KAMADA GROUP

Assistant Professor: *KAMADA,Yoshiaki*

Cell growth (increase in cell mass) and proliferation (increase in cell number) are highly linked with the cell's perception of its nutritional environment. Tor (target of rapamycin) protein belongs to a family of phosphatidylinositol kinase-like protein kinases and plays a central role in controlling cell growth. Specifically, Tor signaling couples nutrient signals to various growth-related processes. Tor protein forms distinct Tor complexes, TORC (Tor complex) 1 and TORC2. TORC1 regulates rapamycin-sensitive branches of the TOR pathway, such as translation initiation, ribosome biogenesis, and autophagy. On the other hand, TORC2, whose function is insensitive to rapamycin, is responsible for actin organization and cell integrity.

The aim of this research group is to reveal the molecular mechanisms of how the Tor pathway regulates each phenomenon, and how Tor receives nutrient signals. We have been studying Tor signaling in the budding yeast, *Saccharomyces cerevisiae*, and have found three novel branches of the TOR signaling pathway.

I. TORC1 is involved in the cell cycle at G2/M transition

It is well known that inhibition of protein synthesis causes cell growth and promotes cell cycle arrest at G1 (G0). Rapamycin, a TORC1 inhibitor has the same effect via inactivating TORC1. Little is known, however, about whether or not TORC1 is involved in other stages of the cell cycle. We generated a temperature-sensitive allele of *KOG1*, which encodes an essential component of TORC1. We found that this mutant, as well as yeast cells treated with rapamycin, exhibits mitotic delay with prolonged G2. We further demonstrated that this G2-arrest phenotype is due to mislocalization and resultant inactivation of Cdc5, the yeast polo-kinase. These results suggest that TORC1 mediates G2/M transition via regulating polo-kinase.

II. TORC1 regulates autophagy via Atg1 kinase complex

Autophagy is mainly a response to nutrient starvation, and TORC1 negatively regulates autophagy. The Atg1 kinase and its regulators, i.e. Atg13, Atg17, Atg29, and Atg31 collaboratively function in the initial step of autophagy induction, downstream of TORC1. Atg1 is a Ser/Thr protein kinase, the activity of which is essential for autophagy and is largely enhanced following nutrient starvation or the addition of rapamycin. This regulation involves Atg13. Although Atg13 is phosphorylated in a TORC1-dependent manner under nutrient-replete conditions, it is immediately dephosphorylated in response to starvation or rapamycin treatment. Dephosphorylated Atg13 binds to Atg1 which allows Atg17, Atg29, and Atg31 to associate with Atg1-Atg13 to form Atg1 complex. Atg1 complex formation triggers autophagy at least in two ways. First, through the recruitment of Atg proteins to the pre-autophagosomal structure (PAS), the putative site for autophagosome formation. Second, Atg1 kinase activity is enhanced by Atg1 complex formation. We assume that Atg1 phosphorylates its substrate(s) at the PAS to trigger a downstream event in autophagy. (Figure 1)

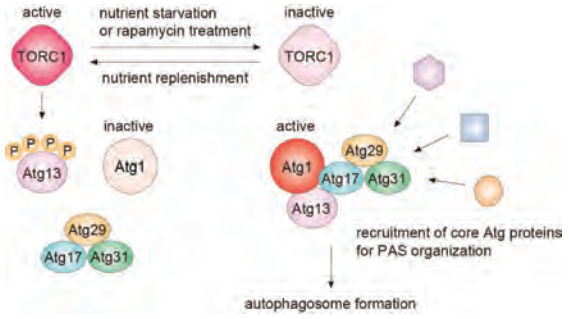

Figure 1. A schematic model of regulation of Atg1 complex by TORC1. When TORC1 is inactivated following nutrient starvation, Atg13 is dephosphorylated. This allows the formation of Atg1 complex among Atg1 and its regulators, followed by the upregulation of the Atg1 kinase activity and assembly of other Atg proteins to the PAS to initiate autophagosome formation.

III. TORC2 phosphorylates Ypk2 kinase to control actin organization

Genetic studies have shown that TORC2 controls polarity of the actin cytoskeleton via the Rho1/Pkc1/MAPK cell integrity cascade. However, the target (substrate) of TORC1 was not yet identified. We found that Ypk2, an AGC-type of protein kinase whose overexpression can rescue lethality of TORC2 dysfunction, is directly phosphorylated by TORC2.

Publication List

〔Original paper (E-publication ahead of print)〕

● Kamada, Y., Yoshino, K., Kondo, C., Kawamata, T., Oshiro, N., Yonezawa, K., and Ohsumi, Y. Tor directly controls the Atg1 kinase complex to regulate autophagy. Mol. Cell Biol. 2009 Dec 7.

〔Review article〕

● Nakatogawa, H., Suzuki, K., Kamada, Y., and Ohsumi, Y. (2009). Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat. Rev. Mol. Cell Biol. *10*, 458-467.

OHNO GROUP

Assistant Professor:	*OHNO, Kaoru*
Technical Assistants:	*HARA, Ikuyo*
	FUKAZAWA, Mitsue
	INAGAKI, Masako

The aim of this laboratory is to research the reproductive hormones in invertebrates and to analyze the mechanisms by which they work. The comparisons of such molecules and mechanisms in various species are expected to provide insights into the evolution oe reproductive hormone systems.

I. Gonadotropins in the starfish, *Asterina pectinifera*

Gonadotropins play important regulatory roles in reproduction in both vertebrates and invertebrates. The vertebrate gonadotropins, LH and FSH are structurally and functionally conserved across various species, whereas no such molecule has been identified in invertebrates. The insect parsin hormonesnare assumed to be the physiological counterpart of LH and FSH in mammals. Some gonadotropic hormones, such as egg development neurosecretory hormone of the mosquito, egg-laying hormone of the sea hare, and androgenic gland hormone of the terrestrial isopod, have been found iw invertebrate species. More recently, an insulin-like peptide was reported to be responsible for the regulation of egg maturation in the mosquito, Aedes aegypti, demonstrating the involvement of insulin signaling in egg maturation among invertebrates.

The gonad-stimulating substance (GSS) of an echinoderm, the starfish, was the very first gonadotropin to be identified in invertebrates. GSS mediates oocyte maturation in starfish by acting on the ovary to produce the maturation-inducing hormone (MIH), 1-methyladenine, which in turn induces the maturation of the oocytes. In this sense, GSS is functionally identical to vertebrate LH, especially piscine and amphibian LHs, acting on the ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of the oocyte. Considering the functional similarity that GSS shares with vertebrate LH, it is very important from an evolutionary point of view to know the chemical and molecular structure of GSS. We cloned the gene encoding GSSdreferred to aminodacid sequence of purified GSS from radial nerves of the starfish, Asterina pectinifera. Interestingly, phylogenetic analyses revealed that it belonged to the insulin/insulin-like growth factor (IGF)/relaxin superfamily and, more precisely, to the subclass of relaxin/insulin-like peptides (Figure 1).

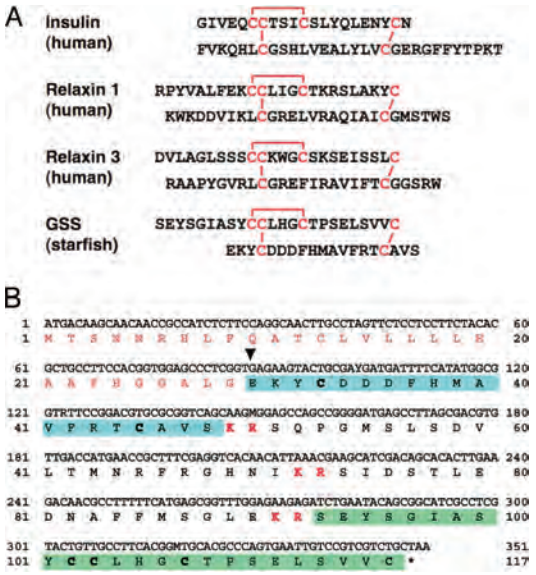

Figure 1. Amino acid sequence of starfish GSS. (A) Comparison of the heterodimeric structure of starfish GSS with those of various representative members of the insulin superfamily. The cysteine bridges are shown in red. (B) Coding DNA sequence and predicted amino acid sequences of GSS. Sequences of A and B chains are shown in green and blue boxes, respectively. Characters shown in red boldface indicate basic dipeptides that are the sites of proteolytic cleavage. Inverted triangle shows the deduced cleavage site of the signal peptide.

II. Search for reproductive hormones in invertebrates

In a collaborative effore with Prof. Yoshikuni's Laboratory of the Kyushu Univ. and Dr. Yamano and Dr. Awaji of the National Research Institute of Aquaculture, Fisheries Research Agency (NRIA), we are searching for reproductive hormones in invertebrates; sea urchin, sea cucumber, oyster, and shrimp. While the collaborators are partially purifying physiological materials which inducg egg maturation from nerve extracts and analyzing them with a tandem mass spectrometer, we are creating and analyzing EST libraries from nerve tissues and developing a databass of the mass analysis performed in this laboratory.

Publication List

〔Original paper〕

- Mita, M., Yoshikuni, M., Ohno, K., Shibata, Y., Paul-Prasanth, B., Pitchayawasin, S., Isobe, M., and Nagahama, Y. (2009). A relaxin-like peptide purified from radial nerves induces oocyte maturation and ovulation in the starfish, *Asterina pectinifera*. Proc. Natl. Acad. Sci. USA *106*, 9507-9512.

TERADA GROUP

Assistant Professor: *TERADA, Rie*
Postdoctoral Fellow: *SHIMATANI, Zenpei*
Technical Assistants: *ASAO, Hisayo*
*SHIMATANI, Zenpei**
MORITOH, Satoru

With the goal of developing an effective molecular breeding method and progressing in our continuing gene function study in rice (*Oryza sativa* L.), an important staple food, we are developing a new technology of gene targeting (GT) mediated by homologous recombination (HR). We have succeeded in gene targeting of *Waxy* based on a strong positive-negative (PN) selection in the cell where HR at the *Waxy* locus took place. Because our GT procedure is applicable to modify various genome regions we have been able to target 15 individual rice genes using the published sequence information. We have also succeeded in making various modifications to the target genes including gene knock-out as well as knock-in targeting of the coding sequence of *GUS* to the targeted gene promoter for analysis of its natural activity. In addition the point mutations on the vector for GT were effectively integrated into the targeted gene locus in *Alcohol dehydrogenase* gene 2 (*Adh2*), and a re-activation system of targeted-*waxy* by Cre-*loxP* recombination (Terada et al., Plant Biotech., in press) was achieved, similar to that used in mouse conditional GT. These results suggest lots of possibilities for creation of ideal mutants for GT-mediated molecular breeding and detailed gene function studies.

I. Generation of blast fungus resistant rice based on effective GT

OsRac1, a homolog of mammalian Rac GTPase, plays an important role in the defense response of plants. Amino acid substitution of the 19th glycine to valine (G19V) alters OsRac1 to be constitutively active (CA) by elimination of its GTPase activity. Increased resistance to blast fungus was detected in random transgenic rice of CA-*OsRac1* driven by 35S promoter. To examine whether the endogenous *OsRac1* shows the same effect or not we generated CA-*OsRac1* using our rice GT procedure. We constructed targeting vectors to induce the G19V substitution through a single point mutation of G to T in the first exon of *OsRac1*. Elimination of the positive marker, *hygromycin phospho transferase* (*hpt*), from targeted *OsRac1* using our Cre-*loxP* system created CA-*OsRac1* at the natural gene locus. Two targeting vectors, pRac1A and pRac1B, were constructed to have homologous sequences of *OsRac1* covering slightly different regions each other in order to insert the *hpt* marker flanked by the *loxP* sites ('floxed') into the first and third intron, respectively (Figure 1). After the G to T substitution by GT the *hpt* is eliminated by the Cre-*loxP* system. A single *loxP* remains in the first or third intron, however, these are expected to splice out. We have succeeded in GT of *OsRac1* using both vectors (Table 1). About 14% of PN selected calli were successfully targeted, giving us the highest efficiency of GT ever obtained. Interestingly in addition to the expected true GT (TGT), one-sided invasion (OSI; HR is caused only at 5' or 3' end of integrated DNA fragment) was frequently detected during GT using either vectors (Table 1). We selected 16 callus lines showing TGT and applied Cre mediated *hpt* elimination. Finally 10 plants regenerated from 4 *hpt* eliminated marker-free lines were grown all of which revealed characteristic semi-dwarf phenotype. Precise G to T substitution and *hpt* elimination in the heterozygous form was confirmed by sequence analyses of obtained plant DNA. Because fungus resistant phenotype is dominant, obtained targeted CA-*OsRac1* plants are under examination for resistance to blast fungus.

This work is a collaboration with Professor Ko Shimamoto in the Laboratory of Plant Molecular Genetics, Nara Institute of Science and Technology (NIST) and supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan Grant-in-Aid for Scientific Research (S) (No. 19108005 to K.S.)

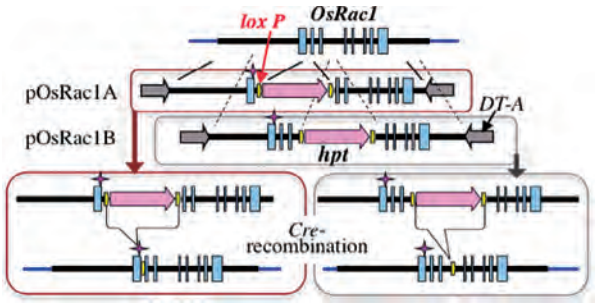

Figure 1. Strategy for creation of CA-*OsRac1*. Star indicates point mutation of G to T.

Targeted gene	PN selected Calli	OSI		TGT	Ratio of TGT/PN (%)
		5'only	3'only	5'+3'	
Waxy	638	0	0	6	0.94
OsRac1 by pRac1A	94	10	37	5	5.5
OsRac1 by pRac1B	80	4	4	11	13.6

Table I. Gene targeting of *OsRac1*

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2009. The former title is indicated by an asterisk (*).

HOSHINO GROUP

Assistant Professor: *HOSHINO, Atsushi*
Technical Assistant: *WATANABE, Seiko*

While genomic structures as well as their genetic information appear to transmit stably into daughter cells during cell division, and also into the next generation, they can actually vary genetically and/or epigenetically. Such variability has had a large impact on gene expression and evolution. To understand such genome dynamisms in eukaryotes, especially in plants, we are characterizing the flower pigmentation of morning glories.

I. The morning glories

Morning glories belong to the genus *Ipomoea* that is the largest group in the family *Convolvulaceae*. Of these, *I. nil* (Japanese morning glory), *I. purpurea* (the common morning glory), and *I. tricolor* have been domesticated well as floricultural plants, and their various spontaneous mutations have been isolated. The wild type morning glories produce flowers with uniformly pigmented corolla, whereas a number of mutants displaying particular pigmentation patterns have been collected (Figure 1). Because flower pigmentation patterns are easily observed, the molecular mechanisms underlying these phenomena provide fine model systems for investigating genome variability.

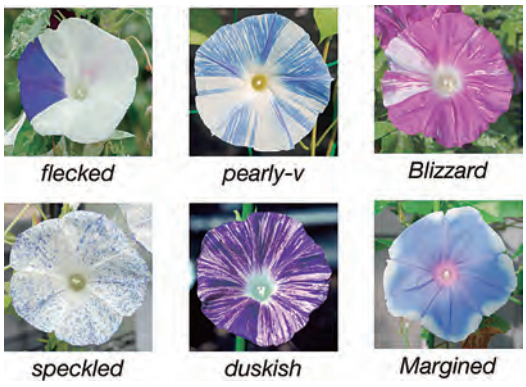

Figure 1. Flower phenotypes of Japanese morning glories.

II. Flower pigmentation patterns

Figure 1 represents examples of such mutants showing particular flower pigmentation patterns. Based on the molecular mechanisms conferring the particular patterns, these mutants can be classified into three groups. The first group includes the *flecked* and *speckled* mutants of *I. nil* that bloom variegated flowers with pigmented spots and sectors on whitish backgrounds. These mutations are caused by the insertions of certain groups of DNA transposons into the genes for flower pigmentation. Recurrent somatic mutations due to transposon excision from the genes result in pigmented spots and sectors in white backgrounds. In the second group, the *pearly-v* mutant of *I. tricolor* and the *duskish* mutant of *I. nil* also have variegated flowers, and epigenetic mechanisms are thought to regulate flower pigmentation. While the mutations in the two groups mentioned above are recessive, *Margined* and *Blizzard* of *I. nil* are dominant mutations. *Blizzard* and *Margined* mutants bloom pigmented corolla with irregular whitish spots and white edge, respectively. It was suggested that non-coding small RNA represses the expression of a pigmentation gene in the whitish parts of the corolla. We are currently characterizing detailed molecular mechanisms of the mutations in the latter two groups.

III. BioResource of morning glories

NIBB is the sub-center for National BioResource Project (NBRP) for morning glory. In this project, we are collecting, maintaining and distributing standard lines, mutant lines for flower pigmentation, and DNA clones from EST and BAC libraries of *I. nil* and its related species. *I. nil* has been one of the most popular floricultural plants since the late Edo era in Japan. It has extensive history of genetic studies and also has many advantages as a model plant; simple genome, large number of mutant lines, and efficient self-pollination. Our collection increased to 200 lines and 100,000 clones by the end of 2009. A web site to integrate information on these resources is under construction (Figure 2).

Figure 2. NBRP morning glory web site.

Publication List

〔Original papers〕

- Hoshino, A., Park, K.I., and Iida, S. (2009). Identification of *r* mutations conferring white flowers in the Japanese morning glory, *Ipomoea nil*. J. Plant Research *122*, 215-222.
- Shimatani, Z., Takagi, K., Eun, C.H., Maekawa, M., Takahara, H., Hoshino, A., Qian, Q., Terada, R., Johzuka-Hisatomi, Y., Iida, S., and Tsugane, K. (2009). Characterization of autonomous *Dart1* transposons belonging to the *hAT* superfamily in rice. Mol. Gen. Genomics *281*, 329-344.

TSUGANE GROUP

Assistant Professor: *TSUGANE, Kazuo*

Although DNA transposons are one of the major components of plant genomes, their transposition is restricted genetically or epigenetically for genome stability. Because insertions of transposons have contributed to the creation of new genes and genome evolution, revealing the genome dynamisms driven by DNA transposons is the purpose of our research.

Gene tagging is an important tool for understanding gene functions. We have constructed mutant rice lines using DNA transposons in order to achieve functional genomics analysis in rice, a model plant for monocots and cereals.

I. An active DNA transposon in rice

The mutable allele *virescent* (or *pale-yellow-leaf variegated, pyl-v*) which displays leaf variegation was caused by the integration of the 607-bp non-autonomous element *nDart1-0* belonging to the *hAT* superfamily into the *OsClpP5* gene encoding the chloroplast protease. In the mutable *pyl-v* allele, somatic excision of *nDart1-0* from *OsClpP5* in the presence of an active autonomous element, *aDart*, results in the pyl-v leaf variegation phenotype, a dark-green sector consisting of somatically reverted cells, due to *nDart1-0* excision, on a pale-yellow background comprising of cells having *nDart1-0* inserted into *OsClpP5* in the homozygous condition. Plants containing the *pyl-v* allele without an active *aDart* element display pale-yellow leaves without variegation; this has been termed as the pale-yellow leaf-stable (pyl-stb) phenotype. In the sequenced Nipponbare genome containing no active *aDart* elements, among the 53 inactive *iDart1* elements, there are 38 putative autonomous *iDart1* elements. Because their putative transposase genes carry no apparent nonsense or frameshift mutations, they are thought to be silenced epigenetically. Therefore we felt it highly likely that an active *aDart* element would be similar in structure to one of these 38 *iDart1* elements. Using map-based cloning, we found that *aDart* in the mutable *pyl-v* plant coincides with one of the 38 *iDart1* elements, *iDart1-27*, residing on chromosome 6 in Nipponbare and that the transcripts of the accumulated transposase gene in the *pyl-v* leaves are predominantly from *Dart1-27*. Two additional smaller transcripts were detected in *pyl-v* (Figure. 1). The major transcripts detected in *pyl-stb* and Nipponbare were derived from non-*Dart1-27* elements, implying that the residual expression of other *Dart1* elements in both *pyl-stb* and Nipponbare would be too weak to act on the *nDart1-0* at *OsClpP5* and lead to their excision even though some of these transcripts might encode an active transposase. While the longest (L) transcripts in *pyl-v* are the *Dart1-27* transcripts having intron 1 at 5'-UTR spliced, the middle (M) and the shortest (S) transcripts were mixtures that had one or two additional introns spliced, respectively, although the exact splicing sites were often different. It is noteworthy that all the shorter transcripts characterized in *pyl-v* were derived from *Dart1-27*, indicating that some of the abundantly expressed transcripts must have undergone further splicing in rice. These findings should facilitate the development of an efficient gene-tagging system in rice and shed light on epigenetic regulatory and evolutionary aspects of autonomous elements in the *nDart*/*aDart* system.

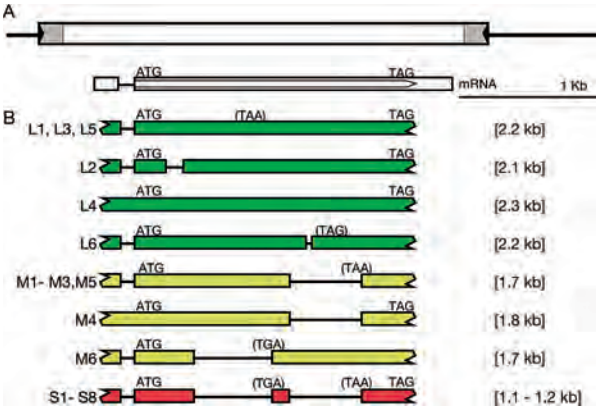

Figure 1 Transcripts of the *Dart1* transposase genes detected in rice. **(A)** Structure of *Dart1-27* in the *pyl-v* genome. **(B)** Schematic representation of the alternatively spliced transposase transcripts observed in rice plants. Three different sized transcripts (L, M, and S) were observed, and their splicing patterns are categorized.

II. Reverse genetic analysis of rice genes

Among active rice DNA transposons, *nDart1-0* and its relatives appear to be more suitable than the others for transposon tagging in rice because (1) their transposition can be controlled under natural growth conditions, *i.e.*, the transposition of *nDart1-0* can be induced by crossing with a line containing an active *aDart* element and stabilized by segregating *aDart*, and (2) *nDart1-0* and its relatives are often found at GC-rich regions in the genome and tend to integrate into promoter proximal genic regions. Because we have also obtained dominant and semi-dominant mutants (Figure 2), *nDart1*-promoted mutant lines can contribute to functional genomics in rice.

Figure 2. Semi-dominant *bushy dwarf tillers* mutant.

Publication List

〔Original paper〕

● Shimatani, Z., Takagi, K., Eun, C.H., Maekawa, M., Takahara, H., Hoshino, A., Qian, Q., Terada, R., Johzuka-Hisatomi, Y., Iida, S., and Tsugane, K. (2009). Characterization of autonomous *Dart1* transposons belonging to the *hAT* superfamily in rice. Mol. Gen. Genomics *281*, 329-344.