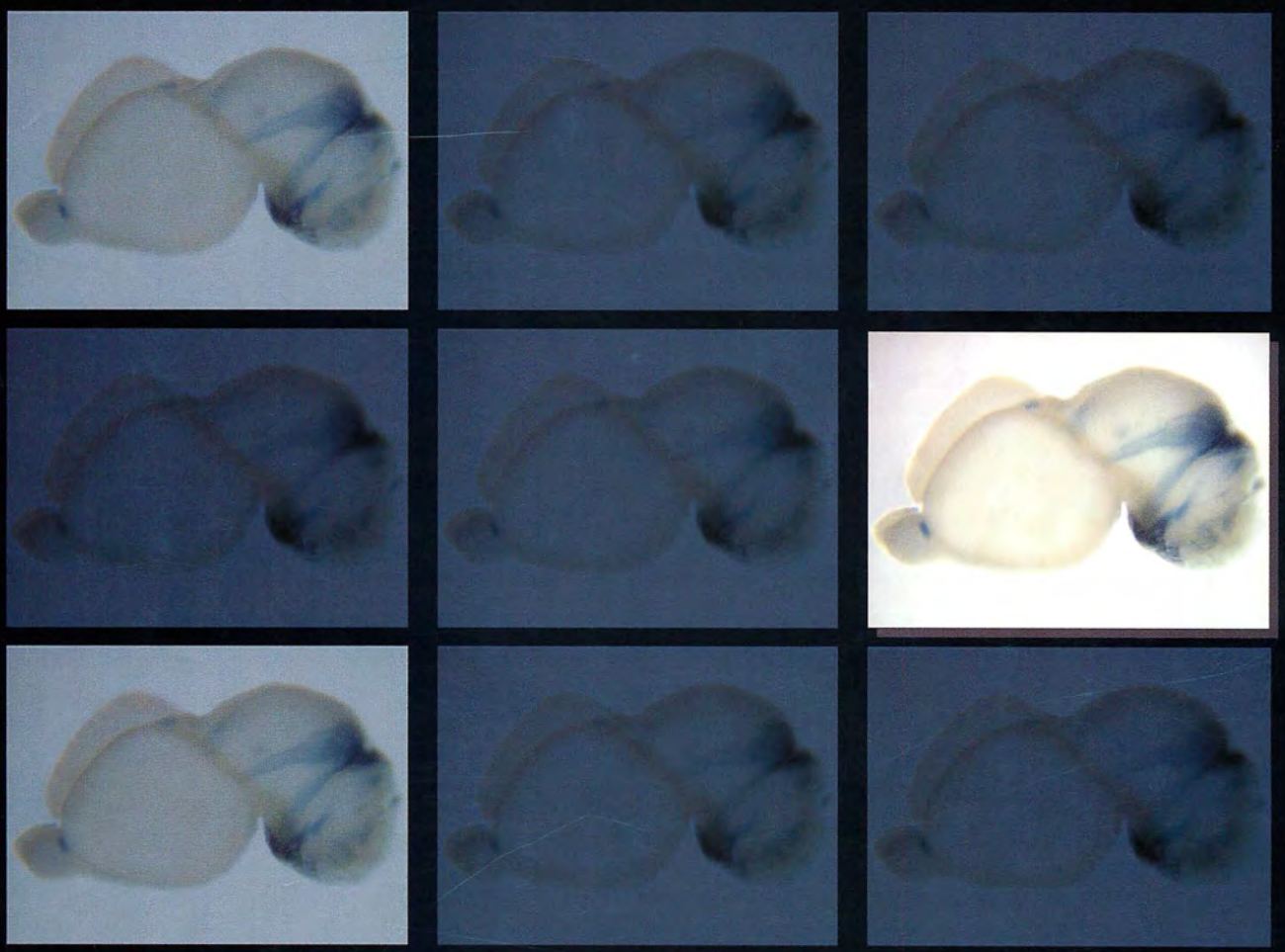
NIBB

NATIONAL INSTITUTE FOR BASIC BIOLOGY



Annual Report

2002

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The cover photograph is the central network of the auditory system visualized in a transgenic mouse line. See also p.28

INTRODUCTION

The National Institute for Basic Biology (NIBB) is a government-supported research institute established in 1977. In 1988, Department of Molecular Biofunction in School of Life Science, the Graduate University for Advanced Studies, was established in the NIBB. The aim of the NIBB is the promotion and stimulation of studies in the field of Biology. As a Center of Excellence (COE), NIBB promotes not only the basic biology but also the modern biological sciences by conducting first-rate research on site as well as in cooperation with other universities and research organizations. Researchers at NIBB investigate cell structure and function, reproduction and development, neuronal and environmental biology, gene expression and regulation, molecular evolution of eukaryotic organisms and integrative and computational biology to elucidate the general and fundamental mechanisms underlying various biological phenomena.

In 2002, there were many doctors who were appointed as scientific staffs in NIBB. On April 1st, Prof. TAKADA, Shinji, who had been a Professor of Kyoto University as well as adjunct-professor of the NIBB, was appointed as the Professor of the Center for Integrative Bioscience (CIB) and made a joint appointment for the NIBB. He was also joint-appointed as a Professor of the Graduate University of Advanced Studies and of Kyoto University.

Prof. MIYATA, Takashi was appointed as a Professor of the Department of Integrative and Computational Biology in the NIBB from Kyoto University on September 1st.

Dr. MOCHIZUKI, Atsushi who was a Research Associate of Kyushu University was appointed as an Associate Professor of the Department of Integrative and Computational Biology in the NIBB on September 1st.

Prof. NAKAMURA, Haruki of Osaka University was also appointed as a Adjunct Professor of CIB on August 1st in place of the former Prof. KANEHISA, Minoru.

Drs. MIZUSHIMA, Noboru, YAMAMOTO, Hiroshi, HORIGUCHI, Gorou (CIB), YAMADA, Kenji, HIYAMA, Takeshi and OGAWA, Hidesato were appointed as Research Associates of the NIBB in 2002.

Drs. JOHZUKA, Masaki returned as a Research Associate of NIBB from the University of California in place of Dr. KODAMA, Ken-ichi, a Research Associate of NIBB.

In congratulation, Dr. YOSHIMORI, Tamotsu, was promoted to a Professor of the National Institute of Genetics in March 1st, 2002. Dr. ITO, Kei, was promoted as an Associate Professor of the University of Tokyo in April 1st, 2002 and stayed in the NIBB as a adjunct-Associate Professor of the NIBB. Dr. HIDAKA, Masumi was promoted as an equivalent position of the Associate Professor of the BioMolecular Engineering Research Institute in Osaka.

Two adjunct Professors Dr. KAMIYA, Ritsu and MABUCHI, Issei and adjunct Associate Professors INABA, Kazuo and ABE, Hiroshi left NIBB in April 31st, 2002 just after the completion of their 5-year

appointments. Three Research Associates left their positions in NIBB to become the equivalent positions in other Universities and Institutes.

The total number of personnels working at NIBB including graduate students and postdoctoral fellows has been kept at approximately 300 for several years.

As a COE of the biological research institute, NIBB is responsible for conducting research projects in cooperation with various research groups. As a part of such cooperative activities, NIBB hosts International Conferences. The 47th NIBB International Conference entitled "Protein Phosphatases in Cellular Signaling Systems" was held in March 13-15 (Prof. M. Noda, organizer) and the 48th NIBB International Conference entitled "Molecular Mechanisms of Sex Differentiation" (Prof. K. Morohashi, organizer) in October 18-20. Those two Conferences were sponsored by the Ministry of Education, Culture, Sports, Science and Technology. In addition, NIBB continues to sponsor interdisciplinary symposia and study meetings on current topics by inviting leading scientists from around the world to the Institute. NIBB also provides a training course in biological sciences for young investigators. To assess our continuing improvement, the activities and future plan of the professor who have spent 10 years at NIBB was subjected to peer review by international scholars in related fields. Prof. NODA, Masaharu was reviewed on the 10-year Evaluation by the committee organized by the Director-General Dr. KATSUKI. The result was accepted by the Advisory Council of the NIBB held in June. We always welcome any suggestions concerning the research activities of NIBB.

KATSUKI, Motoya, D.Sc.
Director-General

ORGANIZATION OF THE INSTITUTE

The National Institute for Basic Biology (NIBB) is an Institute in the Okazaki National Research Institutes (ONRI) that are composed of three independent organizations, NIBB, the Institute for Molecular Science (IMS) and the National Institute for Physiological Sciences (NIPS). They located on a hill overlooking the old town of Okazaki. NIBB was established in 1977 and its activities are supported by Monbukagaku-sho (the Ministry of Education, Culture, Sports, Science and Technology: Mext) of Japan. The Center for Integrative Bioscience that was established as a common facility of the ONRI in 2000 and began in 2001.

Policy and Decision Making

The Director-General oversees the operation of the Institute assisted by two advisory bodies, the Council and the Advisory Committee for Programming and Management. The Council, comprised of distinguished scholars representing various fields of science and culture, advises the Director-General on principles and policies governing the activities and operations of NIBB. The Advisory Committee, comprised of professors within the Institute and an equal number of leading biologists outside NIBB advises the Director-General, upon his request, on planning joint research programs and other important matters in NIBB, as well as on the scientific activities of the Institute. The Council makes a nomination of Director-General and Committee also makes recommendations on the Director-General and on faculty appointments, the Institute's annual budget and future prospects.

Administration

Administration of the Institute is undertaken by the Administration Bureau of the Okazaki National Research Institutes under the direct auspices of the Ministry of Education, Culture, Sports, Science and Technology.

Research

The Institute conducts its research programs through three departments and one laboratory subdivided into 17 divisions and the Center for Integrative and Computational Biology.

Each division has its own research project and is staffed by a professor, an associate professor and two research associates in principle. A division forms an independent project team. Six of the divisions are adjunct and headed by professors who hold joint appointments with other universities. Adjunct divisions have a resident research associate. This arrangement facilitates exchange in research activities in Japan. The Technical Department manages the activities of research technicians and helps to promote research activities of each division and to maintain the common research resources of the Institute. The Department also undertakes the technical education of staffs.

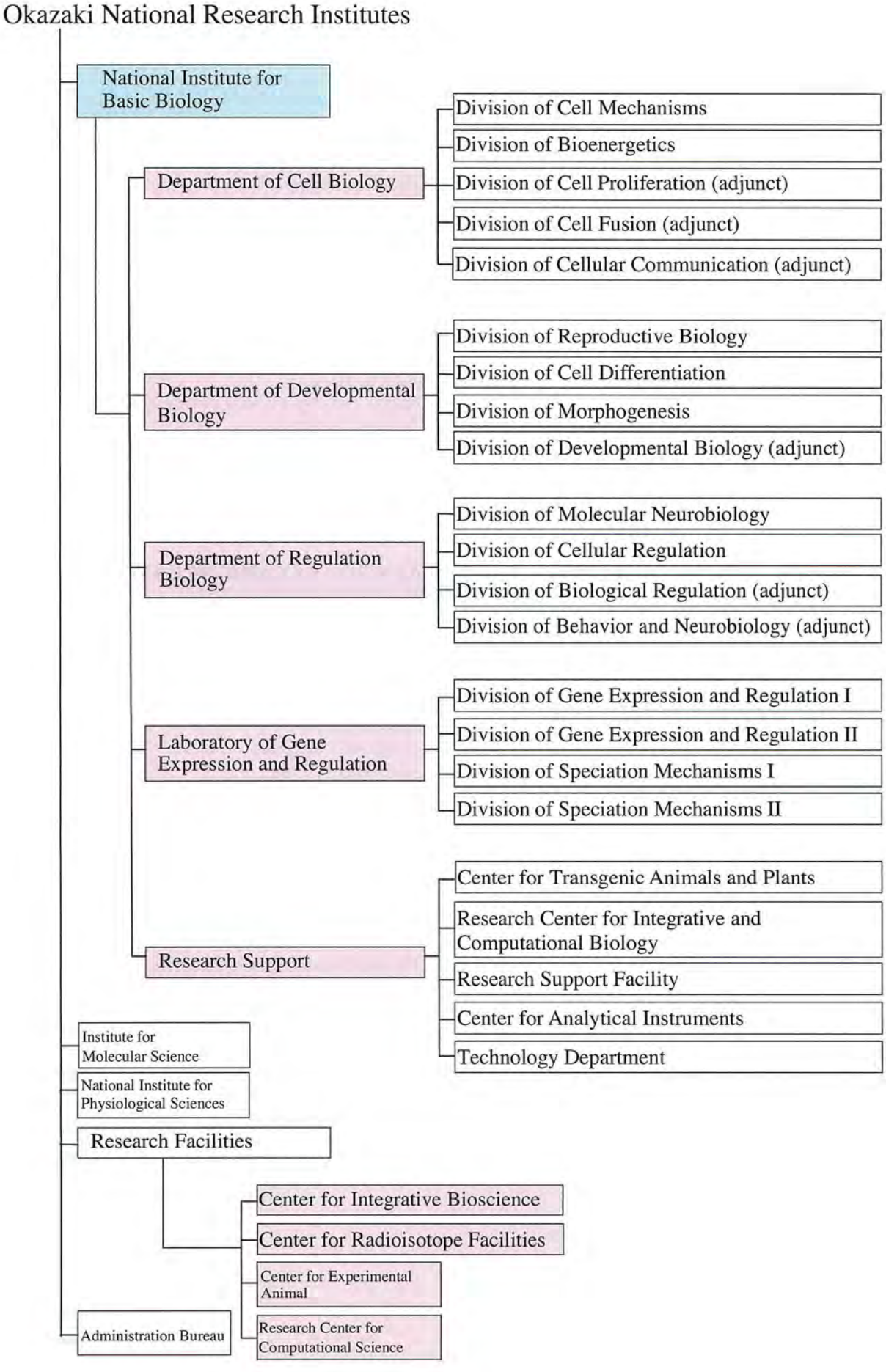
Several members of the Center for Integrative Bioscience jointly work with the NIBB.

Research Support Facilities

The research support facilities of the NIBB consist of the Large Spectrograph Laboratory, the Tissue and Cell Culture Laboratory, the Computer Laboratory, the Plant Culture Laboratory, the Plant Cell Culture Laboratory, the Experimental Farm, the Laboratory of Stress-Resistant Plants and the Center for Transgenic Animals and Plants. In addition, five facilities are operated jointly with NIPS; they consist of the Electron Microscope Center, the Center for Analytical Instruments, the Machine Shop, the Laboratory Glassware Facilities and the Low-Temperature Facilities. The Radioisotope Facilities, the Computer Center and the Animal Care Facilities became common facilities of ONRI.

Campus

The Okazaki National Research Institutes covers an area of 150,000m^2 with four principal buildings. The NIBB's main research building has a floor space of 10,930m^2. Two-thirds of the space was completed in 1982 and the remaining third in June, 1983. The buildings which have the research support facilities were also completed in 1983. A building for the Laboratory of Gene Expression and Regulation (2,577m^2) was built in 1996.


Okazaki National Research Institutes
National Institute for Basic Biology
Department of Cell Biology
Division of Cell Mechanisms
Division of Bioenergetics
Division of Cell Proliferation (adjunct)
Division of Cell Fusion (adjunct)
Division of Cellular Communication (adjunct)
Department of Developmental Biology
Division of Reproductive Biology
Division of Cell Differentiation
Division of Morphogenesis
Division of Developmental Biology (adjunct)
Department of Regulation Biology
Division of Molecular Neurobiology
Division of Cellular Regulation
Division of Biological Regulation (adjunct)
Division of Behavior and Neurobiology (adjunct)
Laboratory of Gene Expression and Regulation
Division of Gene Expression and Regulation I
Division of Gene Expression and Regulation II
Division of Speciation Mechanisms I
Division of Speciation Mechanisms II
Research Support
Center for Transgenic Animals and Plants
Research Center for Integrative and Computational Biology
Research Support Facility
Center for Analytical Instruments
Technology Department
Institute for Molecular Science
National Institute for Physiological Sciences
Research Facilities
Center for Integrative Bioscience
Center for Radioisotope Facilities
Center for Experimental Animal
Research Center for Computational Science
Administration Bureau

MEMBERS OF THE COUNCIL

ISHIGE, Naomichi	Director-General, National Museum of Ethnology
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EGUCHI, Goro	President, Kumamoto University
	(present) Chairman, Board of Directors, Shokei Educational Institution
OHSAKI, Hitoshi	Director-General, Center for National University of Finance
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KISHIMOTO, Tadamitsu	President, Osaka University
SHIMURA, Yoshirou	Director, Biomolecular Engineering Research Institute
	(present) Director, JSPS Liaison Office, Stockholm
SUZUKI, Akinori	President, Akita Prefectural University
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HIDAKA, Toshitaka	Director-General, Research Institute for Humanity and Nature
HOSHI, Motonori	Professor, Keio University
HOTTA, Yoshiki	Director-General, National Institute of Genetics
YAMASHITA, Koujun	Professor, Nagoya University
YOSHIKAWA, Hiroshi	Advisor, JT Biohistory Research Hall
YOSHIDA, Mitsuaki	Director, Banyu Tsukuba Research Institute
YONEYAMA, Toshinao	President, Otemae University
WATANABE, Okitsugu	Director-General, National Institute of Polar Research

MEMBERS OF THE ADVISORY COMMITTEE FOR PROGRAMMING AND MANAGEMENT

AIZAWA, Shinichi	Professor, Kumamoto University
	(present) Group Director, Center for Developmental Biology, RIKEN
OKADA, Kiyotaka	Professor, Kyoto University
KUROSAWA, Yoshikazu	Professor, Fujita Health University
GO, Michiko	Professor, Nagoya University
KOMEDA, Yoshifumi	Professor, Hokkaido University
	(present) Professor, University of Tokyo
KONDO, Hisato	Professor, Osaka University
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HASUNUMA, Kohji	Professor, Yokohama City University
MACHIDA, Yasunori	Professor, Nagoya University
YAMAMOTO, Masayuki	Professor, University of Tokyo
IGUCHI, Taisen	Professor, Okazaki National Research Institutes, Center for Integrative Bioscience.
IIDA, Shigeru	Professor, Okazaki National Institute for Basic Biology
UENO, Naoto	Professor, Okazaki National Institute for Basic Biology
OHSUMI, Yoshinori	Professor, Okazaki National Institute for Basic Biology
NAGAHAMA, Yoshitaka	Professor, Okazaki National Institute for Basic Biology
NISHIMURA, Mikio	Professor, Okazaki National Institute for Basic Biology
NODA, Masaharu	Professor, Okazaki National Institute for Basic Biology
HORIUCHI, Takashi	Professor, Okazaki National Institute for Basic Biology
MURATA, Norio	Professor, Okazaki National Institute for Basic Biology
MOROHASHI, Ken-ichiro	Professor, Okazaki National Institute for Basic Biology
YAMAMORI, Tetsuo	Professor, Okazaki National Institute for Basic Biology

GRADUATE PROGRAMS

The NIBB sponsors two graduate programs.

1. Graduate University for Advanced Studies

NIBB constitutes the Department of Molecular Biomechanics in the School of Life Science of the Graduate University for Advanced Studies. The University provides a three year Ph. D. course. Those who have completed a master's course or equivalent at any university are eligible to apply.

The Department consists of the following Divisions and Fields:

DIVISIONS	FIELDS
Molecular Cell Biology	Biomolecular Systems Cell Dynamics
Developmental Gene Expression and Regulation	Gene Expression Morphogenesis Transgenic Biology
Regulation Biology	Biological Regulation Biological Information

2. Graduate Student Training Program

Graduate students enrolled in other universities and institutions are eligible to conduct research for fixed periods of time under the supervision of NIBB professors.

OFFICE OF DIRECTOR

Director-General: *KATSUKI, Motoya*
Associate Professors: *KODAMA, Ryuji*
UENO, Kohji
Research Associates: *OHNO, Kaoru*

Mechanisms working in the morphogenesis of the lepidopteran wings

KODAMA, Ryuji

Wings of the lepidopteran insects (butterflies and moths) develop from the wing imaginal disc, which is a hollow sac made of simple epithelium. Due to its simple construction, this tissue is a good material to study cellular interactions in the course of morphogenesis.

The outline shape of the adult wing is often different from that of the pupal wing. This difference is brought about by the programmed cell death of the marginal area of the pupal wing. The marginal dying area is called the degeneration region and the internal area, which develop into the adult wing, is called the differentiation region.

The cell deaths in the degeneration region proceeds very rapidly and completes in a half to one day period in *Pieris rapae* or several other species examined. It was shown that the dying cells in the degeneration region have two characteristics common with the apoptotic cell death in mammalian cells. These are i) the presence of apoptotic bodies, which are heavily condensed cells or their fragments engulfed by other cells or macrophages, shown by transmission electron microscopy and ii) the presence of conspicuous accumulation of fragmented DNA evidenced by the TUNEL histological staining. The cells in the degeneration region are actively engulfed by the macrophages in the cavity beneath the wing epithelium. The macrophages seem to be concentrated beneath the degeneration region by the strong adhesion between basal surfaces of the dorsal and ventral epithelium in the differentiation region.

A collaborative work with the laboratory of Dr. K. Watanabe (Hiroshima University) concerns mostly on the development of trachea and tracheole pattern in the swallow tail butterflies. Trachea and trcheoles are both important in delivering air into the wing and their pattern coincide with that of the boundary of degeneration and differentiation zones at the distal end of the wing. According to the observations, the pattern formation of wing epithelium is often dependent on tracheal and tracheole patterns. Basic research on the development of tracheal pattern formation is being done by the scanning electron microscopy and the bright field light microscopy of the fixed or fresh specimens to describe the exact pathway and the time course of the formation of elaborate pattern of trachea and tracheoles and to establish the cytological and developmental relationship between the formation of tracheal pattern and epithelial cell pattern, such as scale cell pattern.

The figure depicts how the tracheoles protrude from the primary trachea at the pre-pupa stage (Fig. 1). The tracheoles are arranged with even spaces within the wing lumen and may closely related with the scale cell pattern formation.

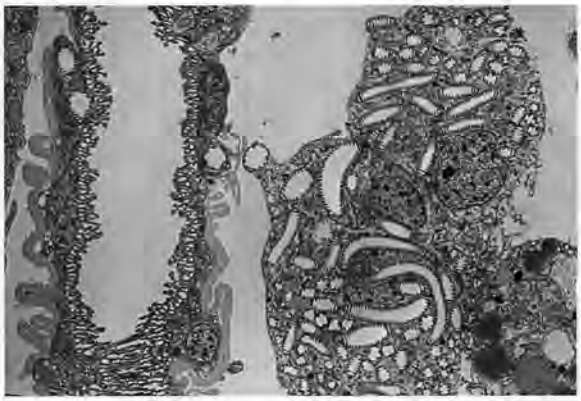

Fig.1. The tracheole cells (on the right) depart from the primary trachea (thick tube on the left) leaving behind tracheole, which was stored within the cytoplasm of the tracheole cell.

In collaboration with the Division of Molecular Neurobiology, the localization of a sodium channel protein was described using immunohistological staining at the level of electron microscopy.

Publication List:

Watanabe, E., T. Y. Hiyama, R. Kodama and M. Noda (2002) Na_x sodium channel is expressed in non-myelinating Schwann cells and alveolar type II cells in mice. *Neurosc. Letters* **330**: 109-113

Control of the distribution of palmitoylated proteins in neuronal growth cones

UENO, Kohji

Signalling proteins such as G proteins and G protein-coupled receptors are modified with palmitate via thioester linkages. Protein palmitoylation is thought to be important in the regulation of signal transduction. We have previously found that protein palmitoylase is expressed in neural cells during mouse embryogenesis. In developing neurons, growth associated protein (GAP)-43 and Go, which are palmitoylated proteins, are mainly concentrated in the growth cones. Addition of an inhibitor of protein palmitoylase to the medium of cultured primary neuronal mouse cells reduces the axonal growth of neurons. From these findings, we speculated that the localization of the palmitoylated proteins in growth cones is critical for the development of axons.

In this study, we are attempting to elucidate the mechanism that determines the localization of the palmitoylated proteins in growth cones. For this analysis, we have established a method to chemically modify a synthetic GAP-43 peptide with palmitate or other fatty acids via a thioester linkage. We have analyzed the interaction of palmitoylated proteins with ERM (Ezrin/Radixin/Moesin) proteins. ERM proteins are thought to be general cross-linkers between plasma membranes and actin filaments. ERM proteins were

analyzed on the basis of evidence that radixin and moesin are concentrated in growth cones. Furthermore, ERM proteins contain a subdomain that possesses a similar structure to the acyl-CoA binding protein, which has an affinity to palmitoyl-CoA. Thus, I guessed that ERM proteins may interact with the palmitoylated proteins. We found that ERM proteins possessed an ability to bind to the chemically-modified palmitoylated peptides.

DEPARTMENT OF CELL BIOLOGY

Chairperson: OHSUMI, Yoshinori

DIVISION OF CELL MECHANISMS
DIVISION OF BIOENERGETICS
DIVISION OF CELL PROLIFERATION (ADJUNCT)
DIVISION OF CELL FUSION (ADJUNCT)
DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

The department consists of two regular divisions and three adjunct divisions. The department conducts studies on molecular dynamics of the cell in eukaryotes such as organelle differentiation, autophagy, cell motility, cytokinesis and neural development.

DIVISION OF CELL MECHANISMS

Professor:	*NISHIMURA, Mikio*
Associate Professor:	*HAYASHI, Makoto*
Research Associates:	*MANO, Shoji*
	YAMADA, Kenji (November 1~)
Technical Staff:	*KONDO, Maki*
NIBB Research Fellow:	*SHIRAHAMA, Kanae*
JSPS Postdoctoral Fellow:	*NITO, Kazumasa (April 1~)*
Graduate Students:	*WATANABE, Etsuko (~March 31)*
	FUKAO, Youichiro
	KAMADA, Tomoe
	HATSUGAI, Noriyuki
Technical Assistants:	*NAKAMORI, Chihiro*
	YAGI, Mina
	YOSHINORI, Yumi (August 1~)
Secretaries	*UEDA, Chizuru*
	KOMORI, Akiko (~March 31)
	IYODA, Yuri (February 15~)

Higher plant cells contain several distinct organelles that play vital roles in cellular physiology. During proliferation and differentiation of the cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur, existing organelles may undergo a transformation of function, while other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organelles) is responsible for flexibility of differentiation events in higher plant cells, the elucidation of regulatory mechanisms underlying organelle transformation are currently studied in this division.

I. Reversible transformation of plant peroxisomes

Dramatic metabolic changes which underlie the shift from heterotrophic to autotrophic growth occur in greening of seed germination. Accompanying these metabolic changes, many constitutive organelles are functionally transformed. Etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes which are microbodies engaged in the degradation of reserve oil via β-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse transition of leaf peroxisomes to glyoxysomes occurs during senescence. The functional transformation between glyoxysomes and leaf peroxisomes is controlled by gene expression, alternative splicing, protein translocation and protein degradation. We now engage in proteomic and transcriptomic analyses of the reversible peroxisomal transition in *Arabidopsis* cotyledons.

II. Intracellular transport of peroxisomal proteins

Enzymes localized in plant peroxisomes are synthesized in the cytosol, and function after their post-translational transport into peroxisomes. Almost all of the peroxisomal matrix proteins are known to contain one of two targeting signals (PTS1 and PTS2) within the molecules. PTS1 is a unique tripeptide sequence found in carboxyl terminus of the mature proteins. The permissible combinations of amino acids for PTS1 in plant cells are [C/A/S/P]-[K/R]-[I/L/M]. In contrast, PTS2 is involved in a cleavable amino terminal presequence of peroxisomal proteins that are synthesized as precursor protein with larger molecular mass. PTS2 consists of a consensus sequence [R]-[L/Q/I]-X5-[H]-[L]. PTS1 and PTS2 are initially recognized by binding with their cytosolic receptors, *At*Pex5p and *At*Pex7p, respectively. Since *At*Pex5p could also bind with *At*Pex7p, they might form a receptor-cargo complex.

Catalase is a peroxisomal protein that have been know to contain no obvious PTS1/PTS2. We recently found that ^{480}QKL of the enzyme function as an alternative peroxisomal targeting signal. Yeast two-hybrid analysis revealed that the signal also binds with *At*Pex5p. The result indicated that catalase is also imported into peroxisomes by PTS1-dependent manner.

Arabidopsis *ped2* mutant is one of the mutants that show resistance to 2,4-dichlorophenoxybutyric acid. *PED2* gene, responsible for this deficiency, encodes a peroxisomal membrane protein, *At*Pex14p. Mutation in the *PED2* gene results in pleiotropic defects on fatty acid degradation, photorespiration, and the morphology of all kinds of peroxisomes by disrupting both PTS1- and PTS2-dependent peroxisomal protein import pathways. Binding assay revealed that *At*Pex14p directly binds with *At*Pex5p, but not with *At*Pex7p. *At*Pex14p controls both PTS1- and PTS2-dependent peroxisomal protein import pathways by binding with the receptor-cargo complex involving *At*Pex5p. These data suggest that the *At*Pex14p has a common role in maintaining physiological functions of all kinds of plant peroxisomes by determining peroxisomal protein targeting.

III. Dynamic morphology and actin dependent movement of plant peroxisomes

To characterize peroxisomes in vivo, we visualized them in cells of various tissues of Arabidopsis by green fluorescent protein (GFP) through the addition of the peroxisomal targeting signal 1 (PTS1) or PTS2. The observation using confocal laser scanning microscopy revealed that the GFP fluorescence signals were detected as spherical spots in all cells of two kinds of transgenic plants. Interestingly, however, abnormal large peroxisomes are present as clusters at the onset of germination, and these clusters disappear in a few days. Moreover, tubular peroxisomes which looked like the dumbbell, as if two peroxisomes were connected with the tubule, were also observed in the hypocotyl. Peroxisomes turned to be motile organelles whose movement might be caused by cytoplasmic flow. The movement of peroxisomes was more prominent in root cells than that in leaves, and divided into two categories: a relatively slow, random, vibrational movement and a rapid movement. Treatment with anti-actin and anti-tubulin drugs revealed that actin filaments involve in the rapid movement of peroxisomes. These findings indicate that peroxisomes undergo dramatic changes in size, shape, number and position within the cell.

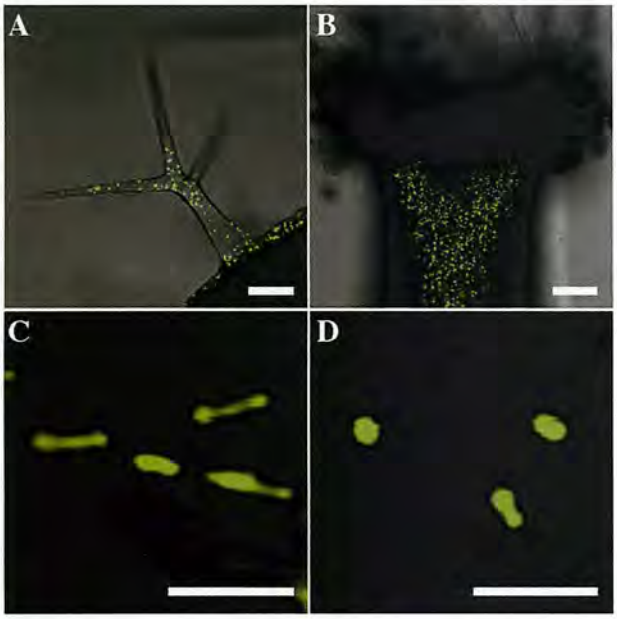

Fig. 1 Confocal microscopic images of various tissues in GFP-PTS1.

(A) and (B) show the merged images with blue light excitation and the difference interference contrast. (A) trichome, (B) pistil. (C) and (D) represent the tubular and the normal-shape peroxisomes, respectively. Each bar indicates 50 μm for (A) and (B), and 5 μm for (C) and (D).

IV. ER derived organelles for transport of proteins to vacuoles.

A novel vesicle, referred to as a precursor-accumulating (PAC) vesicle, mediates the transport of storage protein precursors from endoplasmic reticulum (ER) to protein storage vacuoles in maturing pumpkin seeds. PAC vesicles had diameters of 300 to 400 nm, are derived from ER and contained an electron-dense core of storage proteins. PV72, a type I integral membrane protein with three repeats of epidermal growth factor (EGF)-like motifs, was found on the membrane of the PAC vesicles. We expressed modified PV72 and analyzed their ability to bind to the internal propeptide of pro2S albumin (2S-I), a storage protein precursor, by affinity chromatography and surface plasmon resonance. The recombinant PV72 specifically bound to the 2S-I peptide with a K_D value of 0.2 μM, which was low enough for it to function as a receptor. The EGF-like motifs modulate the Ca^{2+}-dependent conformational change of PV72 to ER bodies are one of ER-derived compartment specific to the *Brassicaceae*, including *Arabidopsis*. ER bodies are rod-shaped structures that are characteristically 5 μm long and 0.5 μm wide. Electron microscopic studies revealed that ER bodies have a fibrous pattern inside and are surrounded by ribosomes (Fig. 2). Fluorescent imaging of ER bodies were observed in transgenic plants of *Arabidopsis* (*GFP-h*) expressing green fluorescent protein fused with an ER retention signal (GFP-HDEL). ER bodies were widely distributed in the epidermal cells of whole seedlings. In contrast, rosette leaves had no ER bodies. We found that wound stress induced the formation of ER bodies in rosette leaves. ER bodies were also induced treatment of methyl jasmonate (MeJA), a plant hormone involved in the defense response against wounding. The induction of ER bodies by MeJa was suppressed by ethylene. ER bodies contained precursors of two vacuolar proteinases, RD21 and VPE. During salt-induced cell death, ER bodies fused with each other and with lytic vacuoles, thereby mediating the delivery of the proteinase precursors directly into the vacuoles. We isolated an *Arabidopsis* mutant, named *nai1*, in which ER bodies were hardly detected in whole plants. ER bodies were concentrated in a 1000 x *g* pellet (P1) after subcellular fractionation. PYK10, a β-glucosidase with an ER-retention signal (KDEL), was specifically accumulated in the P1 fraction of *GFP-h* plants, but not in the P1

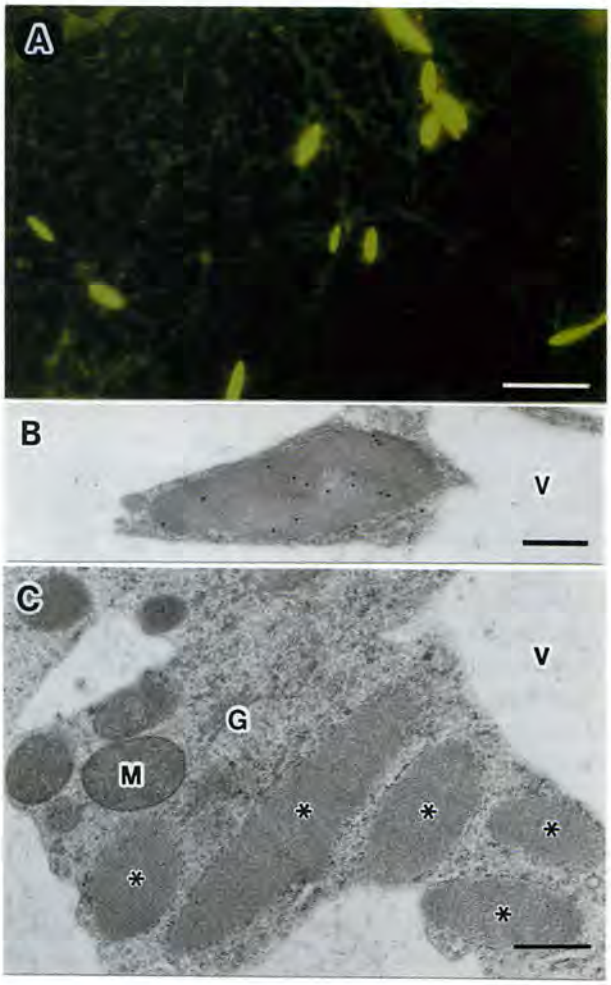

Fig. 2 Rod-shaped bodies that were derived from ER accumulated GFP-HDEL in the epidermal cells of *Arabidopsis* cotyledons.

Fluorescent image of numerous ~5-μm-long bodies in the epidermal cells of 5-day-old cotyledons of the transgenic plants. Bar = 10 μm. (**B**) Immunocytochemistry with anti-GFP antibodies showing that the large spindle-shaped bodies accumulated GFP-HDEL. Bar = 0.5 μm. (**C**) Electron micrograph showing that the bodies had a characteristic fibrous pattern inside and were surrounded by ribosomes. Asterisks show the ER bodies. M, mitochondrion; G, Golgi complex; V, vacuole. Bar = 0.5 μm.

fraction of *nai1* plants. Compare with the accumulation of GFP-HDEL, which was associated with both cisternal ER and ER bodies, the accumulation of PYK10 was much more specific to ER bodies. These findings indicate that PYK10 is the main component of ER bodies. Taken together, these results suggest that the formation of ER bodies is a novel and unique type of endomembrane system in the responses of certain cells to environmental stress.

IV. Role of molecular chaperones in organelle differentiation.

Molecular chaperones are cellular proteins that function in the folding and assembly of certain other polypeptides into oligomeric structures but that are not, themselves, components of the final oligomeric structure. To clarify the roles of molecular chaperones on organelle differentiation, we have purified and characterized chaperonin and Hsp70s and analyzed their roles in the translocation of proteins into chloroplasts.

Previously, we characterized a mitochondrial co-chaperonin (Cpn10) and chloroplast co-chaperonins (Cpn20) and (Cpn10) from *Arabidopsis*. In 2002, we started to characterize molecular chaperones in peroxisomes with collaboration with Dr. A. Kato at Niigata Univ. We found that a novel small heat shock protein (sHsp) localizes in peroxisomes. Molecular and functional characterization of the sHsp is now under experiments.

Publication List:

Fukao, Y., Hayashi, M. and Nishimura, M. (2002) Proteomic analysis of leaf peroxisomes in greening cotyledons of *Arabidopsis thaliana*. *Plant Cell Physiol.* **43**, 689-696.

Hayashi, M., Nito, K., Takei-Hoshi, R., Yagi, M., Kondo, M., Suenaga, A., Yamaya, T. and Nishimura, M. (2002) Ped 3p is a peroxisomal ATP-binding cassete transporter that might supply substrates for fatty acid β-oxidation. *Plant Cell Physiol.* **43**, 1-11.

Hayashi, H., De Bellis L., Kato, A., Hayashi, Y., Nito, K., Hayashi, M., Hara-Nishimura, I. and Nishimura, M. (2002) Molecular characterization of an Arabidopsis acyl CoA synthetase localized on glyoxysomal membranes. *Plant Physiol.* **130**, 2019-2026.

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DIVISION OF BIOENERGETICS

Professor:	*OHSUMI, Yoshinori*
Associate Professor:	*YOSHIMORI, Tamotsu* [1]
Research Associates:	*KAMADA, Yoshiaki*
	NODA, Takeshi
	MIZUSHIMA, Noboru [2]
Technical Staff:	*KABEYA, Yukiko*
NIBB Research Fellow:	*YOSHIMOTO, Kohki*
JSPS Postdoctral Fellow:	*SEKITO, Takayuki*
	SUZUKI, Kuninori
	KIRISAKO, Takayoshi [1]
Postdoctoral Fellow:	*ICHIMURA, Yoshinobu*
	HANADA, Takao
	HANAOKA, Hideki [3]
Graduate Students:	*NARA, Atsuki* [1]
	KUMA, Akiko [4]
	HAMASAKI, Maho [4]
	SHOJI, Shisako [1]
	ONODERA, Jun [4]
Technical Assistant:	*MIWA, Masami*
	TSUKESHIBA Kumi
Secretary	*HARA, Yohko*

[1] *until Mar 2002*
[2] *from Apr2002*
[3] *until Jun 2002*
[4] *Graduate University for Advanced Studies*

I. Background

This division aims to understand the autophagy in respects of its molecular mechanism and its physiological role within higher eukaryotes. Biosynthetic processes and degradation processes are well coordinated to regulate the biological activities, however the study on the latter has been retarded compared to the one on the former and we must shed light on the degradation process to fully understand the cell. Autophagy is a well conserved degradation process in eukaryotes and is a major route for bulk degradation of cytoplasmic constituents in a lytic compartment, lysosome/vacuole.

Upon nutrient starvation, autophagic process starts in the cytoplasm as building up of a membrane structure, an autophagosome, which sequesters a portion of cytosol. Then it is delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by the vacuolar/lysosomal proteases. We had discovered autophagy in a simple eukaryotic model organism, *Saccharomyces cerevisiae* and morphologically described its whole process. We have isolated a set of autophagy-deficient mutants (*apg*), and cloned *APG* genes essential for autophagy.

2. Formation of the Apg12-Apg5-Apg16 multimeric complex is essential for autophagy in yeast.

Autophagy requires a ubiquitin-like protein conjugation system, in which Apg12 is covalently bound to Apg5. In the yeast *Saccharomyces cerevisiae,* the Apg12-Apg5 conjugate further interacts with a small coiled-coil protein, Apg16. The Apg12-Apg5 and Apg16 are localized in the cytosol and pre-autophagosomal structures and play an essential role in autophagosome formation. We showed that the Apg12-Apg5 conjugate and Apg16 form an approximately 350-kDa complex in the cytosol. Because Apg16 was suggested to form a homo-oligomer, we generated an in vivo system that allowed us to control the oligomerization state of Apg16. With this system, we demonstrated that formation of the approximately 350-kDa complex and autophagic activity depended on the oligomerization state of Apg16. These results suggest that the Apg12-Apg5 conjugate and Apg16 form a multimeric complex mediated by the Apg16 homo-oligomer, and formation of the approximately 350-kDa complex is required for autophagy in yeast.

3. Cargo Delivery to the Vacuole Mediated by Autophagosomes

In the yeast *Saccharomyces cerevisiae*, aminopeptidase I (API), a vacuolar hydrolase, is selectively transported to the vacuole via the autophagosome. API forms a cytosol to vacuole targeting (Cvt) complex in the cytoplasm. The complex is engulfed by the autophagosome under starvation conditions. In this study, the Cvt complex is visualized as a dot in the cytoplasm by fluorescence microscopy with API-GFP. The Cvt complex associates with the pre-autophagosomal structure (PAS), which plays a central role in autophagosome formation. In a *cvt19* mutant, which is specifically defective in API transport, but not in autophagy, the Cvt complex forms normally but never associates with the PAS. This indicates that Cvt19p mediates association between the Cvt complex and the PAS.

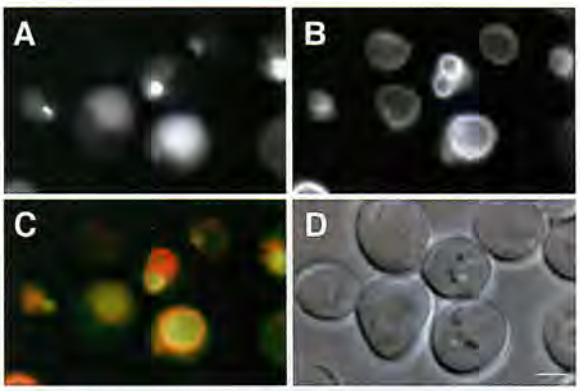

Figure 1 API-GFP labels the vacuoles and dots close to the vacuole. Wild type cells expressing API-GFP under growing conditions. (A) API-GFP, (B) vacuoles labeled with FM4-64, (C) merged image of API-GFP (green) and FM4-64 (red), and (D) Nomarski image. Bar: 2 μm.

4. Studies of mammalian autophagy

We cloned mouse Apg10 and showed that the Apg12 conjugation system is completely conserved between yeast and mammals. In addition, we also cloned a novel Apg5-interacting protein, Apg16L. Although it was considered to be a functional counterpart of yeast Apg16, Apg16L has a large C-terminal region containing a WD repeat domain that is absent from yeast Apg16. This unique feature of Apg16L will provide new insights into role of Apg12-Apg5 in autophagosome formation.

Once an autophagosome is formed, it matures by fusion

with endosomes. Using a dominat negative mutant of mouse SKD1, an AAA ATPase involved in the sorting and transport from endosomes, we showed that SKD1-dependent endosomal membrane trafficking is required for further fusion with lysosome.

To monitor autophagy in vivo, we generated transgenic mice expressing a fluorescent marker for autophagosome. It would be a useful tool for studies on mammalian autophagy.

5. Leaf senescence is accelerated by the disruption of an Arabidopsis autophagy gene.

The molecular machinery responsible for yeast and mammalian autophagy has recently begun to be elucidated at the cellular level, but the role that autophagy plays at the organismal level has yet to be determined. In this study, a genome-wide search revealed significant conservation between yeast and plant autophagy genes. Twenty-five plant genes that are homologous to 12 yeast genes essential for autophagy were discovered. We identified an Arabidopsis mutant carrying a T-DNA insertion within *AtAPG9*, which is the only ortholog of yeast Apg9 in Arabidopsis (*atapg9-1*). *AtAPG9* is transcribed in every wild-type organ tested but not in the *atapg9-1* mutant. Under nitrogen or carbon-starvation conditions, chlorosis was observed earlier in *atapg9-1* cotyledons and rosette leaves compared with wild-type plants. Furthermore, *atapg9-1* exhibited a reduction in seed set when nitrogen starved. Even under nutrient growth conditions, bolting and natural leaf senescence were accelerated in *atapg9-1* plants. Senescence-associated genes SEN1 and YSL4 were up-regulated in *atapg9-1* before induction of senescence, unlike in wild type. All of these phenotypes were complemented by the expression of wild-type *AtAPG9* in *atapg9-1* plants. These results imply that autophagy is required for maintenance of the cellular viability under nutrient-limited conditions and for efficient nutrient use as a whole plant.

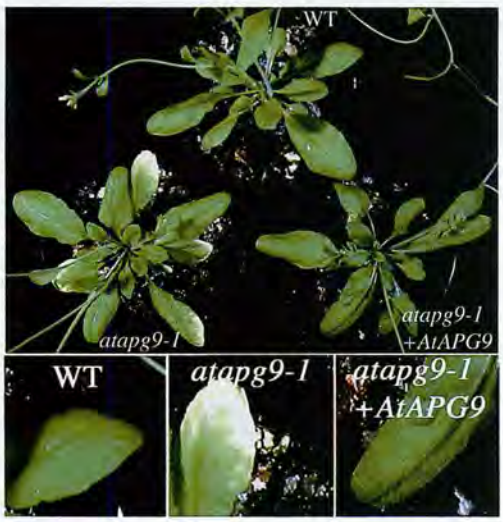

Figure 2 The Arabidopsis during natural leaf senescence. Wild type, *atapg9-1*, and *atapg9-1* plants transformed with wild-type *AtAPG9* gene were grown under standard conditions and photographed on 35 day after germination.

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DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairperson: NAGAHAMA, Yoshitaka

DIVISION OF REPRODUCTIVE BIOLOGY
DIVISION OF CELL DIFFERENTIATION
DIVISION OF MORPHOGENESIS
DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

The department is composed of three divisions and one adjunct division. Department members conduct molecular analysis on various aspects of developmental phenomena including (1) gonadal sex differentiation and gametogenesis, (2) gene regulation in cell differentiation and growth, and (3) molecular basis of body plans.

DIVISION OF REPRODUCTIVE BIOLOGY

Professor:	*NAGAHAMA, Yoshitaka*
Associate Professor:	*YOSHIKUNI, Michiyasu*
Research Associate:	*KOBAYASHI, Tohru*
Technical Staff:	*KOBAYASHI, Hiroko*
JSPS Postdoctoral Fellows:	*IKEUCHI, Toshitaka*
	SHIBATA, Yasuji
	SUDHAKUMARI, Cheni Chery
	WANG, De-shou
Postdoctoral Fellows:	*MATSUDA, Masaru*
	SAKAI, Fumie
	GUAN, Gui-Jin
	SENTHILKUMARAN, Balasubramanian
	LAU, En-Lieng
	PAUL, Bindhu
Graduate Students:	*HORIGUCHI, Ryo (Graduate University for Advanced Studies)*
	KOBAYASHI, Yasuhisa
	FAN, Hai-Guang (Graduate University for Advanced Studies)
	OHMURO, Yuki (Graduate University for Advanced Studies)
	ZHOU, Linyan(Graduate University for Advanced Studies)
Visiting Scientists:	*MATSUDA, Chika*
	MATSUOKA, Yoh
	YANO, Akihiro

The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. Gonadotropins typically act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. Their effects are particularly profound in teleost fishes which provide several excellent models for investigating the basic hormonal mechanisms regulating gonadal sex differentiation and gametogenesis Our research focuses on (1) the identification of regulators and steroidal mediators involved in sex determination, gonadal sex differentiation and gametogenesis, and (2) the mechanisms of synthesis and action of these mediators.

I. Sex-determining gene of medaka

Although the sex-determining gene *SRY/Sry* has been identified in mammals, no comparable genes have been found in non-mammalian vertebrates. To identify such a sex-determining gene, a positional cloning approach is suitable. The medaka, *Oryzias latipes*, has two major advantages for genetic research: a large genetic diversity within the species and the existence of several inbred strains. As in mammals, sex determination in medaka is male heterogametic, although the Y chromosome is not cytogenetically distinct. Alteration of phenotypic sex with no reproductive consequences, and recombination over the entire sex chromosome pair, suggest that there are no major differences, other than a sex-determining gene, between the X and Ychromosomes.To clone positionally the sex-determining region, we generated a Y congenic strain to highlight the genetic differences between the X and Y chromosomes-from inbred strains of medaka. The Y congenic strain has a sex-determining region derived from the HNI-strain Y chromosome on the genetic background of an Hd-rR strain. Using this strain, we had previously constructed a genetic map of the medaka sex chromosome and constructed a BAC (bacterial artificial chromosome) genomic library. We used recombinant breakpoint analysis to restrict the sex-determining region in medaka to a 530-kb stretch of the Y chromosome. Deletion analysis of the Y chromosome of a congenic XY female further shortened the region to 250-kb (Fig.1). Shotgun sequencing of this region predicted 27 genes. Three of these genes were expressed during sexual differentiation. However, only one gene *PG17* was Y specific. The full-length cDNA sequence of *PG17* encodes a putative protein of 267 amino acids, including the highly conserved DM domain. The DM domain was originally described as a DNA-binding motif shared between *doublesex* (*dsx*) in *Drosophila melanogaster* and *mab-3* in *Caenorhabditis elegans*. We thus named it *DMY* (*DM*-domain gene on the Y chromosome). To establish a role for *DMY* during sexual differentiation, we screened wild medaka populations for naturally occurring *DMY* mutants. Two XY females with distinct mutations in *DMY* were found in separate populations (Awara and Shirone). The first heritable mutant - a single insertion in exon 3 and the subsequent truncation of *DMY* - resulted in all XY female offspring. Similarly, the second XY mutant female showed reduced *DMY* expression with a high proportion of XY female offspring. Furthermore, during normal development, *DMY* is expressed only in somatic cells of XY gonads. These findings strongly suggest that the sex-specific *DMY* is required for normal testicular development and is a prime candidate for the medaka sex-determining gene.

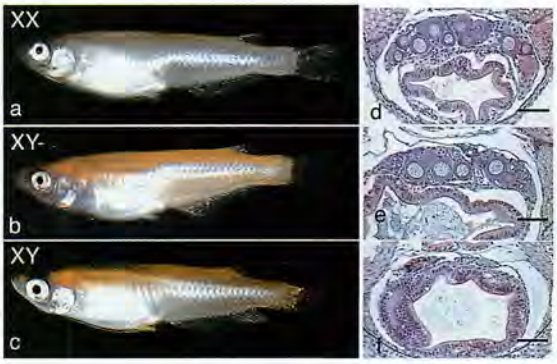

Fig. 1 Characteristics of madaka lacking a part of the Y chromosome (**a-c**). Phenotypes of the congenic strain (XX)(**a**), medaka lacking a part of the Y chromosome (XY^-)(**b**) and the congenic strain (XY)(**c**).Histological cross-sections of medaka fry sampled 30 d.a.h.. XX (**d**) and XY^-(**e**) individuals have ovaries, whereas XY specimen has testes with spermatogonia (**f**). Scale bars, 50 μm.

II. Endocrine regulation of gonadal sex differentiation

Nile tilapia, *Oreochromis niloticus*, is an excellent example of the precise nature of steroidogenic actions during gonadal sex differentiation. In this fish, all genetic female (XX) or male (XY) broods can be obtained by artificial fertilization of normal eggs (XX) and sex-reversed, pseudo male sperm (XX), or normal eggs (XX) and super male sperm (YY), respectively. Fertilized eggs hatch after 4 days at 26℃. On the day of hatching, primordial germ cells (PGCs), are located in the outer layer of the lateral plate mesoderm around the hind gut. At 3 days post-hatching, PGCs are located in the gonadal anlagen after the formation of the coelomic cavity in the lateral plate mesoderm rather than through active migration.

In tilapia, first morphological sex difference is regarded as the number of germ cells between sexes. Second sexual dimorphism in germ cells is the timing for entry of germ cells into meiosis. Recently, we found that two isoforms of *vasa* mRNA and protein are present in tilapia. One (*vas*-s) lacks a part of the N-terminal region found in the other isoform (*vas*). Both isoforms are expressed in oocytes through the embryonic stage when PGCs localize in the lateral plate mesoderm. Thereafter *vas*-s expression increases and *vas* expression becomes undetectable. Expression of both isoforms was observed again after morphological gonadal sex differentiation, irrespective of genotypic sexes. We have also generated medaka transgenic lines with green fluorescent protein (GFP) fluorescence controlled by the regulatory regions of the *olvas* gene in the germ cells. The intensity of GFP fluorescence increases dramatically in PGCs located in the ventrolateral region of the posterior intestine around stage 25 (the onset of brood circulation). Whole-mount *in situ* hybridization and monitoring of ectopically located cells by GFP fluorescence suggest that 1) the increase in zygotic *olvas* expression occurs after PGC specification and 2) PGCs can maintain their cell characteristics ectopically after stages 20-25. The GFP expression persists throughout the later stages in the mature ovary and testis.

In tilapia, mitosis of germ cells begins around 10 days post-hatching in genetic females, but can not be confirmed until after sex differentiation in testes of genetic males. Steroid-producing cells in ovaries, but not testes, at the undifferentiated and differentiating stages express all of the steroidogenic enzymes required for estradiol-17β biosynthesis from cholesterol. Transcripts of estrogen receptors (ER) α and β first appear in both female and male gonads of fry 5-10 days post-hatching. These results, together with evidence of masculinization of genetic females by fadrozole (an aromatase inhibitor) or tamoxifen (an estrogen receptor antagonist), strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. In contrast, the ability of steroid-producing cells to synthesize steroid hormones in the testes only appears at the time of testicular differentiation. Transcripts of androgen receptors are not present in gonads of genetic males during sex differentiation. *DMRT1* is expressed male-specifically in the gonads (Sertoli cells) during sex differentiation, suggesting an important role of *DMRT1* in testicular differentiation.

III. Endocrine regulation of spermatogenesis

Using an organ culture system for eel testes consisting of spermatogonia and inactive somatic cells, we have shown that the hormonal regulation of spermatogenesis in eel testes involves the gonadotropin stimulation of Leydig cells to produce 11-ketotestosterone (11-KT), a potent androgen in fish. In turn, 11-KT activates Sertoli cells to stimulate the production of activin B. Addition of recombinant eel activin B to the culture medium induced proliferation of spermatogonia, producing late type B spermatogonia, within 15 days in the same manner as did 11-KT. cDNAs encoding two androgen receptors (ARα and ARβ) have been cloned, for the first time in any vertebrates, from eel and tilapia testes. In situ hybridization reveals that although both AR mRNAs are present in eel testes prior to HCG injection, only ARα transcripts increase during HCG-induced spermatogenesis suggesting that ARα and ARβ play different roles in spermatogenesis. Activin B binds to activin type I and II receptors on spermatogonia to stimulate de novo synthesis of G1/S cyclins and CDKs leading to the initiation of mitosis. Interestingly, cyclin A1 transcripts are first detected in primary spermatocytes during HCG-induced spermatogenesis in eel testes suggesting an important role for cyclin A1 in the progression to meiosis of male germ cells.

IV. Endocrine regulation of oocyte growth and maturation

Two follicular steroidal mediators, estradiol-17β (oocyte growth) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) (oocyte maturation) were identified in several teleost fishes. Two cell-type models in which the thecal layer provides precursor steroids to the granulosa layer, have been demonstrated for estradiol-17β and 17α,20β-DP production. There is a distinct shift in expression of steroidogenic enzyme genes from cytochrome P450 aromatase (P450arom) for estradiol-17β production to 20β-hydroxysteroid dehydrogenase (20β-HSD) for 17α,20β-DP production in granulosa cells immediately prior to oocyte maturation. The preovulatory surge of LH-like gonadotropin is responsible for the rapid expression of 20β-HSD mRNA transcripts in granulosa cells during oocyte maturation. Two possible transcription factors have been identified: Ad4BP/SF-1 (P450arom) and CREB (20β-HSD). Unlike estradiol-17β (genomic action), 17α,20β-DP binds to a novel, G-protein-coupled membrane receptor (non-genomic action), leading to the *de novo* synthesis of cyclin B, the regulatory component of maturation-promoting factor (MPF), which activates a preex-

isting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase (MO15), thus producing the 34 kDa active cdc2. Upon egg activation, MPF is inactivated by degradation of cyclin B. We showed that the 26S proteasome initiates cyclin B degradation through the first cut of its NH_2 terminus at lysine 57.

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DIVISION OF CELL DIFFERENTIATION

Professor:	*MOROHASHI, Ken-ichirou*
Research Associate:	*SHIMONO, Akihiko*
	ISHIHARA, Satoru
	FUKUI, Yuko
	OGAWA, Hidesato
Technical Staff:	*OKA, Sanae*
NIBB Research Fellow:	*SUZUKI, Taiga*
JSPS Postdoctoral Fellow:	*TSUCHIYA, Megumi*
Postdoctoral Fellow:	*MOHAMAD, Zubair* [1]
	SUGIYAMA, Noriyuki [1]
	MIZUSAKI, Hirofumi [1]
Graduate Student:	*SHIMA, Yuichi* 2)
	BABA, Takahi 3)
	FUKUDA, Takayuki [4]
	KUSAKA, Masatomo [5]
	KOMATSU, Tomoko [5]
	MATSUYAMA, Makoto [5]
	FATCHIYAH [5]
	MORITA, Tomoko [6]
Technical Assistant:	*OWAKI, Akiko* [7]
	KOWA, Hiroe
	SHINOHARA, Yuko [7]
	ISHIKAWA, Azus [7]
	KUSAKA, Hiroko [7]
	SATO, Yuko
Secretary:	*DOUZONO, Akemi* [3]
	YAMANAKA, Tae [3]
	KAWASHIMA, Ryoko
Visiting Scientist:	*HANDA, Yasushi*

[1] *CREST, JST Postdoctoral Fellow*
[2] *Graduate School of Kyushu University*
[3] *Graduate School of Tohoku University*
[4] *Graduate School of Ehime University*
[5] *Graduate University for Advanced Studies*
[6] *Graduate School of Tokyo University*
[7] *CREST, JST Technical Assitant*
[8] *CREST, JST Secretary*

Cell and tissue differentiation proceeds systematically based on orchestrated expressions of a battery of genes. The expressions commence successively along with the passage of time, and consequently a single fertilized egg develops into a variety of tissues and organs, which consist of specialized cells in terms of their structures and functions. Accordingly, it is reasonable to assume that investigation of the mechanisms underlying cell and tissue-specific gene expressions at a molecular level is essential for understanding molecular frameworks for genetic cascades to support cell and tissue differentiation. Based on the concept above, our division of Cell Differentiation has focussed on sex differentiation of the gonads and differentiation of the steroidogneic tissues form the aspect of functions of tissue-specific transcription factors and growth factors.

A number of transcription factors are involved in the process of gonadal differentiation. Some of these factors, such as SRY, WT-1, DAX-1, and SOX-9 have been identified as the products of genes responsible for human diseases that display structural and functional defects in tissues including the gonads. The crucial functions of the other transcription factors such as Ad4BP/SF-1, Emx-2, M33, and Lhx-9 were identified by phenotypes of their gene disrupted mice. In addition, the expression profiles with respect to their distribution and sexual dimorphism strongly suggested the functional significance at the early stage of gonadal differentiation. However, it remains to be clarified how the transcription factors above regulate their target genes transcription and how the genes encoding the transcription factors are regulated. When considering a gene regulatory cascade that supports differentiation and sex differentiation of the gonad, studies of the above two directions are quite important. Based on this concept, we investigated mainly the functions of Ad4BP/SF-1 and Dax-1, and the mechanism of gene regulation encoding these factors.

I. Gene regulatory cascade in the steroidogenic tissue differentiation

When a differentiation process of a tissue is considered, it is possible to assume that certain genes encoding transcription factors are involved in a gene regulatory cascade as the critical components. As a component in the cascades required for adrenocortical and gonadal differentiation, Ad4BP/SF-1 seems to be localized at the upstream of a set of tissue-specific genes including steroidogenic *Cyp* genes, while Ad4BP/SF-1 is localized at the downstream of other transcription factors regulating Ad4BP/SF-1 gene transcription. When considering that upregulation of the components occurs from upstream to downstream of the cascade along with the tissue differentiation and moreover Ad4BP/SF-1 is an essential transcription factor in the adrenocortical and gonadal cascade, identification of the components functioning with Ad4BP/SF-1 and regulating Ad4BP/SF-1 gene transcription is quite important for fully understanding the molecular mechanisms underlying differentiation of the adrenal cortex and gonad. Thus, we have investigated gene regulation of Ad4BP/SF-1 and Dax-1.

Tissue-Specific Enhancers on the *Ad4BP/SF-1* Gene

Based on the aspect above, the regulatory region of the Ad4BP/SF-1 gene has been investigated *in vivo*. A mouse YAC clone (longer than 480 kb) containing whole Ad4BP/SF-1 gene was examined. The long fragment was subcloned into cosmid vector carrying *Lac Z* gene as a reporter gene, and subjected to transgenic assay to examine if they have tissues-specific enhancer element. Our survey for the genomic DNA revealed that particular regions in the Ad4BP/SF-1 gene are responsible for the gene expression specific for the adrenal cortex, ventromedial hypothalamic nucleus, and pituitary. Interestingly, all of them are localized at the intronic regions and the nucleotide sequences are conserved among animal species. The fine structures of these tissue-specific enhancers are investigating.

Wnt4 Signal for Gonad Sex Differentiation

Dax-1 is an orphan nuclear receptor acting as a suppressor of Ad4BP/SF-1, and as an anti-Sry factor in the process of gonadal sex differentiation. The roles of these

nuclear receptors in the differentiation of the gonads and the adrenal cortex have been established through studies of the mutant phenotype in both mice and humans. However, the mechanisms underlying transcriptional regulation of these genes remain largely unknown. We examined the relationship between *Dax-1* gene transcription and the Wnt4 pathway. Reporter gene analysis revealed that *Dax-1* gene transcription was activated by β-catenin, a key signal-transducing protein in the Wnt pathway, acting in synergy with Ad4BP/SF-1. Interaction between β-catenin and Ad4BP/SF-1 was observed using yeast two-hybrid and *in vitro* pull-down assays. The region of Ad4BP/SF-1 essential for this interaction consists of an acidic amino acid cluster, which resides in the first helix of the ligand binding domain. Mutation of the amino acid cluster impaired transcriptional activation of *Dax-1* as well as interaction of Ad4BP/SF-1 with β-catenin. These results were supported by *in vivo* observations using *Wnt4* gene disrupted mice, where *Dax-1* gene expression was decreased significantly in sexually differentiating female gonads. We thus conclude that Wnt4 signaling mediates the increased expression of *Dax-1* as the ovary becomes sexually differentiated.

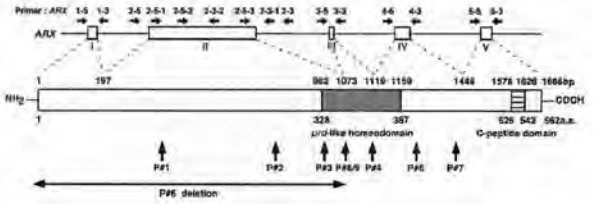
II. Characterization of factors interacting with Ad4BP/SF-1

Arx is essntial for Leydig cell Differentiation.

We have isolated a number of clones by yeast two-hybrid screening using Ad4BP/SF-1, Dax-1, Sox9, Wt-1, Emx-2, and Gata-4 as baits. *Arx*, the *a*ristaless related homeobox gene, is one of the genes interacting with Ad4BP/SF-1. Since the expression of Arx is specific for the gonad and brain from the early developmental stage, the function of Arx was investigated by characterizing phenotype of the gene disrupted mice. In the fetal testes, Arx is expressed in the interstitial cells such as the peritubular myoid cells, tunica albuginea, vessel endothelial cells, and the cell lining beneath the tunica albuginea, but not in Leydig, Sertoli, and germ cells. In the mutant testes, Sertoli, germ cells, tunica albuginea and blood vessels were most likely not affected, whereas the mutant testes were usually characterized by a dysplastic interstitium. MIS, a marker for Sertoli, was clearly detected in the testicular cords of both wild type and mutant testes, whereas expression of Leydig cell marker 3β-HSD was severely diminished in the mutant testis, indicating that Leydig cell differentiation is blocked at a certain stage.

ARX is the responsible gene for XLAG

The structural and functional defects observed in the mutant and the chromosomal localization of *ARX* on Xp22.13 suggested *ARX* as a plausible candidate gene for XLAG (X-linked lissencephaly with abnormal genitalia). To investigate this possibility, we determined nucleotide sequence of the *ARX* in eight XLAG probands, and detected eight mutations. Overall, we found one nonsense mutation, four frame-shift mutations, two misssense mutations, and one larger deletion. Two of the truncated ARX proteins contain the homeodomain but lack the C-peptide (*aristaless*) domain conserved in *prd*-like homeoproteins. Although the function of the domain remains unknown, physiological significance is suggested by our results. Moreover, we identified two missense mutations that lead to amino acid substitutions, R322H and L343Q. Since both amino acids are located in the homeodomain and are highly conserved among the family, it is reasonable to assume that the mutations result in functional failure such as DNA binding.



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DIVISION OF MORPHOGENESIS

Professor:	*UENO, Naoto*
Associate Professor:	*KINOSHITA, Noriyuki*
Research Associates:	*NAKAMURA, Makoto*
	TAKAHASHI, Hiroki
Technical Staff:	*TAKAGI, Chiyo*
	YAMAMOTO, Takamasa
	HYODO, Akiko
	TERASAKA, Chie
	MOROKUMA, Yoshie
	YAMADA, Shigehiro
NIBB Research Fellow:	*MIURA, Jyunko*
Postdoctoral Fellow:	*KITAYAMA, Atsushi*
	MOROKUMA, Junji
Graduate Student:	*KURATA, Tomoko*[1)]
	TAKEUCHI, Masaki[1)]
	IIOKA, Hidekazu[1)]
	MIYAKOSHI, Akira[1)]

[1)] *Graduate University for Advanced Studies*

The complex morphogenesis of organisms is achieved by consecutive cell-to-cell interactions during development. Recent studies suggest that growth factors play crucial roles in controlling such intercellular communications in a variety of organisms. In addition to secretory factors, transcription factors which act cell-autonomously are thought to be essential for the determination of cell fates. Our main interest is to know how pattern formation and morphogenesis during development is regulated by these growth factors and transcription factors. We address this problem using several model animals, including frog, fly and acidian, employing embryology, genetics, molecular and cellular biology, and biochemistry. In addition, we have recently introduced array technology to elucidate precise genetic program controlling early development.

I. Regulation of growth factor diffusion by proteoglycans

During early development, cells receive positional information from neighboring cells to form tissue patterns in initially uniform germ layers. Ligands of polypeptide growth factors such as Wnts and TGF-βs are known to participate in this pattern formation as morphogens. As an initial attempt, we searched for potential cell surface proteoglycans which might interact with morphogens and regulate their diffusion. We identified a gene encoding *Xenopus* glypican-4 (Xgly4), a member of the HSPGs family, and analyzed its role during gastrulation. *In situ* hybridization revealed that *Xgly4* is expressed in the dorsal mesoderm and ectoderm during gastrulation. Xgly4 overexpression and translational inhibition by a Morpholino-oligonucleotide inhibited the gastrulation movements of the embryo and the convergent extension (elongation) of activin-treated animal caps, but did not affect mesoderm induction. Rescue analysis with different mutants of Dsh an essential compomnent of Wnt pathway and Wnt11 demonstrated that Xgly4 function in the non-canonical Wnt pathway, but not in the canonical Wnt/β-catenin pathway for convergent extension movements. Furthermore, we demonstrated that knocking down the levels of Xgly4 inhibits the cell-membrane accumulation of Dishevelled (Dsh), as does the inhibition of Wnt11 in the dorsal marginal zone. Finally, we provided evidence that Xgly4 protein physically binds Wnt ligands. These findings suggest that Xgly4 is serving as a coreceptor for Wnt5A/Wnt11 by presenting these ligands to Frizzled receptor. This hypothesis is further supported by the finding that the causative gene for a zebrafish gastrulation-defective mutant *knypek* encodes a glypican.

II. Gastrulation movement regulated by Wnt signaling

Gastrulation is one of the most important processes during morphogenesis of early embryo, involving dynamic cell migration and change in embryo shape. Almost all animals undergo gastrulation to form the gut. In spite of its importance, the mechanism underlying the event has just begun to be studied at molecular level. During *Xenopus* gastrulation, mesodermal cells migrate to the inside of the embryo and move along the blastocoel roof. One of the important mechanisms for this process is convergent extension. As convergent extension begins, cells are polarized and aligned mediolaterally, followed by the intercalation of these cells. As described above, one of the Wnt signaling pathways, called Wnt/JNK (c-Jun N-terminal kinase) pathway, is shown to be important for the regulation of convergent extension. The pathway is highly conserved among species and initially found to be essential for the establishment of planar cell polarity (PCP) of *Drosophila* wing hair.

We demostrated that *Xenopus prickle* (*Xpk*), a *Xenopus* homologue of a *Drosophila* PCP gene, is an essential component for gastrulation cell movement. Both gain-of-function and loss-of-function of *Xpk* severely perturbed gastrulation and caused *spina bifida* embryos without affecting mesodermal differentiation (Figure 1). We also demonstrated that XPK binds to *Xenopus* Dsh as well as to JNK. This suggests that XPK plays a pivotal role in connecting Dsh function to JNK activation.

Figure 1. Xpk antisense morpholino oligos (mo) inhibit convergent extension movements. Top; uninjected sibling embryo at the tadpole stage, middle panel; mo-Xpk 10-pmol dorsal-injected embryo displaying *spina bifida* and bottom; 4mis mo-Xpk (negative control)-injected embryo showing little effect when injected dorsally .

We are also studying how Wnt signal is transmitted to Dsh from Frizzled receptors. Protein kinase C (PKC) was implicated in the Wnt pathway, but its molecular role was pooly understood. We searched our *Xenopus* EST database for novel PKC genes and identified a novel gene encoding PKCδ. Loss-of-function of PKCδ by a dominant-negative form or Morpholino antisense oligonucleotide has revealed that it is essential for convergent extension during gastrulation. A microscopic analysis showed that PKCδ is requierd for the polarization and morphological change of mesodermal cells in this process. This result suggested that PKCδ might be involved in the Wnt/JNK pathway. It is known that activation of the Wnt/JNK pathway involves translocation of Dsh from cytoplasm to the membrane and activation of JNK. But the molecular mechanism is not clear. We tested whether PKCδ is required for the translocation of Dsh and the activation of JNK, and showed that loss-of-function of PKCδ inhibited both of these events. This result indicates that PKCδ is essential for Dsh function. We also showed that it forms a complex with Dsh and is translocated to the plasma membrane by Frizzled signaling. In addition, activation of PKCδ was sufficient for Dsh translocation and JNK activation. We concluded PKCδ plays an essential role in convergent extension by regulating the localization and activity of Dsh in the Wnt/JNK pathway.

III. Genetic screening for novel DPP/BMP signaling components utilizing *Drosophila* model system

Drosophila is one of the ideal model organisms to dissect signal transduction pathway by genetic methods. We have carried out dominant suppressor screening for two transgenic mutant flies expressed constitutively activated DPP/BMP type-I receptors in wing imaginal discs. We isolated 19 suppressor mutants, *Suppressor of constitutively activated Dpp singnaling* (*Scad*). Alleles of *punt*, *Mad*, *shn* and *dCrebA* were found in isolated *Scad* mutants. We now focus to study a novel locus *Scad67*. *Scad67* encodes a protein with a SP-RING motif. SP-RING motif was originally found in PIAS-family proteins including Drosophila PIAS homolog Su(var)2-10/Zimp. Homozygous *Scad67* mutants show embryonic to pupal stage lethality. The most severe zygotic mutant shows abnormal embryonic head structure and segmentation defects. Recent studies have shown that PIAS-family proteins function as a E3-SUMO ligase and enhance SUMO conjugation against specific substrates. Scad67 is an evolutionary conserved molecule and we found two Scad67 homologous genes in human and also in other vertebrates. In the culture cell, transiently expressed vertebrate Scad67s are localized in the nucleus and frequently accumulate in the nuclear dots. Typical PML body markers, such as SP100, CBP, SUMO1 are co-localised with Scad67s. These data indicate that Scad67 facilitates PML nuclear body assemble. This PML assemble activity is clearly enhanced in the presence of SUMO1 and eliminated by inducing point mutation in the Scad67 SP-RING domain. These results strongly suggest that Scad67s are novel family of SUMO-E3 ligases and participate some critical role in the SUMO-conjugation reaction in the nucleus both in vertebrate and invertebrate.

IV. Brachyury downstream notochord differentiation in the ascidian embryo

Ascidians, urochordates, are one of the three chordate groups, and the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan. It contains a notochord, which is a defining characteristic of chordate embryo composed of only 40 cells. To understand the morphogenesis in this simple system, we have focused on a gene, *Brachyury*, which is known to play an important role in the notochord development. In ascidian, *Brachyury* is expressed exclusively in the notochord and the misexpression of the *Brachyury* gene (*Ci-Bra*) of *Ciona intestinalis* is sufficient to transform endoderm into notochord. This gene encodes a sequence-specific activator that contains a T-box DNA-binding domain, and in vertebrates, it is initially expressed throughout the presumptive mesoderm and gradually restricted to the developing notochord and tailbud. The phenotype of the *Brachyury* mutants in mice and zebrafish revealed that this gene is essential for notochord differentiation. Our goal is to elucidate the down stream pathway of this important gene in ascidian in order to set the stage for understanding not only the formation and function of the notochord but how this important structure has evolved. We conducted the subtractive hybridization screens to identify potential *Brachyury* target genes that are induced upon *Ci-Bra* overexpression. Out of 501 independent cDNA clones that were induced cDNAs, 38 were specifically expressed in notochord cells. We characterized 20 of them by determining the complete nucleotide sequences and *in situ* hybridization analyses which show the spatial and temporal expression patterns of the cDNAs. These potential *Ci-Bra* downstream genes appear to encode a broad spectrum of divergent proteins associated with notochord formation and function.

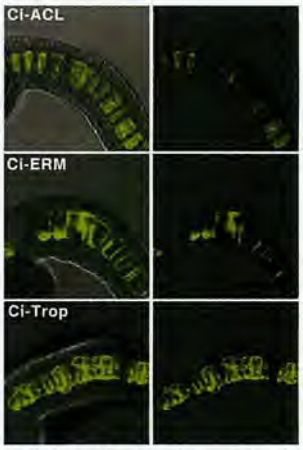

Figure 2. Subcellular localization of electroporated notochord specific genes in ascidian embryos. The embryos were electroporated at one-cell stage with fusion gene containing notochord specific promoter attaced to Ci-ACL-EGFP, Ci-ERM-EGFP and Ci-Trop-EGFP, and grown to the tailbud stage of development. The EGFP fusion proteins shown various subcellular localization in the notochord cells of the tadpole tails.

V. Comprehensive analysis of developmentally regulated genes using macro/microarray

In order to examine the global expression profile during early development of *Xenopus laevis*, we have collected massive EST sequences from three normalized cDNA libraries of early gastrula, neurula and tailbud stage. To date, more than 70,000 ESTs were produced and assembled into 12,447 contigs and 15,361 singlets. Homology analysis shows about 40% of known *X. laevis* proteins are included in these libraries. Moreover, a few thousand novel *X. laevis* orthologue genes which share high similarities ($E<10^{-10}$) with those of other animals are also found. EST sequences and annotational information can be accessed through the web at NIBB *X. laevis* EST database XDB (http://xenopus.nibb.ac.jp/), and cDNAs are available for request.

Using these resources, we generated the NIBB 40k cDNA macroarray, and as an initial case to investigate, we conducted a large-scale gene expression screening of *Xnr-1* regulated genes. As a result, we have isolated 74 (0.16%) *Xnr-1* up-regulated (ratio ≥ 2.0), and 47 (0.10%) down-regulated (ratio ≤ 0.5) independent EST clones. Whole-mount *in situ* hybridization was carried out as secondary screening step, showing high ratio of specific gene expression patterns. These clones encode a variety of signal transduction and transcription regulatory components, and also cytoskeletal components, suggesting dynamic cellular changes in response to the Nodal signals. This also proves DNA array to be an effective screening assay for novel genes which function in the early development of *Xenopus*, utilizing our ESTs.

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DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

Assistant Professor:	*ITO, Kei*
JST Research Fellow:	*AWASAKI, Takeshi (PRESTO)*
JST Postdoctoral Fellow:	*OKADA, Ryuichi (BIRD)*
	KAMIKOUCHI, Azusa (BIRD)
JSPS Postdoctoral Fellow:	*KATO, Kentaro*
Graduate Student:	*TANAKA, Nobuaki (Graduate Univ. Advanced Studies)*
	OTSUNA, Hideo (Nagoya University)

The member of this adjunct division shifted from the Division of Cell Proliferation as of year 2001. The aim of this division is to understand the basic rules by which elaborate neural circuits develop and function. With less than 10^5 neurons, and subject to powerful molecular and genetic techniques, the brain of the fruit fly *Drosophila melanogaster* is a good model system for such study.

I. Comprehensive identification of neural circuits in the adult brain

In spite of the hundred years of efforts using Golgi and other anatomical techniques, the circuit structure of higher-order associative regions of the brain is still essentially unresolved. The GAL4 enhancer-trap system, which is widely used for mutagenesis and gene cloning of *Drosophila*, is also a powerful tool for obtaining a vast array of transformant strains that label specific subsets of brain cells. We screened such strains from a stock of 4500 GAL4 lines made by the *NP consortium*, a joint venture of eight Japanese fly laboratories organized by us.

The database of GAL4 expression patterns in the brain of these strains is the infrastructure for further analysis. Having completed the image database of the confocal sections of the adult brain last year, we started making a similar database for the larval brain and accumulated 59,723 brain images so far.

Analyses of neurons processing visual and olfactory information continue since previous years. For the visual system, we identified in total 44 pathways that connect the optic lobe and the central brain. To investigate the difference of their roles in visual information processing, we blocked the synaptic transmission of identified neurons with the ectopic expression of the dominant mutant form of *Dynamin* GTPase, which blocks the recycling of synaptic vesicles. Flies expressing this transgene in various subsets of the 44 visual pathways were subjected to phototaxis assay, which analyzes the flies' ability to identify the orientation of the light source. The effect was different depending on the color of light source, suggesting that different pathways might convey the visual information of different wavelength ranges.

For olfactory pathways, last year we identified the structures of the projection neurons that transmit information from the first-order olfactory processing site (antennal lobes) to the second-order sites (mushroom bodies and lateral horns). This year we screened the strains that label neurons of these second-order sites. The area of arborization of these neurons showed strong spatial correlation with the distribution of the terminals of the projection neurons. The olfactory code across glomeruli is thus essentially maintained in the neurons of the second-order sites. Like projection neurons, the spatial specification of these neurons were established before adult eclosion and maintained after surgical ablation of the olfactory input.

We also started the characterization of the pathways that convey auditory information. Although the auditory organ of the flies had been identified as the Johnston' Organ, which resides at the base of the antenna, the precise projection patterns of its neurons were not known. We screened the strains that label sensory neurons of this organ and are mapping the distribution of their terminals in the brain.

II. Distribution of neurons that release and receive inhibitory transmitters

Although inhibitory synapses play important roles for information processing, and the GABA is the major neurotransmitter for this function, detail of synaptic connections mediated by GABA is essentially unknown. By combining fluorescent in situ hybridization (FISH) and GFP-immunostaining, we examined the precise distributions of neurons that release and/or receive GABA in the antennal lobe. We found that most of the local interneurons we identified are GABAergic and have GABA receptors. In the case of projection neurons, we found that majority of mACT neurons, but not iACT and oACT neurons, are GABAergic, and that mACT neurons rarely have output sites in the antennal lobe. These findings indicate that, although a wide variety of neurons in the antennal lobe receive inhibitory signals, these signals derive essentially from only the local interneurons.

III. Cell lineage-dependent modular structures in the brain

The brain of *Drosophila melanogaster* is produced by an average of 85 stem cells (neuroblasts) per hemisphere. In previous years, we found that the progeny of each neuroblast project to only a few regions of the adult brain in a stereotypic manner, forming a lineage-dependent circuit module called a clonal unit.

In order to map their distribution, we examined the clonal units in more than 800 adult brains and categorized them. We identified 80 candidate units, among which at least 30 have so far been confirmed to be unique. Further characterization and categorization are under way.

IV. Cell proliferation in the adult brain

Recently, proliferation of neural stem cells in the adult brain was reported in mammals as well as in various insects. To see whether this is also the case in *Drosophila*, we observed the cell proliferation in the adult brain throughout their lifetime (from zero to 50 days after eclosion). Though we found no systematic proliferation of

neuroblasts in old brains, we identified a small number of cells around the antennal nerve that proliferate shortly after eclosion. Immunohistochemistry with a glial cell marker, REPO, and a neuron marker, ELAV, suggests that the proliferating cells are glia. Their progeny becomes either glial cells or the cells of unknown type that is both REPO negative and ELAV negative.

V. Development of identified brain structures

The neural circuits of the adult brain are formed during the larval and pupal stages. Few is known about when and how neurons establish their complicated morphology during pupal metamorphosis. To address this problem, we are tracing the structural change of identified neurons in pupae. An important model circuit for this study is the brain regions called the central complex, whose elaborate structure in the adult brain has long been the scope of anatomical and functional studies. By comparing the image database of the larval and adult brain, we screened for the strains that label putative central complex structure already in larvae. The resulting strains are under detailed investigation to reveal the time course of the central complex composition.

VI. Mechanisms underlying the remodeling of neural circuits

Little is known about the genetic mechanisms that regulate the remodeling of neural circuits during development and maturation of the brain. To address this question, we analyze the larval mushroom body (MB), whose neurons dynamically change the pattern of axon branches during metamorphosis. This process consists of two phases: the degeneration or retraction of existing axon branches, and the re-extension and re-forming of new axon branches (Figure 1A). A single cell labeling of MB neurons revealed that, following the elimination of synapses on the axon branch, axon fibers break and degenerate from proximal to distal subregions of the branches. The synapses lose expression of Fasciclin II (FasII) prior to their elimination (Figure 1B), and the ectopic expression of FasII in the larval MB suppresses the elimination of synapses. Ectopic expression of dominant negative form of Rac, on the other hand, suppresses the degeneration of axon fibers without affecting the synapse elimination. These indicate that the degradation of FasII in the synapses causes the elimination of synapse, whereas the activation of Rac causes the degeneration of axon fibers.

IV. Contribution to the science community

As a joint venture with German and US research groups, we maintain *Flybrain*, a web-based image database of the *Drosophila* nervous system (http://flybrain.nibb.ac.jp). Another database maintained here, mainly for Japanese-speaking fly researchers, is *Jfly* (http://jfly.nibb.ac.jp). Archives of research-related discussions, experimental protocols, images and movies are provided.

Publication List:

Hayashi, S., Ito, K., Sado, Y., Taniguchi, M., Akimoto, A., Takeuchi, H., Aigaki, T., Matsuzaki, F., Nakagoshi, H., Tanimura, T., Ueda, R., Uemura, T., Yoshihara, M., and Goto, S. (2002). GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps. *Genesis* **34,** 58-61.

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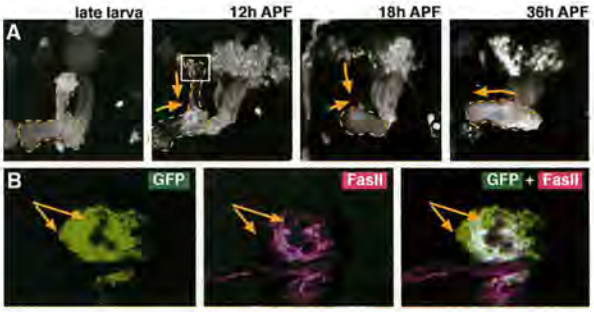

Figure 1. A: Remodeling of mushroom body neurons during early pupal stages. The neurons are labeled by the induced expression of GFP. The vertical and medial branches are pruned during the first 24 hours of the pupal stage (arrows in the second and third panels.) After that, the fibres re-extend only to the medial direction (arrow in the fourth panel.) B: Close-up of the eliminating synapses at the tip of the vertical lobe (white rectangle in the second panel of A.) Arrows show the regions of branches where FasII (magenta, labeled with anti-FasII antibody) disappear before the elimination of synapses of the labeled neurons (green, GFP).

DEPARTMENT OF REGULATION BIOLOGY

Chairperson: NODA, Masaharu

DIVISION OF MOLECULAR NEUROBIOLOGY
DIVISION OF CELLULAR REGULATION
DIVISION OF BIOLOGICAL REGULATION
AND PHOTOBIOLOGY (ADJUNCT)
DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

The department is composed of two regular divisions and two adjunct divisions. The study of this department is focused on molecular mechanisms for the development of central nervous systems in vertebrates, and also on molecular mechanisms for the response of plants toward external environments, such as light, temperature and salinity.

DIVISION OF MOLECULAR NEUROBIOLOGY

Professor:	*NODA, Masaharu*
Research Associates:	*SHINTANI, Takafumi*
	SAKUTA, Hiraki
	HIYAMA, Takeshi (Nov. '02 ~)
Technical Staff:	*TAKEUCHI,Yasushi*
NIBB Research Fellow:	*FUKADA, Masahide*
JSPS Postdoctoral Fellow:	*SUZUKI, Ryoko*
Postdoctoral Fellows:	*FUJIKAWA, Akihiro*[1]
	KATO, Akira[1]
	NAKAMURA, Takahiro[1]
Graduate Students:	*HIYAMA, Takeshi (~ Oct. '02)*
	TAMURA, Hiroshi
	OHKAWARA, Takeshi
	TAKAHASHI,Hiroo
	IHARA, Masaru
	TORIUMI, Shigeru
Visiting Scientists:	*YUASA, Junichi*[2]
	WATAKABE, Ikuko
	YAMAMOTO, Shoichi[3] *(~Apr. '02)*
Secretary:	*KODAMA, Akiko*
Technical Assistant:	*AYABE, Yuko*
JST Technical Staff:	*MIZOGUCHI, Masae*
	GOTO, Megumi
	YAMADA, Kaoru
	MATSUI, Mie

[1] *CREST, JST.* [2] *PREST, JST*
[3] *from SHIONOGI & Co. LTD.*

We have been studying the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system. It covers all the major events including the patterning and growth of the nervous system, neuronal determination, axonal navigation and targeting, synapse formation and plasticity, and neuronal regeneration, especially in the visual system. The scope of our interests also encompasses various functions of the matured brain including sensation, behavior, learning and memory.

I. Molecular mechanism of neural development (Regional specification in the retina and topographic retinotectal projection)

Topographic maps are a fundamental feature of neural networks in the nervous system. Understanding the molecular mechanisms by which topographically ordered neuronal connections are established during development has long been a major challenge in developmental neurobiology. The retinotectal projection of lower vertebrates including birds has been used as a readily accessible model system. In this projection, the temporal (posterior) retina is connected to the rostral (anterior) part of the contralateral optic tectum, the nasal (anterior) retina to the caudal (posterior) tectum, and likewise the dorsal and ventral retina to the ventral and dorsal tectum, respectively. Thus, images received by the retina are precisely projected onto the tectum in a reversed manner.

Since 1992, we have been devoting our efforts to searching for molecules with asymmetrical distribution in the embryonic chick retina, and to characterization of their roles in the topographic retinotectal projection. We identified 33 molecules along the nasotemporal axis and 20 molecules along the dorsoventral axis, with various asymmetrical expression patterns in the developing retina. We elucidated the primary structures of all these cDNA clones and examined their expression patterns during development. These included many novel molecules together with the known molecules: transcription factors, receptor proteins, secretory factors, intracellular proteins, and so on.

We first identified two winged-helix transcriptional regulators, CBF-1 and CBF-2, expressed in the nasal and temporal retina, respectively. Misexpression experiments using a retroviral vector showed that these two transcription factors determine the regional specificity of the retinal ganglion cells, namely, the directed axonal projections to the appropriate tectal targets along the anteroposterior axis. Secondly, we identified a novel retinoic acid-generating enzyme, RALDH-3, which is specifically expressed in the ventral region of the retina, together with a dorsal-specific enzyme RALDH-1.

Furthermore, we recently identified a novel secretory protein, Ventroptin, which has BMP-4 neutralizing activity. *Ventroptin* is expressed in the retina with a ventral high-dorsal low gradient at early stages. This expression pattern is complementary to that of BMP-4. At later stage (E6~), a nasal high-temporal low gradient expression pattern is also detected. *Ventroptin* thus shows a double-gradient expression profile along the dorsoventral and nasotemporal axes. Misexpression of *Ventroptin* altered expression patterns of several topographic genes: *BMP-4, Tbx5* and *cVax* along the dorsoventral axis and *ephrin-A2* along the nasotemporal axis. Consistently, in these embryos, projection of the retinal ganglion cell axons to the tectum was also changed along the both axes. The topographic retinotectal projection along the dorsoventral and anteroposterior axes thus appears to be controlled not independently but in a highly concerted manner by Ventroptin.

To understand the molecular mechanism underlying the specificity of precise neuronal connections, detailed information on the behavior of developing axons during their pathway selection and target recognition is essential. For such studies, several genetic approaches to the laveling and visualizing of neural projections in mice using transgenic or knock-in techniques have been reported. We employed GAP-lacZ as an axon-targeted reporter protein constructed by fusing the membrane-anchoring domain of the GAP-43 protein to lacZ. The reporter gene was introduced into the genome under the control of a promoter element of Brn3b transcription factor to establish transgenic mouse lines. The individual lines thus generated afforded clear images of specific axonal pathways of the visual (Fig. 1), vomeronasal, pontocerebellar, and auditory systems. The reporter protein labelled the entire axonal process as well as the cell body of developing and mature neurons on staining with X-gal. We showed that these lines facilitate the developmental and anatomical study of these neural systems. Moreover, this strategy must be applicable to a variety of neural systems by using various specific promoter

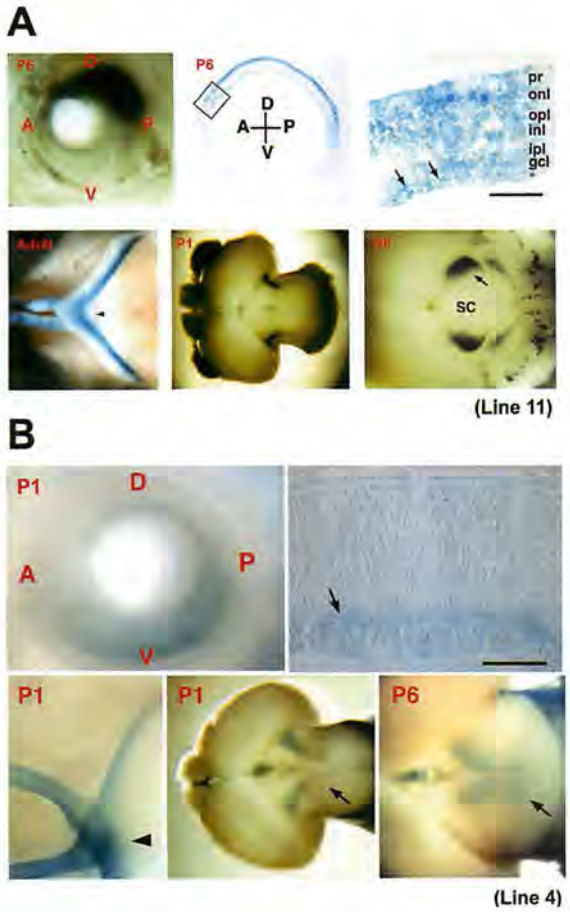

Fig.1. (A) The axonal pathway of dorsal retinal ganglion cells (RGCs) visualized in mouse line 11. The axons project to lateral regions of the superior colliculus (SC) after crossing at the chiasma. (B) The axonal pathway of ventral RGCs visualized in mouse line 4. The axons project to the medial SC.

elements.

Currently, with respect to the identified topographic molecules, we are conducting misexpression experiments using chick embryos and generating knock-out and transgenic mice to elucidate the molecular functions. We expect that our studies will lead to dissection of the molecular mechanism underlying the retinal patterning and topographic retinotectal projection, and ultimately to uncovering the basic principles for establishing complicated but extremely precise neural networks.

II. Physiological roles of protein tyrosine phosphatase receptor type Z (Ptprz)

Protein tyrosine phosphorylation plays crucial roles in various aspects of brain development and brain functions. In 1994, we found that Ptprz/PTPζ/RPTPβ, a nervous system-rich RPTP, is expressed as a chondroitin sulfate proteoglycan in the brain. The extracellular region of PTPζ consists of a carbonic anhydrase-like domain, a fibronectin-type III-like domain and a serine-glycine-rich region, which is considered to be the chondroitin sulfate attachment region. There exist three splice variants of this molecule: A full-length transmembrane form (PTPζ-A); a short transmembrane form (PTPζ-B); and a soluble form (PTPζ-S) which is also known as 6B4 proteoglycan/phosphacan. PTPζ is expressed from the early developmental stage to the adulthood in neurons as well as astrocytes. This suggests that this gene plays variegated roles in the brain development and brain function.

We found in 1996 that PTPζ binds pleiotrophin/HB-GAM and midkine, closely related heparin-binding growth factors which share many biological activities. The chondroitin sulfate portion of PTPζ is essential for the high affinity binding (Kd = ~0.25 nM) to these growth factors, and removal of chondroitin sulfate chains results in a marked decrease of binding affinity (Kd = ~13 nM). We further revealed that chondroitin sulfate interacts with Arg^{78} of midkine. Pleiotrophin and midkine on the substratum stimulated migration of neurons in the glass fiber assay and Boyden chamber cell-migration assay. Experiments using various midkine mutants with various affinities for PTPζ indicated that the strength of binding affinities and the neuronal migration-inducing activities are highly correlated. These results suggest that PTPζ is involved in migration as a neuronal receptor for pleiotrophin and midkine.

To identify the substrate molecules of PTPζ, we have recently developed the yeast substrate-trapping system. This system is based on the yeast two-hybrid system with two essential modifications: Conditional expression of v-src to tyrosine-phosphorylate the prey proteins and screening using a substrate-trap mutant of PTPζ as bait. Using this system, we successfully isolated a number of candidate clones for substrate molecules or interacting molecules. We found that PTPζ interacts with PSD-95/SAP90 family members, SAP102, PSD-95/SAP90 and SAP97/hDlg, which are concentrated in the central synapses mediating protein-protein interactions to form large synaptic macromolecular complexes. Here, the C-terminus of PTPζ binds to PSD-95/SAP90 proteins through the second PDZ domain. This suggests that PTPζ is involved in the regulation of synaptic function. Furthermore, we identified GIT1/Cat-1 as a PTPζ substrate. It is known that Cat-1 regulates Pak, a serine threonine kinase which serves as a target for the small GTP-binding proteins, Cdc42 and Rac, and is implicated in a wide range of cellular events including the cell adhesion and cell morphological change. Pleiotrophin, PTPζ and GIT1/Cat-1 might regulate the neuronal migration and neurite extension by controlling the Pak signaling pathway. We are continuing efforts to characterize the other candidate clones.

In addition, to study the physiological roles of PTPζ *in vivo*, we generated PTPζ-deficient mice in which the PTPζ gene was replaced with the LacZ gene in 1997. We are currently studying the phenotype of PTPζ-deficient mice biochemically, anatomically, physiologically and ethologically, and have already found abnormalities in behavior, learning and memory, etc. Very recently, we found that PTPζ is expressed in gastric epithelial cells and functions as the receptor for VacA toxin secreted from

Helicobactor pylori. Surprisingly, PTPζ-deficient mice are resistant to VacA and do not thereby develop gastric ulcers, in contrast to the wild-type mice.

III. Physiological roles of Na_x sodium channel

Sodium-level sensing system in the brain is essential for the regulation of the sodium and water balance in body fluids. Previously, we demonstrated that *Na_x* (also called *Na_x2/NaG*) gene is expressed in the circumventricular organs and *Na_x*-deficient mice ingest salt in excess. In *Na_x*-deficient mice, *c-fos* expression in the subfornical organ (SFO) and vascular organ of the lamina terminalis (VOLT) was markedly elevated as compared with wild-type mice. Stimulation of the SFO/VOLT of wild-type animals by infusion of a hypertonic sodium solution into intracerebroventricule (ICV) leads to avoidance of salt intake. In contrast, *Na_x*-deficient mice did not show such aversion.

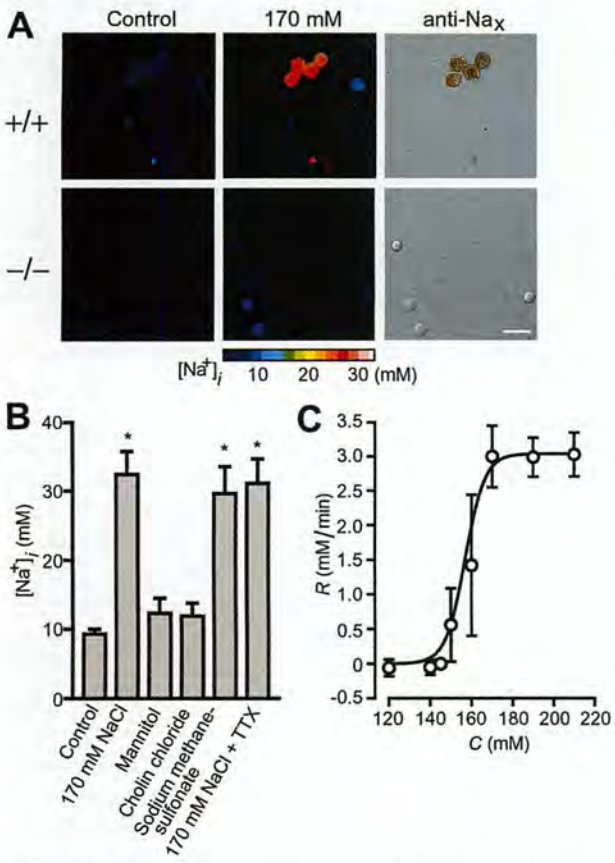

Fig. 2. Sodium-concentration sensitivity is lost in SFO neurons in the *Na_x*-null mutants. (A) Pseudocolor images showing the $[Na^+]_i$ of the cells in the control and high sodium solutions. Scale bar, 50 μm. (B) The $[Na^+]_i$ response is dependent on $[Na^+]_o$, but not on extracellular $[Cl^-]_o$ or osmotic pressure. Na_x is TTX resistant. (C) Relationship between the $[Na^+]_i$ increase rate (R) and $[Na^+]_o$. R_{Max} = 3.04 mM/min, $C_{1/2}$ = 157 mM, and a = 4.67 mM.

We showed that Na_x is a sodium channel which is sensitive to the increase of extracellular sodium level: Entry of sodium ions occurred in the Na_x-immunopositive neurons from wild-type mice, in response to a rise of the extracellular sodium concentration (Fig. 2). In contrast, these responses were not observed in Na_x-immunonegative cells or neurons of *Na_x*-deficient mutant origin. Transfection of *Na_x* cDNA conferred the sodium sensitivity on *Na_x*-deficient cells. All of the GABA-immunopositive neurons isolated from the SFO responded to the extracellular sodium increase. Based on these findings, we propose that GABAergic inhibitory neurons expressing *Na_x* control the activity of the SFO and suppress the salt-intake behavior of animals under thirst conditions.

In addition, we examined the localization of Na_x throughout the visceral organs at the cellular level. In visceral organs including lung, heart, intestine, bladder, kidney and tongue, a subset of Schwann cells within the peripheral nerve trunks were highly positive for Na_x. An electron microscopic study indicated that these Na_x-positive cells were non-myelinating Schwann cells. In the lung, Na_x-positive signals were also observed in the alveolar type II cells, which actively absorb sodium and water to aid gas exchange through the alveolar surface. It was thus suggested that the Na_x sodium channel is involved in controlling the local extracellular sodium level through sodium absorption activity.

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Hiyama, T.Y., Watanabe, E., Ono, K., Inenaga, K., Tamkun, M.M., Yoshida, S. and Noda, M. (2002) Na_x channel involved in CNS sodium-level sensing. *Nature Neurosci.*, **5**, 511-512.

Watanabe, E., Hiyama, T.Y., Kodama, R. and Noda, M. (2002) Na_x sodium channel is expressed non-myelinating Schwann cells and alveolar type II cells in mice. *Neurosci. Lett.*, **330**, 109-113.

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DIVISION OF CELLULAR REGULATION

Professor:	*MURATA, Norio*
Associate Professor:	*MIKAMI, Koji*
Research Associates:	*SUZUKI, Iwane*
	YAMAMOTO, Hiroshi
Technical Staffs:	*IINUMA, Hideko*
	YAMAGUCHI, Katsushi
Monka-sho Foreign Scientist:	
	MUSTARDY, Lázsló [1]
NIBB Research Fellow:	*KANESAKI, Yu*
JSPS Research Fellow:	*SHIVAJI, S* [2]
JSPS Post-Doc Fellows:	*SULPICE, Ronan* [3]
	JOGADHENU, S. S. Prakash [2]
Postdoctral Fellows:	*OHNISHI, Norikazu*
	TAKAHASHI, Shunichi
	KANASEKI, Toku
	ALLAKHVERDIEV, Suleyman I. [4]
Visiting Scientists:	*INABA, Masami* [5]
	LOS, Dmitry A. [6]
	SERGEYENKO, Tatiana [6]
	LYUKEVICH, Alexander A. [6]
	SHAPIGUZOV Alexey [6]
	SZALONTAI, Balazs [1]
	PANICHIKIN Vladimir [7]
	KWON, Ohoak [8]
	HÜBSHMANN, Thomas [9]
	ALLAHVERDIYEVA, Yagut [10]
	HALL, John J. [11]
	PAITHOONRANGSARID, Kalayanee [12]
	DULAI, Sandor, [1]
	CHINTALAPATI, Suresh Kumar [2]
Technical Assistants:	*OKADA, Akiko*
	KOIKE, Yukari
	KAIDA, Satomi
Secretaries:	*ODA, Keiko*
	KUBOKI, Yuko
	KAWAI, Masayo
	ODA, Yuko
Graduate Students:	*FERJANI, Ali*
	SUZUKI, Shingo

[1] *from the Biological Research Center, Szeged, Hungary*
[2] *from the Centre for Cellular and MolecularBiology,Hyderabad, India*
[3] *from Université de Rennes 1, Rennes, France*
[4] *from the Institute of Basic Biological Problems, Pushchino, Russia*
[5] *from Ehime University, Matsuyama, Japan*
[6] *from the Institute of Plant Physiology, Moscow, Russia*
[7] *from Moscow State University, Moscow, Russia*
[8] *from Korea Basic Science Institute, Taejeon, Korea*
[9] *from Humboldt Universität Berlin, Berlin, Germany*
[10] *from Institute of Botany, Baku, Azerbaijan*
[11] *from University of Durham, Durham, England*
[12] *from National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand*

The major thrust of the research efforts of this division is directed towards understanding the signal transduction mechanisms operating in plants and microorganisms with respect to perception of various kinds of stress that arise due to changes in environmental conditions, with particular emphasis on extreme temperatures and salinity. In addition, techniques are being developed for transformation of plants so that they gain the ability to acclimate to and tolerate such stress conditions. In 2002, significant progress was made in the following areas using cyanobacteria as a model system.

I. Membrane rigidification enhances the cold inducibility of gene expression

Our previous experiments have indicated that the expression of the cold-inducible *desA* gene in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which encodes the Δ12 fatty acid desaturase, is induced by catalytic hydrogenation of fatty acids in plasma-membrane lipids. Thus, we hypothesized that changes in membrane fluidity/rigidity are primary signal of cold stress and are perceived by a cold sensor that is localized at plasma membranes. To evaluate this hypothesis, we examined the effect of genetic rigidification of membrane lipids on the cold inducibility of gene expression using DNA microarrays.

We first inactivated the *desA* and *desD* genes for Δ12 and Δ6 desaturases, respectively, in *Synechocystis* by targeted mutagenesis. The resultant *desA*$^-$/*desD*$^-$ cells contained only mono-unsaturated lipid molecules, whereas wild-type cells contained poly-unsaturated lipid molecules. DNA microarray analysis demonstrated that in *desA*$^-$/*desD*$^-$ mutant cells a large number of genes were induced by cold than in wild-type cells. Fourier transform infrared (FTIR) spectroscopy demonstrated that the replacement of poly-unsaturated membrane lipids by mono-unsaturated ones rigidified the membrane lipids. Thus, we conclude that the rigidification of membrane lipids enhances the cold-inducibility of gene expression.

By contrast, the heat inducibility of gene expression was unaffected by mutation of the *desA* and *desD* genes, indicating that heat inducibility of gene expression is not triggered by changes in membrane rigidification. These findings suggest that the change in membrane fluidity/rigidity is involved in the mechanism of cold perception but not of heat perception in *Synechocystis* cells. [Inaba *et al.* (2003) *J. Biol. Chem.*, in press].

II. Multi-stress sensor Hik33 in *Synechocystis*

An increase in osmotic pressure leads to a decrease in cell volume (plasmolysis) by efflux of water from the cell. To recover from the plasmolysis, cells perceive a signal of hyperosmotic stress to maintain physiological cellular osmolarity *via* the expression of osmostress-inducible genes. Screening of *Synechocystis* mutant library of histidine kinases by DNA microarray technique identified a histidine kinase, Hik33, as an osmosensor. Our previous results have demonstrated that Hik33 is a cold sensor in *Synechocystis*.

DNA microarrays were employed to examine whether Hik33 perceives cold and osmostress differently. The results indicated that Hik33 regulates the expression of a large number of osmostress-inducible genes, although 66% of Hik33-regulated genes encoded proteins of unknown function. Hik33-regulated genes are involved in the structural maintenance of cell wall and membranes and the regulation of phosphate transport, photosynthesis, signal

transduction, gene expression, and folding and turnover of proteins (Fig. 1). Although Hik33 regulates the cold-inducible expression of genes which are involved in the regulation of photosynthesis and gene expression, it is clear that Hik33 regulates distinct sets of genes under osmostress and cold conditions with the exception of a small number of genes whose expression was induced by both osmostress and cold (Fig. 1). Therefore, we conclude that Hik33 senses cold and osmostress differently to regulate the expression of distinct sets of genes in a stress-specific manner [Mikami *et al.* (2002) *Mol. Microbiol.* **46**, 905-915].

Recently, a histidine kinase, NblS, which is a putative homologue of Hik33 in *Synechococcus elegatus* PCC 7942, was identified as a sensor of strong light and nutrient stress. Moreover, Hik33 was originally identified as DspA, a chemical sensor of drugs such as inhibitors of photosynthesis. These results suggest that Hik33 might also recognize strong light, nutrient stress and chemicals. Confirmation of this possibility is in progress with DNA microarray technique.

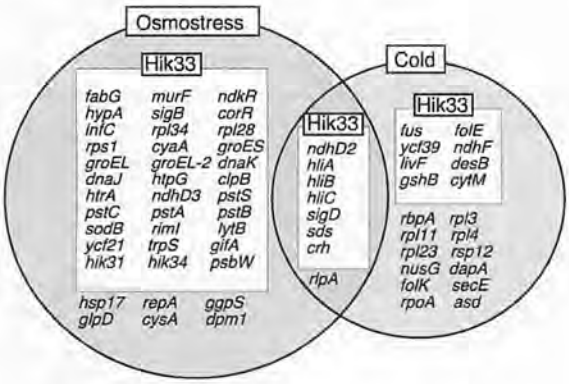

Fig. 1. Osmostress-inducible and cold-inducible genes that are regulated by Hik33. Large and small circles enclose genes whose expression is induced by osmostress and cold, respectively. Rectangles in these circles enclose genes whose expression is regulated to a greater or lesser extent by Hik33 in cells under osmostress and under cold. Genes outside rectangles appeared to be not regulated by Hik33 in terms of their responses to the respective stresses. The rectangle in the overlapping region of the two circles encloses genes whose Hik33-regulated expression was observed under both kinds of stress.

III. Glucosylglycerol, a compatible solute, sustains cell division under salt stress.

When the organisms are exposed to a sudden increase in the external concentration of salts, they accumulate compatible solutes, such as trehalose and glycinebetaine, as a result of synthesis *de novo* or uptake from the environment. *Synechocystis* cells accumulate glucosylglycerol (GG) and sucrose as the compatible solutes under salt stress. While the molecular mechanism for GG biosynthesis including regulation of the expression of the *ggpS* gene, which encodes glucosylglycerol phosphate synthase, has been intensively investigated, the role of GG in protection against salt stress remains poorly understood. We studied the role of GG in the tolerance to salt stress, and found that salt stress inhibited cell division and significantly increased cell size in Δ*ggpS* mutant cells [Ferjani *et al.*, (2003) *Plant Physiol.* in press; Fig. 2]. Electron microscopy revealed that, in Δ*ggpS* cells, separation of daughter cells was incomplete and aborted division could be recognized by the presence of a structure that resembled a division ring (Fig. 2). Addition of GG to the culture medium protected Δ*ggpS* cells against salt stress and reversed the adverse effects of NaCl on cell division and cell size. These observations suggest that GG is important for salt tolerance and thus for the proper division of cells under salt stress conditions.

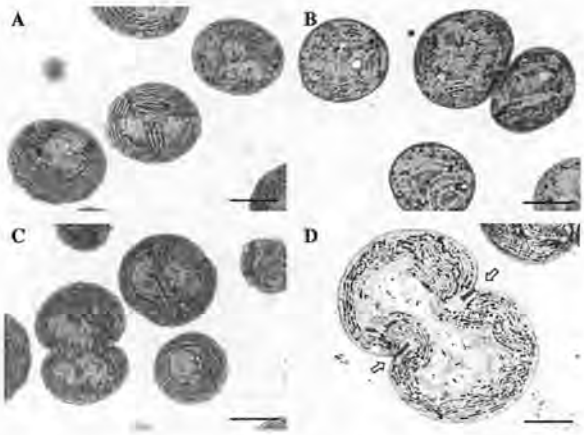

Fig. 2. Effects of 450 mM NaCl on the ultrastructure of wild-type (WT) and Δ*ggpS* cells of *Synechocystis*, as examined by electron microscopy. The cells that had been grown with 20 mM NaCl were cultured in the presence of 20 mM or 450 mM NaCl for three days. (A) WT cells in 20 mM NaCl; (B) WT cells in 450 mM NaCl; (C) Δ*ggpS* cells in 20 mM NaCl; (D) Δ*ggpS* cells in 450 mM NaCl. Arrows in (D) indicate a division ring-like structure at the cell's equator. Each scale bar represents 1 μm.

IV. Salt stress inhibits the repair of photosystem II by suppressing the activities of transcriptional and translational activity

Strong light impairs the photosynthetic apparatus, in particular that of photosystem II (PSII), *via* a process known as photodamage or photoinhibition. However, photodamaged PSII can be repaired, and the repair process involves the replacement of the damaged D1 protein (D1), that is a reaction center protein of PSII, by light-dependently synthesized D1 *de novo*.

Under natural conditions light and salt stress are major environmental factors that limit the efficiency of photosynthesis. However, we have found that the effects *in vivo* of light and salt stress on PSII are completely different in *Synechocystis* [Allakhverdiev *et al.* Plant Physiol. (2002) 130, 1443-1453]. Strong light induced photodamage to PSII, whereas salt stress inhibited the repair of the photodamaged PSII and did not accelerate damage to PSII directly. The combination of both stresses inactivated PSII more rapidly as a consequence of their synergistic effects.

Radioactive labeling of cells revealed that salt stress inhibited the synthesis of proteins *de novo* and, in particular, the synthesis of the D1. DNA microarray analysis indicated that the light-induced expression of various genes was suppressed by salt stress. These results suggest that salt stress inhibits the repair of PSII via suppression of the activities of the transcription and translational machinery (Fig. 3).

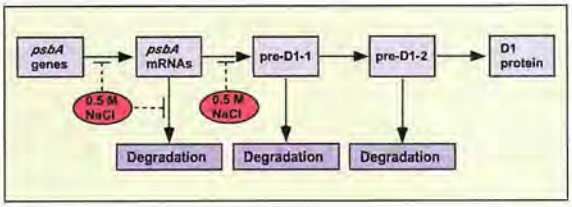

Figure 3. A hypothetical scheme for the inhibition of the D1 protein synthesis *de novo* by salt stress.

List of publication:

(1) Original articles

Allakhverdiev, S.I, Nishiyama, Y., Miyairi, S., Yamamoto, H., Inagaki, N., Kanesaki, Y., Murata, N. (2002) Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of *psbA* genes in *Synechocystis*. *Plant Physiol.*, 130, 1443-1453.

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DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

Professor (Adjunct): *WADA, Masamitsu*
Associate Professor(Adjunct):
KIYOSUE , Tomohiro
Research Associate: *KIKUCHI, Kazuhiro*
NIBB Research Fellow: *KASAHARA, Masahiro*
JSPS Postdoctral Fellow: *CHRISTENSEN, Steen*
PRESTO Fellow: *KAGAWA, Takatoshi*
BRAIN Postdoctoral Fellow:
OGURA, Hironobu
MEXT Postdoctoral Fellow:
TAKAHASHI, Fumio
Graduate Students: *OIKAWA, Kazusato*
(Tokyo Metropolitan University)
SUETSUGU, Noriyuki
(Tokyo Metropolitan University)

Plants respond to light, as an environmental factor to optimize development and regulate other physiological phenomena. Phytochrome and blue light receptors, such as cryptochrome and phototropin (phot), are the main photoreceptors for plant photomorphogenesis. The goal of our research is to elucidate the photoperception and the signal transduction pathways of photomorphogenesis.

I. Chloroplast relocation movement

One of our major subjects is chloroplast photo-relocation movement, which is thought to be one of the simplest phenomena in this field. We use the fern *Adiantum capillus-veneris* and the moss *Physcomitrella patens* as model plants for our cell biological approach since the gametophytes are very sensitive to light and the organization of the cells is very simple. We also use *Arabidopsis* mutants to identify the genes regulating chloroplast photo-relocation movement.

1- 1 *Arabidopsis*

Chloroplasts accumulate at the cell surface under weak light and escape from the cell surface to the anticlinal wall under strong light to optimize photosynthesis. The significance of chloroplast avoidance movement, however, has been uncertain. We analyzed the influence of strong light using several mutants which are defective in avoidance movement in *Arabidopsis*. One of the mutants is *phot2*, defective in the blue-light photoreceptor phototropin. The other mutant is *chup1* which is hypothesized to lack some part of the movement mechanism. Both mutants showed severe damage of leaves when irradiated with strong light for more than 10 hr. The reactive oxygene-scavenging capacity in these mutant leaves, especially the activities of scavenging enzymes, did not differ between mutant and wild-type plants. This suggests that the chloroplast avoidance response is a very important physiological response in plants under high light conditions.

1- 2 *Adiantum phy3*

Adiantum phytochrome3 (PHY3) is a unique chimeric protein with a phytochrome structure in the N-terminal half and a phototropin structure in the C-terminal half. *PHY3* gene analysis of EMS-induced *rap* (*r*ed light-induced *ap*hototropic) mutants of *Adiantum* and complimentation testing revealed that phy3 is the photoreceptor of red light-induced phototropism and chloroplast photorelocation movement. Phy3 also mediates these phenomena in *Adiantum* sporophytes as well as gametophytes. Phylogenic analyses of *PHY3* genes in various ferns suggest that this gene is only found in advanced ferns and is not present in primitive ferns. Enhancement of the photosensitivity of ferns, likely an additional function of phy3, may have contributed to fern evolution and differentiation.

Phy3 has not yet been shown to function as a blue light photoreceptor.

1- 3 *Adiantum phot2*

Adiantum mutants which do not show chloroplast avoidance movement under strong blue light have been isolated. Gene analysis of these mutants and complimentation tests with *PHOT2* gene reveal that phot2 is a photoreceptor of blue light-induced avoidance movement of chloroplasts in *Adiantum*, as in the case of *Arabidopsis*. Particle bombardment of modified *PHOT2* genes into *phot2* mutants show that LOV2 domain of phot2 functions as a chromophore binding domain but LOV1 domain does not. Gene deletions of *PHOT2* from 3' region also show that an important domain of phot2 exists within the C-terminus.

II. Gene targeting and gene silencing

In order to elucidate the role of genes in *Adiantum* and rice whose function is unknown, we have tried to establish new methods for gene targeting in these organisms.

2- 1 *Miniature transposable element*

Miniature inverted-repeat transposable elements (MITE) have been found in wide range of organisms but active MITEs have not been identified. We found a new class of MITEs in rice and named them *miniature Ping* (*mPing*). *mPing* elements are activated in cells derived from anther culture and excise efficiently from original sites to reinsert into new loci. *mPing*-associated *Ping* element which has a putative transposase sequence was also found and shown to transpose within the rice genome.

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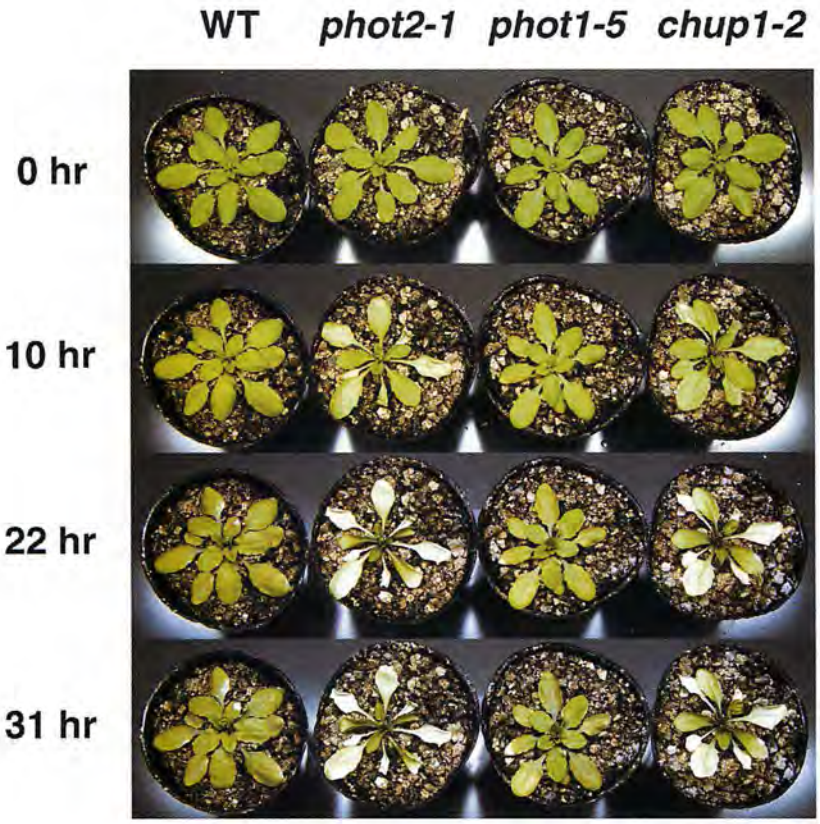

Figure. Phenotypes of plants exposed to continuous strong light. Wild-type, *phot2-1*, *phot1-5* and *chup1-2* mutant plants (4 weeks old) were irradiated with white light at 1400 μmol m^{-2} s^{-1} and were photographed before (0 hour) and after irradiation for 10, 22 and 31 hours. Leaves of *phot2-1* and *chup1-2* plants showed some signs of bleaching after 10 hours of treatment but were severely bleached and necrotic after 22 hours. In contrast, wild-type and *phot1-5* plants withstood this light stress over the 31 hours time period of the treatment.

DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

Professor:	*MURAKAMI, Fujio*
Associate Professor:	*NAKAFUKU, Masato*
Research Associate:	*TAMADA, Atsushi*
Postdoctoral Fellow:	*HANAMURA, Kenji* [1]
	TANIGUCHI, Hiroki [4]
	HATANAKA, Yumiko [4]
	KUMADA, Tatsuro [4]

The vertebrate nervous system contains a large number of neurons that are connected to each other by specific axonal projections. We are interested in how the complex but precise neuronal network, which is indispensable for functioning of the nervous system, is constructed during development. The goal of our research in this division is to elucidate the cellular and molecular mechanisms underlying the neuronal network formation in vertebrates, with current focuses on the mechanisms of axon guidance and neuronal migration.

I. Mechanism of Axonal Guidance

During development of the nervous system, growing axons are guided through specific pathways to correct targets. Our research interest focuses on the cellular and molecular mechanisms of axon guidance in the vertebrate nervous system. We are particularly inter-ested in how guidance mechanisms operating in differ-ent phases of axonal growth are assembled to generate the complex but precise axonal wiring patterns in the brain. To address these issues, we are first trying to understand axonal wiring patterns during development with reference to the structural organiza-tion of the brain. Using whole-mounted preparations of embry-onic rat brains, we will reveal whole axonal trajectories by labeling with neuronal tracers, such as DiI, or by immunohistochemical staining with antibodies against molecular markers expressed in a subset of neurons. We will next explore the axonal guidance mechanisms generating these wiring patterns. Following questions will be addressed. 1) What structures along the path-way have key roles in axonal guidance? 2) What guidance cues exist in these structures? Are these attractive cues or repulsive cues? Are these short-range cues or long-range cues? 3) What molecules are responsible for these cues? 4) How do multiple guid-ance mechanisms work in concert to generate specific wiring patterns? To answer these questions, we use neuroanatomical techniques, in vitro culture techniques including dissociated cell, explant and whole-mount cultures, and biochemical and molecular biological techniques.

II. Mechanism of Neuronal Migration

A variety of neurons migrate from their birthplace to the position where they finally settle. Neuronal migration in the vertebrate central nervous system occurs both along the radial axis and along the tangential axis of the neural tube. Another interest of our research focuses on the cellular and molecular mechanisms un-derlying the radial and tangential neuronal migration. In many regions of the central nervous system, neurons migrate radially from the ventricular zone where they are born toward the pial surface. We are investigating the mechanisms of radial migration, using neurons in the neocortex as a model system. In addition to the radial migration, some neurons in the brain, such as interneurons in the forebrain, move tangentially for a long distance. We are also investigating how tangentially migrating neurons are guided for a long distance through specific routes to the final positions, using neurons that migrate from the rhombic lip at the dorsal rim of the hindbrain, such as cerebellar granule cells and precerebellar neurons.

To address these issues, we developed in vitro culture systems that reconstruct migratory events occurring in vivo. We labeled migrating neurons with green fluorescent protein (GFP) by transplantation of small piece of explant taken from a transgenic rat expressing GFP or by introducing GFP cDNA into limited regions of the brain by electroporation. Slices of the brain or flat-mounted brain preparation are then cultured on perme-able membrane filters. These culture systems enable us to analyze migration pattern of neurons in real time (Fig. 1). Using these in vitro culture systems, we are currently investigating the cellular and molecular mechanisms of neuronal migration.

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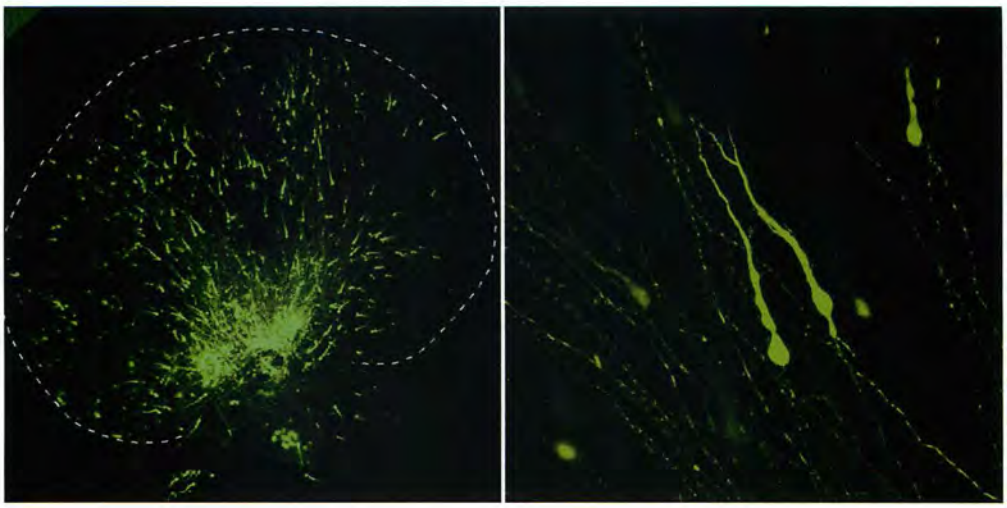

Figure 1. Migrating neocortical neurons visualized with green fluorescent protein (GFP) in vitro.

(Left) Slice culture of rat neocortex that was introduced with GFP cDNA into the ventricular zone by electroporation. A broken line shows the outer margin of the slice. GFP-positive neurons were moving radially from the ventricular zone (bottom) toward the pial surface (top).

(Right) High power view of labeled migrating neurons. Leading processes of neurons extended toward the pial surface.

LABORATORY OF GENE EXPRESSION AND REGULATION

Chairperson: IIDA, Shigeru

DIVISION OF GENE EXPRESSION AND REGULATION I
DIVISION OF GENE EXPRESSION AND REGULATION II
DIVISION OF SPECIATION MECHANISMS I
DIVISION OF SPECIATION MECHANISMS II

The laboratory consists of four regular divisions and conducts researches into regulatory mechanisms of gene expression in microorganisms, plants and animals.

DIVISION OF GENE EXPRESSION AND REGULATION I

Professor:	*IIDA, Shigeru*
Research Associates:	*TERADA, Rie*
	HOSHINO, Atsushi
	TSUGANE, Kazuo
Technical Staffs:	*FURUKAWA, Kazuhiko*
	TANAKA-FUKADA, Sachiko
NIBB Research Fellow:	*CHOI, Jeong-Doo*
JSPS Postdoctoral Fellow:	*PARK, Kyeung-Il*
Postdoctoral Fellows:	*URAWA, Hiroko**
	*JOHZUKA-HISATOMI Yasuyo***
	ISHIKAWA, Naoko
Visiting Scientist:	*CHAREERAT Mongkolsiriwatan[1)]*
Graduate Student:	*OHNISHI, Makoto[2)]*
Technical Assistants:	*MORITA, Yasumasa*
	NAGAHARA, Miki
	SAITOH, Miho
Secretaries:	*HOSHINO Maki****
	*SANJO Kazuko*****

**until May, 2002*
***from February, 2002*
****until May, 2002*
*****from April, 2002*

[1)]from Kasetsart University
[2)]Graduate University for Advanced Studies

The main interest of the group is in understanding the biology of the dynamic genome, namely, genome organization and reorganization and its impact on gene expression and regulation. Although there are many elements affecting organization and reorganization of the genomes, we are currently focused on mobile genetic elements in general and plant transposable controlling elements in particular. Since plant transposable elements are known to be associated with both genetic and epigenetic gene regulations, we are characterizing various aspects of genetic and epigenetic gene regulations. In addition, we are also undertaking reverse genetic approaches in order to elucidate the nature of dynamic genome in plants.

I. Spontaneous mutants in the Japanese morning glory.

The Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*), displaying blue flowers, is believed to be originated from southeast Asia and has an extensive history of genetic and physiological studies. The plant had been introduced into Japan from China in about 8th century as a medicinal herb, seeds of which were utilized as a laxative, and has become a traditional horticultural plant in Japan since around 17th century. A number of its spontaneous mutants related to the color and shape of the flowers and leaves have been isolated, and about 10% of these mutants carry mutable alleles conferring variegated phenotypes. All of the mutants available are spontaneous mutants and most of them were isolated more than 60 years ago. Several lines of evidence indicate that an *En/Spm*-related transposable element *Tpn1* and its relatives, which we termed *Tpn1*-family elements, are common spontaneous mutagens in the plant. Indeed, we previously identified four mutable alleles for flower pigmentation, *flecked-1*, *flecked-2*, *speckled* and *purple-mutable* (*pr-m*), which are caused by integration of non-autonomous *Tpn1*-related elements, *Tpn1*, *Tpn9*, *Tpn2* and *Tpn4* into the genes for antocyanin biosynthesis, *DFR-B*, *ANS*, *CHI* and the gene for a vacuolar Na^+/H^+ exchanger *InNHX1*, respectively. An apparent stable *r-1* allele conferring white flowers was also previously found to be caused by insertion of a non-autonomous *Tpn1*-family element, *Tpn3*, into the *CHS-D* gene encoding a chalcone synthase for anthocyanin biosynthesis. The transposition of these non-autonomous elements is mediated by a *Tpn1*-related autonomous element, which appears to be subjected to epigenetic regulations. Thus the phenotype of the white flowers in the *r-1* mutant due to very rare excision of *Tpn3* from the *CHS-D* gene is likely to be the result from epigenetic inactivation of the autonomous element.

We are able to identify two mutations in the *UF3GT* gene encoding UDP-glucose:flavonoid 3-O-glucosyltransferase, which is the first step to form stable anthocyanin pigments accumulating in the vacuole. One of them carries a *Tpn1*-related non-autonomous element *Tpn10* having integrated into its promoter region, and the other contains an insertion of 4 bp, which generates a termination codon in the cording region. Another mutant displaying maroon flowers was found to carry a 4-bp insertion within the coding region of a newly isolated glucosyltransferase gene. The mutant is likely to be deficient in a transglucosylation process from anthocyanidin 3-glucose to the poly-acylated and glucosylated anthocyanin pigments accumulated in the petal vacuole. In addition to these newly identified mutations in the genes encoding the enzymes involved in anthocyanin biosynthesis pathway, mutants bearing white flowers were found to carry two different 7 bp insertions at the same site within the coding region of a regulatory protein containing the WD40 motif. Both insertions causing frameshifts would result in truncated regulatory protein. These 7 bp insertions are likely to be footprints generated by excision of a *Tpn1*-related transposable element. The results imply that 4 bp insertions detected in the two different glucosyltransferase genes are also footprints generated by excision of *Tpn1*-related elements, which are spontaneous mutagens in the Japanese morning glory.

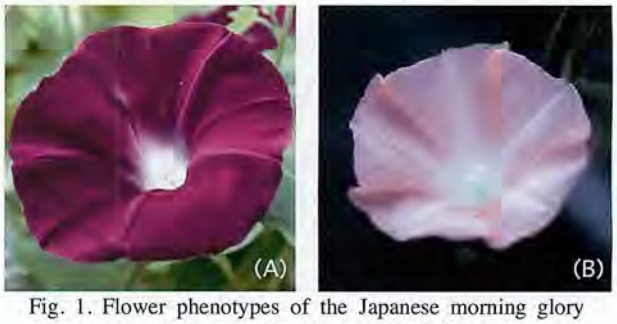

Fig. 1. Flower phenotypes of the Japanese morning glory carrying the wild-type (A) and mutant (B) *UF3GT* gene in the very similar genetic background.

II. Spontaneous mutants in the common morning glory.

The common morning glory (*Ipomoea purpurea* or *Pharbitis purpurea*), originating from Central America, was introduced to Europe probably in the late 17th century and several cultivars with different color of flowers were already recorded in the late 18th century. Previously, we identified that the mutable *flaked* allele of the plant for flower variegation is caused by the insertion of the transposable element *Tip100* into the *CHS-D* gene. The 3.9-kb *Tip100* element belongs to the *Ac/Ds* family.

We are able to identify two mutant lines showing white seeds and palely pigmented flowers were found to produce drastically reduced amounts of the transcripts of the *IPMYC3* gene, a homologue of the *an1* gene for the bHLH transcriptional factor that regulates flower and seed pigmentation in petunia. The genomic sequences revealed that both mutants carry two insertions of transposable elements into the *IPMYC3* gene, which comprises 8 exons. In one mutant, two copies of *Tip100* belonging to the *Ac/Ds* family are inserted into the 2nd intron and the 7th exon. The other mutant contains two different *Mu*-related transposable elements in the 2nd exon and the 5th intron. Moreover, the latter mutant appears to carry an additional mutation for flower pigmentation.

III. Spontaneous mutants in the morning glory (*Ipomoea tricolor*).

Like *Ipomoea purpurea*, the morning glory (*Ipomoea tricolor*) is also originated from central America. The wild type plant produces bright blue flowers, which is one of the most important floricultural plants in both the United States and Europe. We have identified a mutant displaying white flowers carries about 11.5 kb insertion into the 5th intron of the *DFR-B* gene. The junction sequences of the insertion site revealed that the 5th intron of the *DFR-B* gene contains a 0.7 kb *MITE*-like element and that the 11.5 kb sequence is integrated in the middle of the element. Neither terminal inverted repeats of the insertion nor its target site duplication was detected, and the transcription of the *DFR-B* gene appears to be blocked efficiently within the inserted sequence.

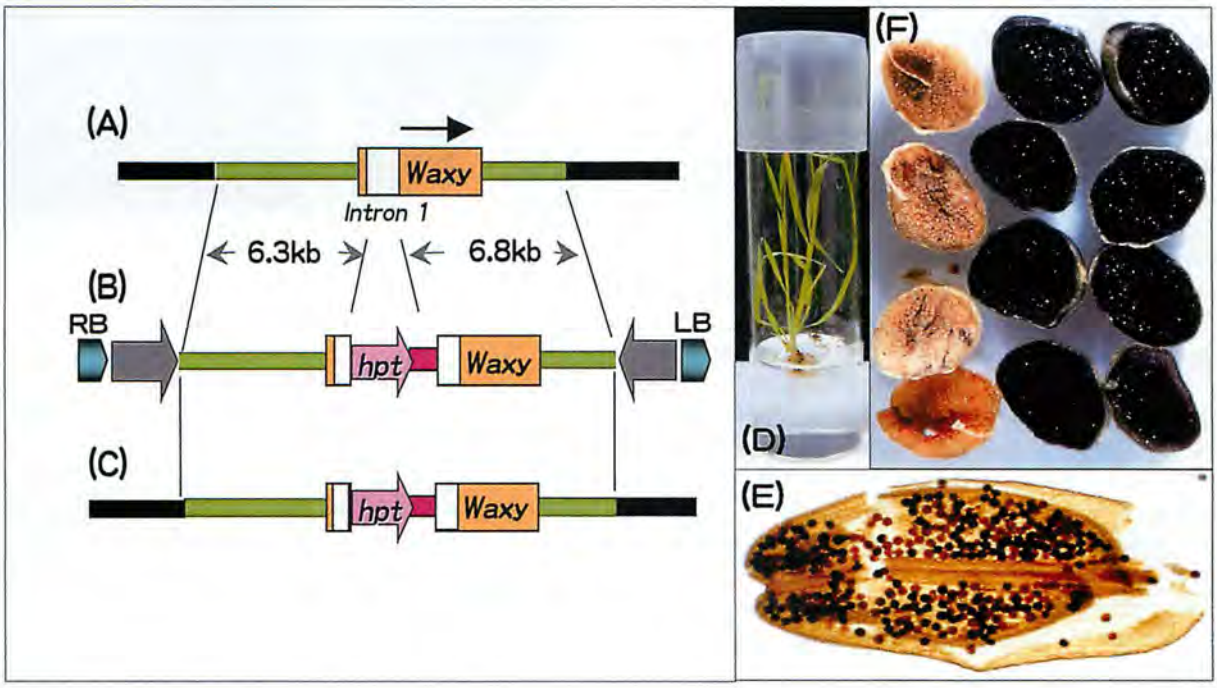

Fig. 2. Strategy for the targeted disruption of the *Waxy* gene by homologous recombination and the *Waxy* phenotype of the targeted rice plant. (A) The genomic structure of the wild-type *Waxy* gene region. (B) The structure between the left and right borders (LB and RB) of the T-DNA region on the targeting vector used. (C) The structure of the targeted *waxy* gene by homologous recombination. The white rectangles in the orange *Waxy* box represent the *Waxy* intron 1 sequence, and the green bars indicate the sequences corresponding to the *Waxy* flanking segments carried by the targeting vector. *DT-A* and *hpt* indicate the negative and positive selection markers employed. (D) A fertile target rice plant. (E) The *Waxy* phenotype in pollen. (F) The *Waxy* phenotype in endosperm. The *Waxy* and *waxy* phenotypes are indicated by the dark and light brown staining with iodine, respectively.

IV. Targeted gene disruption by homologous recombination in rice.

Rice (*Oryza sativa* L.) is an important staple food for more than half of the world's population. It is a model plant for other cereal species because of several characteristics: its small genome of about 430 Mb; the similarity of its sequences and constellation of the genes with other cereals; the availability of large expression sequenced tag (EST), full-length cDNAs and the entire genome sequence of both *Japonica* and *Indica* subspecies; and its efficient use of *Agrobacterium*-mediated transformation. A comparison of the genome sequences of these *Japonica* and *Indica* subspecies with that of *Arabidopsis* indicates that a large proportion of rice genes hove no recognizable homologues in *Arabidopsis*. Under these circumstances, the development of a method to study gene function by modifying genomic sequences precisely becomes extremely important, and gene targeting is a powerful tool of such reverse genetics. In higher plants, however, the method is far from a common practice.

We have succeeded to develop an efficient and reproducible procedure for gene targeting in rice, based upon the following components: (a) optimization of *Agrobacterium*-mediated transformation of embryogenic rice calli, (b) utilization of strong positive/negative selection, and (c) stringent PCR screening for targeted allele. As a model gene to be targeted, we chose the *Waxy* gene encoding granule-bound starch synthase, a key enzyme in amylose synthesis, because its mutations affect the quality and quantity of rice grain and because the associated phenotype in pollen and in endosperm can easily assessed by simple iodine staining. All of the six independently obtained fertile transgenic rice plants from six experiments were heterozygotes at the *Waxy* locus: a wild-type *Waxy* allele and a targeted recombinant *waxy* allele. These transgenic plants segregated into a 3:1 ratio, confirming to the Mendelian pattern of inheritance. Neither ectopic targeting (integration of the sequence produced by homologous recombination into a site other than the correctly targeted site in the genome) nor ectopic integration (integration by nonhomologous endjoining) of the selective marker gene could be observed. Southern blot analysis revealed that the targeted *waxy* region of around 35 kb comprised the anticipated structure and that the drug-resistant marker used for selecting transformants was integrated only within the targeted *waxy* gene in the genome. Sequencing analysis of the junction regions confirmed that precise somatic homologous recombination occurred to generate the rice plants carrying the targeted *waxy* gene. We are currently examining whether the strategy we used is applicable to obtain various gene-targeted or knockout lines of rice.

V. Characterization of mutable virescent allele in rice.

Leaves of seedlings in the *virescent* mutant of rice (*Oryza sativa* L.) are initially pale yellow green due to partial deficient in chlorophyll and gradually become green with the growth of the mutant. The mutable *virescent* plant (*yl-v*), displaying pale green leaves with dark green sectors, was isolated among progeny of a hybrid between *Indica* and *Japonica* rice plants. The leaf variegation is regarded as a recurrent somatic mutation from the recessive pale green to the pigmented revertant allele. During repeated crossing with the *Japonica* rice T-65, an apparent stable *virescent* mutant, *yl-stb*, the seedlings of which bear pale green leaves, was obtained. Although the *yl-stb* mutant could not grow on soil, we have succeeded in growing it with in vitro culture during its critical juvenile phase. Preliminary HPLC analysis indicated accumulation of a carotenoid intermediate in the *yl-stb* mutant. The mutable *virescent* allele was mapped in the short arm of the rice chromosome 3.

Fig.3. Variegated leaves of the mutable *virescent* (*yl-v*) mutant of rice.

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DIVISION OF GENE EXPRESSION AND REGULATION II

Professor:	*HORIUCHI, Takashi*
Research Associate:	**HIDAKA, Masumi*
	KOBAYASHI, Takehiko
	JOHZUKA, Katsuki
Technical Staff:	*HAYASHI, Kohji*
JSPS Research Fellow:	***Austen RD Ganley*
Post Doctoral Fellows:	*KODAMA, Ken-ichi*
Graduate Student:	*SERIZAWA, Naomi*
	WATANABE, Takaaki
Visiting Fellows:	*UJIIE, Yoshifumi*

** to Biomolecular Engineering Research Institute, 6-2-3, Furuedai, Suita, Osaka, 565-0874, JAPAN (Sep, 1, 2002-).*
*** from Nov. 30, 2002*

The genomes of higher organisms contain significant amounts of repetitive sequences, which in general, are unstable. At present, neither the physiological function(s) of repeated sequences nor the mechanism controlling the instability is fully understood. To clarify these aspects, we are pursuing the following themes using *E. coli* and *S cerevisiae*: (1) An amplification mechanism of repeated sequences or genes, especially rRNA repeated genes, (2) a mechanism of replication fork block-dependent recombination, a key reaction that increases or decreases the number of repeats, and (3) development of *in vivo* artificial gene amplification systems. Structural and functional analyses of the *E. coli* genome are also being carried out. In 2002, work on the following three subjects has advanced our knowledge of the dynamics and structure of the genome.

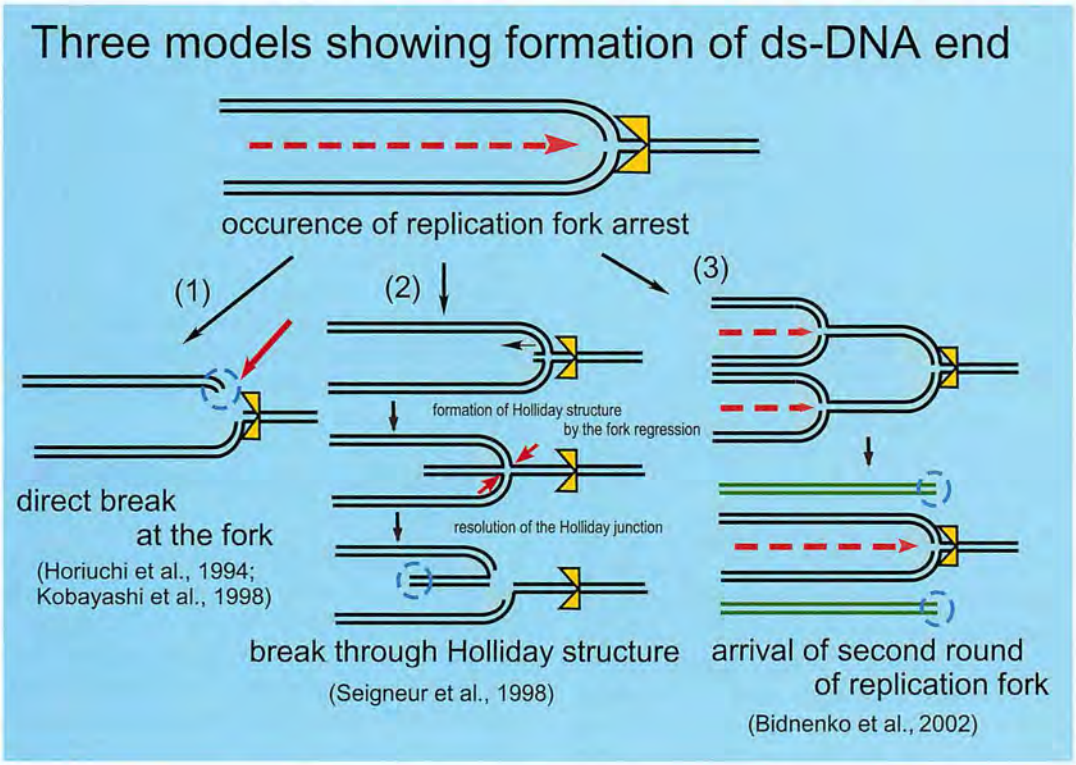
I. Unequal sister-chromatid recombination induces the instability of the rDNA in *Saccharomyces cerevisiae*.

The yeast *S. cerevisiae* carries approximately 150 copies of the ribosomal RNA gene (rDNA) in tandem repeats on chromosome XII. Transcription of the rDNA is carried out specifically by RNA polymerase I (Pol I), one of the three kinds of eukaryotic RNA polymerases. The absence of an essential subunit of Pol I in *rpa135* deletion mutants causes a gradual decrease in the rDNA repeat number to approximately half the normal level and reintroduction of the missing *RPA135* gene induces a gradual increase in repeat number back to the normal level. This contraction/expansion requires the *FOB1* gene protein (Fob1p) which is also required for replication fork blocking activity at the replication fork barrier (RFB) site. The RFB site is located in a non-transcribed spacer region, called NTS1, in each rDNA unit. Using this artificial induction system of amplification, we tested the effects of several mutations on the pattern of copy number increase. The gene *RAD52* was essential for the increase in rDNA repeats. *mre11* mutant cells showed a partial reduction in the amplification of rDNA. In a *sir2* mutant, the rate of amplification increased by more than two-fold, although the copy number was not stabilized. The hyper-recombinogenic phenotype in the mutant was dependent on *FOB1*. In addition, the loss of *SIR2* function reduced the association of Mcd1p, a factor involved in sister-chromatid cohesion, with the non-transcribed spacer region, NTS1, in the rDNA repeats. A temperature sensitive mutant of the cohesin complex, *smc1-2*, had an approximately 8-fold increase in the loss of a *URA3* marker, which is integrated in the rDNA repeats, at the semi-permissive temperature. These results suggest that the establishment of sister-chromatid cohesion would affect the efficiency of the unequal sister-chromatid recombination to change the copy number of rDNA after the double strand break at the DNA replication fork arrested at the RFB site (see Figure).

II. Transcription-dependent recombination in yeast rDNA.

In *Escherichia coli*, we previously identified a chromosome-derived *Eco*RI fragment, as a recombinational hotspot, HotH, which contains the 3' end of an rRNA operon, *rrnD*. Under HotH active conditions, the progress of the DNA replication fork was retarded in the latter part of the *rrnD* operon, the transcriptional orientation of which is opposite to that of the fork. This result suggests that the collision between the replication fork and rRNA gene transcription may be responsible for retardation. Inactivation of the promoter of the *rrnD* operon simultaneously reduced the retardation of fork movement and HotH activity. These results indicate that HotH activation is caused by the collision between the DNA replication fork and the reverse-oriented *rrnD* transcription.

The rRNA gene (rDNA) in *S. cerevisiae* has a tandem repeat structure, whose copy number is approximately 150 copies. The replication fork barrier (RFB) site is located near the 3'-end of the 35S rDNA of each rDNA unit. This site inhibits the progression of the DNA replication fork coming from the opposite direction of the 35S rDNA transcription. The gene *FOB1* was previously shown to be required for the replication fork blocking activity at the RFB site and also later for the change in the copy number of rDNA through unequal sister chromatid recombination. Another function of the RFB site may be to avoid a collision between 35S rDNA transcription and DNA replication. Although a collision has never been observed in the wild type or even in a *fob1* mutant with 150 copies of rDNA, it could be detected in a *fob1* derivative with a reduced number of rDNA copies (approximately 20) by using two-dimensional agarose gel electrophoresis. This result suggests that while each rDNA of 150 copies may be weakly transcribed or a small fraction of the rDNA may be actively transcribed, most of the reduced copies are probably extensively transcribed. The collision was dependent on the transcription of 35S rDNA by Pol I. In addition, under these *FOB1* defective conditions, transcription apparently enhanced recombination to change the copy number of rDNA and also to produce the extra-chromosomal rDNA circles (ERCs) whose accumulation is known to be a cause of aging. These results suggest that such a transcription-dependent



Three models explaining the formations of a double stranded (ds) DNA end are shown. When the fork is blocked for some reason, any of the following three mechanisms possibly work. (1) A nick or a ds-break is introduced at either sister-chromatid. This is thought to occur when the fork is blocked at the RFB site in rDNA repeats in yeast (see text). (2) When the fork is arrested, two newly synthesized sister-chromatid DNA strands start to pair with each other, leading to regression of replication fork, resulting in a formation of the Holliday structure. A resolvase enzyme, such as RuvC in *E. coli*, resolves the structure, producing a ds-DNA end. (3) When the second round of DNA replication reaches the previously arrested fork, two linear ds-DNA molecules with ends are produced. This event is believed to occur in prokaryotes, in which multi-round replications are allowed.

collision may function as a recombination trigger, such as introducing a ds-break shown in Figure, for multiplication of a single copy gene that is intensely transcribed in general.

III. Complete genomic sequence of the *E. coli* K12 W3110 strain

Escherichia coli is one of the organisms that has been most extensively analyzed physiologically, biochemically and genetically. Of all *E. coli* strains, *E. coli* K12 W3110 has probably been used most frequently as the wild-type strain in these experiments. Here, we have determined the complete nucleotide sequence of the genome of strain W3110 by mainly using lambda phages of Kohara's bank. Previously a US group determined the genomic sequence of another K12 wild-type derivative, MG1655. Both strains were derived from a common ancestor strain, W1485, approximately 50 years ago. After comparing the two sequences , the following results were obtained.

The total number of nucleotides in the W3110 genome is 4660170 bp. There were 349 bp conflicts between the sequences of W3110 and MG1655. Re-sequencing of the conflict sites by a PCR method using their genomic DNAs as templates revealed that only eight sites (9 bp) were true conflicts. Seven of them are base-change type conflicts and one is a two base frame-shift. All of these differences reside within genes, seven in ORFs and one in a 23S rRNA gene.

Next we investigated the number and the type of insertion sequence (IS) elements in collaboration with Dr. Ohtsubo's laboratory (Tokyo Univ.). Thirteen different IS elements were identified. While 12 IS elements are present in the W3110 genome and not in MG1655, one IS element is in MG1655, but not in W3110.

These results indicate that the DNA sequence is almost perfectly conserved in bacterial cells under stock culture conditions and in Kohara's lambda phage bank, and during growth through repeated vegetative cycles10. Under stock conditions, which include transfers from the stock to fresh plates and *vice versa*, IS transposition events seem to occur more frequently than base-changes.

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DIVISION OF SPECIATION MECHANISMS I

Professor:	*YAMAMORI, Tetsuo*
Research Associate:	*KOMINE, Yuriko*
	WATAKABE, Akiya
	KITSUKAWA, Takashi
Technical Staff:	*OOSAWA, Sonoko*
	SAWADA, Kaoru
	Miki, Kazuhiko
Technical Assistant:	*ISHIKAWA, Takako*
NIBB Postdoctoral Fellow:	*SI, Xuaohui*
Postdoctral Fellow:	*HATA, Katsusuke*
	SAKATA, Shuzo
Graduate student:	*KOMATSU, Yusuke*
	TAKAHATA, Toru
Secretary:	*HAYASHI, Hitomi*

Our research is focusing to understand mechanisms underlying memory and evolution of the brain. For one approach to understand these questions, we are studying the genes that are expressed in specific areas of the primate neocortex. Using differential display method, we obtained three genes that showed marked differences within areas of the primate neocortex. Our second approach is to understand informational processing in the brain underlying learning behaviors by examining gene expression. Here, we report our findings focusing on those of the year of 2002.

I. Genes expressed in specific areas of the neocortex

The neocortex, most remarkably evolved in primates, plays the major role in higher functions of the brain. It is known to be divided into distinct functional and anatomical areas and has been a matter of debate to what extent the area of the neocortex is genetically and environmentally determined. It is also puzzling why, during the evolution of mammals, the neocortex was most markedly expanded while the number of the genes in mammal phyla were little changed. To access these questions, we studied gene expression within different areas of the neocortex.

1) In collaboration with Professor Hiroyuki Nawa (Nigata university), we used the DNA macroarray technique to examine gene expression in the areas of human prefrontal, motor and visual cortexes. We found almost all the genes among 1088 genes examined showed only less than a factor of two in the difference of their expressions. Only one gene showed more than three fold difference and another one was between two and three fold difference within the three areas. These results suggest that the genes that are expressed among different areas of the human neocortex are very similar. However, the question remained whether there are any genes that show marked differences within areas of the neo-cortex.

2) In order to answer this question, we employed differential display methods and found at least three genes that indicated area specific expressions.

i) One gene, designated occ1, is specifically expressed in the occipital cortex, particularly in V1, in the primate brain. Furthermore, the expression of occ1 turned out to be activity dependent, because, in the monocularly deprived monkeys injected with TTX into one of the eyes, the expression of occ1 is markedly decreased in the ocular dominance column of the primary visual cortex (V1).

ii) The other gene that showed marked difference within the neocortex, is *gdf7*, a member of BMP/TGF-β family, which is specifically expressed in the motor cortex of African green monkeys. We are currently examining the detailed expression pattern of the gene.

iii) The third gene that we designated tentatively as 134G was preferentially expressed in association and higher sensory areas in the neocortex (Komatsu et al., Society for Neuroscience 32nd Annual Meeting, 2002).

3) We have also further isolated several area specific genes with RLCS (Restriction Landmark cDNA Scanning). We are now characterizing these genes to reveal the mechanisms that form neocortical areas.

Fig. 1 Expression pattern of occ1 in the visual cortex.

In situ hybridization pattern of occ1 in the primate visual cortex. occ1 is markedly expressed in the layer IVcβ and moderately in the layers of II, III and IVα in area V1. The boundary between V1 and V2 is shown by an arrow.

II. Gene expression under a declarative and a non-declarative memory

In order to study informational processing underlying the declarative and the non-declarative memory at molecular and cellular levels in the brain, we employed c-Fos mapping techniques. There have been an increasing number of studies using c-Fos as markers to examine neuronal activities since c-Fos induction by electrical stimulation was found by Morgan and Curran. However, many sensory stimuli per se are now known to cause c-Fos induction. So, we should be very careful to distinguish the c-Fos expression that is caused by learning process from that caused by sensory stimuli. For this purpose, it is necessary to use behavioral systems that are able to distinguish the two. Although a few behavioral systems in rodents have been successfully used for physiology, animal behavior and recently for analyses of knockout mice, little behavioral systems in fact distinguish them. Therefore, we prepared ourselves for using two behavioral systems, each of which represent a declarative and a non-declarative memory.

In one system, we collaborated with professor Yoshio

Sakurai (Kyoto University) who developed audio-visual discrimination task (AVD-task). In this task, a rat was asked to choose either an audio cue (a high or low tone) or a visual cue (a light from the right or the left) to obtain a food pellet (Fig. 2). We found that the visual and audio tasks enhanced the specific expression of c-Fos in the visual and audio cortices, respectively. Among the early visual and auditory pathways examined, c-Fos was specifically induced in the cortices but not in the earlier pathways, suggesting the neural modulation of the neocortex depending on the types of the tasks. Interestingly, the task-dependent c-Fos expression was only observed in excitatory neurons in the relevant sensory cortices.

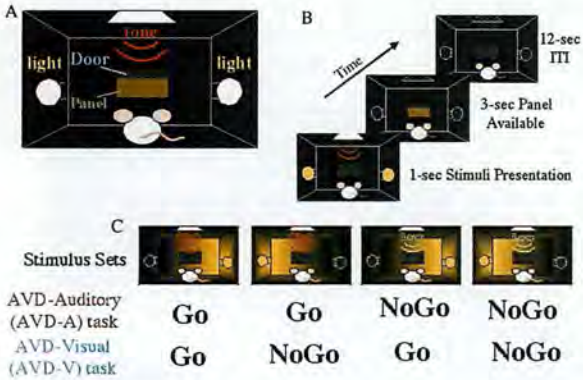

Fig.2. AVD tasks.

A) A rat faces to a panel with a visual cue (a light from right or left) or an auditory cue (a high or low tone) was shown to push the panel with the time sequence shown in (B). The combination of possible cues by auditory and visual tasks to obtain a food pellet is shown in (C). (See Sakata et al., Eur. J. Neurosci., 15, 735-743, 2002, for the detail of the tasks)

The other task we developed is a wheel running system in which a water-deprived mouse is asked to run to obtain water in front because the wheel with pegs is turning to the other direction (Kitsukawa et al., Society for Neuroscience 32nd Annual Meeting, 2002). The pegs can be changed with various patterns as desired. The task required for the mouse thus can be regarded to represent a procedural learning. We examined various areas of brains following to the change of the peg pattern. Among the areas examined, we found marked c-Fos expression in the striatum. The striatum, which is composed of projection neurons and several distinguished types of interneurons, is known to play an important role in a reward-based learning. The characterization of these subtypes of interneurons has been progressed. However, the roles in behavioral tasks have been little known. We hope our system combined with c-Fos mapping technique solve these questions.

III CNTF is specifically expressed in the developing pineal gland

CNTF, a member of the IL-6 family, attracts quite attentions of developmental neuroscientists because it shows various effects on neurons and glias. CNTF knockout mice, however, only indicate moderate motor neuron deficiency in the adult, but no apparent phenotype in the development. In order to explore the function of the IL-6 family, we extensively examined the expression of members of the family and their receptors and found the specific expression of CNTF in the embryonic pineal glands and eyes. This was to our knowledge for the first time to show a clear expression of CNTF in the development. Next question to be asked is the functional role of CNTF in the pineal development. Because of no reported phenotype of CNTF knockout mice in their development, it has been difficult to know the developmental roles of CNTF if any. In fact, we confirmed that there seemed no apparent difference in the pineal development of CNTF knockout mice compared to that in wild type mice. This is presumably because the CNTF gene knockout was compensated by other CNTF-like factors.

We therefore studied cultured pineal organs in rodents to ask if there were any effects of CNTF on them following to a previous study that shows that cultured neonatal pineal organs develop photoreceptor-like cells (Araki, 1992). We found that CNTF inhibits photoreceptor-like cells (Hata et al., Society for Neuroscience 32nd Annual Meeting, 2002). Our observation raises an interesting possibility that CNTF plays a critical role in the pineal development to suppress a certain phenotype such as photoreceptors. We are currently working to prove it.

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DIVISION OF SPECIATION MECHANISMS II

Professor:	*HASEBE, Mitsuyasu*
Associate Professor:	*MURATA, Takashi*
Assistant Professor:	*FUJITA, Tomomichi*
Technical Staff:	*SUMIKAWA, Naomi*
NIBB Research Fellow:	*MISHIMA, Misako (-March 31)*
	HIWATASHI, Yuji
JSPS Research Fellow:	*NISHIYAMA, Tomoaki*
	SATOH, Yoshikatsu
Graduate Students:	*KOBAYASHI-ARAKAWA, Satoko*
	SAKAKIBARA, Keiko
	SAKAGUCHI, Hisako (Shinshu Univ.)(-March 31)
	NAKAMURA, Tohru (Niigata Univ.)(April 1-)
Technical Assistant:	*TANIKAWA, Yukiko*
	BITOH, Yoshimi
	NARUSE, Mayumi
	AOKI, Etsuko
	WATANABE, Kyoko (-Feb. 4)
	YANO, Kana (-Nov. 8)
	KABETANI, Keiko (Feb. 1-)
	OGURA, Youko (Nov. 1-)
Secretary:	*KABEYA, Kazuko*
Visiting Scientists:	*Jean-Pierre Zrÿd [1](-Jan. 18)*

[1]from Université de Lausanne, Lausanne, Switzerland

All living organisms evolved from a common ancestor more than 3.5 billion years ago, and accumulated mutations on their genomes, which caused the present biodiversity. The traces of evolutionary processes are found in the genomes of extant organisms. By comparing the genomes of different organisms, we can infer (1) the phylogenetic relationships of extant organisms and (2) the genetic changes having caused the evolution of morphology and development. The inferred phylogenetic relationships give important insights on problems in various fields of evolutionary biology, and our group focuses on biogeography, evolution of morphological traits, and systematics in wide range of taxa. On the evolution of morphology and development, we aim to explore genetic changes led the evolution of plant body plan. We selected Arabidopsis (angiosperm), Gnetum (gymnosperm), Ginkgo (gymnosperm), Ceratopteris (pteridophyte), Physcomitrella (bryophyte), and some green algae as models to compare the gene functions involved in development of both reproductive and vegetative organs in land plants.

I. Molecular phylogeny of the sundews *Drosera*

Carnivorous plants have long attracted the attention of botanists, because of their highly specialized morphology and curious trapping mechanisms. However, their evolutionary processes are still unknown. The sundew genus *Drosera* consists of carnivorous plants with active flypaper traps and contains nearly 150 species mainly distributed in Australia, Africa, and South America, with some species in the Northern Hemisphere. In addition to the confusion of intrageneric classification of *Drosera*, intergeneric relationships among *Drosera* and two other genera in the Droseraceae with snap traps, *Dionaea* and *Aldrovanda* are problematic. We conducted phylogenetic analyses of DNA sequences of the chloroplast gene *rbcL* for 59 species of *Drosera* covering all sections except one. These analyses revealed that 5 sections in 11 including 3 monotypic sections are polyphyletic. Combined data of the *rbcL* and 18S rDNA sequences were used to infer phylogenetic relationships among *Drosera*, *Dionaea*, and *Aldrovanda*. This analysis revealed that all *Drosera* species form a clade sister to a clade including *Dionaea* and *Aldrovanda*, suggesting that snap traps of *Aldrovanda* and *Dionaea* are homologous despite their morphological differences. MacClade reconstructions indicated that multiple episodes of aneuploidy occurred in a clade including mainly Australian species, although chromosome numbers in other clades are not so variable. *D. regia* native to South Africa and most species native to Australia were basally clustered, suggesting that *Drosera* originated in Africa or Australia. The *rbcL* tree indicates that Australian species expanded their distribution to South America, and then to Africa. Expansion of distribution to the North Hemisphere from the South Hemispere occurred in a few different lineages.

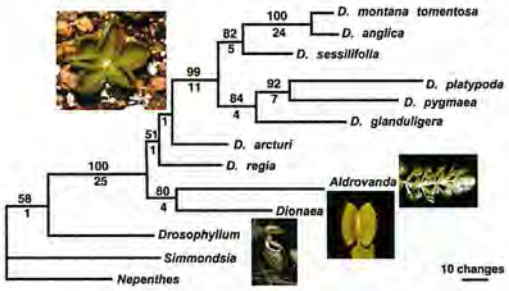

Figure.1. The most parsimonious tree resulting from parsimony analysis of the combined sequences of *rbcL* and 18S rDNA. Branch lengths correspond to the number of nucleotide substitutions (ACCTRAN optimization). Numbers above branches represent bootstrap values more than 50% of 10,000 bootstrap replicates, and numbers below branches are decay indecies (Bremer, 1988).

II. Evolution of reproductive organs in land plants

A flower is the most complex reproductive organ in land plants and composed of sepals, petals, stamens, and gynoecium. Female haploid reproductive cells are covered with a sporangium (nucellus) and two integuments, and further enclosed in a gynoecium. Male haploid reproductive cells (pollens) are covered with a sporangium (pollen sack). On the other hand, gymnosperms and ferns have simpler reproductive organs than angiosperms and lack sepals and petals. Female sporangia (nucellus) of gymnosperms are covered with only one integument. Sporangia of ferns have no in-teguments and are naked on the abaxial side of a leaf.

The development of floral organs is mainly regulated by A-, B-, C-function genes, which are members of the MIKC-type MADS-box genes. These genes are transcription factors containing four domains, MADS, I, K, and C domains. MADS-box genes of angiosperms are divided into more than 10 groups based on the gene tree. The

FLO/LEAFY gene is the positive regulator of the MADS-box genes in flower primordia.

What kind of changes of the MADS-box genes caused the evolution of the complex reproductive organs in the flowering plant lineage? Comparisons of MADS-box and FLO/LFY genes in vascular plants suggest that the following sequential changes occurred in the evolution of reproductive organs. (1) Origin of MIKC-type MADS-box genes. (2) The number of MADS-box genes increased, and the three ancestral MADS-box genes that later generate A-, B-, C-functions genes were likely originated before the divergence of ferns and seed plants. (3) Specification of MADS-box gene expression in reproductive organs occurred in seed plant lineage. (4) The ancestral gene of the AG group of MADS-box genes acquired the C-function before the divergence of extant gymnosperms and angiosperms. (5) The gene duplication that formed the AP3 and PI groups in MADS-box genes occurred before the diversification of extant gymnosperms and angiosperms. (6) The ancestral gene of angiosperm A-function gene was lost in extant gymnosperm lineage. (7) LFY gene became positively regulate MADS-box genes in the common ancestor of angiosperms and gymnosperms after the divergence of ferns and seed plants. (8) Spatial and temporal patterns of A-, B-, C-function gene expression were established in the angiosperm lineage.

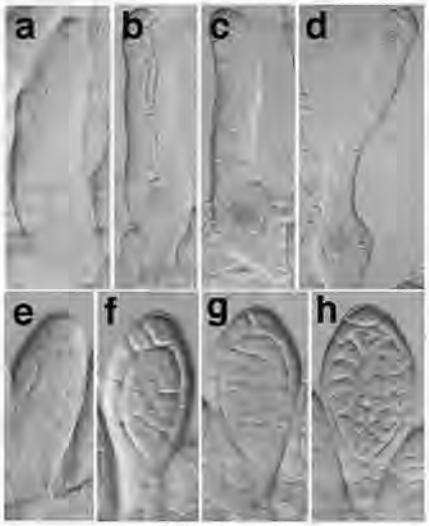

Figure 2. Expression of *Physcomitrella patens* MADS-box gene. GUS activity of PpMADS1-uidA protein was detected in transgenic *Physcomitrella patens*. Localization of PpMADS1-GUS protein in an archegonium (**a-d**), and an antheridium (**e-h**) is shown.

MIKC-type MADS-box genes have been reported only from vascular plants, but not from non-vascular plants. To investigate the origin of MIKC-type MADS-box genes, eight MADS-box genes (*PPM1-PPM5* and *PPMADS1-3)* were characterized in the moss *Physcomitrella patens* (Henschel et al. 2002). Phylogenetic reconstructions and comparison of exon-intron structures revealed that these moss genes represent two different classes of homologous, yet distinct MIKC-type MADS-box genes. We named them MIKC^{C}-type and MIKC*-type genes. MIKC^{C}-type genes are abundantly present in all land plants, and include the A-, B-, and C-function genes. In contrast, *LAMB1* from the clubmoss was identified as the only other MIKC*-type gene published so far. Our findings strongly suggest that the last common ancestor of mosses and vascular plants contained at least one MIKC^{C}- and one MIKC*-type gene.

III-I. Evolution of vegetative organs

The ancestor of land plants was primarily haploid. The only diploid cell was the zygote, which immediately underwent meiosis. It is believed that early during land plant evolution, zygotic meiosis was delayed and a multicellular diploid sporophytic generation be-came interpolated into the life cycle. In the early stages of land plant evolution, sporophytes are epiphytic to gametophytes, as observed in extant bryophytes. During the course of evolution, both generations started to grow independently at the stage of pteridophytes. Finally gametophytes became much reduced and epiphytic to sporophytes in seed plant lineage. Molecular mechanisms of development in a diploid generation have been well studied in some model angiosperms, but we have scarce information on those in a gametophyte generation. For example, mosses have leaf- and stem-like organs in their haploid generation, but it is completely unknown whether similar genes involved in angiosperm leaf and stem development are used in the gametophytic generation of mosses or not. To understand the evolution of body plans in diploid and haploid generation at the molecular level, we focus on the comparison of molecular mechanisms governing shoot development between Arabidopsis and the moss *Physcomitrella patens*. Physcomitrella is known by its high rate of homologous recombination and suitable for analyze gene functions using the gene targeting, and should be a good model lower land plants.

III-II. Characterization of homeobox genes in the moss Physcomitrella patens

Homeobox genes encode transcription factors involved in many aspects of developmental processes including shoot development in angiosperms. The homeodomain-leucine zipper (HD-Zip) genes, which are characterized by the presence of both a homeodomain and a leucine zipper motif, form a clade within the homeobox superfamily and previously re-ported only from vascular plants. We isolated 10 HD-Zip genes from P. patens (*Pphb1-10* genes). Based on a phylogenetic analysis of the 10 Pphb genes and previ-ously reported vascular plant HD-Zip genes, all the Pphb genes except *Pphb3* belong to three of the four HD-Zip subfamilies (HD-Zip I, II, and III), indicating that these subfamilies originated before the divergence of the vascular plant and moss lineages. Pphb3 is sister to HD-Zip II subfamily, and has some distinctive characteristics, including the difference of a1 and d1 sites of its leucine zipper motif, which are well conserved in each HD-Zip subfamily. Comparison of the genetic divergence of representative HD-Zip I and II genes showed that the evolutionary rate of HD-Zip I genes was faster than HD-Zip II genes.

The moss homologs of *SHOOTMERISTEMLESS* and *ZWILLE* genes, which are involved in Arabidopsis shoot development, have been cloned and their characterization is in progress.

III-III. Establishment of enhancer and gene trap lines in the moss *Physcomitrella patens*

We also established enhancer and gene trap lines and tagged mutant libraries of *P. patens* to clone genes involved in the leafy shoot development in haploid generation. Elements for gene-trap and enhancer-trap systems were constructed using the uidA reporter gene with either a splice acceptor or a minimal promoter, respectively. Through a high rate of transformation conferred by a method utilizing homologous recombination, 235 gene-trap and 1073 enhancer-trap lines were obtained from 5637 and 3726 transgenic lines, respectively. Expression patterns of these trap lines in the moss gametophyte varied. The candidate gene trapped in a gene-trap line YH209, which shows rhizoid-specific expression, was obtained by 5' and 3' RACE. This gene was named *PpGLU*, and forms a clade with plant acidic alpha-glucosidase genes. Thus, these gene-trap and enhancer-trap systems should prove useful to identify tissue- and cell-specific genes in Physcomitrella.

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RESEARCH SUPPORT

CENTER FOR TRANSGENIC ANIMALS AND PLANTS
RESEARCH CENTER FOR INTEGRATIVE
AND COMPUTATIONAL BIOLOGY
RESEARCH SUPPORT FACILITY
THE CENTER FOR ANALYTICAL
INSTRUMENTS (managed by NIBB)
TECHNOLOGY DEPARTMENT

CENTER FOR TRANSGENIC ANIMALS AND PLANTS

Head: *NODA, Masaharu*
Associate Professor: *WATANABE, Eiji*
Technical Staff: *IINUMA, Hideko*
Supporting Staff: *YASUDA, Mie*
UNOU, Satsuki
SHIMIZU, Naoki
NOGUCHI, Yuuji

I. Research supporting activity

NIBB Center for Transgenic Animals and Plants was established in April 1998 to support researches using transgenic and gene targeting techniques in NIBB. We are now planning on the construction of the center building.

The expected activities of the Center are as follows:

1. Provision of information, materials and techniques to researchers.
2. Equipment of various instruments to analyze mutant animals and plants.
3. Development of novel techniques related to transgenic and gene targeting technology.

II. Academic activity

We are studying the functional role of Nax sodium channel in collaboration with Division of Molecular Neurobiology. Nax was long classified as a subfamily of voltage-gated sodium channels (NaChs) that serve to generate action potentials in electrically excitable cells such as neuronal and muscle cells. Comparing with the other NaChs, however, Nax has unique amino acid sequences in the regions, which are known to be involved in ion selectivity and voltage-dependent activation and inactivation, suggesting that it must have specific functional properties. To clarify the functional role of Nax, Nax-deficient mice were generated and the physiological phenotypes have been examined. Behavioral studies suggested that the Nax channel plays an important role in the central sensing of body-fluid sodium level and regulation of salt intake behavior. Ion imaging and electrophysiological studies *in vitro* also suggested that Nax is an extracellular sodium-level sensitive sodium channel. More recently, we found that Nax is expressed in non-myelinating Schwann cells and alveolar type II cells in addition to the neurons and ependymal cells in the circumventricular organs (CVOs). Nax is thus likely to be involved in reception of sodium-level in body fluids at the CVOs and sodium absorption in the visceral nervous system and in the lung.

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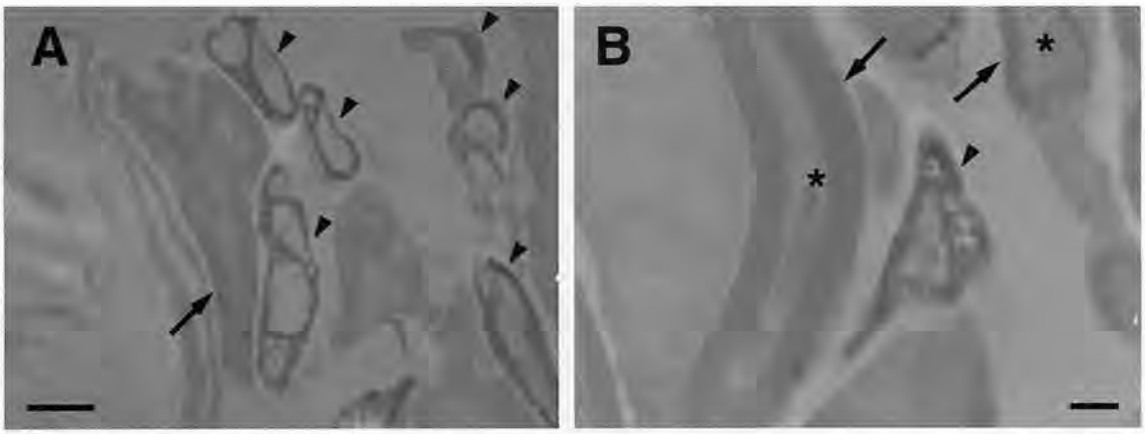

Fig. 1 Nax sodium channel is expressed in non-myelinating Schwann cells.

Immuno-electron micrographs of a region underneath fungiform taste buds stained with anti-Nax antibody. Arrowheads indicate immuno-positive non-myelinating Schwann cells. Arrows indicate the myelin sheath, and asterisks indicate myelinated axons. Scale bars: 1 μm in (A) and 400 nm in (B), respectively. We also found the Nax-expression in the alveolar type II cells. Nax expressed in these cells is likely to be involved in sodium absorption in the peripheral nervous system and in the lung.

RESEARCH CENTER FOR INTEGRATIVE AND COMPUTATIONAL BIOLOGY

Head: *HORIUCHI, Takashi*
Professor: *MIYATA, Takashi*
(kyoto University)

The neutral theory of molecular evolution, the basic theory for explaining evolutionary changes at the molecular level, claims that most evolutionary changes at the molecular level are caused by random drift of neutral mutants. This is sharply contrasted to the evolution at the organismal level, where it is generally thought that natural selection is the major driving force by which evolutionary changes occur. How can we explain the evolutionary changes at the two levels in a unified way. This is the major problem that remains unsolved for molecular evolutionists. As a first step for understanding the final problem, we are investigating relationships of evolutionary diversifications between molecular and organismal levels, based on molecular phylogenetic approach.

I. Divergence pattern of animal gene families and relationship with the Cambrian explosion

In multicellular animals, a variety of gene families involved in cell-cell communication and developmental control have evolved through gene duplication and gene shuffling, basic mechanisms for generating diverse genes with novel functions. Each of these animal gene families has originated either from a few ancestral genes which are shared with plants and fungi or from an ancestral gene created uniquely in animal lineage. The major groups of bilateral animals are thought to have diverged explosively at or prior to the Vendian - Cambrian boundary. No direct molecular evidence has been provided to date as to whether the Cambrian explosion was triggered by a dramatical increase in the number of genes involved in cell-cell communication and developmental control either immediately prior to or in concert with the Cambrian explosion.

A molecular phylogeny-based analysis of several animal-specific gene families has revealed the gene diversification by gene duplication occurred during two active periods interrupted by a long intervening quiescent period. Intriguingly, the Cambrian explosion is situated in the silent period, indicating that there is no direct link between the first burst of gene diversification and the Cambrian explosion itself. The above result also suggests the importance of gene recruitment as a possible molecular mechanism for morphological diversity.

II. Episodic divergence of eukaryote-specific genes and evolution of Giardial membrane bounded organella

To understand a question of whether divergence of eukaryote-specific gene families recognized commonly in all eukaryotes by gene duplication and domain shuffling proceeded intermittently during evolution, as found in animal-specific gene families, a further molecular phylogeny-based analysis has been conducted for kinesin family as a typical example for eukaryote-specific gene families. We have cloned and sequenced cDNAs encoding kinesins and kinesin-related proteins (KRPs) Giardia lamblia, the most primitive protist that represents the earliest branching among extant eukaryotes and have obtained 13 kinesin-related cDNAs, some of which are likely homologs of vertebrate kinesins involved in vesicle transfer to ER, Golgi, and plasma membrane. A phylogenetic tree of the kinesin family revealed that most gene duplications that gave rise to different kinesin subfamilies with distinct functions have been completed before the earliest divergence of extant eukaryotes. This suggests that the complex endomembrane system has arisen very early in eukaryotic evolution, and diminutive ER and Golgi apparatus recognized in the Girdial cells, together with the absence of mitochondria, might be characters acquired secondarily during evolution of parasitism. To understand the divergence pattern of the kinesin family in the lineage leading to vertebrates, seven more Unc104-related cDNAs have been cloned from sponge, amphioxus, hagfish, and lamprey. The divergence pattern of animal Unc104/KIF1 subfamily is characterized by two active periods in gene duplication interrupted by silent periods of considerable long, but not proceeded gradually: Thus, in the eukaryotic lineage leading to vertebrates, the divergence of kinesin family might occurred episodically, as demonstrated by at least three periods of extensive gene duplications, instead of proceeding gradually. Extensive subfamily-generating duplications in ancient times before the earliest branching among extant eukaryotes may have implications to the evolution of Giardial membrane bounded organella.

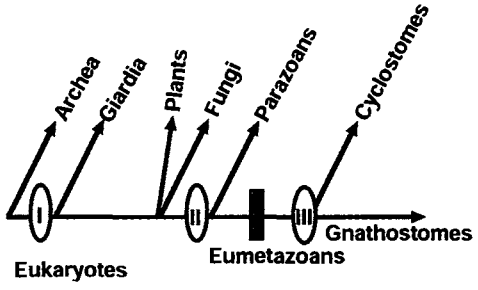

Figure 1. Episodic divergence of kinesin family in eukaryotic lineage leading to vertebrates. Circles I, II and III, periods in which extensive gene duplications occurred. In the period I, basic kinesin genes corresponding to subfamilies with different structures and functions were created by gene duplication and domain shuffling. In the later periods II and III, the multiplicity of members in the same subfamily increased by further gene duplications. Box, the Cambrian explosion.

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Associate Professor: *MOCHIZUKI, Atsushi*
JSPS Postdoctral Fellow: *TOHYA, Shusaku*
Graduate Student: *TAKANO, Ryohji*

I. Directionality of Stripes Formed by Anisotropic Reaction-Diffusion Models.

The pattern formation of animal coating has been studied mathematically by a pair of partial differential equations, named a reaction-diffusion (RD) model. By the model, starting from an initial distribution very close to uniformity, a spatial heterogeneity emerges and a stable periodic pattern is formed spontaneously. This simple mechanism suggests that the reaction of a small number of chemicals and their diffusion might create stable non-uniform patterns. When we analyze the model in a two dimensional plane, striped patterns in addition to spotted patterns often emerge. This was considered as the basic mechanism explaining the stripe patterns observed among animal coating.

We focused on the directionality of the stripes. Most of the stripes observed in the fish skins are either parallel or perpendicular to their anterior-posterior (AP) axis. The direction of stripes is considered of importance in the behavioral and ecological viewpoints. However, very little is known about the mechanisms that makes the strong directionality either in the actual fish skin or in the theoretical models. The standard RD model doesn't determine the direction of stripes. To explain the directionality of stripes on fish skin in closely related species, we have studied the effect of anisotropic diffusion of the two substances on the direction of stripes, in the cases in which both substances have the high diffusivity in the same direction.

We also studied the direction of stripes in more general situations in which the diffusive direction may differ between the two substances. We derived a formula for the direction of stripes, based on a heuristic argument of unstable modes of deviation from the uniform steady state. We confirm the accuracy of the formula by computer simulations. When the diffusive direction is different between two substances, the directions of stripes in the spatial pattern change smoothly with the magnitude of anisotropy of two substances. When the diffusive direction of the two substances is the same, the stripes are formed either parallel to or perpendicular to the common diffusive direction, depending on the relative magnitude of the anisotropy. The transition between these two phases occurs sharply.

Figure 1. The stripe formation in the skin of *Genicanthus*. These two species are closely related.

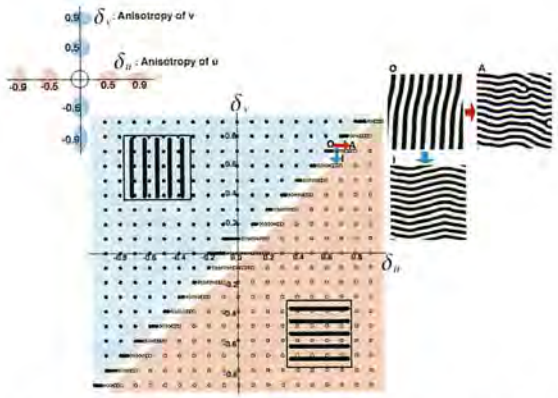

Figure 2. Summary of the direction of stripe patterns obtained by the anisotropic diffusion model. Horizontal and vertical axes indicate anisotropy of activator and that of inhibitor, respectively. The left-upper corner indicates the distortion of diffusion range. Each point indicates the direction of the observed stripe: horizontal; vertical; or not-determined. The direction is determined only by the difference between anisotropies. A small difference in diffusion anisotropy can alter the final pattern to one with opposite directionality. Note the transition of the stripes by changing the anisotropies from O to A (or I).

II. Comparative Study of Circadian Clock Models

Many species living in an environment fluctuating with a strong daily rhythm have evolved an internal clock. According to molecular biological studies of circadian clock, many genes and proteins are involved in the biochemical dynamics generating their stable rhythm. Although the identity of genes and proteins may differ between species, the basic mechanism is the same -- there is one or more genes whose products may enter to the nucleus and then suppress the transcription of their own gene(s). This negative feedback with a long time delay can generate the circadian rhythm.

In this study, we attempt to identify those aspects of biochemical systems for the circadian rhythm which promote sustainable oscillation. We discussed models of different complexity, and examined the effects of cell compartmentalization, protein modification steps in chemical reactions, having multiple clock genes in a cell, and cooperativity in the inhibition of transcription and in the protein transport. To derive general biological conclusions, we focused on mathematical analyses of relatively simple models, rather than computer simulations of complex realistic models.

First, using a Lyapunov function, we proved under very general conditions that two-variable models always converge to a stable equilibrium, implying that additional structures are needed for the model to generate a sustainable rhythm. Second, we compared several models of different complexity using the Routh-Hurwitz criteria of stability. We showed that a sustainable oscillation is more likely to occur; if the cell is compartmentalized so that the proteins need to be transported from the cytosol to the nucleus; if the proteins have to be modified before entering the nucleus; if the kinetics of transcription inhibition or the transport to the nucleus have a nonlinear depend-

ence on the substrate concentration (cooperativity); or if the products of two clock genes form a heterodimer that suppresses both of their own genes.

Specifically we analyzed the local stability mathematically for the cases: (1) there is the reaction reverse to protein modification, (2) there are two clock genes, per and tim, instead of one -- which have been analyzed only by computer simulations; and also the cases (3) the protein transport to the nucleus has cooperativity, which has not been examined before.

III. Pattern formation of the cone mosaic in the zebrafish retina

In teleost fish, there are several subtypes of cone cells, which are sensitive to different wave-lengths of light. In retinas of some species of teleost fish, regular arrangements of cone cells are observed, where each subtype of cone cells appears periodically in the two-dimensional retinal sheet. Different patterns are observed in different species. These patterns are called "cone mosaics." The biological mechanism of the pattern formation is still under examination. Some species show quite different mosaic patterns between peripheral region and more central region in the retina, which suggests mobility of cone cells in retinal space.

We have studied the mechanism of the pattern formation by using mathematical models. In this study, I examined a process of cell-cell interaction to generate the regular mosaic pattern -- namely cell rearrangement. In the model, cells have already been determined as to their final subtypes, but they change their locations in the pattern formation process and the cell movement is affected by their neighbours. I study the conditions for generating mosaic pattern.

First, an assumption regarding "double cone" is shown to be necessary, whereby the two component cells of a double cone behave as if they are a single cone, i.e. a green cone and a red cone are never detached during the pattern formation process. Second, the correct pattern is derived by computer simulation only when the chosen magnitude of cell adhesion is appropriate. I determine the conditions of adhesive strength for generating the observed pattern. These conditions lead to the testable predictions.

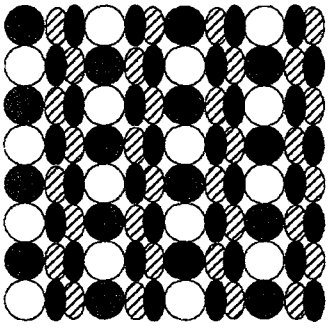

Figure 3. The example of obtained pattern by cell rearrangement model. The obtained patterns were completely the same as the actual zebrafish retina, when the used adhesion was appropriate.

I also compared the condition of cell-cell interaction for generating the mosaic pattern with the different model, the fate transition model. In the fate transition model, cell differentiation regulated by interaction of neighbouring cells is assumed to be responsible for the mosaic pattern. The condition for obtaining the focal pattern is looser in the cell rearrangement model than in the fate transition model.

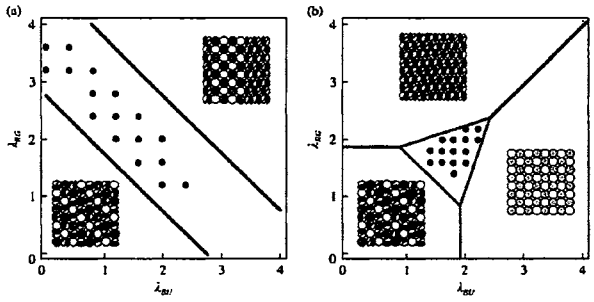

Figure 4. The successful values of adhesion parameters in cell rearrangement model (a) and fate transition model (b). The successful region in cell rearrangement model distribute linearly. On the other hand, horizontal and vertical values have to be balanced in fate transition model. The line segments indicate the boundaries between the region of zebrafish pattern and that of non-zebrafish patterns, which are determined by analytical method. The lines explain the distribution derived computer simulation very well.

IV. Formation and Maintenance of Distinctive Cell Patterns by Co-expression of Membrane-bound Ligands and Their Receptors

We showed that graded or checkerboard-like cell patterns, and segmental domains along a body axis, can be generated by cell behaviors involving differences in intercellular repulsion. A membrane-bound signal transduction system mediating contact-dependent cell interactions includes membrane-bound ligands (ephrins) and their receptors with tyrosine-kinase activity (Eph proteins). These molecules mediate both repulsive and attractive interactions under bilateral threshold control: Cells expressing the receptors adhere to a surface bearing a critical density of ligand reciprocal to the density of receptor, but are repelled by a surface with other densities of ligand. The present paper extends this model: General membrane-bound ligands (not always ephrins) and their receptors are assumed to be co-expressed in a single cell under bilateral threshold control. Computer simulations of cell pattern formation showed that when co-expression of the ligand and receptor is reciprocal, the cells self-organize into a pattern of segmental domains or a graded cell arrangement along the body axis. The latter process interprets positional information in terms of protein molecules. When co-expression of the two species of molecules is not always reciprocal, the cells generate various patterns including checkerboard and kagome (star) patterns. The case of separate expression of ligands and receptors in different cells is also examined. The mechanism of differences in cell repulsion is compared to the differential cell adhesion hypothesis, which has been used to explain cell sorting.

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Ryohji, T., Mochizuki, A. & Iwasa, Y. Possibility of Tissue Separation Caused by Cell Adhesion. *J. Theor. Biol.* (In Press)

RESEARCH SUPPORT FACILITY

Head of Facility: *MURATA, Norio*
Associate Professor: *WATANABE, Masakatsu*
MIKAMI, Koji
(Stress-Resistant Plants)
Research Associates: *HAMADA, Yoshio*
(Tissue and Cell Culture)
UCHIYAMA, Ikuo
(Computer)
Technical Staff: *HIGASHI, Sho-ichi*
(Large Spectrograph)
MIWA, Tomoki (Computer)
NANBA, Chieko
(Plant Culture, Farm, Plant Cell)
NISHIDE, Hiroyo
(Computer)
ITO, Makiko
(Large Spectrograph.-June 2002)
ICHIKAWA. Chiaki
(Large Spectrograph. November 2002-)
YAGI, Eri (Tissue and Cell Culture. June 2002 -)
HARADA, Miyuki (Computer)
MAKIHARA, Nobuko
(Computer)
SUZUKI, Keiko
(Plant Culture, Farm, Plant Cell)

I. Facilities

1. Large Spectrograph Laboratory

This laboratory provides, for cooperative use, the Okazaki Large Spectrograph (OLS), which is the largest spectrograph in the world, dedicated to action spectroscopical studies of various light-controlled biological processes. The spectrograph runs on a 30kW Xenon arc lamp and has a compound grating composed of 36 smaller individual gratings. It projects a spectrum of a wavelength range from 250nm (ultraviolet) to 1,000nm (infrared) onto its focal curve of 10m in length. The fluence rate (intensity) of the monochromatic light at each wavelength is more than twice as much as that of the corresponding monochromatic component of tropical sunlight at noon (Watanabe et al., 1982, *Photochem. Photobiol.*, 36, 491-498).

A tunable two-wavelength CW laser irradiation system is also available as a complementary light source to OLS to be used in irradiation experiments which specifically require ultra-high fluence rates as well as ultra-high spectral-, time-and spatial resolutions. It is composed of a high-power Ar-ion laser (Coherent, Innova 20) (336.6-528.7 nm, 20W output), two CW dye lasers (Coherent, CR599-01) (420-930nm, 250-1000mW output), A/O modulators (up to 40MHz) to chop the laser beam, a beam expander, and a tracking microbeam irradiator (up to 200 $\mu m\ s^{-1}$ in tracking speed, down to 2 μm in beam diameter) with an infrared phase-contrast observation system.

2. Tissue and Cell Culture Laboratory

Various equipments for tissue and cell culture are provided. This laboratory is equipped with safely rooms which satisfy the P2/P3 physical containment level. This facility is routinely used for DNA recombination experiments.

3. Computer Laboratory

Computer laboratory maintains several computers to provide computation resources and means of electronic communication in this Institute. Currently, the main system consists of three servers and two terminal workstations: biological information analysis server (SGI Origin 2000), database server (Sun Enterprise 450), file server (Sun Enterprise 3000), data visualization terminal and molecular simulation terminal (both are SGI Octanes). Some personal computers and color/monochrome printers are also equipped. On this system, we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database construction for the Institute members.

Computer laboratory also provides network communication services in the Institute. Most of PCs in each laboratory as well as all of the above service machines are connected each other with local area network (LAN), which is linked to the high performance multimedia backbone network of Okazaki National Research Institute (ORION). Many local services including sequence analysis service, file sharing service and printer service are provided through this LAN. We also maintain a public World Wide Web server that contains the NIBB home pages (http://www.nibb.ac.jp).

4. Plant Culture Laboratory

There are a large number of culture boxes, and a limited number of rooms with environmental control for plant culture. In some of these facilities and rooms, experiments can be carried out at the P1 physical containment level under extraordinary environments such as strong light intensity, low or high temperatures.

5. Experimental Farm

This laboratory consists of two 20 m^2 glass-houses with precise temperature and humidity control, three green houses (each 6 m^2) at the P1 physical containment level, a small farm, two greenhouses (45 and 88 m^2) with automatic sprinklers. The laboratory also includes a building with storage and work space.

6. Plant Cell Laboratory

Autotrophic and heterotrophic culture devices and equipment for experimental cultures of plant and microbial cells in this laboratory. A facility for preparation of plant cell cultures including an aseptic room with clean benches, is also provided.

7. Laboratory of Stress-Resistant Plants

This laboratory was found to study transgenic plants with respect to tolerance toward various environmental stresses. It is located in the Agricultural Experimental Station of Nagoya University (30 km from National Institute for basic Biology). The laboratory provides a variety of growth chambers that precisely control the conditions of plant growth and dacilities for molecular biological and physiological enaluations of transgenic plants.

The laboratory is also a base of domestic and international collaborations devorted to the topic of stress resistant transgenic plants.

II. Research Activities

1. Faculty

The faculty of the Research Support Facility conducts its own research as well as scientific and administrative public services.

(1) Photobiology: Photoreceptive and signal transduction mechanisms of phototaxis of unicellular algae are studied action spectroscopically (Watanabe 1995, In "CRC Handbook of Organic Photochemistry and Photobiology") by measuring computerized-videomicrographs of the motile behavior of the cells at the cellular and subcellular levels. Photoreceptive and signal transduction mechanisms of algal gene expression were also studied by action spectroscopy.

A novel blue-light receptor with an effector role was found from *Euglena glacilis* (Fig. 1; Iseki *et al.* 2002, *Nature* **415**, 1047-1051): *Euglena gracilis*, a unicellular flagellate, shows blue-light type photomovements. The action spectra indicate the involvement of flavoproteins as the photoreceptors mediating them. The paraflagellar body (PFB), a swelling near the base of the flagellum has been considered as a photosensing organelle for the photomovements. To identify the photoreceptors in the PFB, we isolated PFBs and purified the flavoproteins therein. The purified flavoprotein (ca. 400 kDa), with noncovalently bound FAD, seemed to be a heterotetramer of a- and b-subunits. Predicted amino acid sequences of each of the subunits were similar to each other and contained two FAD-binding domains each followed by an adenylyl cyclase catalytic domain. The flavoprotein showed an adenylyl cyclase activity, which was elevated by blue-light irradiation. Thus, the flavoprotein (PAC, photoactivated adenylyl cyclase) can directly transduce a light signal into a change in the intracellular cyclic AMP level without any other signal transduction proteins.

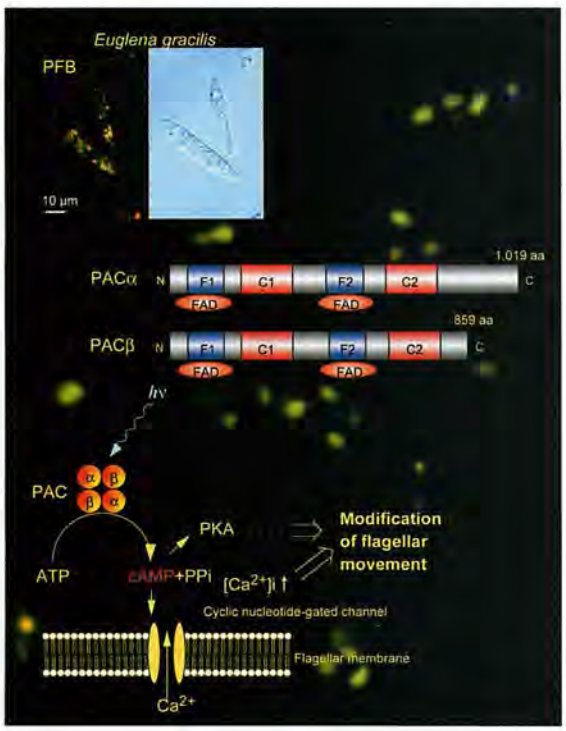

Fig.1 Photoactivated adenylyl cyclase (PAC): a novel blue-light receptor mediating photoavoidance in *Euglena gracilis*.

(2) Developmental Biology: Replacement of the ankyrin repeats of mouse Notch2 gene with E.coli b-gactosidase gene induces early embryonic lethality around E10.5. The lethality was suggested due to defects in extraembryonic tissues, because the mutant embryo grew and differentiated further in vitro. Histological examination and in situ hybridization analysis with trophoblast subtype-specific probes revealed that the development of giant and spongiotrophoblast cell layers are normal in the mutant placenta, while vasculogenesis in the labyrinth layer apperaed compromised at E9.5. Since the lethality was circumvented by production of chimeric mice with tetraploidy wild type embryos, we concluded that the embryonic lethality is due to defect in growth and/or differentiation of labyrinthine trophoblast cells. The mutant embryo, however, could not be rescued in the tetraploid chimeras beyond E12.5 because of insurfficient development of umbilical cord, indicating another role of Notch2 signaling in the mouse development. Chimeric analysis with diploid wild type, however, revealed contribution of mutant cells to these affected tissues by E13.5. Thus, Notch2 are not cell autonomously required for the early cell fate determination of labyrinthine trophoblast cells and allantoic mesodermal cells, but plays an indispensable role in the further formation of functional labyrinth layer and umbilical cord.

(3) Computational Biology: Comparative genomics is a useful approach to find clues to understanding complex and diverse biological systems from rapidly growing genome database. We have constructed a workbench system for comparative analysis of microbial genomes named MBGD. Since the number of completed microbial genome sequences has now become more than a hundred, we have enhanced the efficiency of the database greatly to treat such large number of genomes. One of the unique features of MBGD is to allow users to create their own orthologous classification table with specified set of organisms. By this approach, MBGD accommodates comparative analysis for both closely related and distantly related genomes.

We also continue to develop an automated method for orthologous grouping among multiple genomes, which is a key component of MBGD. In addition to splitting fusion genes into orthologous domains, we are also trying to enhance the algorithm to incorporate the information of gene arrangement on each genome.

2. Cooperative Research Program for the Okazaki Large Spectrograph.

The NIBB Cooperative Research Program for the Use of the OLS supports about 20 projects every year conducted by visiting scientists including foreign scien-tists as well as those in the Institute.

Action spectroscopical studies for various regulatory and damaging actions of light on living organisms, bio-

logical molecules, and organic molecules have been conducted (Watanabe, 1995, In "CRC Handbook of Organic Photochemistry and Photobiology". pp, 1276 - 1288).

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THE CENTER FOR ANALYTICAL INSTRUMENTS

(managed by NIBB)

Head of Facility: *MOROHASHI, Ken-ichirou*
Technical Staffs: *MORI, Tomoko*
MAKINO, Yumiko
TAKAMI, Shigemi
NAKAMURA, Takanori
Technical Assistant: *MORIBE, Hatsumi*

The Center serves for amino acid sequence analysis, and chemical syntheses of peptides and nucleotids to support researchers in NIBB and NIPS. Newly installed instrument in 2002 is Confocal Laser Scanning Microscope and Environmental Scanning Electron Microscope. Instruments of the Center can be used by researchers outside the Institute upon proposal.

Figure 1. MALDI/TOF-MS.

Figure 2. Environmental Scanning Electron Microscope.

Representative instruments are listed below.

Protein Sequencers (ABI Procise 494, 473A)
Amino Acid Analyzer (Hitachi L8500A)
Peptide Synthesizers (ABI 433A, 432A)
Plasmid Isolation Systems (Kurabo PI-100Σ, PI-50)
Automatic Nucleic Acid Isolation System (Kurabo NA-2000)
DNA Sequencers (ABI PRISM 310, ABI PRISM 3100, ABI 377)
DNA/RNA Synthesizers (ABI 394, 392)
Thermal Cyclers (Perkin Elmer PJ-9600, Takara TP-300)
Integrated Thermal Cyclers (ABI CATALYST Turbo 800)
Particle Delivery System (Bio-Rad BiolisticPDS-1000/He)
Gas Chromatograph (Shimadzu GC-14APF-SC)
Glycoprotein Analysis System (Takara Glyco-Tag)
High Performance Liquid Chromatographs (Shimadzu LC-10AD, LC-6AD, Waters 600E)
Integrated Micropurification System (Pharmacia SMART)
Flow Cytometer (Coulter EPICS XL)
Biomolecular Interaction Analysis Systems (Pharmacia BIACORE 2000, Affinity Sensors IAsys)
Laboratory Automation System (Beckman Coulter Biomek 2000)
NMR Spectrometer (Bruker AMX-360wb)
EPR Spectrometer (Bruker ER-200D)
GC/Mass Spectrometer (JEOL DX-300)
MALDI/TOF-MS (Bruker Daltonics REFLEX Ⅲ)
Inductively Coupled Plasma Atomic Emission Spectrometer (Seiko SPS1200A)
Spectrofluorometers (Hitachi 850, Simadzu RF-5000)
Spectrophotometers (Hitachi 330, 557, Varian Cary 5G, Perkin Elmer Lambda-Bio)
Microplate Luminometer (Berthold MicroLumat LB 96P)
Time-resolved Fluorescence Microplate Reader (Pharmacia DELFIA Research)
Microplate Readers (Corona MTP-120, MTP-100F)
Spectropolarimeter (JASCO J-40S)
FT-IR Spectrophotometer (Horiba FT-730)
Laser Raman Spectrophotometer (JASCO R-800)
Bio Imaging Analyzers (Fujifilm BAS 5000, BAS 2000)
Fluorescence Bio Imaging Analyzer (Takara FMBIO)
Electrophoresis Imaging Systems (BIOIMAGE)
Microscopes (Carl Zeiss Axiophot, Axiovert)
Environmental Scanning Electron Microscope (PHILIPS XL30 ESEM)
Confocal Laser Scanning Microscope (Leica TCS SP2)

TECHNOLOGY DEPARTMENT

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Common Facility Group

Chief : FURUKAWA, Kazuhiko

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NANBA, Chieko (Subunit Chief)
NISHIDE, Hiroyo
IINUMA, Hideko
SUZUKI, Keiko (Technical Assistant)
MAKIHARA, Nobuko (Technical Assistant)
YAGI, Eri (Technical Assistant)
ICHIKAWA, Chiaki (Technical Assistant)
YAMAMOTO, Mamiko (Technical Assistant)
HARADA, Miyuki (Technical Assistant)

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MAKINO, Yumiko
TAKAMI, Shigemi
NAKAMURA, Takanori
MORIBE, Hatsumi (Technical Assistant)

Glassware Washing Facility
(KATO, Yousuke)
(FURUKAWA, Kazuhiko)

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Cell Biology Group
KONDO, Maki (Unit Chief)
KABEYA, Yukiko

Developmental Biology Group
TAKAGI, Chiyo
OKA, Sanae

Regulation Biology Group
YAMAGUCHI, Katsushi
TAKEUCHI, Yasushi

Gene Expression and Regulation Group I
MIWA, Tomoki (Unit Chief)
TANAKA, Sachiko (Subunit Chief)
HAYASHI, Koji
MOROOKA, Naoki

Gene Expression and Regulation Group II
OHSAWA, Sonoko (Unit Chief)
SAWADA, Kaoru (Subunit Chief)
SUMIKAWA, Naomi
MIKI, Kazuhiko

Integrated Bioscience Group
MIZUTANI, Takeshi
UTSUMI, Hideko
NODA, Chiyo
KONDO, Makiko (Technical Assistant)

The Technology Department is a supporting organization for researchers and research organization within the NIBB. The Department develops and promotes the institute's research activities and at the same time, maintains the research functions of the institute.

The department is organized into two groups: one, the Common Facility Group, which supports and maintains the institute's common research facilities and the other, the Research Support Group, which assists the research activities as described in individual reports.

Technical staffs participate, through the department, in mutual enlightenment and education increase their capability in technical area. Each technical staff is proceeded to the fixed division usually and they support the various research with their special biological and biophysical techniques.

The Department hosts an annual meeting for technical engineers who work in various fields of biology at universities and research institutes throughout Japan. At this meeting, the participants present their own activities and discuss technical problems. The Proceedings are published soon after the meeting.

CENTER FOR INTEGRATIVE BIOSCIENCE
(jointly managed by NIBB)

Head: NAGAYAMA, Kuniaki

DEPARTMENT OF DEVELOPMENT, DIFFERENTIATION AND REGENERATION
- DIVISION OF DEVELOPMENTAL GENETICS
- DIVISION OF MOLECULAR & DEVELOPMENTAL BIOLOGY

DEPARTMENT OF BIO-ENVIRONMENTAL SCIENCE
- DIVISION OF BIO-ENVIRONMENTAL SCIENCE
- DIVISION OF PLANT DEVELOPMENTAL GENETICS
- DIVISION OF BIOINFORMATICS

The center is jointly managed by NIBB and two other institutes in Okazaki, IMS (Institute for Molecular Science) and NIPS (National Institute for Physiological Sciences). The following projects will be the main focus of the center: 1) Development, Differentiation and Regeneration. 2) Strategic Methodology. 3) Bio-Environmental Science.

DIVISION OF DEVELOPMENTAL GENETICS

Professor:	*KOBAYASHI, Satoru*
Research Associate:	*MUKAI, Masanori*
Technical Staffs:	*NODA, Chiyo*
NIBB Research Fellow:	*SATO, Kimihiro*
JSPS Postdoctral Fellow:	*SHIGENOBU, Shuji*
Postdoctoral fellow:	*AMIKURA, Reiko*
Graduate Students:	*HAYASHI, Yoshiki*
	UNNAI, Kohei [1)]
	KITADATE, Yu [1)]
	HAYASHI, Makoto [1)]
Technical Assistant:	*UEDA, Yoshiko*
	TSUCHIYA, Naomi
	ARITA Kayo
Secretary:	*SUZUKI, Masako*

[1)] *Graduate School of Biological Sciences, University of Tsukuba*

The sperm and egg, or the germ cells are the specialized cells, which can transmit the genetic materials from one generation to the next in sexual reproduction. All the other cells of the body are somatic cells. This separation of germ and somatic cells is one of the oldest problems in developmental biology. In many animal groups, a specialized portion of egg cytoplasm, or germ plasm, is inherited by the cell lineage which gives rise to germ cells. This cell lineage is called germline. The germline progenitors eventually migrate into the gonads, where they differentiate as germ cells when the organisms are physically matured. Earlier investigators have demonstrated that germ plasm contains maternal factors required and sufficient for germline formation. In the fruit fly, *Drosophila*, this cytoplasm is histologically marked by the presence of polar granules, which act as a repository for the maternal factor required for germline formation. Our molecular screens have identified several factors stored in the polar granules. One of the factors is mitochondrial large rRNA which functions to form the germline progenitors, or pole cells. The other is *nanos* mRNA, which is required for pole cell differentiation.

I. Role of Mitochondrial Ribosomal RNAs in Pole Cell Formation

Ultrastructural studies have shown that the germ plasm is basically composed of polar granules and mitochondria. While the primary roles of the mitochondria are oxidative phosphorylation and biosynthesis of many metabolites, it has now become evident that they are also involved in germline formation.

In *Drosophila*, pole cell formation requires the function of mitochondrial ribosomal RNA in germ plasm. We have previously reported that mitochondrial large rRNA (mtlrRNA) and small rRNA (mtsrRNA) are both transported from mitochondria to polar granules. This transportation occurs during early embryogenesis, when mitochondria are tightly associated with polar granules in germ plasm, and it depends on the function of the maternally-acting gene, *tudor*, that is known to be required for pole cell formation. Mitochondrial rRNAs remain on the polar granules until pole cell formation and are no longer discernible on the granules within pole cells. Reduction of the extra-mitochondrial mtlrRNA amount results in the failure to form pole cells and injection of mtlrRNA is able to induce pole cells in embryos whose ability to form these cells has been abolished by uv-irradiation. These observations clearly show that the extra-mitochondrial mtlrRNA on polar granules has an essential role in pole cell formation, presumably cooperating with mtsrRNA.

Recently, we found that mitochondrial rRNAs form mitochondrial-type of ribosomes on polar granules, cooperating with mitochondrial ribosomal proteins. This suggests the possibility that the protein(s) essential for pole cell formation is produced by the mitochondrial-type of ribosomes. To address this issue, we examined the effect of *Chloramphenicol* and *Kasugamycin* on pole cell formation. *Chloramphenicol* and *Kasugamycin* are known to inhibit mitochondrial (prokaryotic)-type of translation. When these antibiotics were injected into the posterior pole region of early embryos, pole cell formation was severely affected. In contrast, *Chloramphenicol* and *Kasugamycin* treatment did not affect somatic cell formation at a dose we used. These observations strongly suggest that the mitochondrial-type of translation system must be intact for the embryos to form pole cells.

II. Role of Nanos protein in pole cell differentiation

Pole cells differ from the soma in regulation of mitosis and transcriptional activity. Pole cells cease mitosis at gastrulation and remain quiescent in the G2 phase of the cell cycle throughout their migration to the gonads, while somatic cells continue to proliferate during the rest of embryogenesis. Furthermore, pole cells are transcriptionally quiescent until the onset of gastrulation, although transcription is initiated in the soma during the syncytial blastoderm stage. Consistent with this, RNA polymerase II (RNAP II), but not RNA polymerase I, remains inactive in early pole cells. Thus, the ability to express zygotic mRNA-encoding genes is suppressed only in pole cells in early embryos.

Among the maternal components of germ plasm, Nanos (Nos) is essential for the germline-specific events occurring in pole cells. nos mRNA is localized in the germ plasm during oogenesis, and is translated in situ to produce Nos protein after fertilization. Nos is only transiently present in the posterior half of embryos during the preblastoderm stage, and is required there for posterior somatic patterning. Nos in the germ plasm is more stably inherited into the pole cells at the blastoderm stage, remaining detectable in these cells throughout embryogenesis. Pole cells that lack Nos (*nos*$^-$ pole cells) are unable to follow normal germline development; they fail to migrate properly into the embryonic gonads, and consequently do not become functional germ cells. In nos$^-$ pole cells, mitotic arrest at G2 phase is impaired, and they undergo premature mitosis. Furthermore, *nos*$^-$ pole cells fail to establish and/or maintain transcriptional quiescence, and ectopically express somatically-transcribed genes, including *fushi tarazu* (*ftz*), *even-skipped* (*eve*) and *Sex-*

lethal (*Sxl*).

Nos represses translation of mRNAs with discrete RNA sequences called Nos response elements (NREs). In the pathway leading to posterior somatic patterning, Nos acts together with unlocalized Pumilio (Pum) protein to repress translation of maternal *hunchback* (*hb*) mRNA. This translational repression is mediated by binding of Pum to NREs in the 3'-untranslated region (UTR) of hb mRNA. In pole cells, Nos also acts with Pum to regulate germline-specific events. Pum, like Nos, is required in pole cells for their migration to the gonads.

We found that Nos, along with Pum, represses translation of importin α2 (impα2) mRNA in early pole cells. The impα2 mRNA contains an NRE-like sequence in its 3'-UTR and encodes a *Drosophila* importin α homologue that plays a role in nuclear import of karyophilic proteins. We found that Nos inhibits expression of a somatically-transcribed gene, *ftz*, in pole cells by repressing Impα2-dependent nuclear import of a transcriptional activator for ftz, Ftz-F1. Furthermore, the expression of another somatic gene, *eve*, and RNA Polymerase II activity are also repressed by Nos in pole cells through its effects on Impα2-dependent nuclear import.

The above results raise the question whether the pole cells lacking Nos (*nos*$^-$ pole cells) are able to differentiate into somatic cells. However, it is difficult to study their developmental fate, since Nos also represses apoptosis of pole cells, and almost all of *nos*$^-$ pole cells are eliminated until at least the end of embryogenesis. To overcome this problem, we used Df(3L)H99, a deletion for three genes required for apoptosis. Introduction of the H99 deficiency results in nos$^-$ pole cells being escaped from apoptosis. We transplanted the *nos*$^-$ H99$^-$ pole cells into normal embryos and observed their behavior, and found that some of *nos*$^-$ H99$^-$ pole cells were able to differentiate as somatic cells. This suggests that pole cells have the ability to differentiate as somatic cells, but its ability is inhibited by Nanos activity.

III. Comprehensive analysis of genes expressed in *Drosophila* gonad

Pole cells migrating into the gonads are specified to be the primordial germ cells (PGCs). It has been believed that zygotic genes expressed in pole cells within the gonads are required for their fate specification. To explore the regulatory mechanism of germline specification, we attempted to identify genes expressed in pole cells and/or in somatic cells within the gonad by a comprehensive approach. From the embryos carrying EGFP-vasa transgene that express GFP only in pole cells, we isolated the gonads by using fluorescence-activated cell sorting (FACS), and costructed a gonad cDNA library. Each cDNA clone was sequenced from both 5' and 3' ends, and these Expression Sequence Tags (ESTs) were computationally condensed into sequence clusters, which were then subjected to whole-mount *in situ* hybridization (WISH). As of July 2002, approximately 10,000 of ESTs were generated. The WISH analysis identified more than 30 genes that were expressed predominantly in the gonads. In addition, we found gonad-specific splicing form in some transcripts. These transcriptome data will allow us to illustrate genetic networks governing the germline specification.

IV. *sva53*, a Maternal Gene Required for Meiosis

It has been believed that maternal factors localized in germ plasm may ultimately trigger germline-specific events, such as meiosis. We have isolated an X-linked maternal mutation, *sva53* that affects meiosis. Pole cells that were formed in the embryos derived from *sva53* homozygous germline clone (*sva53* pole cells) were able to develop into the oocytes, but they failed to execute meiosis. We also found that the germline-specific expression of *vasa* gene was severely affected in *sva53* pole cells. These results suggest that the maternal factor encoded by *sva53* gene may activate gene expression, which is essential for meiosis. In order to identify *sva53* gene, we mapped *sva53* mutation to 200 kb-genomic region of 11C by using duplications and deficiencies. Within this region, we found a gene encoding a Zn-finger transcription factor, of which mRNA is maternally supplied into embryos.

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Inoue, S., Shimoda, M., Nishinokubi, I., Siomi, M., Okamura, M., Nakamura, A., Kobayashi, S., Ishida, N. and Siomi, H. (2002) A Role for the *Drosophila* Fragile X-Related Gene in Circadian Output. *Current Biology* **12**, 1331-1335.

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DIVISION OF MOLECULAR & DEVELOPMENTAL BIOLOGY

Professor:	*TAKADA, Shinji*
Research Associate:	*KOSHIDA, Sumito*
Technical Staff:	*UTSUMI, Hideko*
NIBB Research Fellow:	*OHBAYASHI, Norihiko*
Postdoctoral Fellow:	*KAWAMURA, Akinori*
	MUROYAMA, Yuko
Graduate Student:	*YAMAGUCHI, Yoshifumi*[1)]
	ISHIDA, Mio[1)]
	OKITA Akiyoshi[1)]
	OGURA Satoshi[1)]
Technical Assistant:	*OHBAYASHI, Akiko*
	ODA, Ritsuko

[1)] *Graduate School of Biostudies, Kyoto University*

The research interest of this laboratory is to understand molecular mechanism of the vertebrate development. Particularly, roles of cell-to-cell signals, including members of Wnt and fibroblast growth factor (FGF) families, are characterized. Evidence indicated that each signal is involved in many aspects of the vertebrate development. For instance, we have revealed that Wnt-3a, a members of Wnt family, plays essential roles in a number of aspects of the mouse development, including somite development, neural crest formation and neural development. However, cellular and molecular mecha-nisms how a cell signaling molecule regulates these different events. Thus, we are focusing on precise functional analysis of cell-to-cell signals and identifica-tion of target genes induced by these signals. In addition, to reveal molecular networks in which these signals are involved, we are also trying genetical approach with the zebrafish.

I Roles of Wnt signals during embryogenesis

The Wnt family of genes that encode cysteine rich secreted proteins consists of at least 15 members in the vertabrate. It has already been shown that some of them are expressed and play important roles during gasturulation. For instance, we showed that Wnt-1 and Wnt-3a, which are expressed in the most dorsal region within the developing central nervous system, direct specification of the dorsal interneurons. Analysis of mouse embryos lacking both Wnt1 and Wnt3a and culture of explants from the neural plate indicated that these Wnt signals promote generation of the most dorsal subclass of the interneuron, called D1 and D2, at the expense of that of more ventral subclass, called D3 (FIG. 1).

Wnt-3a is also expressed in the primitive streak ectoderm during gastrulation and in the tailbud in later development of the mouse. For dissecion of the complex developmental events regulated by Wnt-3a signaling in these regions, it is important to identify genes regulated by this signal. It has already been demonstrated that T (Brachyury) is a direct target of Wnt-3a in the anterior primitive ectoderm, which is fated to give rise to the paraxial mesoderm, suggesting that Wnt-3a modurates a balance between mesodermal and neural cell fates via T.

To gain more insight into roles of Wnt signaling during embryogenesis, we searched for potential target genes of this signaling by an induction gene trap screening in mouse ES cells. In at least three ES cell clones among 794 clones screened, expression of beta-geo reporter genes was dramatically changed in response to the conditioned medium of Wnt-3a expressing cells. The expression analysis of the reporter genes in embryos generated from these ES cell clones revealed that the spatiotemporal expression patterns of these reporter genes were well correlated to those of several Wnt genes. These results suggested that an induction gene trap approach is effective for screening of target genes of Wnt signaling during embryogenesis.

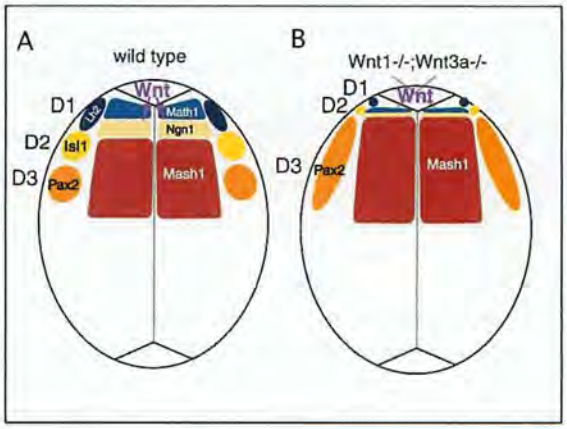

FIG.1. Summary of the phenotype of dorsal interneuron development in Wnt-1$^{-/-}$/ Wnt-3a$^{-/-}$ embryos at E10.5. D1, D2, and D3 interneurons are indicated by the expression of LH2, Isl1, and Pax2, respectively. The progenitors of interneurons are also subdivided by the expression of Math1, Ngn1, and Mash1, respectively. Absence of Wnt-1 and Wnt-3a leads to diminished development of D1 and D2 neurons and to a compensatory increase of D3 neurons.

II Roles of Fgf signals in bone development

The Fgf family of cell signaling molecules is composed of at least 22 members in the mouse. Although Fgf signaling has been implicated in the bone development, study on null mutant mice have not yet fully shown the role of this family in the skeletal development.

As an attempt to identify a candidate Fgf gene essential for bone formation, we have examined the expression of mouse Fgf18 gene using in situ hybridisation and demonstrated expression during calvarial and long bone development. Furthermore, to investigate the role of Fgf18 in vivo, we generated a null allele of the Fgf18 locus by homologous recombination in ES cells. In Fgf18-deficient mouse embryos generated by gene targeting, progress of the suture closure is delayed (FIG. 2). Furthermore, terminal differentiation of the osteoblast is specifically delayed in developing calvaria and long bones. Proliferation of calvarial osteogenic mesenchymal cells is transiently decreased. On the other hand, the number of proliferating and differentiated chondrocytes is increased in the limbs. Fgf18 is thus essential for distinct aspects of cell

proliferation and differentiation in bone formation. Thus, Fgf18 plays essential roles in the osteogenesis and the chondrogenesis of the mammal and regulates differently cell proliferation and differentiation in these two processes.

To reveal the molecular mechanism that regulates the osteogenesis and the chondrogenesis, we are currently analyzing function of several genes, which act on these processes with Fgf-18.

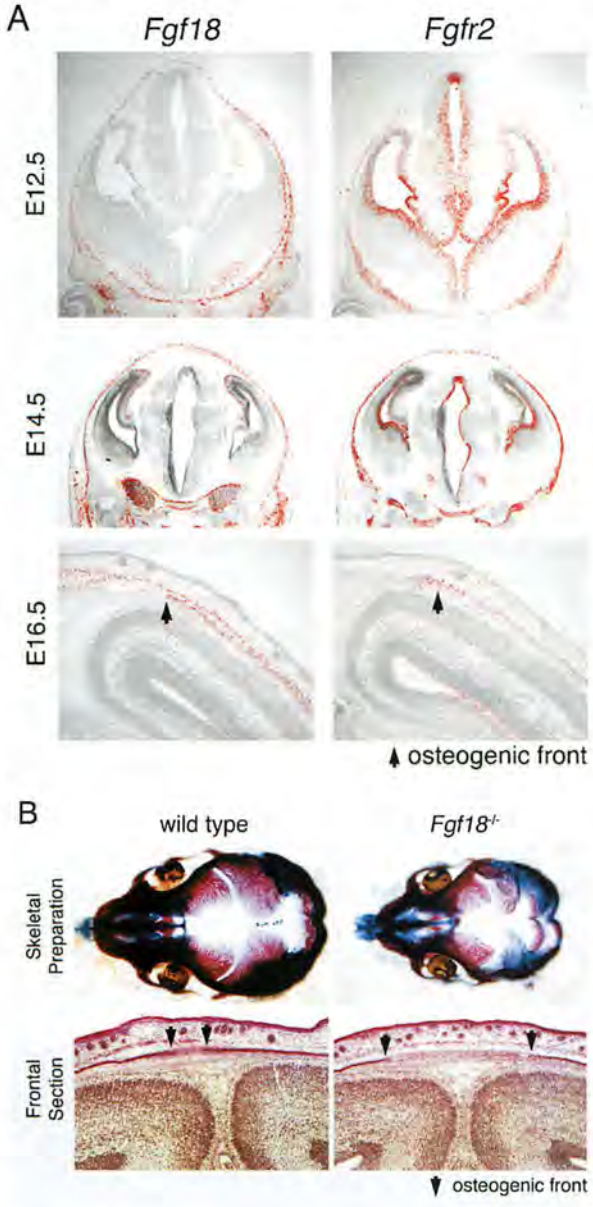

FIG.2 Fgf18 is required for normal bone development. (A) Expression of Fgf18 and its receptor, Fgfr2, in the calvarial bone development. The expression of Fgf18 is first detected in osteogenic mesenchymal cells surrounding the brain at E12.5. At this stage, Fgfr2 is expressed in ventral mesenchyme, where ossification is initiated. In later stages, Fgf18 is also expressed in differentiating osteoblasts while Fgfr2 is expressed intensely in cells at the osteogenic front. (B) The phenotype of Fgf18$^{-/-}$ embryos in the calvarial bone development. In the absense of Fgf18, calvarial ossification is delayed.

III Screening of mutations affecting mesoderm development in zebrafish

To identify genes involved in several aspects during early embryogenesis of the vertebrate, we have performed screening of zebrafish mutants induced by ENU, a chemical mutagen. Until now, we have screened 630 of F2 families and found a number of mutants whose phenotypes are different from those already reported. For instance, some of these mutants displayed defects in the somite development. Cloning of genes that are responsible for these defects is in progress.

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DIVISION OF BIO-ENVIRONMENTAL SCIENCE

Professor:	*IGUCHI, Taisen*
Associate Professor:	*WATANABE, Hajime*
Research Associate:	*KATSU, Yoshinao*
Technical Staff:	*MIZUTANI, Takeshi*
Institute Research Fellow:	*SONE, Kiyoaki*
Graduate Students:	*KHONO, Satomi* [1)]
	MIYAGAWA, Shinichi
	NAGATA, Emiko
Visiting Scientist:	*KATO, Hideo* [2)]
Technical Assistants:	*KAJIYAMA, Chieko*
	SUZUKI, Atsuko
	KOBAYASHI, Kaoru
	KOBAYASHI, Mika
	TAKAHASHI, Eri
	ITAMOTO, Misaki
	HINAGO, Megumi
Secretary:	*IMAIZUMI, Taeko*

[1)] *Graduate School of Yokohama City University*
[2)] *from Nihon Bioresearch Inc.*

Synthetic chemicals found in the environment have the capacity to disrupt endocrine system development and function in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ER) and evoke estrogenic effects. Early evidence that estrogenic chemicals could pose a threat to human health during development came from studies of diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments have demonstrated that exposure of animals to sex hormones during perinatal life can cause permanent and irreversible alterations of the endocrine and reproductive systems as well as the immune system, nervous system, bone, muscle, and liver in both sexes. Although many of these chemicals may bind to ER and evoke estrogenic effects in wildlife and humans, the effects of estrogen are not well understood even now. Thus, understanding the effects of sex hormones at the molecular level, especially during development, is very important to resolve these problems.

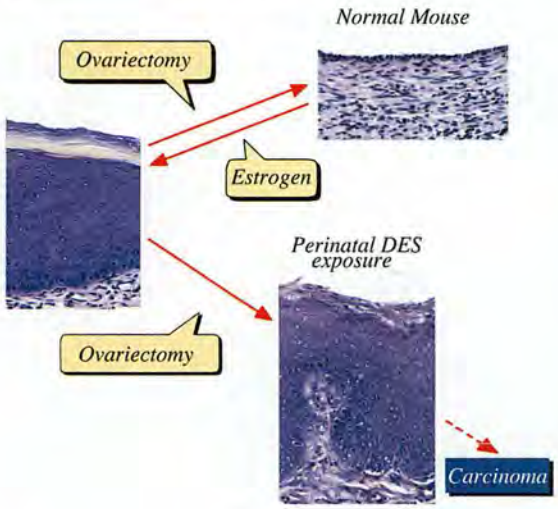

FIG. 1

I. Estrogen-induced irreversible changes

Perinatal sex-hormone exposure has been found to induce lesions in reproductive tracts in female mice. The possible relevance of the mouse findings to the development of cancer in humans has been emphasized. In the early seventies, a close correlation between occurrence of vaginal clear cell adenocarcinoma in young women and early intrauterine exposure to DES was demonstrated. Many chemicals released into the environment have the potential to disrupt endocrine function in wildlife and humans. Some of these chemicals induce estrogenic activity by binding to ER. The neonatal mouse model has been utilized especially to demonstrate the long-term effects of early sex hormone exposure on the female reproductive tract. Neonatal treatment of female mice with estrogens induces various abnormalities of the reproductive tract: ovary-independent cervicovaginal keratinization, adenosis, uterine hypoplasia, epithelial metaplasia, oviductal tumors, polyovular follicles (PF) and polyfollicular ovaries. Female reproductive tracts in mice exposed prenatally to estrogen show altered expression of Hoxa genes and Wnt genes and the analysis of knockout mice lacking Hoxa-10 or Wnt7a show uterine hypoplasia. The growth response of neonatally DES-exposed reproductive organs to estrogen is reduced, as are ER levels and epidermal growth factor (EGF) receptor levels, in addition to other hormone receptor levels.

Estrogenic compounds such as bisphenol A (BPA) and nonylphenol as well as dioxins and PCBs were found in the human umbilical cord. BPA can easily cross the placenta and enter the fetus in Japanese monkey and mice. BPA can be found in fetal brain, testis and uterus when given to pregnant mice and monkeys. Neonatal exposure to a high BPA dose induced ovary-independent vaginal changes, PF and infertility lacking corpora lutera. Prenatal exposure to a low BPA dose induced acceleration of vaginal opening in the offspring. Thus, the developing mammal is sensitive to exposure to estrogenic agents.

In order to clarify the molecular mechanisms of these effects, we are studying changes in gene expression patterns induced by perinatal exposure to chemicals or estrogen using differential display and DNA microarray

FIG. 2 Fluorescence image of an array

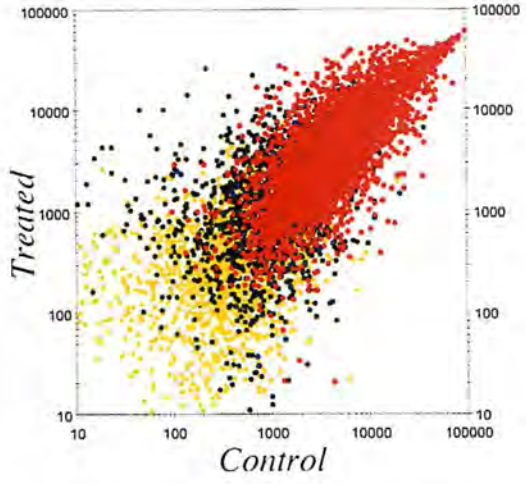

FIG.3 Scatter plot of average expression levels in control and chemical-treated uterus

techiniques. We have found genes possibly related to the ovary-independent changes by differential display. We also have clustered groups of genes that are responsive to estrogenic stimuli in uterus by using the DNA microarray system. We need to understand the molecular background of the critical period during development, the low dose effect of estrogenic chemicals and the molecular metabolism of hormones and hormone-like agents in animals including humans.

II. Effect of estrogen on amphibian and fishes

During embryogenesis, exogenous estrogen exposure induces abnormal sex differentiation and the abnormal bone formation in African clawed frog, *Xenopus laevi*, the cyprinodont fish, mummichog (*Fundulus heteroclitus*) and mosquitofish (*Gambusia affinis*). To analyze the function of estrogen, we have isolated cDNA clones of ERα and β from *F. heteroclitus*, *G. affinis* and American alligators. The estrogen-responsive genes must play important roles. We try to isolate the estrogen-responsive genes to understand the molecular physiology of estrogen action. Japanese tree frog (*Hyla japonica*) takes water through ventral skin. We found that sex steroids and endocrine disruptors interfere with water absorption through ventral skin in frogs. Further, using the amphibian and fish as model animals we aim to analyze the effects of numerous chemicals released into the environment on endocrine system function in wildlife.

III. Molecular Target Search

Abnormalities caused by endocrine disrupting chemicals are reported but the molecular mechanisms of the effects are not well studied. Although estrogen receptor is one of the strongest candidates possibly responsible for the endocrine disrupting function of many chemicals, it alone cannot explain the variety of phenomena induced by endocrine disrupting chemicals. Thus, we are also looking for new target molecules that may be responsible for endocrine disruption. In parallel, we also are studying the ligand-binding mechanisms of nuclear receptors to hormones and other chemicals using Surface Plasmon Resonance technology.

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DIVISION OF PLANT DEVELOPMENTAL GENETICS

Associate Professor:	*TSUKAYA, Hirokazu, Ph.D.*
Research Associate:	*HORIGUCHI, Gorou, Ph.D.*
Technical Staff:	*KONDO, Makiko*
	TAKABE, Eriko
Post Doctoral Fellow:	*CHO, Kiuhyon, Ph.D.*
Graduate Student:	*KOZUKA, Toshiaki*
	NARITA, Noriyuki
Secretary:	*KABEYA, Kazuko*

Diversity of plant form is mostly attributable to variation of leaf and floral organs, which are modified leaves. The leaf is the fundamental unit of the shoot system, which is composed with leaf and stem. So the leaf is the key organ for a full understanding of plant morphogenesis. However, the genetic control of development of these shapes had remained unclear. Recently, studies of leaf morphogenesis has been in a turning point, after our successful application of the techniques of developmental and molecular genetics to it, using model plants, *Arabidopsis thaliana* (L.) Heynh (reviewed in Tsukaya 2002a, b).

In a determinate organ, a leaf, number of leaf cells is not necessarily reflected on leaf shape or, in particular, leaf size. Genetic analyses of leaf development in *Arabidopsis* shows that a compensatory system(s) act in leaf morphogenesis and an increase of cell volume might be triggered by a decrease in cell number. Thus, leaf size is, at least to some extent, uncoupled from the size and number of cells by the compensatory system(s). Based on these facts, a new perspective on understanding of mechanisms for leaf morphogenesis, Neo-Cell theory, is proposed (Tsukaya, 2002a; Tsukaya, 2003). Neo-Cell theory is the Cell theory which stipulated positional cooperation of the behavior of cells. In short, Neo-Cell theory hypothesizes as follows: cells are the unit of morphogenesis; however, each cell is also controlled by factors that govern the morphogenesis of the organ of which the cells are a part.

Focusing on mechanisms that govern polarized growth of leaves in a model plant, *Arabidopsis thaliana*, we found that the two genes act independently to each other on the processes of polar growth of leaves: the *ANGUSTIFOLIA* (*AN*) gene regulates width of leaves and the *ROTUNDIFOLIA3* (*ROT3*) gene regulates length of leaves. The *AN* gene controls the width of leaf blades and the *ROT3* gene controls length. Cloning of the *AN* gene (Kim et al., 2002) revealed that the gene is a member of *CtBP* (C-terminal binding protein) gene family which had been found from animal kingdom. In the animal kingdom, CtBPs self-associate and act as a co-repressors of transcription. We found that also the AN protein can self-associate in yeast two-hybrid system (Kim et al. 2002). Furthermore, microarray analysis suggested that the *AN* gene might regulate the expression of certain genes, for example, the gene involved in formation of cell walls, *MERI5*. We also found that the abnormal arrangement of cortical MTs in *an-1* mutant leaf cells appeared to account entirely for the abnormal shape of the cells. It suggested that the *AN* gene might regulate the polarity of cell growth by controlling the arrangement of cortical MTs.

The *ROT3* gene was cloned by us in 1998. Transgenic experiments proved that the *ROT3* gene regulates leaf-length without affect on leaf-width via biosynthesis of steroids (Kim et al., 1999). In relation to it, we recently revealed that a steroid hormone, brassinosteroid, controls both proliferation and expansion of leaf cells (Nalaya et al., 2002). In addition, by studying role of light condition in leaf development, we found that the length of leaf petioles was related exclusively to genetic control of the length of individual cells in all leaf morphological mutants examined. In contrast, both the size and the number of cells were affected by the mutations examined in leaf blades. Studies of the roles of light signal perception in leaf development are now underway.

Apart from the aspects of leaf expansion, we also analyzed genes for identification of leaf primordia. The *AS1*, *AS2* and *BOP* genes are needed for determinate growth of leaf. Molecular and anatomical analysis of the *AS2* gene has been carried out in collaboration with a research team of Prof. Machida, Nagoya University (Endang et al., 2001; Iwakawa et al. 2002). *AS2* gene is a member of a novel gene family, including 42 putative genes in *Arabidopsis* genome, encoded proteins with cysteine repeats and a leucine zipper (Iwakawa et al. 2002). The gene product also represses the expression of class 1 *knox* homeobox genes in leaves (Endang et al. 2001; Iwakawa et al. 2002), as known for *AS1*. We also isolated and analyzed a novel mutant, *bop* (blade-on-petiole) mutant, which strongly enhances the *as2* phenotype, in collaboration with a reaseach team of Prof. Nam, POSTECH, Korea (Ha et al. 2003). Three class I *knox* genes, namely, *KNAT1*, *KNAT2* and *KNAT6*, were misexpressed in the leaves of the *bop1-1* mutant as in those of the *as2* mutant. The *bop1* single mutant results in ectopic, lobed blades along the adaxial side of petioles of the cotyledon and foliage leaves. We, thus, suggest that *BOP1* promotes or maintains a developmentally determinate state of leaf cells through regulation of class I *knox1* genes (Ha et al. 2003).

On the other hand, we are trying to identify molecular mechanisms which distinguish developmental pathway of leaves from that of shoots by studying tropical plants with 'fuzzy' morphology. For such purposes, we introduced tropical plants having queer developmental program for leaf morphogenesis, namely, *Chisocheton*, *Guarea* and *Monophyllaea*, as materials for the studies. The indeterminate compound leaves of members of the genus *Chisocheton* in Southeast Asia and of the genus *Guarea* in the New World and Africa, in the family Meliaceae, are unique and can develop indeterminately as a result of the activity of the leaf apical meristem, which can function very similarly to a shoot apical meristem. We performed a molecular phylogenetic study of these genera, and the result suggested that indeterminate program in the leaves of members of these two genera might have evolved only once in Meliaceae (Fukuda et al. 2003).

In addition, we are interested in environmental adapta-

tion of leaves, from view point of biodiversity. For example, thermograph analysis of function of downy bracts of a so-called snowball plant, *Saussurea medusa* that habits in alpine zone of Yunnan, China, revealed that the downy bracts of *S. medusa* have two functions: thermal insulation to protect the inside of flowers and the accumulation of heat on the upper surfaces of the inflorescence (Tsukaya et al. 2002a). Anatomical analyses of various types of morphological adaptation of leaves against certain kinds of environments were also performed (Tsukaya, 2002d, e, f, g). In particular, leaf index, relative length of leaf to width, is also the most diverse factor of leaf shape, and is affected by environmental factors in some plants (Tsukaya, 2002c; Tsukaya et al. b). Are *AN* and *ROT3* genes are involved in regulation of adaptive change of leaf index in natural condition? Are these genes the responsible for evolution of rheophytes? So called "Evo/Devo" study of leaf morphogenesis is also one of our research project in NIBB.

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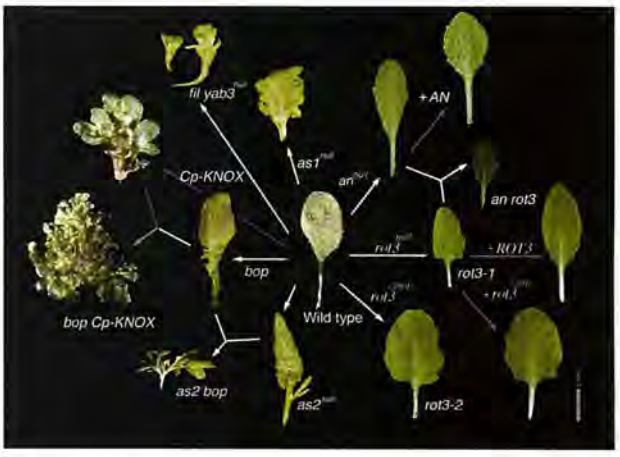

Leaf shape control by various genes in *Arabidopsis thaliana*.

DIVISION OF BIOINFORMATICS

Professor (Adjunct): *NAKAMURA, Haruki (Osaka University)*

The aim of this laboratory is structural bioinformatics covering molecular modeling and design of proteins and other biological macromolecules: Development of a new database, eF-site, for protein surface geometry with the physicochemical properties, and identification of protein functions using the database, and Development of new algorithms and softwares for large scale simulation calculations by parallel computers to examine free energy landscapes of biomolecular systems.

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CENTER FOR RADIOISOTOPE FACILITIES (CRF)

Head:YAMAMORI, Tetsuo
(Professor, concurrent post)

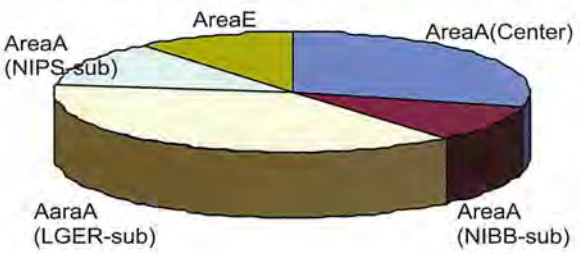
Associate Professor: *OGAWA, Kazuo*
(Radiation Protection Supervisor)
Technical Staffs: *MATSUDA, Yoshimi*
(Radiation Protection Supervisor)
KATO, Yosuke
(Radiation Protection Supervisor)
MOROOKA, Naoki
(Radiation Protection Supervisor)
Supporting Staff: *ITO, Takayo*
IIDA, Yumi
KATAGIRI, Izumi

I. Research supporting activity

Technical and supporting staffs of the CRF are serving the purchase of radioisotopes from JRA (Japan Radioisotope Association) and the transfer of radioisotope wastes to JRA. The physical maintenance of the controlled areas where radioisotopes are used is also one of our business.

In this summer (July 1, 2002), a new radioisotope facility opened at the area E about 1km apart from the area A where four controlled areas have been built: Center, NIBB-sub, LGER (Laboratory of Gene Expression and Regulation)-sub, and NIPS (National Institute for Physiological Science)-sub. Each facility is maintained under the two radiation protection supervisors. Matsuda and Kato are named to the supervisors in the area A, and Ogawa and Morooka in the area E. Ito and Iida support them at the area A, and Katagiri at the area E.

Users going in and out the controlled areas counted by the monitoring system are 6,700 persons in 2002. The items in each controlled area is presented in Figure 1.



Actual counts

Area A	
Center	2,359
NIBB-sub	956
LGER-sub	3,320
NIPS-sub	924
Area E	700
total	6,700

Figure 1. Percentage of users going in and out the controlled areas in 2002.

II. Academic activity

Academic activity by teaching staff is focused on the analysis of the structure and function of a dynein motor protein. Dyneins are a group of microtubule-activated ATPases that serve to convert chemical energy into mechanical energy and divided into axonemal and cytoplasmic dyneins. Figure 2 shows the localization of two isoforms of dynein in the outer arms of sperm axonemes (Ogawa et al., 1977) and the mitotic apparatus of cleaving egg (Mohri et al., 1976) visualized by anti-axonemal dynein (Fragment A) antibodies.

The native dyneins are very large and range in molecular mass up to 1 to 2 mega Da. They are complex proteins containing heavy, intermediate, and light chains defined by the molecular mass. Our present project is the molecular cloning of polypeptides contained in outer arm dynein of sea urchin sperm flagella to understand the mechanism how dynein interacts with microtubules, resulting in producing the force.

Outer arm dynein consists of two heavy chains with ATPase activity. The motor activity is closely related to this polypeptide. The first successful molecular cloning of this huge polypeptide (520 kDa) was performed in our laboratory in 1991. Since then cDNA clones for axonemal and cytoplasmic dyneins have been isolated in a variety of organisms. The sequences of heavy chains, without exception, contain four P-loop motives referred to as ATP-binding sites in the midregion of the molecules. Figure 3A and B draw the structure of heavy chain deduced from the amino acid sequence (Ogawa, 1992). Outer arm dynein contains three intermediate chains (IC1, IC2, and IC3) that range in molecular mass from 70 to 120 kDa. IC2 and IC3 were cloned by Ogawa et al. (1995) and contain the WD repeats in the carboxy-terminal halves of the molecules. By contrast, IC1 is not a member of the WD family. IC1 has a unique sequence such that the N-terminal part is homologous to the sequence of thioredoxin, the middle part consists of three repetitive sequences homologous to the sequence of NDP kinase, and the C-terminal part contains a high proportion of negatively charged glutamic acid residues (Ogawa et al., 1996). Thus, IC1 is a novel dynein intermediate chain distinct from IC2 and IC3 and may be a multifunctional protein.

Six light chains with molecular masses of 23.2, 20.8, 12.3, 11.5, 10.4, and 9.3 kDa are associating with outer arm dynein. We have already isolated cDNA clone of five LCs. LC1 (23.2 kDa) and LC3 (12.3 kDa) are highly homologous to mouse Tctex2 and Tctex1, respectively, which are encoded by the t complex region that is involved in transmission ratio distortion (TRD), male sterility and the development of germ cells. Our finding raises the possibility that axonemal dynein proteins are involved in this phenomenon. TRD may be caused by the dysfunction of multiple axonemal dynein proteins.

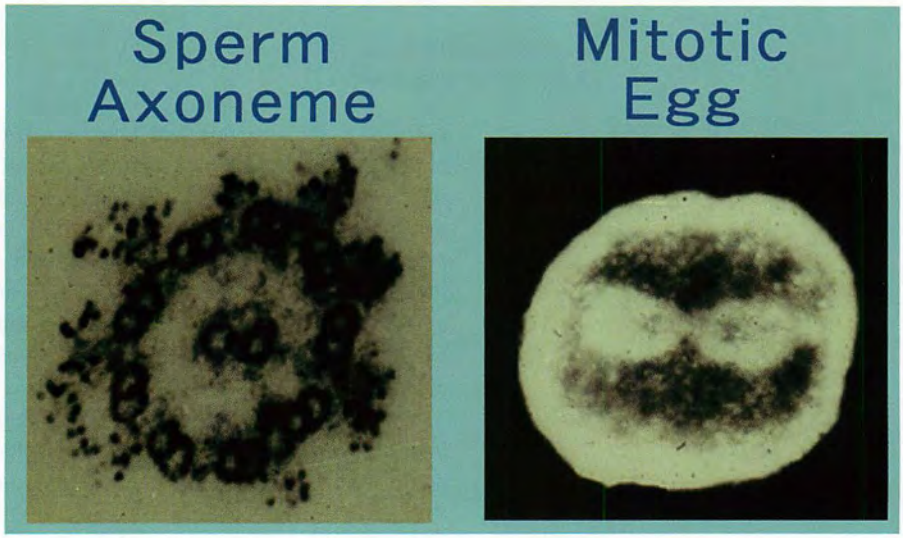


Figure 2. Localization of two dynein isotypes on outer arm of sperm axonemes and mitotic apparatus of cleaving egg.

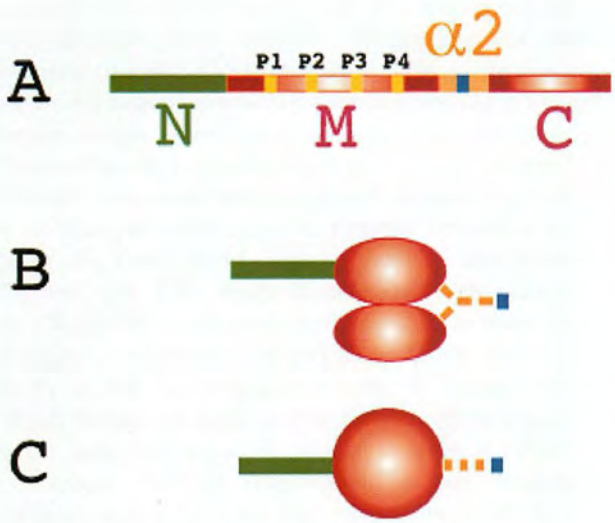
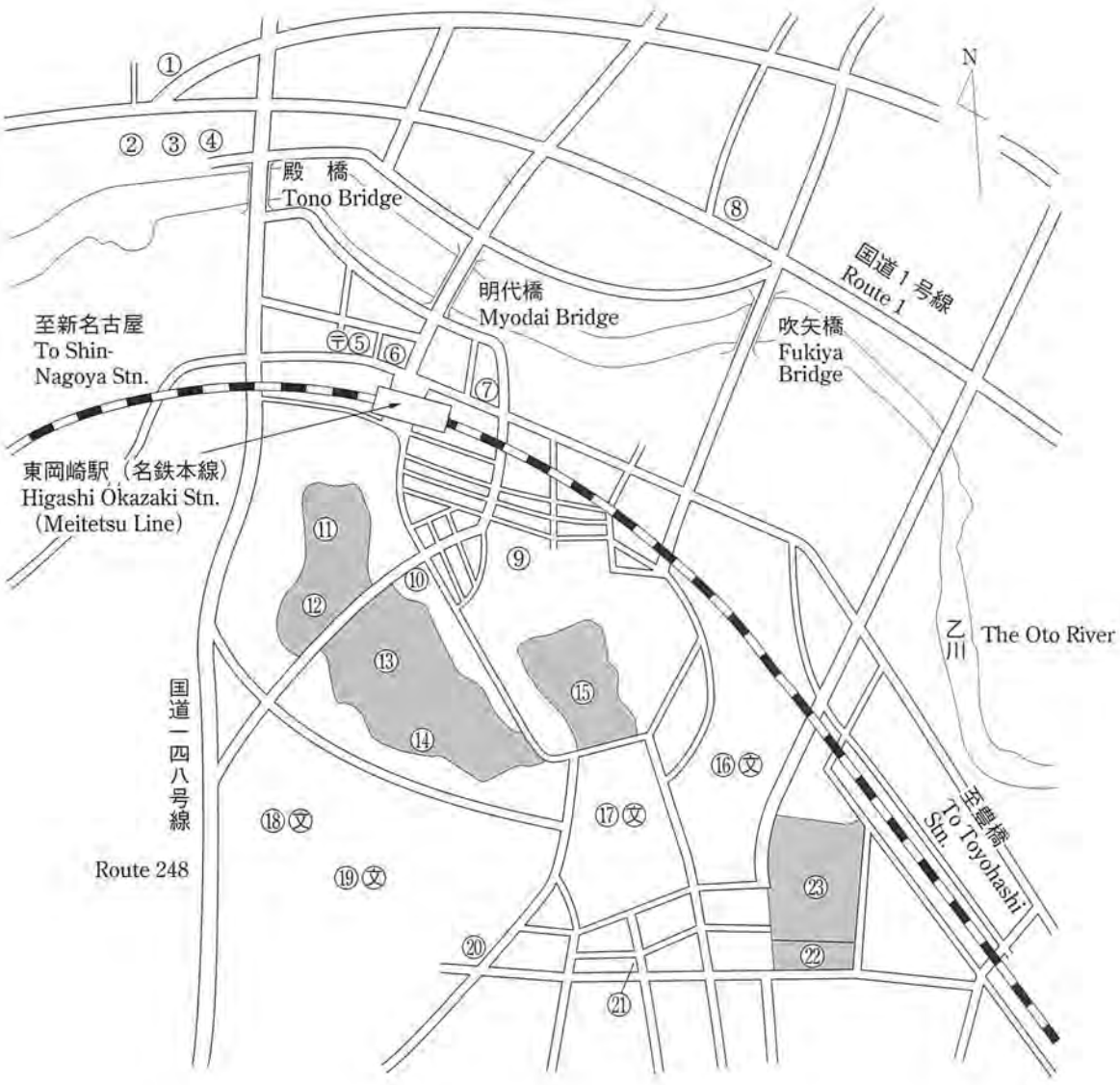


Figure 3. Structure of dynein heavy chain. A; Analysis of amino acid sequence of heavy chain reveals that it consists of three major domains referred to as N, M, and C from the N-terminus. B and C; M and C domains make larger domain (motor domain) by intramolecular association.



① 名鉄岡崎ホテル
Meitetsu Okazaki Hotel

② 岡崎城
Okazaki Castle

③ 岡崎ニューグランドホテル
Okazaki New Grand Hotel

④ 岡崎グランドホテル
Okazaki Grand Hotel

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Post Office

⑥ 銀行
Bank

⑦ スーパーマーケット“ユニー”
Super Market “Uny”

⑧ 市役所
Okazaki City Office

⑨ 六所神社
Rokusho Shrine

⑩ 岡崎国立共同研究機構（看板）
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National Institute of Basic Biology

⑫ 生理学研究所
National Institute for Physiological Sciences

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Administration Bureau

⑭ 分子科学研究所
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Center for Integrative Bioscience

National Institute for Basic Biology
Okazaki 444-8585, Japan

NATIONAL INSTITUTE
FOR BASIC BIOLOGY

Nishigonaka 38, Myodaiji, Okazaki 444-8585 Aichi, Japan

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