins and BCL2A1**

Prolactin-related proteins (PRPs) are specific proteins of the growth hormone/prolactin (GH/PRL) family in bovine placenta. A full-length cDNA for two new members of bovine PRPs, bPRP-VIII and -IX were identified, and their placental localization and quantitative expression were examined.

New bPRP-VIII and -IX were identified from bovine placentome. Localization and quantitative gene expression in the placenta were respectively investigated by *in situ* hybridization and real-time RT-PCR methods. Recombinant proteins of these genes were produced by a mammalian HEK293 cell expression system. Full-length bPRP-VIII and -IX cDNA were respectively cloned with 909 and 910 nucleotide open-reading-frames corresponding to proteins of 236 and 238 amino acids. The predicted bPRP-VIII amino acid sequence shared about 40 to 70% homology with other bPRPs, and bPRP-IX had about 50 to 80% homology of others. The two new bPRPs were detected only in the placenta by RT-PCR. mRNA was primarily expressed in the cotyledon and intercotyledonary tissues throughout gestation. An *in situ* hybridization analysis revealed the presence of bPRP-VIII and -IX mRNA in the trophoblastic binucleate and/or trinucleate cells. bPRP-VIII mRNA was observed in the extra-embryonic membrane on Day 27 of gestation, however, no bPRP-IX mRNA was observed in the extra-embryonic membrane in the same stage of pregnancy by quantitative real-time RT-PCR analysis. Both new bPRP genes were possible to translate a mature protein in a mammalian cell expression system with approximately 28 kDa in bPRP-VIII and 38 kDa in bPRP-IX.

Their different temporal and spatial expressions suggest a different role for these genes in bovine placenta during gestation.

A full-length cDNA for the bovine BCL2 antiapoptotic family member, BCL2-related protein A1 (BCL2A1) was identified. Spaciotemporal expression profiles of BCL2A1 suggested the implication to trophoblast cell proliferation and differentiation during pregnancy. We cloned a full-length bovine BCL2A1 cDNA with 725 nucleotides and an open-reading frame corresponding to a protein of 175 amino acids. The predicted amino acid sequence shared 78% homology with human BCL2A1. All BCL2 homology domains (BH1, BH2, BH3, and BH4) in bovine BCL2A1 were conserved as in other mammalian BCL2A1. In

![Fig. 8](image)
*In situ* hybridization for BCL2A1, BAX, CASP3 and PL in bovine placenta
the placentomes, in situ hybridization revealed that the BCL2A1 expression was localized in binucleate cells expressing various pregnancy-specific molecules like placental lactogen. BCL2-associated X protein (BAX) was also expressed in binucleate cells (Fig. 8). Quantitative real-time RT-PCR detection showed a high-level expression of BCL2A1 in the conceptus at Day 21 of gestation, and it was expressed and increased in the extra-embryonic membrane, cotyledon, and intercotyledon from implantation to term. BAX expression intensity increased with progression of gestation and remained elevated in postpartum. Caspase-3 protein (CASP3) and mRNA (CASP3) were detected from late gestation to postpartum in placenta as well as in the results of TUNEL detection. It is likely that apoptosis of binucleate cells is regulated by the balance of the BCL2A1 and BAX. This molecule is a new candidate for anti-apoptotic maintenance of the binucleate cells that support placental functions throughout gestation in bovine.

Molecular Biology and Immunology Department

Production and characterization of allospecific anti-bovine CD34 monoclonal antibody

CD34 is a cell-surface glycoprotein that is specifically produced in hematopoietic and nonhematopoietic stem/progenitor cells. In order to facilitate the study on bovine hematopoiesis, we produced a monoclonal antibody (mAb) against bovine CD34 (boCD34) designated as N21 by using mouse cells producing recombinant boCD34 as antigens. The mAb N21 stained relatively high percentages (23% in average) of bone marrow mononuclear cells (BMMNCs) from 4 neonatal Holstein calves, but did not stain BMMNCs from the other 6 calves. Cell sorting experiments showed that hematopoietic progenitor cells such as colony-forming unit-granulocyte macrophage (GM-CFU) and burst-forming unit-erythroid (BFU-E) (Fig. 1) were enriched in N 21+ (thus boCD34+) cell fraction, suggesting that boCD34 was produced specifically in hematopoietic progenitor cells. To examine the cause of the difference in reactivity to mAb N21 among the calves, the sequence in the coding region for boCD34 of each calf was determined. As a result, 4 single-nucleotide polymorphisms within the coding region were found; 3 of them lead to amino-acid changes, S159F, W261R, and A276V, respectively. The CD34 mutants each of which represented one of the observed alleles were produced in HeLa cells by DNA transfection, and the reactivity of mAb N21 to these mutants was assessed; mAb N21 reacted to W261 allele, but not to R261 allele. This assignment of immunodominant amino-acid residue well explained the correlation between genotype and reactivity to mAb N21 of the calves examined. Taken together, mAb N21 was shown to recognize boCD34 in an allospecific manner, discriminating a single amino-acid change. This mAb would not only facilitate the identification and characterization of bovine hematopoietic progenitor cells, but be used as an allelic cell-surface marker in allogeneic transplantation studies using the cattle.

Fig. 1
A bovine BFU-E (day 12) grown in methylcellulose Colony was unstained. (magnification 40 x)
Animal and cell culture models for the study of mammalian immune system

Microglia have critical roles in development, homeostasis and pathogenesis in the brain. As immune surveillant cells, microglia respond to various extracellular signals such as neuronal injury or infection, and secrete several proteases, neurotrophic factors and various cytokines. Wiskott-Aldrich syndrome protein (WASP), the gene product responsible for the Wiskott-Aldrich immunodeficiency syndrome, acts as an important adaptor molecule in T cell receptor signaling. However, little attention was paid on the roles of WASP in microglial activation. So, we have established a microglial cell line from WASP-dominant negative transgenic mice. These microglial cells produced lower levels of inflammatory cytokines, such as tumor necrosis factor alpha and interleukin 6 than the wild-type microglia, after stimulation with bacterial lipopolysaccharide (LPS). Furthermore, LPS-stimulated WASP-microglia showed less neuronal killing in coculture system. Concomitantly, release of nitric oxide was reduced in WASP-microglia (Fig. 2). These results strongly suggest that WASP has important roles in the signal transduction pathway in LPS-activated microglia. In addition, we have established immortalized microglial cell lines from the transgenic mice overexpressing prion-protein, to study the mechanisms of neuropathogenesis and possible involvement of microglial cells in prion diseases. These cell lines are highly susceptible to various strains of mouse-adapted scrapie and BSE prions. We demonstrated that both expression and activation of P2X7, one of the ionotropic receptor channels for ATP, were increased in microglial cells after persistent infection with scrapie ME-7 strain. Further studies will be needed to determine whether the changes in P2X7 properties are observed in prion-infected animal brain.

Innate Immune System in Insects

A short antibacterial peptide was designed and synthesized on the basis of the active sites of defensins. The 9-mer peptide, ALYLAIRRR-NH₂, was tested to determine whether it can suppress the proliferation of methicillin-resistant Staphylococcus aureus (MRSA) in vivo and in vitro evaluation of the peptide against MRSA was carried out histopathologically. Silk sutures pretreated or non-treated with the peptide were embedded in the back skin of mice for 24h. Sutures treated with the peptide showed strong inhibition of the proliferation of MRSA judging from examination by light microscopy of biopsy samples. On the contrary, non-treated sutures showed numerous Gram-positive loci. For in vitro experiments, silk fibroin films containing different concentration of the 9-mer peptides were prepared. MRSA seeded on the culture plates was covered with a transparent fibroin film and incubated at 37°C for 24h. No MRSA colonies were detected under the films containing the 9-mer peptide, whereas many colonies appeared under the control film without the peptide (Fig. 3). These results suggest this synthetic antibacterial peptide is a useful lead peptide for development of novel therapeutic agents against infection with antibiotic-resistant bacterial pathogens.

Antibacterial peptide defensin isoform A was previously isolated from the midgut contents of Ornithodoros moubata blood-fed
females. However, not only defensin A, but also three other defensin isoforms showed gene expression in the midgut, suggesting the possibility that these antibacterial peptides are secreted into the midgut lumen. To further understand tick immune mechanisms, the involvement of antibacterial peptides in midgut defense was investigated. Three antibacterial peptides with molecular masses near defensin isoforms B, C and D were detected in the midgut contents of blood-fed females. Enzyme-linked immunosorbent assay analysis revealed that the antibacterial peptides in the midgut contents cross-reacted with defensin A antibodies and increased as a response to blood feeding. Simultaneously, the antibacterial activity of the midgut contents was enhanced by blood feeding. Secretion of antibacterial peptides into the midgut lumen and an increase in the peptide concentration following blood feeding was also confirmed. These findings further support the hypothesis that antibacterial peptides play an important role in the midgut defense of ticks.

An antibacterial peptide was isolated from a lepidopteran insect, Spodoptera litura. The molecular mass of this peptide was determined to be 4489.55 by matrix assisted laser desorption/ionization-time of flight mass (MALDI-TOF-MS) spectrometry. The peptide consists of 42 amino acids and the sequence has 69-98% identity to those of moricin-related peptides, antibacterial peptides from lepidopteran insects. Thus, the peptide was designated S. litura (Sl) moricin. Sl moricin showed a broad antibacterial spectrum against Gram-positive and negative bacteria. Sl moricin gene was inducible by bacterial injection and expressed tissue specifically in the fat body and hemocytes. Furthermore, the solution structure of Sl moricin was determined by two-dimensional (2D) 1H-nuclear magnetic resonance (NMR) spectroscopy and hybrid distance geometry-simulated annealing calculation. The tertiary structure revealed a long a-helix containing eight turns along nearly the full length of the peptide like that of moricin, confirming that Sl moricin is a new moricin-like antibacterial peptide. These results suggest that moricin is present not only in Bombyx mori but also in other lepidopteran insects forming a gene family.

**Development of mammalian selection markers**

We have developed a series of new mammalian cell surface marker fusion genes using a streptavidin gene from Streptomyces avidinii as an antigen. The fusion genes are intended to use as selection markers to separate transformed mammalian cells rapidly without any toxic effect on cell growth. Two different length of the streptavidin gene was used: a longer fragment contains the native bacterial signal sequence which the shorter fragment lacks. To express the streptavidin antigen on mammalian cell surface, the streptavidin gene was sandwiched by a mammalian signal sequence and a transmembrane sequence at N-terminus and C-
terminus, respectively. A signal sequence and a trans-membrane sequence from either the mouse H2-K gene or the mouse Kit gene were used. Some constructs contain the EGFP gene sequence next to the streptavidin gene sequence. Totally eight kinds of fusion genes were constructed in this study. To examine the expression of the fusion genes on mammalian cell surface, a series of plasmids encoding the fusion protein were transfected into the HeLa cells. Expression of the fusion protein on cell surface was observed for any of the constructs. Then, an antibody-mediated immunomagnetic separation methodology was applied to separate transformed cells. Transfected cells were incubated with a polyclonal antibody against streptavidin, and the antibody bound cells were pulled out using a paramagnetic beads coupled with the corresponding secondary antibody. Highly pure population of transformed cells was separated (Fig. 4). Then, effects of fusion proteins on cell growth were assayed. Cell proliferation rate of transformed HeLa cells were compared with that of the untransformed HeLa cells. No significant difference of cell growth was observed in any of the fusion genes. The property of eight fusion genes developed in this study was found to be similar. These results suggest that the fusion genes and the immunomagnetic separation protocol are useful for various transformation applications.

Fig. 4
Expression of fusion protein on HeLa cells transfected with a plasmid encoding the FSAH2K-EGFP fusion protein
(A) Localization of streptavidin antigen on the surface of a transformed cell. Serial images captured at 5-μm intervals on the z-axis.
(B) Transfected HeLa cells. Red staining indicates transformed cells.
(C-E) Immunomagnetically separated cells, same field.
(C) Light-interference view.
(D) Cells immunostained with anti-streptavidin antibody.
(E) EGFP-positive cells.
Scale bar: 10 mm (A) and 100 mm (B-E).
It has been thought that olfactory information processing in the insect brain is mediated by dynamic modulation of groups of neurons firing synchronously. We characterized correlations of firing among several single unit activities by simultaneous recordings from the antennal lobe (AL) and mushroom body (MB) to odor stimuli in the male cockroach (*Periplaneta americana*). We found odor-dependent synchronous activity correlations between the AL and the MB. Cross-correlograms of activities of AL neuron pairs indicated that they had common excitatory synaptic input, shared reciprocal synaptic input and monosynaptic inhibitory input in the AL circuit. Cross-correlograms of activities of neuron pair obtained between the AL and the MB revealed direct monosynaptic connection from AL neuron (presumptive projection neuron: PN) to MB neuron (presumptive intrinsic Kenyon cell: KC). Each PN formed several different neural circuits functionally connected to KCs. Cross-correlograms of the KC pairs suggested that the cells received common excitatory synaptic input from the PNs. These results indicate that some functional synaptic connections in a group of neighboring AL neurons are formed depending on odor quality. A group of PNs may encode information of specific odor by sending their synchronized spikes from AL to MB. The results support the view that odor representation can be accomplished by ensemble networks.

### Induction of anhydrobiosis in isolated fat body tissue from an insect

Some organisms can stand almost complete dehydration. Such a biological state without water is referred to as anhydrobiosis. Since anhydrobiotic organisms are in zero metabolism, they can be in dormant eternally unless water is given. The Sleeping Chironomid, *Polypedilum vanderplanki* (Diptera, Chironomidae) is only insect species enables to enter anhydrobiosis. A question is whether or not the central nervous system is involved in the induction mechanism. We excised tissues from the larvae followed by complete dehydration in different media to determine the ability for trehalose synthesis and the viability of these tissues after rehydration. Only fat-body tissues produced a large amount of trehalose in certain medium upon desiccation (Fig. 2). We also found that the fat body tissues could be preserved in a dry state at room temperature for an extended period of more than 18 months in viable form. Thus we have confirmed that the central nervous system is not involved in the induction of anhydrobiosis, even in this complex multicellular organism. This is an important novel finding because insect diapause is strictly under regulation of the central nervous system. This gives us a great encouragement for developing dry preservation technology in tissues and cells.

### Novel flavonoids isolated from the green cocoon shell of the silkworm, *Bombyx mori*

Two flavonoids containing the L-proline moiety, 6-C-[(2S,5S)-prolin-5-yl] quercetin...
(prolinalin A) and 6-C-{(2S,5R)-proline-5-yl] quercetin (prolinalin B), were isolated from the green cocoon shell of the silkworm, *Bombyx mori*. Extract of the green cocoon shell (160 g) was applied to a solid-phase extraction cartridge. The elute was applied to Toyoperl HW-40F column chromatography and further purified by reversed-phase HPLC. Prolinarin A (3.2 mg) and prolinalin B (1.5 mg) were obtained. Their structural elucidation was achieved by application of acid hydrosis and spectroscopic methods (Fig. 3). These compounds were not found in the leaves of mulberry (*Morus alba* L.), the host plant of the silkworm, suggesting that the flavonoids are metabolites of the insect. This is the first time that flavonoids with an amino acid moiety have been found as naturally occurring compounds.

**Morphology of foretarsal ventral surfaces of Japanese *Papilio* butterflies**

Comparison of ventral surface of foretarsa among Japanese *Papilio* butterflies showed that the shapes of fifth foretarsi, numbers and localization of chemosensilla for contact chemicals, and spines in these areas were closely related to both phylogeny and behavior of these species. The results basically supported the classification that Japanese *Papilio* species are divided into five subgenera -- *Papilio* (*P. machaon*), Princeps (*P. xuthus* and *P. demoleus*), Achillides (*P. maackii* and *P. bianor*), Menelaides (*P. helenus*, *P. polyes*, *P. protenor* and *P. macilentus*) and Iliades (*P. memon*). Moreover, foretarsal morphology of female also corresponded to the physical features of their preferable host plant leaves. The specific character of female *P. machaon*, *P. macilentus* and *P. maackii* seems to relate to the area of distribution the species and their hostplants.
Visual ‘pinpoint’ location associated with pheromonal cue in males of the black chafer
*Holotrichia loochooana* loochooana (Coleoptera: Scarabaeidae)

Females of the black chafer *Holotrichia loochooana* loochooana (Sawada) (Coleoptera: Scarabaeidae) release anthranilic acid, which functions as sex attractant pheromone for males and aggregation pheromone for females. When a white lure treated with anthranilic acid was placed next to an untreated black lure in the field, males were observed to make pinpoint landings significantly more frequently on the latter (Fig. 5). When the distance between the two lures was increased from 0 cm to 20 cm, frequency of pinpoint landing onto the untreated black lures significantly decreased while that onto the treated white lures slightly increased. When the lures were further separated to 2-m intervals, males approached only to the treated lures regardless of the color but significantly more frequently landed on the black ones than on the white. These observations demonstrated that males locate and land on a female by visual cues after reaching the vicinity by olfaction.

Relationship between imprinting disorder and overweight at birth in bovine somatic cell nuclear transfer clones

Genomic imprinting, which is the parental-origin-specific gene regulation mechanism in mammals, plays essential roles in development and growth. The mechanisms discriminating between paternal and maternal genes are not clear yet but DNA methylation as epigenetic marker is thought to be a major clue for their recognition. DNA methylation for parental imprinted markers persists in somatic cells after fertilization, and is erased and re-established in germ cells according to sex. Recent remarkable technique of somatic cell nuclear transfer (SNT) cloning made us to reproduce animals without germ cell transmission and fertilization. But survival rate of SNT clone at birth is still miserable. One possibility of this inefficiency has been thought to be epigenetic disorder. Weight of bovine SNT clones at birth are usually heavier than average and the neonatal survival rate is low.

We had determined bovine imprinting genes and identified several genes expressed paternally monoallelic in Holstein × Japanese black families. Based on the information, we successively investigated allelic expressions for polymorphic imprinting genes in adult
Japanese black SNT clones. Surprisingly, almost half of healthy and well-grown adult bovine SNT clones had imprinting disorder, i.e. bi-allelic expressions. Some of them were also confirmed fertile. These results were clearly indicated that imprinting disorder was common for bovine SNT clones with no apparent abnormality in adulthood. But our further analysis revealed the relationship between overweight at birth and bi-allelic disorder (Fig. 6). Our results indicate that the management of imprinting will crucial for effective SNT clone reproduction.

**Protein-permeable scaffold of a collagen vitrigel membrane useful for reconstructing crosstalk models between two different cells**

We recently succeeded in converting a soft and turbid disk of collagen gel into a strong and transparent gel-membrane by utilizing a concept for the vitrification of heat-denatured proteins and named the novel gel in a stable condition for “vitrigel”. The collagen vitrigel membrane involving a nylon frame can be easily handled with tweezers, consequently it functions as a scaffold excellent for three-dimensionally culturing cells on double surfaces of it. Here, we investigated the molecular permeability of the collagen vitrigel membrane in time-course using glucose and serum proteins. The glucose added to one compartment penetrated into another compartment via the collagen vitrigel membrane and the glucose concentration of each compartment came nearly up to a plateau level within 24 hours. Serum proteins with not only low molecular weight but also high one more than 100 kDa passed gradually through the collagen vitrigel membrane (Fig. 7). These
data suggest the protein-permeable scaffold of collagen vitrigel membrane is useful for the reconstruction of crosstalk models between two different cells.

**Electroencephalogram (EEG) changes with eyelid movements in piglets**

It is known that the amplitude and rhythm of the EEG is largely altered in response to opening and closing eyes. In this study, a wireless recording system was applied to examine EEG activity with or without opening eyes in unrestrained, male Landrace piglets. Electrodes and telemetry devices were implanted under halothane anesthesia. Recordings were performed while lying at rest, following recovery from the surgical operation. In the absence of eyelid movement, slow waves with large amplitude appeared in the EEG. The power of the delta (1-3.9 Hz) and theta (4-7.9 Hz) activities were larger than that of the alpha (8-12.9 Hz) and beta (14.1-25 Hz) (Fig. 8). While eyelid movement was present, faster waves with small amplitude were recorded in the EEG trace. With the eyelid movement, the power of the alpha and beta activities was stronger than that of the delta and theta (Fig. 9). According to the power spectral analysis of the EEG, the delta and theta activities, which appeared in the absence of eyelid movement, were replaced with faster alpha and beta activity once eyelid movement appeared. These findings strongly suggest arousal in the piglets while lying at rest.
The role of glucose as a metabolic regulator of hypothalamic gonadotropin-releasing hormone pulse generator activity in goats

We examined the relative importance of blood glucose vs. free fatty acids as a metabolic signal regulating gonadotropin-releasing hormone (GnRH) release as measured electrophysiologically by multiple-unit activity (MUA) in the arcuate nucleus/median eminence region in ovariectomized, estradiol-treated goats. MUA was recorded before, during, and after: 1) cellular glucoprivation by intravenous infusion of 2-deoxy-D-glucose (2DG); 2) peripheral hypoglycemia in response to intravenous infusion of insulin; and 3) cellular lipoprivation induced by intravenous infusion of sodium mercaptoacetate (MA), and effects on the interval of characteristic increases in MUA (MUA volleys) were examined. Infusion of the highest dose of 2DG increased the mean interval between MUA volleys, whereas the lower doses of 2DG had no effect on volley interval (Fig. 10). The MUA volley intervals lengthened as insulin-induced hypoglycemia became profound. There was a negative correlation between MUA volley intervals and blood glucose concentrations during insulin infusion, and coinfusion of glucose with insulin returned the MUA volley interval to a normal frequency. Infusion of MA alone or MA with 2DG did not increase MUA volley intervals. These findings demonstrate that glucose availability, but not fatty acids, regulates the GnRH pulse generator activity in the ruminant. Glucose is considered a key metabolic regulator that fine-tunes pulsatile GnRH release.

Molecular and evolulional analyses of insects

Oligo array analyses of silkworm larval skin RNAs identified some genes whose expression is repressed in a translucent skin mutant, oa or od. We estimated a magnitude of...
nucleotide diversity of molybdenum cofactor sulfase (og) gene using 15 *Bombyx mori* regional races. We also estimated that of mitochondrial cox 1 gene and comparatively analyzed these results. To detect transposition event of *Bombyx* MITE-like transposon, Organdy, we carry out excision assay on specific insertion sites.

**Genetics and evaluation of silkworm stocks**

Bisexually reproductive tetraploid strains were established with high fertility in the *Bombyx mori*. It was reconfirmed that the female sex was determined by the presence of W chromosome in the tetraploid and triploid offspring of the strains. Our database search of the deleted sequence found in one of the intersex strain, *Isx-2*, showed no significant homology to the previously registered genes. In normal cells we detected fragments of mRNA transcribed from the deleted region, but it was difficult to clone full length mRNA. We obtained BAC clones contain the deleted sequence. The female specific splicing of *Bmdsx* gene was increased in the cultured male cells transfected with one of these BAC clones. Mapping of homeotic mutant genes was examined by a three-point test. Three homeotic mutant genes belonging to *E*-pseudoalleles and *Nc*, *E*<sup>66L</sup>, *E*<sup>6C</sup>, *E*<sup>4C</sup> and *Nc* were arranged in order from the proximal side of the sixth linkage chromosome. The new mutant gene, “maternal brown egg of Shimizu” (*b-2*) was confirmed to be a novel mutant as a member of recessive pseudoalleles in the *Bombyx mori*.

As a trial in order to develop longterm preservation of silkworm eggs, ovary transplantation using fourth instar larva of silkworm was technically too difficult to keep alive. A correlation between total amount of sugar alcohols contained in eggs and viability in two-years preservation of silkworm eggs was low score.

**Insect-plant interactions**

We have cloned a cDNA for laccase from the salivary glands of the green rice leafhopper, *Nephotettix cincticeps*. While many insect laccases have been identified in cuticle, we know only an example of laccase of salivary gland origin. To characterize the enzymological properties, we expressed a salivary laccase as a recombinant protein. *E. coli* cells harboring the expression plasmid produced laccase as a soluble protein in the cytoplasms. Laccase activity was detected when copper chloride was added to *E. coli* culture medium, suggesting the presence of a cupper center essential for the oxidase activity.

We have started to isolate brown planthopper resistant genes from rice using map-based cloning method. “Norin PL10” has a resistance gene (*Bph 3*) which is originated from a Sri Lanka variety ‘Rathu Heenati’. The chromosome segment of ‘Rathu Heenati’ was found in the chromosome 4 of Norin PL10, between the two SSR (Simple Sequence Repeat) makers, RM6487 and RM3735.

We found that the latex of a wild fig species, *Ficus septica* that grows in Ishigaki Island, Okinawa, showed a strong toxicity to the Eri silkworm, *Samia ricini*. When the Eri silkworms were fed the artificial diet containing 2% of mulberry latex, growth inhibition was observed, and when the diet containing more than 10% of mulberry latex was fed, high mortalities were observed. We also discovered that the mulberry leaves are toxic to insect except the silkworm, *Bombyx mori*, and that the latex ingredients, three sugar mimic alkaloids, which are well known as glycosidase inhibitors, and unknown high-molecular-weigh factor(s) are responsible for the defense of mulberry leaves against herbivorous insects.

**Natural enemies**

*Neoseiulus womersleyi* is one of the most important predators of spider mites of the genus *Tetranynchus* in Japan. We found that *N. womersleyi* learns a specific blend of several volatiles emitted from prey-infested plants and is attracted to the volatile complex. The predatory mite has intra-specific variation in its olfactory response. The predator showing strong olfactory response could reach prey-infested plants more efficiently than the mites with weak olfactory responses did. Genetic
analysis using microsatellite markers showed that a population of 40-60 adult females per generation would be sufficient to conserve the genetic diversity of the wild populations. Minute pirate bug, Orius strigicollis, is one of the most effective predators of small insect pests, such as thrips. We evaluated genetic differentiations among wild populations of this species using microsatellite DNA markers and found that no difference among populations 100 km apart, suggesting that this species has large gene pools in the field. We also analyzed polymorphic DNA regions of mitochondrial and nuclear DNAs amplified from Japanese Orius species. Phylogenetic analyses based on nucleotide sequences showed that these DNA regions can be used to examine genetic interrelationships among species and strains. Based on the results, we developed PCR primers to amplify polymorphic DNAs from many other insects.

**Symbiotes**

About 70,000 expressed sequence tags were generated from the brown planthopper, Nilaparvata lugens, and new oligo-microarray was designed based on a sequence clustering analysis of the planthopper ESTs. A microarray-analysis-room was newly set up for silkworm- and planthopper-arrays. The silkworm-array is used by more than 12 domestic research groups and planthopper-array by three groups. RNAi protocol was successfully applied to the planthoppers and is useful for the functional analyses of the planthopper genes. Nucleotide sequence analyses of ribosomal RNA and mitochondria were performed in culicoides species in Japan, and the species were identified based on the nucleotide sequences.

We found two key amino-acid positions affecting adaptability for heterologous over-expression of termite cellulases (432 amino-acids, a member of the glycoside-hydrolase family 9) which generally resist against recombinant production. By introducing amino-acid mutations at the two positions, the termite cellulases were over-expressed in E. coli.

The internal ribosome entry site (IRES) in the intergenic region of Plautia stali intestine virus binds with 80S ribosomes in the absence of eukaryotic initiation factors. This indicates that the IRES-mediated protein synthesis would be possible in a complete reconstituted system, containing 80S ribosome, mRNA, tRNAs, aminoacyl tRNA synthetases (AARSs), elongation factors, ATP, and GTP. We prepared translation apparatus from eggs of brine shrimp and reconstituted translation was examined. Our AARSs were inactivated during the purification procedure. If stable AARSs are purified, eukaryotic reconstituted translation system would be available with the IRES.

We found the infectivity against rice yellow dwarf-phytoplasma was different between regional strains of green rice leafhopper, Nephotettix cincticeps, and thereby were able to build the experiment system of virulence mechanism of phytoplasma insect pathogens.

It was examined by measuring the initial quantity of Anomala cuprea entomopoxvirus (AcEPV) fusolin gene in the ectoperitrophic area of A. cuprea larvae with a method of real-time quantitative PCR of the gene whether or not a greater number of the AcEPV virions had passed the peritrophic membrane (PM) after the feeding of spindles mixed with spheroids compared with that after the feeding of spheroids only. The experimental results indicated that a greater number of AcEPV virions had passed through the disintegrated sites generated in the PM by the spindles, and that they had reached the ectoperitrophic area and then had entered the midgut epithelium within 6 hr after the administration of the spindles.

Resistance of the silkworm to Cry1Ac toxin of Bacillus thuringiensis was related to recessive major gene. We selected two silkworm lines on the susceptibility against entomogenous fungus, Beauveria brongniartii, infection. One line was resistant and the other was susceptible. First filial generation (F1) of two lines showed resistance and half of backcrosses between F1 and susceptible line showed resistance. These results suggested that the resistance of the silkworm against B. brongniartii infection was related to dominant major gene.
Development of biosensors and related materials focusing on the immobilization of chemical recognition molecules

We have been reported that liposome containing insect sensory organ extracts and the membrane potential sensitive dye is a new type biosensor. We immobilized the liposome on a photodiode and measured membrane potential changes triggered by salt stimulation (Fig. 1 Left). The photodiode output was in proportion to the concentration of salt solution. We also constructed the other type of biosensor that uses a planar lipid bilayer membrane which is used for immobilization of receptor proteins from insect sensory organs. This biosensor is fabricated by combining receptors with a field effect transistor (FET). Our FET device could detect the membrane potential changes stimulated by salt solution (Fig. 1 Under). Lactose immobilized silk fibroin was indicated a better substrate for fibroblast culture than no-treated silk fibroin by the results of mice fibroblast cultivation study. We established the lipofection method which transfect plasmid DNA transiently into silkgland cells of silkworm larva, and we optimized the experimental conditions.

Development of measurement and recording methods for obtaining bio-physical information on insects

Multichannel microelectrodes are being developed in order to record action biopotentials of insects. Silicon was conventionally used as the electrode material and microelectrodes were fabricated by anisotropic etching and reactive ion etching. Because electrode microprobe shape and size were very difficult to control, we proposed a novel pin-shaped multichannel microelectrode that used epoxy-based UV sensitive photoresist as the electrode material. Analysis of the electrical properties of this type electrode showed that it has properties excellent enough to record insect biopotentials. We could record muscle action potentials from a flapping silk moth using our epoxy-based multichannel microelectrodes (Fig. 2).
Development of functional materials such as fine chemical using chitin and fibroin etc.

The porous microsphere was prepared by the addition of the lithium bromide. The dissolution rate from the microsphere of the theophylline was quickly finished further than non-pore microsphere. The dissolution rate was rapider as particle size was smaller. Oleic acid, palmitic acid and ricinoleic acid were extracted from the beetle larva cuticula by the supercritical carbon dioxide (Fig. 3).

Antheraea pernyi silk fibroin films were incubated with Protease Type XXI at 37°C, to investigate the degradation behaviour in an in vitro model system. The enzyme-resistant fractions of films were collected after incubation time of 17 days, and analyzed by FT-Raman spectroscopy. The intensity ratio \( I_{850}/I_{830} \) between the bands at 850 and 830 cm\(^{-1}\) is sensitive to the hydrogen-bonding state of the \( \text{Y} \) phenoxy group. The \( I_{850}/I_{830} \) intensity ratio decreased from 1.55 to 1.30 upon biodegradation of silk films, suggesting a more buried state of Tyrosine (Tyr) residues in the biodegraded film. This feature can be explained assuming that a certain amount of exposed Tyr residues, initially present in the amorphous film domains more accessible to protease, were lost with the peptide fragments removed by proteolytic cleavage.
Characterization of silk fibroin and other biopolymers from chemical and physical respect and development of their applications to wound covering materials and others

The primary structure of sericin A was determined by RT-PCR; it consists of 1,271 amino acid residues with a high serine content up to 44% in molar ratio, and additionally, the presence of repetitive sequences with 86 and 8 amino acids per repeat was revealed. The sequences were predicted less preferable to form β-sheet structure compared to the 38-amino acid repeats that constitute sericin M. This is consistent with the experimental result using cast films; sericin M tends to favorably form β-sheet structure through humidity compared to sericin A. Electron microscopic observations of liposomes showed that newly constructed silk fibroin recovered from B. mori posterior silk gland quickly penetrates phospholipids membranes without bursting them. Observations of the planar lipid bilayer membrane showed that the regenerated silk fibroin after autoclaving penetrates phospholipids membrane as well.

New process for forming the film of hornet silk using the harmless solvent system has been developed. This hornet silk film was found to exhibit the quite low cell adhesion activity when the film coexists with serum. This finding indicates that the hornet silk film is useful for the biomaterials. The sequence of a silk protein from Japanese spider Nephila clavata was determined from a partial cDNA clone. The repeating unit was composed of several segments such as a polyalanine, glycine-glicine-Xaa with Xaa being alanine, tyrosine, glutamine or leucine. Circular dichroism (CD) studies showed the stable secondary structures of spider silk in silk gland.

The compressive modulus of silk fibroin sponge influenced growth rate of fibroblasts cultured in the sponge, and the higher cell growth rate was observed in the softer silk sponge. The secondary structure changes of silk fibroin in the sponge construction during storage were observed by 13C solid-state NMR measurement.

Insect Biotechnology and Sericology Department

Building of the data retrieval system for the genetic resources of silkworm

The database on the preserved silkworm races that have been maintained as the genetic resources by National Institute of Agrobiological Sciences is presently disclosed on the following: http://ss.nises.affrc.go.jp/nisesDB/bombygen/tablemaster-eng.html in English (Table 1 and Fig. 1). This database is configured with a hierarchical tree structure. It is troublesome to find the target characteristic races. So, the retrieval function was added to supplement the faults and to correspond quickly. Some database softwares which have retrieval function on the Web are commercially available. But we did not use these database softwares for providing security of the private server. The program function was added on the retrieval system configured with HTML by using JavaScript. Still more, the retrieval system was composed of the preserved silkworm races and the commercial races. The system made it possible to gain access to the preserved silkworm races and to the commercial silkworm races either in Japanese or English for many users.

Improvement and application of mapping systems in the silkworm, Bombyx mori

Molecular linkage map has been improved and finally 330 EST-cDNA clones have been mapped on 28 linkage groups. The methods for
### Table 1 Menu of Commercial silkworm races

**Genetic Resources of Silkworm**

(Commercial Silkworm Races)

#### Classification of Genetic Resources and the Number of the Variety

<table>
<thead>
<tr>
<th>Selective Item</th>
<th>Number</th>
<th>Selective Item</th>
<th>Number</th>
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<tr>
<td>Egg Selection</td>
<td>2</td>
<td>Cocoon Filament Selection</td>
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</tr>
<tr>
<td>Larva Fitness</td>
<td>41</td>
<td>Filament</td>
<td>7</td>
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<tr>
<td>Growth Velocity</td>
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<tr>
<td>High Temperature Resistance</td>
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<td>Healthiness</td>
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<td>Cocoon Weight</td>
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<td>Parent Silkworm for Hybridization</td>
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<tr>
<td>Selection</td>
<td>4</td>
<td>Japanese</td>
<td></td>
</tr>
<tr>
<td>Character (Sericin Cocoon/Naked Pupa)</td>
<td>8</td>
<td>Chinese</td>
<td>68</td>
</tr>
<tr>
<td>Cocoon Neatness</td>
<td>3</td>
<td>European</td>
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</tr>
<tr>
<td></td>
<td>Cycle-Cross</td>
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</table>

The silkworm races can be retrieved according to the characteristics of cocoon.

#### Characteristics of Genetic Resources

<table>
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<tr>
<td>Growth Velocity</td>
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<td>Japanese</td>
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<tr>
<td>High Temperature Resistance</td>
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<td>Chinese</td>
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<td>Cocoon Weight</td>
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<td>European</td>
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<tr>
<td>Selection</td>
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<td>Cycle-Cross</td>
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<tr>
<td>Character (Sericin Cocoon/Naked Pupa)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cocoon Neatness</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The target Commercial silkworm races are retrieved according to the indicated conditions.

(Please select values of characteristics of cocoons.)

- Hatchability (per all eggs) (%) All
- Missing larvae ratio All
- Reliability percentage (%) All
- Double cocooning silkworms ratio All
- The number of cocoons per liter All
- Cocoon weight (g) All
- Cocoon shell weight (g) All
- Cocoon shell ratio All
- Weight of cocoon filament (g) All
- Raw silk ratio All
- Length of cocoon filament (m) All
- Size of cocoon filament (d) All
- Reliability percentage (d) All
- Neatness (points) All

---

**Fig. 1**

Selection of quantitative characteristics (Commercial)
linkage analysis and mapping that were very efficient for the organisms without crossing over in one sex were developed. Scanning linkage analysis (SLA) is the method for linkage analysis using the same backcross segregants to know where genes are linked. BCMAP is the mapping method for the same individuals of the backcross segregants. These methods were introduced for making the molecular genetic map of EST-cDNA clones in the silkworm, *Bombyx mori*. The newly developed EST-cDNA clones were examined by Southern blot hybridization to clarify whether those showed effective RFLP or not. The clones showing effective RFLP were used for SLA and BCMAP. Finally new 70 clones were added to the map this year.

The markers on the map and the methods were introduced to analyze many kinds of characters like resistant genes against BT-toxin, polyphagous gene and etc. The molecular markers on the map was also used to make homozygote of p and +p genes on the second chromosome. As shown on Fig. 1, p/+ and +/+ were identified by using very closely linked RFLP marker.

**Induction of cell-differentiation of insect cultured cell by drags, and its application**

Characterization of new insect cultured cell lines was analyzed. BmN4 cells that was derived from ovary tissues was induced strongly to fat cells by treatment of the three complex drags, Insulin, Dexamethason and IBMX. Particularly, Dexamethason promoted to accumulate the fat in the cells. We analyzed the gene related to form the fat. The gene was designated to the BmFABP1. This gene’s promoter region was analyzed in detail. We discovered the suppression-region of expression of the gene. By a method of gel sift assay, we clarified the existence of protein that combined to origo DNA that contained the partial sequences of the BmFABP1 gene in the extracts of BmN4 cells. We established the insect cell culture system of Baculovirus gene expression by using *Bombyx mori* serum-free cultured cell line, NIAS-Bm-Ke1. To produce a high amount of the gene product of luciferase, *Bombyx mori* heat-treated serum has to be contained in culture medium (see p12, Figs. 1 and 2; p13, Fig. 3). As an experimental system, clone cell lines from *Bombyx mori* cell line (NISES-BoMo-Cam1) was selected.

**Utilization of transposon for the construction of transgenic insects and application for the analysis of insect genes**

The technologies that developed a transgenic insect for these five years has been joined and the standard methods for making the transgenic silkworm, including the injection method for the preblastodermal embryos, marker genes for the screening of the transgenic silkworm and non-diapausing strains for the injection, have been authorized. The method development has been shown to be very useful and gave very high efficiency of transformation.
rate compared to the previous methods, and many different transgenic silkworms that introduced many different foreign genes were produced. Especially, the GAL4/UAS strains using yeast to control the gene expression of the introduced foreign gene was very effective for the study of gene function and for the production of useful materials. The efficient production of recombinant proteins in the transgenic silkworm has been shown to be possible and to be useful as the new developed system for it. In addition, jump starter strains have been constructed for the enhancer trap in the silkworm. The strains have been shown to be used to transpose the mutator gene in the silkworm and can be used for the production of useful proteins. Furthermore, the method for the analysis of brain and nervous system using GAL4-RNAi has been confirmed and the transgenic insects that introduced human FMR1 gene was constructed.

**Newly developed mulberry cultivar “Ayanobori” with high leaf quality and productivity**

Recently, the sericulture is on the decline in Japan. However, the improvement of the productivity in mulberry field is still of importance for the development of sericulture. We have developed a new mulberry cultivar “Ayanobori” (Fig. 4), which was selected out of seedlings obtained by crossing the mother “Wasemidori” and the father “Hayatesakari Tetraploid”. Its resistance to dwarf disease has
been studied at two experiment stations, in Tokushima and Kagoshima prefectures, for five years since 1996. Local adaptability has been tested at two experiment stations, in Gunma and Miyazaki prefectures, for four years since 1998. “Ayanobori” was found to be superior in leaf yield and lodging resistance to the control cultivar “Shinichinose”. In order to officially register “Ayanobori” as a new variety, an application has been made to the Ministry of Agriculture, Forestry and Fisheries of Japan, and the application has been announced officially since June, 2005. “Ayanobori” has the following characteristics:

“Ayanobori” is a triploidy cultivar belonging to Morus alba L. Its form is characterized by moderate expansion resulting in tolerance to lodging. The shoots are longer in length and nearly the same in the number of shoots, respectively, as compared to “Shinichinose”. The sprouting time in spring is a few days earlier than in the case of “Shinichinose”. The young shoots grow vigorously and uniformly after sprouting. Resprouting after intermediate pruning in summer and autumn is also vigorous. The yield is high in both normal planting and dense planting. In the silkworm rearing both with mulberry shoots and by the artificial diet added with the “Ayanobori” leaf powder, the leaf quality is almost the same in “Ayanobori” as in “Shinichinose” whose leaf quality is said to be pretty high.

“Ayanobori” is resistant to dwarf disease, but rather sensitive to bacterial blight and powdery mildew as compared to “Shinichinose.” The injury of shoot tips to cold is less pronounced than in “Shinichinose”. “Ayanobori” has a good rooting ability; production of sapling by hard-wood cutting is easy.

Taken together, “Ayanobori” can be easily cultivated in wide areas from Kyushu district to Tohoku district of Japan and is adaptable to both normal and densely planted cultivations in summer and spring pruning. Introduction of “Ayanobori” would lead to improve the productivity in mulberry field. Since “Ayanobori” has a high quality of leaves, “Ayanobori” might be able to contribute to improve the artificial diet suitable for production of useful substances using transgenic silkworm.

Breeding and utilization of a new silkworm race having special features

The sericin produced by the sericin cocoon strain “SERICIN HOPE” was experimentally used to food and cosmetic items. Furthermore, we are developing another two sericin cocoon strains, which simultaneously secrete functional colored ingredients with sericin (Fig. 5). “SERICIN FLAVO”, whose flavonol ingredients in the green cocoon strongly inhibit oxidation, was released as a commercial race. A yellow sericin cocoon strain (provisional name: “SERICIN CAROTENE”) produced 50mg of sericin cocoon containing 2.5mg of carotenoids per larva. The yellow cocoon shows the stronger inhibition of tyrosinase activity and less anti-oxidation than the SERICIN FLAVO cocoon. These ingredients also appeared to suppress some bacterial growth. These additional activities of the SERICIN HOPE cocoon were effective even in gel and emulsion states for hours, because the ingredients were slowly released into the water solution. Based on these findings, the colored sericin produced by SERICIN FLAVO and SERICIN CAROTENE is expected to be widely used as a new functional cosmetic material.

A strain having twice as much sericin solubility in 60°C water as ordinary races was found in the silkworm genetic resources. Differences in cocoon filament intensity were

![Fig. 5](image-url)  
Three kinds of cocoons produced by sericin cocoon race series  
left, SERICIN HOPE; center, SERICIN CAROTENE; right, SERICIN FLAVO.
also surveyed in the genetic stocks. The strongest cocoon filament showed an intensity of over 4.5 g/d, or 1 g/d higher than ordinary races. These strains might be useful resources for breeding new silkworm races.

**Chemical and physical modifications of silk and the development of characteristic silk products**

$^{13}$C solution and solid-state NMR analyses were employed to investigate native structure and degradation pattern of silk sericin. Sericin samples were prepared from Sericin Hope silkworm, which secrets almost exclusively sericin. The $^{13}$C solution NMR spectra of native sericin solution obtained from Sericin Hope larvae exhibited that sericin was largely random coil structure before spinning. The structural changes by thermal degradation were followed by $^{13}$C solution NMR measurements. The $^{13}$C NMR spectrum after thermal degradation exhibited obvious peak splitting at Asp C$\alpha$, C$\beta$ and C$\gamma$ peaks. This observation indicated that Asp residues might be a weak point toward hydrolysis.

It was examined to form the artificial skein and blood tube using cocoon filaments. The basement of artificial skein was weaved by using single cocoon filament which reeled up from a cocoon (Fig. 6), the artificial blood tube basement was made by the formation of knitting and winding with cocoon filaments. In order to produce the silk tube except sericin, it was degummed (dissolving sericin), the fibroin solution was put on the tube, and the fibroin was fixed with alcohol treatment.

As the changing treatment of the fiber’s characteristics, the knit materials made of silk, cotton and wool were treated with high temperature. The activated value of the antibacterial activity was investigated. As a result, the value of silk was the biggest among them.

The silk thread characteristics of the silkworm races, “Koishimaru”, “Ohkusa” and “Shokoh” were investigated. As a result, it was recognized that they were very similar to the silk characteristics of Edo period (1603-1867).

The flexible type silk reeling machine was developed to produce five kinds of thick silk fibers, that is, the high bulk silk, the special flat silk, the combination silk yarn of high bulk silk and special flat silk, the super thick silk and the covered silk yarn. Those thick silk fibers are produced by reeling from large number of cocoons, for example, from around 200 cocoons to 300 hundred cocoons per one thread. Using these fibers, jackets, sweaters and shawls were produced; those products show each specific characteristic on the aspects of bulkiness, luster and texture.

![Fig. 6](image)

Artificial skein basement weaved by using single cocoon filament for the warp and weft filaments
The Plant Science Division consists of the following five departments, and is actively engaged in multidisciplinary researches.

Scientists at the Molecular Genetics Department are engaged in studies of the structure and function of plant genomes, genes, their products, and their networks, which are involved in various agriculturally important traits, and also the mechanisms regulating expression of these genes.

Those at the Biochemistry Department are involved in researches on the three-dimensional structure of proteins, and structure-function relationships of proteins involved in response to hormones and other biotic signals in plant cells.

Plant Physiology Department is engaged in analyses of molecular mechanisms of important physiological processes in plants including photosynthesis, morphogenesis such as leaf and floral organ development, the symbiotic process of nitrogen fixation, mechanisms of defense against plant pathogens, and tolerance against environmental stresses.

The Plant Biotechnology Department is developing new techniques for next-generation plant biotechnology and also producing novel transgenic crops with superior traits which conventional breeding techniques can not produce.

The Institute of Radiation Breeding is developing new technologies utilizing radiation for plant breeding, the creation of plant genetic resources through mutation induction, and the elucidation of gene expression mechanisms in plant mutants.

Major topics in each department are described the next page.

Molecular Genetics Department

The research activities of this department are mainly focused on the analysis of the structure and function of rice genome, genes and their products, the development of tools and resources for functional analysis of rice genes, and the mechanisms regulating gene expression. Major topics in the fiscal year 2005 are described as follows.

DNA methylation analysis

DNA methylation is an epigenetic modification that a methyl group is added to the 5th carbon of pyrimidine ring of cytosine. It is informative to systematically scan genome-wide changes of methylation through each developmental stage, and to precisely analyze interactions between methylation status and gene expressions. Restriction landmark genome scanning (RLGS) is a two-dimensional electrophoresis of genomic DNA, which visualizes thousands of loci. We improved RLGS method to detect methylated sites directly. The isoschizomers, MspI and HpaII that recognize the same sequence (CCGG) but have different methylation sensitivity were employed. We detected 22 spots on both RLGS patterns (MspI and HpaII) in Arabidopsis thaliana ecotype Columbia. In comparison of them, 18% of the spots were polymorphic, which indicated the methylation of C^5mCGG sites. And, 52 and 54 restriction enzyme sites were also analyzed in two other ecotypes, Wassilewskija and Landsberg erecta, respectively. Consequently, 15% of the 52 common sites showed methylation polymorphism among the three ecotypes. Almost all the restriction sites analyzed in this
research were located in or near genes. This improved RLGS method is readily applicable to practical analyses of methylation dynamics in an un-sequenced species and even in a cloned animal/plant.

**Gene family-oriented rice gene annotation**

After the completion of rice genome sequence by the International Rice Genome Sequence Project (IRGSP) and the collection of over 32,000 rice full-length cDNA clones and their complete sequence analysis by the Rice full-length cDNA Consortium, next challenging target is the comprehensive annotation of rice genes and their functional analyses. For the establishment of bioinformatics platform for rice gene annotation, Rice genome annotation program (RAP) has been initiated in December 2004. RAP activity is the human curated annotation of the gene structure generated by the mapping and alignment of full-length cDNA clones, individual EST clones and combiner EST sequences to the rice genome sequence. Currently about 25K locus have been assigned on the IRGSP built 3 Pseudomolecules (See RAP-DB: http://rapdb.lab.nig.ac.jp/ ). In RAP-2 annotation meeting held in February 2006 in Tsukuba, 580K single pass sequences from 380K full-length cDNA clones (FL-ESTs) were incorporated for the mapping and alignment. As pointed out in RAP-2 discussion meeting held in November 2005 in Manila, next direction of rice gene annotation is toward the gene family specific- and much deeper gene function-related annotation.

Along with the future direction of rice gene annotation, our research team has also focused some gene families, such as calcium or Ca2+ related proteins in signal transduction pathways (Nagata et al. 2004, 2005), and the membrane protein. In this year we have mainly focused to the membrane transport protein families.

Cells maintain their biological activities by importing and exporting various materials. Supplementation with energy, materials, and substrates and efflux of salts, drugs, and ions are necessary to maintain biological activity in prokaryotic and eukaryotic cells. On the other hand, environmental situations within cells differ among organisms: unicellular organisms cannot control the ion concentrations outside the cell, but multicellular eukaryotes (especially animals) can precisely regulate the ion concentrations of their environments within micro molar ranges. Therefore, we can expect organisms to differ the gene numbers, structure, and functions according to their biological abilities and/or environmental situations. Because transport activities are necessary in most tissues at distinct levels, we expected that the transcripts of most transmembrane transporters would be contained within the standard materials (including various developmental stages, tissues, and plants stimulated with various treatments) held in full-length cDNA libraries. We searched for ortholog with known membrane transport genes by using the 32,127 full-length cDNA data for rice and also *Arabidopsis* and rice genomic sequence data. We used the BLASTX program to search for sequence homologies at the amino-acid level. Because membrane transport proteins have specific structural features, the identification of orthologs is clear from computer calculations. There have been many precise reports of individual transporter protein families (e.g. Pao et al. 1998; Sanchez-Fernandez et al. 2001; Eng et al. 1998; Mäser et al. 2001; Wipf et al. 2002), but we have little overall information about whole transport systems. We tried to examine the topics and selection of total membrane transport systems, as indicated by the overall outline of gene diversity in organisms. Comparison of membrane transport genes indicated that these genes are examples of the evolutionary diversity of homeostasis systems in organisms. The increase in the ratio of membrane transport genes was smaller than in other gene categories (*e.g.* transcription factors, metabolism) in higher eukaryotes (350-850). Usually, according to the complexity of the organisms, the gene numbers increases drastically by divergence, evolution and duplication of the genes. Therefore, the indispensable number to retain the cell membrane transport homeostasis
seems 300-350 and the increases of the genes are strongly limited by selection pressures. Especially the numbers of the pump system genes are 70-170. This indicates that, the active transport systems (= the system that operates structure material concentration against a physicochemical situations) are strongly controlled its working situations. Many of the newly diverged genes of higher animals and plants are channels and secondary transporters works by adapting already existing concentration gradients.

**Phenotype catalog of Tos17 insertion mutants**

Functional identification of rice genes is an important target after whole genome sequencing of rice. Gene disruption is one of the most powerful methods for this purpose. The endogenous retrotransposon in rice, *Tos17*, is only active in cultured cell, and is transposed with 'copy and past' manner. This characteristic is suitable for gene disruption system. Because the copy number of original *Tos17* in japonica rice Nipponbare is two, detection of newly transposed *Tos17* by hybridization and isolation of flanking region of *Tos17* are relatively easier than other high-copy number of transposons. Furthermore, because *Tos17* is an endogenous retrotransposon, gene disruption line with *Tos17* is same as spontaneous mutation lines for handling mutant plants and is not under regulation of the genetically modified organism (GMO). This enables large scale phenotyping in the field. Currently, more than 50,000 insertion lines have been produced and stocked.

In the project of MAFF ‘Isolation and Functional Analysis of Useful Rice Genes and Development of Techniques for Their Utilization’, large scale phenotyping proceeded with collaboration of 7 laboratories for 3 years project. From 1000 to 3000 lines were observed in each laboratory for a year. It was difficult to keep the quality of data without standard. We chose standardized vocabularies for phenotyping. Their consist 12 categories, germination, growth, leaf color, leaf shape, culm shape, mimic response, tillering, heading date, flower, panicle, sterility, and seed. The categories were divided into 53 sub category with phenotype ID numbers for description more detailed phenotypes. The phenotype ID system enables to input phenotype data into barcode handy terminal in the field. Even using the standardized phenotyping system, personal bias for observation is still remaining. *e.g.*, determination between dwarf and semi-dwarf phenotypes, yellow green and pale green phenotypes. To solve the personal bias problem, photo images were also obtained in the field. All phenotype data with phenotype ID and photo images have been stored into web-based relational database with PostgreSQL and CGI script written in perl language.

Descriptions of special phenotypes were saved into free style comment box. Phenotyping of 50,000 insertion lines is almost finished. Nearly half of insertion lines showed at lease one phenotype. Most frequently appeared phenotype was dwarf and sterile (including partial sterile). Pigmentation mutant also relatively frequently appeared. For the low frequency but characteristic phenotype, narrow leaf, brittle culm, viviparous, abnormal shoot, abnormal tillering, abnormal flower, *etc.*, were observed. Isolation and characterization of the responsible gene of these phenotypes will be helpful to design new shape of rice for improvement of photosynthesis, yield, resistance of plant pathogens, etc.. Currently, more than 172,000 phenotype descriptions and 58,000 photo images are maintained with the relational database and stored data is still growing. We are also constructing the database for disruption site by *Tos17* on the rice genome. Mutant lines can be searched by BLAST search ([http://tos.nias.affrc.go.jp](http://tos.nias.affrc.go.jp)). When the candidate of the knock-out line is detected, phenotype description with photo images will be listed. Seed of mutant lines is distributed by rice genome resource center.

**A major QTL, qSH1, for shattering habit in rice**

Loss of seed shattering habit is one of the critical events during domestication of rice plant, because the ‘easy-to-shatter’ trait in wild
relatives results in severe reduction in yield. Therefore, seed shattering habit is one of the most important agronomic traits in rice breeding. To reveal a molecular basis of seed shattering habit in rice, we genetically analysed naturally occurring variations in seed shattering among cultivars. We first performed a QTL (Quantitative Trait Locus) analysis between a shattering-type *indica* variety, Kasalath, and a non-shattering type *japonica* cultivar, Nipponbare (Fig.1A). Five QTLs were detected on five chromosomes of rice in an F2 population of the cross between Kasalath and Nipponbare (Fig. 1B). The QTL with the largest effect, *QTL of seed shattering in chromosome 1* (*qSH1*), explained about 68.6% of the total phenotypic variation in the population (Fig. 1B). We made a near-isogenic line (NIL) that contained a chromosome segment from Kasalath at the *qSH1* locus in a Nipponbare background. The NIL exhibited the formation of a complete abscission layer between the pedicel and spikelet at the base of the rice seed (Fig. 1C), while no abscission layer was observed in Nipponbare at all (Fig. 1C). This indicated that Kasalath allele of *qSH1* was involved in the formation of abscission layer.

A large-scale linkage analysis of 10,388 plants segregating at the *qSH1* region was performed for the fine mapping of *qSH1*. We finally defined functional natural variation in 612 bp between the flanking markers *qSH1*-F and *qSH1*-H and found only one single nucleotide polymorphism (SNP) within this region (Fig. 2A). Gene prediction using the Nipponbare rice genome and the Kasalath genome at the *qSH1* locus predicted no distinct open-reading frame (ORF) in the candidate FNP region. However, we found one ORF that was an ortholog of the *Arabidopsis REPLUMLESS* (*RPL*) gene encoding a BEL1-type homeobox (Fig. 2B), located 12 kb away from the SNP. The *RPL* gene is required for pod shatter and is involved in the formation of a dehiscence zone.

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**Fig. 1**

*qSH1* is required for formation of the abscission layer at the base of the rice grain
(A) Seed shattering habits of rice panicles. Left: non-shattering-type cultivar, Nipponbare. Right: shattering-type cultivar, Kasalath.
(B) Chromosomal locations of QTLs for seed shattering degree, based on an F2 population from a cross between Nipponbare and Kasalath. *qSH1* is marked on chromosome 1 with the nearest DNA marker (C434).
(C) Photo of a rice grain. White box indicates position of abscission layer formation.
To verify the function of candidate gene, we introduced ten of 10- to 25-kb Kasalath genomic fragments scanning the predicted ORF and the SNP into the non-shattering Nipponbare cultivar (Fig. 2C). Only transgenic lines that contained the Kasalath fragment with both the ORF and the SNP exhibited complete seed shattering, although one fragment, which contained a full ORF region but not the SNP, partly complemented the phenotype (Fig. 2C).

**In situ** hybridization analysis revealed that, in Nipponbare, the ORF was expressed in the rachis meristem (Fig. 2D), but it was not expressed in the provisional abscission layer (Fig. 2D). On the other hand, in the NIL the ORF was expressed at the stage of establishment of the rachis meristem (Fig. 2E), the stage of differentiation of glumes and formation of the provisional abscission layer at the base of the spikelet (Fig. 2E), and the stage of rapid elongation of rachis and branches (Fig. 2E). We therefore concluded that this RPL ortholog was the *qSH1* gene and that the identified SNP affected only the spatial mRNA expression pattern of *qSH1*, which was lost at the abscission layer in Nipponbare.

**Flowering-time control of rice**

We have previously identified that a novel B-type response regulator, *Ehd1*, promote flowering mainly under short-day conditions (Doi *et al.*, 2004). *Ehd1* encodes a B type response regulator, which consists of one receiver and one GARP DNA binding domain. *Ehd1* mRNA was induced by short-day treatments and exhibited two peaks a day; one before dawn and the other after dawn. This indicates that both photo signal transduction
and circadian clocks may control the *Ehd1* mRNA. To reveal the molecular mechanism further, we here over expressed several *Ehd1* cDNA derivatives driven by a maize *UBQ* promoter. The results have clearly shown that over-expressing a full length *Ehd1* cDNA induces flowering of rice under short-day conditions (SD). Interestingly, an *Ehd1* derivative, termed IGR, which lost only the receiver domain of *Ehd1*, significantly promoted flowering of rice under SD. In contrast, other two *Ehd1* derivatives, which lost C terminal region after the GARP domain and which lost the N terminal region before the GARP did not promote and repress flowering at all. These indicate that several functional domains may exist in the *Ehd1* gene product. In addition, over expression of the full length *Ehd1* and IGR did not flowered early under long-day conditions. Therefore, post transcriptional regulation of *Ehd1* mRNA and/or the modification of *Ehd1* gene product would be the target for long-day repression of flowering in rice. Exogenous applied cytokinin induced A type response regulators in rice.

Under the same conditions, *Hd3a* mRNA, a downstream gene of *Ehd1*, was not affected by the application regardless of the functional *Ehd1*. This suggests that *Ehd1* may not receive cytokinin signaling in rice. Therefore, what kind of chemical signals are received by *Ehd1* receiver domain is an open question.

**A novel gene that is up regulated by gibberellin is involved in rice growth**

The plant hormone gibberellin (GA) plays an important role in regulating many physiological processes in the growth and development of plants, including seed germination, shoot and stem elongation, and flower development. To understand how GA stimulates leaf sheath elongation, a cDNAs microarray containing 4000 clones randomly selected from a rice cDNA library prepared from seedlings treated with GA$_3$ was analyzed to identify new members involved in cell elongation. A novel GA enhanced gene, designated as *OsGAE1*, was identified using microarray analysis of GA regulated genes. *OsGAE1* expressed in a dose

**Fig. 3**

Histochemical localization of GUS activities in transgenic rice plants expressing GUS gene driven by the *OsGAE1* promoter region

Leaf sheath longitudinal sections (a), young seedlings (b) and panicle (c) of transgenic rice plants were incubated with GUS staining solution for 2-12 h. SAM; shoot apex meristem. PL; primary leaf.
and time course-dependent manner with minimum expression at 1 µM GA₃ and maximum expression at 50 µM GA₃ starting from 1 h and peaked at 24 h after GA₃ treatment. OsGAE1 expression was up regulated by GA₃ at transcript level while no significant effect was observed for other hormones. OsGAE1 was expressed in *Escherichia coli* with N-terminal His tag and the recombinant protein migrated at 38 kDa, slightly larger than the predicted 29 kDa, during SDS-polyacrylamide gel electrophoresis. Anti-OsGAE1 antibodies immunoreacted with a protein of 40 kDa in rice leaf sheath. OsGAE1 expressed mainly in growing leaf sheath and callus compared to leaf and root. *In situ* hybridization and OsGAE1 promoter analysis revealed that OsGAE1 expressed in shoot apex meristem and young primary leaves (Fig. 3). Northern blot, Western blot and GUS activities revealed that OsGAE1 transcript and translation are up regulated by GA₃. Transgenic rice expressing OsGAE1 in antisense orientation exhibited severely affected vegetative and reproductive growth (Fig. 4). The transgenic plants were 55 to 70 % short compared to control. These results suggest that OsGAE1 is differentially expressed in rice leaf sheath in relation to GA₃ and it encodes a functional protein which is involved in GA regulated growth and development of rice.

**Fig. 4**
Phenotype of transgenic rice constitutively expressing OsGAE1 in antisense direction
a) The transgenic plants were grown in an isolated greenhouse and photographed two months after transfer to soil.
b) Antisense transgenic plants exhibited stem bifurcation usually at 2nd or 3rd node.
c) Spikes of antisense transgenic plants remained in the leaf sheath of flag leaf.
Structural biology

X-ray crystallographic analysis of proteins

Crystal structure studies of several biologically important proteins have been carried out. α-Galactosidases catalyze the hydrolysis of α-linked galactosyl residues from galacto-oligosaccharides and polymeric galacto-(gluco)mannans. The crystal structure of Mortierella vinacea α-galactosidase I was determined at 1.6 Å resolution (Fig. 1). The structure consisted of a catalytic domain comprising a (β/α)₈-barrel structure and a C-terminal domain made up of eight β-strands containing a Greek key motif. Owing to the high resolution X-ray data, four carbohydrate chains were observed in one α-galactosidase I molecule and their structures were identified to be high mannose type. α-Galactosidase I seemed to form a tetramer around the crystallographic four-fold axis.

The crystal structure of elapid snake toxins pseudochetoxin (PsTx) and pseudocin (Pdc) have been determined at around 2 Å resolution. These proteins belong to the cysteine-rich secretory protein family isolated from the snake venom and target cyclic nucleotide-gated ion channels. The structure consisted of an N-terminal domain that has a fold similar to the group 1 plant pathogenesis-related proteins and a cysteine-rich C-terminal domain. The multidomain structure seemed to play an important role to recognize the target proteins.

3D-structure of barnacle cement protein, Mrcp-20k

Structure determination by X-ray crystallography and/or NMR spectroscopy is the powerful tool for research of proteins with unknown functions because protein function is strictly regulated by three-dimensional (3D) structure. Barnacle cement proteins, which are secreted for underwater adhesion, were recently isolated and cloned. The molecular functions of these proteins in adhesion are not to be established. We have determined the 3D-structure of one of these proteins, Mrcp-20k, in solution by NMR spectroscopy in order to obtain insight into its biological function.

Mrcp-20k contains 32 cysteine residues. They are assembled in regular repetitive positions in the primary structure, leading to

Fig. 1
The ribbon model of the crystal structure of Mortierella vinacea α-galactosidase I
Two catalytic residues, disulfide bridges and the sugar chains were shown as black ball-and-stick drawings in red, yellow and gray color, respectively.

Fig. 2
Structure of Mrcp-20k
Six homologous units are numbered, and the boundaries of each repeats are marked by dotted lines. Cystines are shown in yellow sticks.
the prediction of six repeated sequences with low homology. The NMR analyses of the protein revealed the repetitive structure with six homologous units corresponding to the six repeats. The calculated structure revealed that the conformation of the N-terminal part in each unit is supported by two disulfide bonds, while the C-terminal part forms a β-hairpin structure (Fig. 2). The C-terminal part of each unit has one more disulfide bond with the exception of the fifth and sixth units. The second unit has an additional β-hairpin. In the whole structure of the protein, the C-terminal three units formed a novel triangular domain (Fig. 2). The central part of the domain was supported by disulfide bonds and the three β-hairpins were exposed into solvent. Because several atoms in the N-terminal domain, especially in the third unit, were not observable, we modeled the structure of the N-terminal domain based on the similarity between the C-terminal and N-terminal halves of \( rMrcp-20k \). The resulted whole structure consists of two triangular domains which were connected by a linker sequence. The unique structure determined by us should accelerate further biochemical studies which lead to identification of the precise molecular function of \( M rcp-20k \).

**Molecular analysis of signal perception and transduction**

**Interaction between rice and rice blast fungus: Identification of a secretory protein from rice blast fungus as catalase**

Rice blast is one of the most important diseases for rice production in Japan as well as in the world. Recently, the whole genome sequence of the fungus was reported, providing an excellent model system for the analysis of pathogenic interaction between fungi and higher plants, in combination with a variety of the genome resources of rice. A common feature in the defense responses by the host plant is the rapid production of reactive oxygen species (ROS), which is believed to be a signal to the following events such as expression of defense related genes. However, the overall understanding of the biological significance of ROS is not sufficient. For example, ROS produced by the host plants has been postulated to be toxic to the invading microbes, however, some pathogenic bacteria and fungi show strong viability in the presence of ROS. *Botrytis cinerea*, a necrotic pathogen in a variety of crops, secretes several kinds of catalases which likely play important roles to avoid toxic activity of ROS. However, in the infection process of hemi-autotrophic fungi including rice blast fungus, no conclusive evidence on the role of ROS has been shown. To address the question, we examined ROS-degrading activities in rice blast fungus and found a stable catalase activity in the culture filtrate of the fungus. The activity was detected in the 6 different field isolates, which was likely to be carried in a single polypeptide because the in gel activity was detected in the presence of sodium dodecylsulfate, in contrast to catalase from mammals and higher plants that is homotetramer of 60kDa subunit. As shown in the figure (Fig. 3), antibody against bovine catalase did not react to any secreted polypeptide from rice blast fungus, supporting the idea that the catalase is phylogenetically distinct from that of higher organisms. We are going to identify the gene(s) encoding the catalase and analyze the role of the catalase by reverse genetics approach.

![Fig. 3](image-url) Western blot analysis of the total secreted proteins from rice blast fungus
Ten mg of total protein secreted into the culture media of P91-15B (lane 3) and INA86-137 (lane 4), together with 50 ng of purified bovine catalase (lane 1) and 10 mg of protein of culture media (lane 2). As shown here, no bands were detected by anti-bovine catalase antibody.
**Proteomic analysis**

Based on the proteomic approach, mainly silkworm have been analyzed using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry or ion-trap mass spectrometry coupled with high-performance liquid chromatography after the separation by two-dimensional polyacrylamide gel electrophoresis or two-dimensional high-performance liquid chromatography. The results obtained by proteomic analysis, the silkworm proteome database has been constructed using Make 2 ddb II software linking with silkworm genome data. In addition to these conventional approaches, new methods and apparatus have been developed for protein analysis, native two-dimensional electrophoresis to identify the protein interactions on gels, on-target sequential biochemical reactions for de novo sequences or identification of amino acid modifications after the measurements of proteins in a high-mass range, and the direct protein analysis which were extracted from a small tissue section using a mass spectrometry. Evidences of differential expression of proteins in the small tissues were showed by the help of hierarchical cluster analysis. Proteins were not only distributed with a gradient, but also they were specifically expressed in the part of tissue.

**Analysis of auxin-signaling pathway**

Synthetic peptides corresponding to the C-terminus of auxin-binding protein 1 (ABP1) have been shown to function as auxin agonists. The identification of a C-terminal receptor would be key importance in analyzing the auxin-signaling pathways. To define the receptor, photoaffinity crosslinking studies were performed and isolated two types of maize proteins as candidates for the receptor. One was a GPI-anchored plasma membrane protein (termed C-terminal peptide-binding protein 1, CBP1). CBP1 was found to be a copper-binding protein, and is highly homologous to the proteins functioning on cell elongation processes relating to plant cell polarity such as *Arabidopsis* SKU5, SKS6 and tobacco NTP303. Furthermore, a null mutation of the *ABP1* gene has been shown to disturb the directional cell elongation at the early embryogenesis. The present results indicate that an ABP1-CBP1 pathway may contribute to directional cell growth processes relating to cell polarity among the auxin-mediated cell expansion processes.
The research activities of our department are mainly focused on the elucidation of molecular mechanisms of important physiological processes in plants. The major topics in fiscal 2005 are as follows.

**Photosynthesis and carbon metabolism**

**Identification of critical residues for substrate recognition of CtpA, an integral protease for assembly of oxygen-evolving complex of photosystem II.**

Carboxyl-terminal processing protease A (CtpA) is an indispensable protease for assembling of the photosynthetic oxygen evolving machinery, which performs proteolytic processing of the precursor form of the D1 protein, a pivotal subunit of photosystem II. The CtpA is well known to have very narrow substrate specificity, which is one of the common properties of proteases involved in regulation of cellular processes. In order to approach molecular basis of the high specificity, we examined each residue of the spinach CtpA by several concepts that tendency of conservation in the CtpA family, distance from the catalytic center and accessibility of substrates. I400, V416 and Y419 were extracted as candidate residues involved in substrate recognition of the protease (Fig. 1A).

We introduced a series of site-directed mutations about these residues of the spinach *CtpA* gene and transformed the mutagenized genes into *ctpA* deleted *Synechocystis* sp. PCC 6803 cells, which helps rapid evaluation of effects of each mutation onto the CtpA activity. These analyses revealed that V416 and Y419 are not so important for the CtpA action, because these positions could be accepted various substitutions without deactivation of the CtpA. In contrast, I400 is likely to be critical for CtpA activity. It is noteworthy that I400T, a relatively mild substitution, significantly abolishes the activity and then *Synechocystis* cells with the mutant gene could not grow on photoautotrophic medium (−glucose). In contrast, V416T cells did not abolish the CtpA activity and then the mutant as well as WT cells grew well on the same medium.

![Fig. 1 (A)](image)

Three dimensional structure of the CtpA and positions of our target residues

Catalytic center residues, S372 and K397 were colored with red and blue, respectively. CtpA has two grooves close to the catalytic center. I400, V416 and Y419 (colored with orange) emerged on surface of the narrow groove.

![Fig. 1 (B)](image)

Photoautotrophic growth test of the several mutants *Synechocystis* cells carrying the I400T mutant CtpA gene could not grow on photoautotrophic medium (−glucose). In contrast, V416T cells did not abolish the CtpA activity and then the mutant as well as WT cells grew well on the same medium.
photoautotrophic medium (Fig. 1B). It clearly suggested that hydrophobic environment supplied from this aliphatic residue is required for maintenance of the CtpA activity. It is most probable that the 1400 is a key residue for substrate recognition of the CtpA and contributes the high specificity of the protease.

**Genetic analysis of C3-C4 intermediate photosynthesis**

C3-C4 intermediate plants have lower activities of photorespiration than C3 plants. We investigated structural and photosynthetic characteristics in leaves of reciprocal hybrids differing in genome constitution produced between *Moricandia arvensis* (C4; MaMa) and *Brassica oleracea* (C3; CC). We found that the hybrids had intermediate features between the parents and expressed more strongly the C3-C4 intermediate characteristics with an increase in the constitution ratio of the MaC genome. The same pattern of expression of the C3-C4 intermediacy was found between the hybrids that the parents were reciprocally exchanged. We conclude that the C3-C4 intermediate characteristics are inherited in the hybrids depending on the constitution ratio of the parent genomes and there is no evidence of maternal inheritance in these characteristics.

**Isolation of genes that are differentially expressed during C3 and C4 leaf development**

C4 leaves have a specialized structural feature, Kranz anatomy, that differs from C3 leaves. *Allotheropsis semialata* is a unique grass including both Kranz (C4) and non-Kranz (C3) forms within this one species. We used subtractive hybridization to isolate genes that are differentially expressed during leaf development of the two forms. Northern blot analyses of the subtracted clones revealed that six and 13 genes were C4- and C3-abundant, respectively, most of which showed high similarities to genes involved in mechanisms of regulation, signaling and transport, carbon metabolism, and protection from abiotic or biotic stress. We are now attempting to characterize these genes more in detail to elucidate possible involvements in C4 leaf development.

**Identification and physiological analyses of a locus for rice yield potential**

We analyzed quantitative trait loci (QTLs) for the ratio of filled grains, a yield component, in backcrossed inbred lines of ‘Nipponbare’ and ‘Kasalath’. Only one QTL (rg5), with a positive Kasalath allele, was detected across environments. In a near-isogenic line (NILrg5) with a Kasalath chromosome segment containing *rg5*, carbohydrate storage capacity before heading or sink activity was significantly higher than in Nipponbare (control). The ratio of filled grains and yield per plant were significantly higher in NILrg5 than in Nipponbare, by 5% (*P* < 0.01) and 15% (*P* < 0.05), respectively. These results suggest that *rg5* improves carbohydrate storage capacity and keeps sink activity higher in the reproductive stage, and consequently increases yield potential.

**Light response**

**Distinct functions of different phytochrome species in rice**

We have isolated phytochrome B (*phyB*) and *phyC* mutants from rice (*Oryza sativa*) and have produced all combinations of double mutants. Seedlings of *phyB* and *phyB phyC* mutants exhibited a partial loss of sensitivity to continuous red light (Rc) but still showed significant deetiolation responses. The responses to Rc were completely canceled in *phyA phyB* double mutants. These results indicate that phyA and phyB act in a highly redundant manner to control deetiolation under Rc. Under continuous far-red light (FRc), *phyB* mutants showed partially impaired deetiolation, and *phyA phyC* double mutants showed no significant residual phytochrome responses, indicating that not only phyA but also phyC is involved in the photoperception of FRc in rice. Interestingly, the *phyB phyC* double mutant displayed clear R/FR reversibility in the pulse irradiation experiments, indicating that both
phyA and phyB can mediate the low-fluence response for Lhcb gene expression (Fig. 2). Rice is a short-day plant, and we found that mutation in either phyB or phyC caused moderate early flowering under the long-day photoperiod, while monogenic phyA mutation had little effect on the flowering time. The phyA mutation, however, in combination with phyB or phyC mutation caused dramatic early flowering.

Salt tolerance

Isolation of cation transporter genes functioning in salt tolerance

Some cation transporters are known to mediate ion homeostasis in cytoplasm and contribute to the salt tolerance in plants. Finding a novel cation transporters involved in the salt tolerance is important to unravel and improve the salt tolerance in crops. In this purpose, we carried out the screening of a rice full-length cDNA expression library through functional complementation in yeast. We found a cDNA clone encoding the rice homologue of the *Arabidopsis* potassium channel protein KAT1 (OsKAT1) suppressed the salt-sensitive phenotype of yeast strain G19 that lacks a major component of Na⁺ efflux (Fig. 3). The intracellular K⁺ contents under salt stress conditions increased in OsKAT1-expressing yeast during the logarithmic growth phase, suggesting the enhancement of K⁺ uptake by OsKAT1. At the late linear phase, however, the transformants accumulated lesser amounts of Na⁺ than mutant cells whereas the K⁺ amounts of these cells were almost the same. Intracellular Na⁺/K⁺ ratio in the OsKAT1 expressing cells was maintained at a steady level throughout the culture, whereas that in the mutant cells changed dramatically. These results suggest that OsKAT1 may participate in maintenance of cytosolic ion homeostasis during salt stress and protect the cells from Na⁺. The function of OsKAT1 observed in yeast is expected to take place also in rice.

Disease resistance

BTH-inducible WRKY transcription factor plays a critical role in blast resistance of rice

The signaling pathway mediated by salicylic acid (SA) plays a critical role in activating defense responses in some dicot plants. Benzothiadiazole (BTH) is one of the chemicals known as ‘plant activator’ that protect plants from diseases by activating the SA signaling pathway in a manner termed ‘priming’. BTH is effective also in rice, however,
little is known about the roles of SA and BTH in the defense system in rice. To investigate the molecular mechanisms underlying the BTH-induced defense program in rice, we first analyzed gene expression profiles by microarray following BTH application to rice plants and revealed a number of BTH-responsive genes including WRKY transcription factor genes. The WRKY genes were induced within 3 h after BTH application, preceding some pathogenesis-related (PR) genes. RNAi-mediated silencing of one of them, OsWRKY45, compromised BTH-induced resistance to a compatible race of rice blast fungus (Fig. 4A), indicating that this transcription factor plays a critical role in blast resistance of rice. Moreover, overexpression of OsWRKY45, driven by the ubiquitin promoter, markedly enhanced the blast resistance (Fig. 4 B and C). The OsWRKY45-overexpressing rice plants showed upregulation of PR1b and PR2 genes when grown in a growth chamber, but no upregulation of these genes was observed when grown in a greenhouse, suggesting some environmental factor(s) that influences the PR-gene expression. Only small adverse effects of the transgene expression on the growth were observed in these transformants. These results suggest that the overexpression of OsWRKY45 mimicked the ‘priming’ effect of BTH in activating the defense program in rice.

Assessing real time variability in various genome regions
All organisms need a system to protect them from various pathogens, by differentiating them from their self-components. Also each plant line has several disease resistance (R) genes to recognize infection of specific pathogens through their corresponding avirulence gene products, directly or indirectly, and induce various defense reactions, including hypersensitive death of the infected cells. This recognition system of the pathogen molecules is
analogous to vertebrates’ immunity system based on the recognition of pathogen’s specific molecule (antigen) with the highly variable antibody: immunoglobulins. However, although plants lack special diversifying system of the R-genes as the case of vertebrate globulin molecules, we have shown that the genome regions around the R-genes are high in the mutation frequencies (2004 Annual Report), especially in the base substitution rate. In order to clarify whether this accumulation of variability around the R-genes are due to the simple selective accumulation, or reflecting the higher mutation rate in this region, we have assessed the real-time mutation (base-substitution) rate in the *Arabidopsis* genome and checked the correlation of this with the distance from the R-genes. β-glucuronidase (GUS) genes with a stop codon mutation was transformed into *Arabidopsis*, and the transformants were checked for their insertion sites in the genome. As shown in the Fig. 5, the somatic mutation during the individual development was assessed by the development of revertant of the GUS genes as the blue spot of digested X-Gluc. Some correlation of higher substitution rate with the R-gene was suggested, and more confirmation is now being done. If this was confirmed, it will become the first case of verification of the plant R-gene mutation development system.

**Catalytic activation of plant MAPK phosphatase NtMKP1 by its physiological substrate SIPK**

In plants, two MAPKs, wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK), are key molecules of signal transduction upon wound and disease responses. The activity of MAPKs is strictly regulated via phosphorylation by an upstream MAPK kinase. Conversely, MAPKs are dephosphorylated and inactivated by protein phosphatases. Previously, we identified NtMKP1 (MAPK phosphatase) as a novel calmodulin (CaM)-binding protein. Here, we investigated the interaction between NtMKP1 and substrate MAPKs or CaMs. NtMKP1 inactivated SIPK through dephosphorylation. CaM interacted with NtMKP1, but did not activate its phosphatase activity. NtMKP1 is composed of four domains: a dual-specificity phosphatase catalytic domain, a gelsolin homology domain, a CaM-binding domain, and C-terminal domain. Deletion analysis revealed that the N-terminal non-catalytic region of NtMKP1 interacted with SIPK and was essential for inactivating SIPK, whereas the CaM-binding and C-terminal domains were dispensable. Moreover, the phosphatase activity of NtMKP1 was increased strongly by the binding of SIPK, but weakly by WIPK, another MAPK. The strong activation of NtMKP1...
phosphatase activity by SIPK depended partially on the putative common docking domain of SIPK. On the other hand, conversion of Lys41 and Arg43 of NtMKP1 to Ala (K41A/R43A) abolished the interaction with SIPK. Expression of constitutively active MAPK kinase in *Nicotiana benthamiana* induced activation of SIPK and cell death. Simultaneous expression of either NtMKP1 (Fig. 6) or NtMKP1 L443R, which was unable to bind CaM, compromised the constitutively active MAPK kinase-induced responses, whereas that of NtMKP1K41A/R43A did not. These results indicate that the regulation of NtMKP1 activity by SIPK binding, but not by CaM binding, is important for the function of NtMKP1.

**Symbiotic nitrogen fixation**

A novel Fix- symbiotic mutant of *Lotus japonicus, Ljsym105*, shows impaired development and premature deterioration of nodule infected cells and symbiosomes

Nitrogen-fixing symbiosis between legume plants and rhizobia is established through complex interactions between two symbiotic partners. To identify the host legume genes that play crucial roles in such interactions, we isolated a novel Fix- mutant *Ljsym105* from a model legume *Lotus japonicus* MG-20 by somaclonal mutation through extensive culture of suspension cells followed by regeneration of the plant. The *Ljsym105* mutants displayed nitrogen deficiency symptoms after inoculation with *Mesorhizobium loti* under nitrogen free conditions, but their growth recovered when supplied with nitrogen-rich nutrients (Fig. 7A). The mutant *Ljsym105* formed an increased number of small and pale-pink nodules (Fig. 7B). Nitrogenase (acetylene reduction) activity per nodule fresh weight was low but retained more than 50% of that of the wild type nodules. Light and electron microscopic observations revealed that the *Ljsym105* nodule infected cells were significantly smaller than those of wild type plants, contained enlarged symbiosomes with multiple bacteroids, and underwent deterioration of the symbiosomes prematurely as well as disintegration of the whole infected cell cytoplasm (Fig. 7C and D). These results indicate that the ineffectiveness of the *Ljsym105* nodules is primarily due to impaired growth of infected cells accompanied with the premature senescence induced at early stages of nodule development. We delimited the *Ljsym105* locus in a few hundred kb region on the upper portion of chromosome 4, and are in progress towards molecular identification of the *Ljsym105* gene.

Fig. 7

(A, B) Growth and nodulation phenotypes of wild type MG-20 (left) and *Ljsym105* (right) plants inoculated with *M. loti*. Bars = 10 mm (A) and 4 mm (B). (C, D) Micrographs of nodule infected cells of wild type MG-20 (C) and *Ljsym105* (D). Wild-type nodule infected cells are densely packed with bacteroids, whereas *Ljsym105* infected cells show less-dense and aggregated bacteroids (arrow). U, uninfected cells; V, vacuoles. Bars = 10 µm.
The research activities of our department are mainly focused on the development of basic studies related to plant biotechnology as well as the generation of novel transgenic crops with superior traits which conventional breeding techniques can not produce. The major topics in fiscal 2005 are as follows.

**Characterization of transcription factors responsible for endosperm-specific expression of seed proteins**

Cis-regulatory elements involved in the endosperm-specific regulation of cereal storage protein genes have been mainly characterized by producing stable transgenic plants or by transient expression assays using particle bombardment. A number of consensus sequences such as the prolamin box (P-box), GCN4, AACA and ACGT motifs, among others, have been identified as elements that are critical in determining the endosperm specificity of cereal seed storage protein genes. A conserved element, referred to as the endosperm box, located 300 basepairs upstream of the transcriptional start site, has been found in many cereal prolamin storage protein genes. The endosperm box has a bifactorial motif that is composed of the P-box (TGTAAAG) and the GCN4 motif (TGA(G/C)TCA), which are separated by less than 10 nucleotides.

We characterized here the transcription factor which is involved in binding to the P-box. This Dof zinc finger protein called RPBF (rice prolamin box binding factor) has been isolated from rice cDNA EST clones containing the conserved Dof domain by examining their trans-activation abilities with storage protein promoters (Fig. 1). RPBF is found as a single gene per haploid genome. Comparison of RPBF genomic and cDNA sequences revealed that the genomic copy is interrupted by one long intron of 1892 bp in the 5' noncoding region. We demonstrated by transient expression in rice callus protoplasts that the isolated RPBF trans-activated several storage protein genes via an AAAG target sequence located within their promoters, and with methylation interference experiments that additional AAAG-like sequences in promoters of genes expressed in maturing seeds were recognized by the RPBF protein. Binding was sequence-specific, since mutation of the AAAG motif or its derivatives decreased both binding and trans-activation by RPBF. Synergism between RPBF and RISBZ1 recognizing the GCN4 motif (TGA(G/C)TCA) was observed in the expression of many storage protein genes. Over-expression of both transcription factors gave rise to much higher levels of expression than the sum of individual activities elicited by either RPBF or RISBZ1 alone (Fig. 2). Furthermore, mutation of recognition sites suppressed reciprocal trans-activation ability, indicating that there are mutual interactions between RISBZ1 and RPBF. The RPBF gene is predominantly expressed in maturing endosperm and coordinately expressed with seed storage protein genes, and is involved in the quantitative regulation of genes expressed in

![Fig. 1](image-url)
Molecular analysis of rice/blast interactions

Plants activate self-defense systems to resist pathogens upon sensing them. There are several different classes of resistance: non-host resistance, variety-specific resistance mediated by R (resistance) genes, and basal (or general) resistance. Among these, basal resistance is not strong enough to avoid infection completely but understanding it at the molecular level is important to manipulate the durability of disease resistance.

It is believed that basal resistance is established after plants perceive invading microbes through non-specific elicitors generated during infection process. One of them is fragmented chitin (V-acetylchitooligosaccharide), which derives from a common component of fungal cell walls. We have focused on the contribution of chitinolytic enzymes and their products, chitin elicitors, in rice/blast interactions. Previous studies revealed that chitin elicitor causes various defense-related cellular responses and induces blast resistance, and overexpression of chitinase results in enhanced fungal disease resistance in many species. Clarification of the signal transduction pathway starting with chitin elicitor will result in a better understanding of basal resistance lied in rice/blast interactions.

This year we evaluated involvement of the chitin-elicitor binding protein (CEBiP) in the basal resistance. Expression of CEBiP was repressed by RNAi and using leaf sheaths of the stable transformants we scored blast disease resistance. As a result, we observed that growth of blast fungus was promoted in the CEBiP RNAi lines 48 hr after inoculation. This result indicates that recognition of chitin elicitor contributes to the expression of basal resistance in rice/blast interactions.

Transgenic crops expressing P450 monooxygenases (CYP) for phytoremediation

The increased use of pesticides and herbicides on the agricultural scene has caused serious problems for plants, fish, insects, mammals, and sometimes to humans. These chemicals are usually removed from the environment by natural degradation and degradation by bacteria and plants. To decrease the load on the environment, the enhancement of degradation of these chemicals by plants should be as effective as the reduction in their usage.

Phytoremediation is the use of living plants to remove and or detoxify organic and inorganic compounds. It is a possible method for reducing the risks of exposure of people and the environment to pesticides. Current physical clean-up methods such as removing contaminated soil from a site and chemical remediation treatments are very costly and sometimes environmentally destructive. Plants can remove organic pollutants, including pesticides, by root and leaf uptake of contaminants, biochemical degradation and subsequent accumulation of non-phytotoxic...
metabolites in plant tissue.

Cytochrome P450 monoxygenase plays an important role in the oxidative metabolism of xenobiotics in higher plants as well as in mammals. The enzyme system on microsomes consists of many P450 species and a few NADPH-cytochrome P450 oxidoreductase molecules. Agrochemicals including herbicides were metabolized by P450 species, conjugated with glutathione or sugars, and compartmentalized into vacuoles, or cell walls, in plant body. The oxidation by P450 species is considered to be the limiting step of metabolism of foreign chemicals.

Rice genome contained 246 P450 genes, but the P450 species involved in xenobiotic metabolism have not been studied well yet. However, mammalian P450 species involved in the xenobiotic metabolism have a high activity to metabolize various herbicides with different modes of action and in different chemical functional groups.

We produced three types of transgenic rice plants expressing human P450 species, CYP1A1 or CYP2B6 or three P450s, CYP1A1, CYP2B6 and CYP2C19 simultaneously. The transgenic rice plants had an enhanced ability to metabolize herbicides with different chemical structures owing to the introduced P450s. The chemicals were supposed to be absorbed by the transgenic plants in the fields and metabolized rapidly into non-phytotoxic compounds. Therefore, they exhibited a remarkable cross-tolerance toward various herbicides.

The transgenic rice plants expressing P450s involved in xenobiotic metabolism are quite useful for reducing the residual herbicides in plants. In addition, they can be used for phytoremediation of various chemicals that are widespread in agricultural environments.

**Development of male sterile transgenic crops for the gene confinement in transgenic crops**

The possibility of gene transfer from transgenic corps to wild relatives or non-transgenic crops has often been cited as an environmental and consumers’ concern. Commercialized transgenic crops have already been confirmed food and feed safety and influence on biodiversity by authorities. However, gene confinement technology must be important to achieve co-existence and utilization of future generations of transgenic crops.

We have been developing to develop male sterile transgenic crops using several molecular approaches for gene confinements. We cloned tapetum or anther specific expressed genes from *Brassica oleracea* and *B. rapa* and isolated promoter region by 5’-Race. Then tissue and time specificity of isolated promoters were confirmed by GUS expression. We could not detect GUS expression except in anther using several promoters such as BoA3, BoMS2, BrA6, BrMS2 (Fig. 3 A). But other promoters induced GUS expression not only tapetum and anther but in petal, ovule and other tissues. We have also isolated nine genes, which have the potentiality of inducing male sterility, including ribonuclease, protease, apoptosis related genes and phytohormone biosynthesis related genes from *Brassica* genus. And we constructed vectors to combine the promoters and those isolated gene. Subsequently we demonstrated effects of combination of promoters and those...
isolated genes in transgenic *Arabidopsis thaliana*. Analysis of transgenic *Arabidopsis thaliana* revealed that expression of protease in tapetum effectively induced male sterility (Fig. 3B). We will introduce those constructions to *Brassica* crops and investigate the stability of male sterility by the cultivation in semi-closed green house and in natural condition such as an isolated field.

**Comparative genomic approach to agronomically important genes in wheat and barley**

Colinearity in gene content and order between rice and closely related cereal crops has been as a powerful tool for gene identification. Using a comparative genomic approach, we have identified the rice genomic region syntenous to the region of the short arm of wheat chromosome 2D, on which a QTL locus for *Fusarium* head blight (FHB) resistance is located. Utilizing markers known to reside near the FHB QTL and data from wheat genetic maps, we have identified the syntenous region of the short arm of rice chromosome 4 to the FHB QTL locus on wheat 2DS. Fifteen predicted rice genes with similarity to known disease-related genes have been identified in an approximately 6-Mb rice sequence spanning the syntenous region. Rice sequences of these putative genes were used in BLAST searches to identify wheat expressed sequence tags (ESTs) exhibiting significant similarity. RT-PCR analysis and gene mapping of wheat homologues to rice disease-related genes in this region revealed a possible candidate for the FHB QTL on wheat chromosome 2DS. We also applied this approach to explore the genes for heading of wheat. Wheat and rice are closely related species to each other, but rice plant shows a heading under short-day condition, while long-day condition stimulates the heading of wheat. We just cloned several genes orthologous to rice heading-related genes, and analyzed the expression profile of them.

**Construction of macroarray system for survey genes regulated by HrpG or HrpX**

To survey genes regulated by HrpG or HrpX, we constructed a DNA macroarray system consisting of 2,384 of genomic DNA fragments of strain T7174 (MAFF311018) of *Xoo*. It comprised about 95.5% of the whole genome DNA. Using this macroarray system, it was confirmed that the *hrp* gene cluster (about 87k to 120k region of the *Xoo* genome) was upregulated in wild type strain under *hrp*-inducing medium, while it was not up regulated in the ΔhrpX and ΔhrpG mutants (Fig. 4). Moreover, it was revealed that thirteen genomic regions were regulated by HrpG or HrpX. To check that expression of genes within these regions were really controlled by the HrpG or HrpX, a real-time quantitative RT-PCR system was used. Finally, six genes (XOO0037, XOO0078, XOO1388, XOO2263, XOO4042 and XOO4134) were newly identified. In addition, two genes, XOO2263 and XOO4134 contained a plant-inducible-promoter (PIP) box, which was a consensus sequence of HrpX regulons, upstream region of putative initiation codon. These obtained results showed that this macroarray system was useful tool for genome-wide gene expression analysis in *Xoo*.

![Schematic representation of regions with up- and down-regulated gene expression in hrp inducing condition (ΔhrpX / wild type strain)](image-url)
Institute of Radiation Breeding

The research activities of Institute of Radiation Breeding are focused on the development of new strains of seed-propagated, vegetatively propagated and woody crops through mutation by the application of various forms of irradiation. Mutations are induced by the following radiation sources: Gamma Field and the Gamma Greenhouse for γ-ray chronic irradiations to the growing plants, and Gamma Room for γ-ray acute irradiations to seed, bulbs, tubers, scions and in vitro materials. The institute is also involved in the development of new technologies for plant breeding mainly utilizing γ-ray and ion beam irradiation and chemical mutagens, including the elucidation of gene expression mechanisms in mutants. The institute provides irradiation service and cooperative research at the request of universities, private industries, prefectural experiment stations, and incorporated agencies of Ministry of Agriculture, Forestry and Fisheries. The major topics in fiscal 2005 are as follows.

Characterization of Chlorophyllide a Oxygenase (CAO) in Rice

Chlorophyll (Chl) plays a central role in capturing light energy for photosynthesis. Land plants contain two types of chlorophyll, Chl a and Chl b. The Chl a is a component of the core and the peripheral antenna complexes. The Chl b is contained only in peripheral antenna complexes, the light-harvesting chlorophyll a/b-protein complexes (LHCPs). The LHCPs are classified into light-harvesting complex of photosystem I (LHCl) and the light-harvesting complex of photosystem II (LHCII). The only structural difference between Chl a and Chl b is a methyl group on a C7 of the tetapyrrole in Chl a and a formyl group at the corresponding site in Chl b. The key enzyme involved in Chl b synthesis is chlorophyllide a oxygenase (CAO). It catalyzes a 2-step oxygenase reaction that converts chlorophyllide a (Chlde a), a precursor of Chl a, to Chlde b. The CAO has a mononuclear iron-binding motif and a Rieske binding motif, which are thought to be important for its activity. Null mutants of CAO in Arabidopsis thaliana lack Chl b and have pale green leaves.

We isolated two pale green rice mutants, “YM-15” and “G-52”. These mutants were induced from cultivars “Hitomebore” and “Reimei” by gamma ray-irradiation. We extracted chlorophyll from mature leaves of these mutants with methanol and analyzed the chlorophyll composition. The analysis revealed that both mutants lack Chl b, suggesting that they have defects in Chl b synthesis. The most likely candidate for the mutated gene in these mutants is CAO.

To examine the CAO sequence in these mutants, we searched for CAO homologs in the rice genome database by using the Arabidopsis CAO sequence as a template in a BLAST search. Unexpectedly, we found two CAO homologs, which are tandemly repeated with a 6.6-kbp interval in a BAC clone, OSJNBa0057L21. The predicted amino acid sequences of these genes, OSJNBa0057L21 Predgene09 (CAO-09) and Predgene11 (CAO-11), show 86.7% sequence similarity to each other, and 74.2% and 70.0% similarity, respectively, to Arabidopsis CAO. The full-length clones corresponding to CAO-09 and CAO-11 (J013001P04 and 001-114-D11, respectively) have been registered in the rice full-length cDNA database, suggesting that both genes are expressed. Sequence analysis of CAO-09 in the mutants revealed that “YM-15” has a 1-bp deletion in the 2nd exon, which results in a frame-shift and a premature stop codon. In “G-52”, a 3-bp deletion was found in the 7th exon of CAO-09. On the other hand, no mutation was observed in the coding region of CAO-11 in either mutant. These observations suggest that CAO-09 encodes CAO.

To verify this hypothesis, we searched for
retrotransposon *Tos17*-induced mutants that disrupt these two CAO-like genes within the *Tos17* insertion mutant database. We found three mutants (ND0017, NE1005, ND3004) of *CAO-09* and one (ND1064) of *CAO-11*. We selected plants homozygous for each allele and analyzed their phenotypes. Consistent with the analysis of "YM-15" and "G-52", homozygotes of all three mutations for *CAO-09* showed pale green phenotype and lacked Chl \( b \). On the other hand, the homozygotes of the *CAO-11* mutation did not show a pale green phenotype and contained a normal level of Chl \( b \). The 3-bp deletion in *CAO-09* of G-52 results in a deletion of the lysine residue (K), which is located in the mononuclear iron-binding site in CAO. This residue is not necessarily highly conserved in proteins containing mononuclear-iron-binding sites, but is limited to arginine or lysine, both of which are positively charged amino acids, among CAOs of distantly related species. Complete loss of Chl \( b \) in G-52 suggests that this positively charged residue is important for CAO activity or stability.

The rice genome is estimated to have many duplicated genes, which are thought to have redundant functions. However, the example described here suggests that not many of such genes are functionally redundant, even if they are expressed.

**Comparison of the Mutation Inducing Effect between Ion Beams and Gamma Rays**

Mutation induction is a useful method for plant breeding and the type of mutagenic treatment is an important factor for successful...
results.

One unique characteristic of ion beam irradiation is their high level of linear energy transfer (LET) and the potential to focus that high energy on a target site. As a consequence, ion beam irradiation can induce a high level of mutagenic effect. For this reason, there is an expectation for a higher degree of effect on DNA and a higher level of mutation induction when compared to gamma rays having a low level of LET. However, the use of ion beam irradiation as a mutagen for mutation breeding has not been examined. Therefore, we have investigated the nature of ion beams as a treatment for mutation breeding and compared it with gamma ray irradiation treatments.

Seeds of the rice variety, cv. ‘Hitomebore’ were placed on 6 cm diameter petri dishes and irradiated with 220 MeV carbon-ion beams ($^{12}$C$^{5+}$, LET; 121 keV/µm); 320 MeV carbon-ion beams ($^{12}$C$^{6+}$, LET; 86 keV/µm); and 100 MeV helium-ion beams ($^{4}$He$^{2+}$, LET; 9 keV/µm) generated by an AVF-cyclotron. Gamma rays were applied to dry seed at dosage rate of 10 Gy per hour in a gamma-room.

The effect of mutation induction was different among irradiation treatments (Table 1). Within the irradiation dose range utilized in this experiment, the mutation frequency per M1 spike using 100 MeV helium-ion beams was the highest at 12.4 %. The frequency was 10.4 % using 320 MeV carbon-ion beams, 9.0 % using carbon-ion beams, and 8.4 % using gamma rays.

The relative frequency and type of chlorophyll mutations (albino, xantha, viridis and others) generated by each treatment, are shown in Table 2. Among the four irradiation treatments, the spectrum of chlorophyll mutations also did not indicate significant differences in treatments. From these results, the relative frequency of each type of chlorophyll mutation induced by radiation was the same without regard to the type of radiation.

### Table 1 The effect of ion beam and gamma ray irradiation on mutation induction

<table>
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<th>Treatment</th>
<th>Dose (Gy)</th>
<th>Chlorophyll mutation frequency per M1 spike (%)</th>
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<tr>
<td>220 MeV carbon-ion beams</td>
<td>40</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.0</td>
</tr>
<tr>
<td>320 MeV carbon-ion beams</td>
<td>100</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.4</td>
</tr>
<tr>
<td>100 MeV helium-ion beams</td>
<td>250</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.1</td>
</tr>
<tr>
<td>Gamma rays</td>
<td>300</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>7.7</td>
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<td>150</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.3</td>
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### Table 2 The relative frequency and type of chlorophyll mutant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>albina</th>
<th>xantha</th>
<th>viridis</th>
<th>others</th>
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<tbody>
<tr>
<td>220 MeV carbon-ion beams</td>
<td>53.6</td>
<td>8.7</td>
<td>25.0</td>
<td>12.8</td>
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<tr>
<td>320 MeV carbon-ion beams</td>
<td>50.4</td>
<td>11.7</td>
<td>25.1</td>
<td>12.8</td>
</tr>
<tr>
<td>100 MeV helium-ion beams</td>
<td>47.7</td>
<td>15.0</td>
<td>24.6</td>
<td>12.6</td>
</tr>
<tr>
<td>Gamma rays</td>
<td>52.5</td>
<td>8.2</td>
<td>30.6</td>
<td>8.7</td>
</tr>
</tbody>
</table>

**In vitro culture of a periclinal chimera for obtaining non-chimera plantlets at a high frequency**

The generation and development of plant chimeras, following irradiation, is a commonly encountered obstacle for obtaining a complete mutant in the mutation breeding especially for vegetatively propagated crops. Among the three types of chimera, sectorial, periclinal and mericlinal chimeras, a periclinal chimera is most stable. The induced mutant comprising the periclinal chimera is not easily separated during the natural development, even if simple cutting-back techniques are employed. In vitro culture techniques are believed to be able to isolate a complete mutant from a chimera. In our study, we demonstrated that only one genotype was separated from the other genotype in the chimera.

In 2001, we identified a periclinal
cytochimera among the mulberries (*Morus alba* L.) consecutively irradiated since 1979 in the Gamma Field. The chimera has both tetraploid cells and original diploid cells. The tetraploid cells were localized in the L1 layer of the shoot apical meristem (SAM) and diploid ones were identified in the L2 and L3 layers. Generally, angiosperm, such as mulberry, has a SAM constructed with three cell layers, commonly called L1, L2, and L3 from the epidermal cells to central cell layer. In addition, the L1 cells develop into the epidermal cells of a plant body while L2 and L3 develop into the internal plant cells. The macro-morphological phenotypes of the cytochimera were found to be similar to the original diploid cultivars. The sizes of the stomata guard cells, which are derived from the L1, were larger than that of the original diploid. The longitudinal section of the SAM indicated tetraploid L1 and diploid L2 and L3 cell layers (Fig. 2). We induced the regenerated plantlets via *in vitro* direct adventitious bud formation by culturing immature leaves of the cytochimera. By applying a flow cytometric analysis, we recognized that most of the regenerated plants were tetraploid suggesting that they tended to be derived from the L1 cells. Other regenerated plantlets retained the original chimerism. There was no plantlet derived from only the L2 or L3 cells among the regenerated plantlets. In this case, we successfully obtained the complete tetraploid from the cytochimera by the *in vitro* culture method.

Similar results were obtained on a mutant cultivar of Japanese pear (*Pyrus pyrifolia* Nakai), cv. ‘Osa Gold’. The cultivar is a black-spot-disease-resistant mutant derived from cv. ‘Osa Nijisseiki’ that is known as a self-compatible mutant derived from cv. ‘Nijisseiki’ in which self compatibility is caused by the complete deletion of *S* gene. However, PCR analysis of cv. ‘Osa Nijisseiki’ reported that the original wild-type (self-incompatible) cells have been retained and are maintained in a chimera state (Sassa, et al. 1997). In our studies, we found that cv. ‘Osa Gold’ is also a chimera similar to cv. ‘Osa Nijisseiki’ because a weak amplified fragment of *S* gene was identified by PCR analysis (Fig. 3). Using *in vitro* culture methods, we successfully obtained regenerated plantlets via adventitious buds that were induced on the leaves of *in vitro* cultured winter buds. The PCR analysis indicated that most of the plantlets were wild type (Fig. 3). The other regenerated plantlets were chimera similar to the cv. ‘Osa Nijisseiki’, judging from the PCR fragment pattern. In this case, we were not successful in obtaining the non-chimera cell plantlets by the *in vitro* culture method.

These results indicate that by *in vitro* culture of a periclinal chimera, we can obtain non-chimera plantlets at a high frequency or not at all. *In vitro* culture techniques are not always a ‘veritable panacea’. We need to develop new and more robust techniques having application to any types of chimeras.

**Fig. 2.** Longitudinal sections of SAMs of a diploid wild type (left) and cytochimera with tetraploid cells in L1 layer

**Fig. 3.** PCR analysis of the original chimeric pear, cv. Osa Gold, and regenerated plantlets (1-4) after *in vitro* culture

Regenerated plantlet 1: same as original cv. Osa Gold; 2-4: same as Nijisseiki, from which Osa Gold is derived

*S* 2, 4, 5: Self incompatible gene alleles 2, 4, 5
## List of Publication

### Original papers

#### 1) Western language

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<th>Title</th>
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Main meetings related NIAS

The 13th International Genetic Resources Workshop on Rice Genome and Plant Genetic Resources

NIAS sponsored the 13th NIAS International Workshop on Genetic Resources as parts of symposium sessions during the 10th International Congress of SABRAO (The Society for the Advancement of Breeding Research in Asia and Oceania) which was held on August 22-23, 2005 in Tsukuba. In this WS, two research highlights on “Rice Genome: Challenges and Opportunities” and “Perspectives of Utilization and Conservation of Plant Genetic resources” was discussed by 16 contributors (7 from outside Japan).

The NIAS-COE International Symposium: “Present Status of Studies for Utilization of Insect Properties”

The NIAS-COE International Symposium was held on October 4-5, 2005, at Tsukuba International Congress Center (Epocal Tsukuba). The purpose of this symposium was to discuss recent progress in the fields of utilization of insect properties involved in Baculoviruses, gene expression, cell lines, tissue engineering, silk hydrogel, insect immunity, trypanosoma, development, and gene expression, 13 oral presentations, 6 from overseas and 7 from Japan, were given. This symposium stimulated the activities of many participants on utilization of insect properties and gave several new ideas as to the next step.

International Workshop on Animal Genome Analysis 2005: “Analysis of MHC, MHC-related genes and disease resistance for animal breeding and selection”

International workshop on animal genome analysis 2005 was held under the title “Analysis of MHC, MHC-related genes and disease resistance for animal breeding and selection” on November 9, 2005 at Jitensha Kaikan in Tokyo with 81 participants. Eight invited speakers (1 from overseas and 7 Japanese) gave presentations on genome structure and function in infection defence of MHC, MHC-related genes.

44th Gamma-Field Sympo (Mito, July 12-13, 2005)

2005 Silk Summer Seminar in Okaya (July 28-29, 2005)

Rice Genome Sympo (Tsukuba, March 22, 2006)
# Executive Members and Research Staff Members

*(as of March 31, 2006)*

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<td><strong>Environmental Physiology Laboratory</strong></td>
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<td><strong>Disease Physiology Laboratory</strong></td>
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<td><strong>Plant Biotechnology Department</strong></td>
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<td><strong>Institute of Radiation Breeding</strong></td>
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Members of NIAS Evaluation Committee

(as of March 31, 2006)

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<tr>
<th>Name</th>
<th>Institution</th>
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<tbody>
<tr>
<td>Ueda Ryu</td>
<td>National Institute of Genetics</td>
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<tr>
<td>Kiguchi Kenji</td>
<td>Shinshu University</td>
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<td>Kouno Tomohiro</td>
<td>Tokyo University of Agriculture</td>
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<td>Takeda Kazuyoshi</td>
<td>Okayama University</td>
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<td>Nishimura Ikuko</td>
<td>Kyoto University Graduate School of Science</td>
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<td>Hirai Atsushi</td>
<td>Meijyou University</td>
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<td>Sakaki Yoshiyuki</td>
<td>Riken Genomics Sciences Center</td>
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<td>Sano Hiroshi</td>
<td>Nara Institute of Science and Technology</td>
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</tbody>
</table>
### FINANCIAL OVERVIEW

**Fiscal Year 2005 (April 2005 - March 2006)**

<table>
<thead>
<tr>
<th>TOTAL BUDGET</th>
<th>12,157,405</th>
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</table>

#### OPERATING COSTS | 4,742,596 |

- **Personnel** (407)*
  - President (1)
  - Vice President (2)
  - Auditor (2)

- Administrators I (87)**
- Administrators II (42)**
- Researchers (273)

  * Number of persons shown in ( )
  ** General administration
  *** Field management and transportations

- Administrative costs | 487,021 |
- Facilities Improvement Expense | 104,477 |

#### RESEARCH PROMOTION COSTS | 7,414,809 |

- Research Grant from MAFF | 2,990,821 |
- Entrusted Research Expenses from MAFF | 3,354,059 |
- Entrusted Research Expenses from MEXT | 396,625 |
- Entrusted Research Expenses from others | 673,304 |

*MAFF: Ministry of Agriculture, Forestry and Fisheries
*MEXT: Ministry of Education, Culture, Sports, Science and technology

![Pie chart showing budget allocation](image)
Location: How to access to our National Institute of Agrobiological Sciences (NIAS)

Transportation

From Tokyo: Take the JR Joban Line from Ueno Station and get off at Ushiku Station. From the West Exit, take the Kantetsu Bus bound for Yatabe-shako, Tsukuba-Daigaku-Chuo, or Seibutsu-Ken-Owashashi-Campus and get off at Norin-Danchi-Chuo. Alternatively, from Yaeu-South Gate at Tokyo Station, take an Highway Bus bound for Tsukuba-san and get off at Norin-Danchi-Chuo.

From Tokyo International Airport (Narita): Take the Kanto-Tetsudo Bus bound for Tsuchiura via Tsukuba Center, and get off at Tsukuba Center and take a taxi.

- Headquarters Area
  1-2-1 Kannondai, Tsukuba, Ibaraki, 305-8602, Japan
  TEL: +81-29-838-7416  FAX: +81-29-838-7408
  http://www.nias.afrjni.go.jp/

- Ohwashi Area
  1-2 Ohwashi, Tsukuba, Ibaraki, 305-8634, Japan
  TEL: +81-29-838-6026

NIAS Institutions outside Tsukuba

- Ohmiya (Inst. Radiation Breeding)
  2425 Kamimurata, Hitachihoubun, Ibaraki, 319-2293, Japan
  TEL: +81-295-52-1138

- Matsumoto (Sericultural Science Lab.)
  1-10-1 Agata, Matsumoto, Nagano, 390-0812, Japan
  TEL: +81-263-32-0549

- Okaya (New Silk Materials Lab.)
  1-4-8 Gouda, Okaya, Nagano, 394-0021, Japan
  TEL: +81-266-22-3664

- Kobuchizawa (Insect Genetics Lab.)
  6585 Kobuchizawa, Yamanashi, 408-0044, Japan
  TEL: +81-551-36-2046