LABORATORY OF BIOLOGICAL DIVERSITY

KAMADA Group

Assistant Professor: *KAMADA,Yoshiaki*

Nutrient is indispensable for cells to survive. Thus, the transmission of nutrient signals is also important. Tor (target of rapamycin) protein plays a central role in controlling cell growth in response to nutritional environments. 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 our research group is to reveal the molecular mechanisms of how Tor receives nutrient signals and how the TORC1/2 pathways regulate each phenomenon. We have been studying Tor signaling in the budding yeast, *Saccharomyces cerevisiae*, and have found three novel branches of the TOR signaling pathway.

I. Cell cycle at G2/M is regulated by TORC1.

TORC1 regulates protein synthesis which is important for promotion of the cell cycle at G1 (G0). 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 (kog1-105),* which encodes an essential component of TORC1. We found that this mutant, as well as yeast cells treated with rapamycin, exhibit 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 phosphorylates Atg13, the molecular switch of autophagy.

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 phosphorylation of Atg13.

We found that Atg13 is directly phosphorylated by TORC1. Phosphorylated Atg13 (during nutrient-rich conditions) loses its affinity to Atg1, resulting in repression of autophagy. On the other hand, under starvation conditions Atg13 is immediately dephosphorylated and binds to Atg1 to form Atg1 complex. Atg1 complex formation confers Atg1 activation and consequently induces autophagy.

We determined phosphorylation sites of Atg13 and generated an unphosphorylatable Atg13 mutant (Atg13-8SA). Expression of Atg13-8SA induces autophagy bypassing inactivation of TORC1, such as starvation treatment or rapamycin. These results demonstrate that Atg13 acts as a molecular switch for autophagy induction (Figure 1).

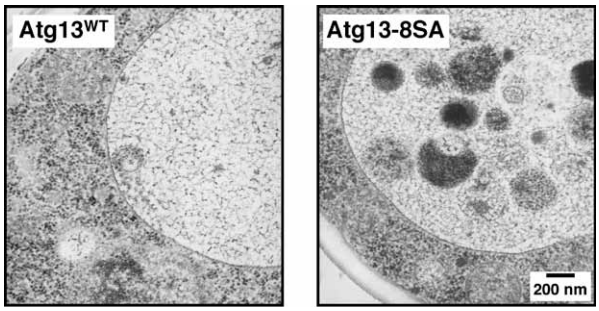

Figure 1. Induction of autophagy by expression of unphosphorylatable form of Atg13 (Atg13-8SA). Images of electron microscopy of $Atg13^{WT}$ (left) and Atg13-8SA (right) expressing cell.

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 TORC2 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〕

- Kamada, Y., Yoshino, K., Kondo, C., Kawamata, T., Oshiro, N., Yonezawa, K., and Ohsumi, Y. (2010). Tor directly controls the Atg1 kinase complex to regulate autophagy. Mol. Cell. Biol. *30*, 1049-1058.

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

- Yoshida, S., Imoto, J., Minato, T., Oouchi, R., Kamada, Y., Tomita, M., Soga, T., and Yoshimoto, H. A novel mechanism regulates H_2S and SO_2 production in *Saccharomyces cerevisiae*. Yeast. 2010 Oct 8.

〔Review articles〕

- Kamada, Y. (2010). Prime-numbered Atg proteins act at the primary step in autophagy. –Unphosphrylated Atg13 can induce autophagy without TOR inactivation. Autophagy *6*, 415-416.
- Kamada, Y., and Ohsumi, Y. (2010). The TOR-mediated regulation of autophagy in the yeast *Saccharomyces cerevisiae*. The Enzymes XXVIII, F. Tamanoi and M.N. Hall, eds. (Academic Press, Elsevier), pp. 143-165.

LABORATORY OF BIOLOGICAL DIVERSITY

OHNO Group

Assistant Professor:	*OHNO, Kaoru*
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	INAGAKI, Masako
	NAKAMURA, Ryoko
	MATSUDA, Azusa
	FUJITA, Miyako

The aim of this laboratory is to research 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 of 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 hormones are assumed to be the physiological counterpart of LH and FSH in mammals. Some gonadotropic hormones, such as the egg development neurosecretory hormone of the mosquito, the egg-laying hormone of the sea hare, and the androgenic gland hormone of the terrestrial isopod, have been found in 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 GSS referred to amino acid 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).

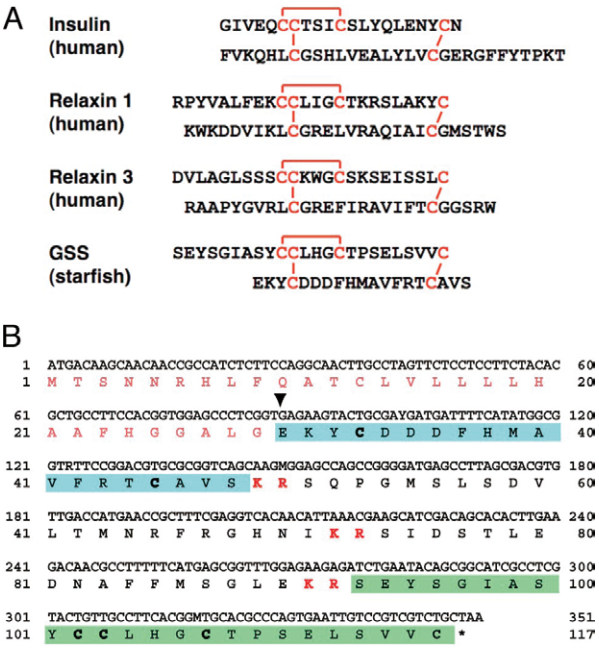

Figure 1. Amino acid sequence of starfish GSS. (A) Comparison of the heterodimeric structure of starfish GSS with those of various representative members of the insulin superfamily. The cysteine bridges are shown in red. (B) Coding DNA sequence and predicted amino acid sequences of GSS. Sequences of 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 effort 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 induce 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 database of the mass analysis performed in this laboratory.

Publication List

〔Original paper〕

- Fujiwara, A., Unuma, T., Ohno, K., and Yamano, K. (2010). Molecular characterization of the major yolk protein of the Japanese common sea cucumber (*Apostichopus japonicus*) and its expression profile during ovarian development. Comp. Biochem. Physiol. A Mol. Integr. Physiol. *155*, 34-40.

LABORATORY OF BIOLOGICAL DIVERSITY

TERADA Group

Assistant Professor: *TERADA, Rie*
Postdoctoral Fellow: *SHIMATANI, Zenpei*

Gene targeting (GT) mediated by homologous recombination (HR) is the most effective tool for generation of mutant plants for both studies of molecular genetics and molecular breeding. Since the first success of our *Waxy* GT in rice (*Oryza sativa* L.) based on a strong positive-negative (PN) selection, we have modified 15 individual rice genes into various forms, not only gene knock-out but also knock-in and a single nucleotide substitution at the target gene locus by our GT method.

I. Generation of blast fungus resistant rice by GT of *OsRac1*.

As an attempt to apply GT to molecular breeding, we have modified rice *OsRac1* to be constitutively active (CA) by amino acid substitution of the 19th glycine to valine (G19V). OsRac1, a homolog of mammalian Rac GTPase, plays an important role in the defense response of plants. Its GTPase activity is increased by G19V substitution through a single point mutation of G to T in the first exon of *OsRac1*. As shown in Figure 1A the point mutation was inserted into *OsRac1* by GT with vectors of pOsRac1A or pOsRac1B. Elimination of the positive marker, *hygromycin phospho transferase* (*hpt*) from targeted *OsRac1* by the Cre-*loxP* system created CA-*OsRac1* at the natural gene locus. Although single *loxP* remains in the first or third intron, it is expected to splice out. We have obtained 5 and 11 of the expected true GT (TGT) callus lines by pOsRac1A and pOsRac1B, respectively. G to T substitution was detected by sequence analysis in all 5 TGT lines by pOsRac1A and in 6 of 11 TGT lines by pOsRac1B, respectively. The substitution efficiency was 100% in TGT by pOsRac1A but was about 45% in TGT by pOsRac1B. Distance between the point mutation and *hpt* positive marker in the vector homology arm is thought to be an important factor for insertion efficiency of point mutation. This result was quite different from the GT at *Alcohol dehydrogenase* gene 2 (*Adh2*) where point mutations near to both ends of the vector homology arms were effectively integrated. Subsequently TGT callus lines were applied to Cre mediated *hpt* elimination. From both TGT with pOsRac1A and with pOsRac1B, each of the two expected marker-free lines were obtained. These 4 callus lines were regenerated to plants and are under analyses for fungal resistance.

II. Insertion of visual markers as fusion genes with *OsMADS* by GT.

Several markers have been developed for visualization of gene functions. We are progressing with visualization of *OsMADS* expression using knock-in targeting with visual markers for the study of flower development. *OsMADS* is a homolog of the *APETALA1* (*AP1*) gene and is predicted to work in the flower meristem under the Florigen Activation Complex, FAC, with Hd3a-14-3-3-OsFD1. We are inserting coding sequences of visual markers, luciferase and m-Orange, into the 3' TAG downstream of *OsMADS* as fusion proteins using our GT method. We constructed two knock-in targeting vectors named as p15L and p15Or (Figure 1B). We obtained 6 TGT lines having luciferase gene that connected to the coding sequence of *OsMADS* (Figure 1B, Table 1). We are now progressing with knock-in GT of the p15Or vector. These works are in collaboration with Professor Ko Shimamoto in the Laboratory of Plant Molecular Genetics, Nara Institute of Science and Technology (NAIST) and are 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.)

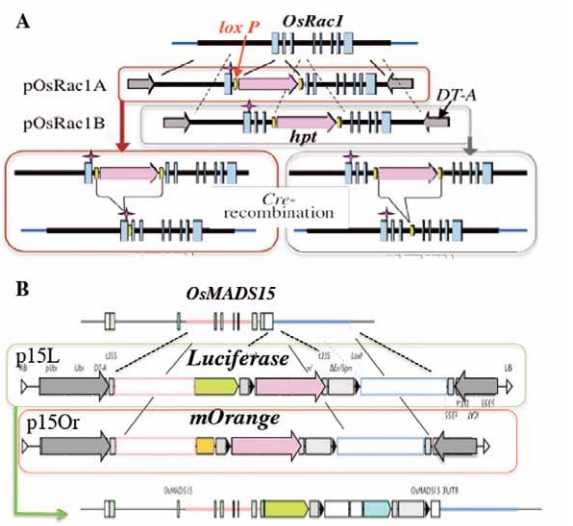

Figure 1. Generation of CA-*OsRac1 and* visualization of *OsMADS* by GT mediated gene modifications.

Targeted gene	PN selected Calli	TGT 5'+3'	Ratio of TGT/PN (%)
OsRac1 by pRac1A	94	5	5.5
OsRac1 by pRac1B	80	11	13.6
OsMADS by p15L	169	6	3.6

Table 1. GT of *OsRac1* and *OsMADS*

Publication List

〔Original paper〕

● Terada, R., Nagahara M., Furukawa K., Shimamoto, M., Yamaguchi, K., and Iida S. (2010). Cre-*loxP* mediated marker elimination and gene reactivation at the *waxy* locus created in rice genome based on strong positive-negative selection. Plant Biotechnology *27*, 29-37.

LABORATORY OF BIOLOGICAL DIVERSITY

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.

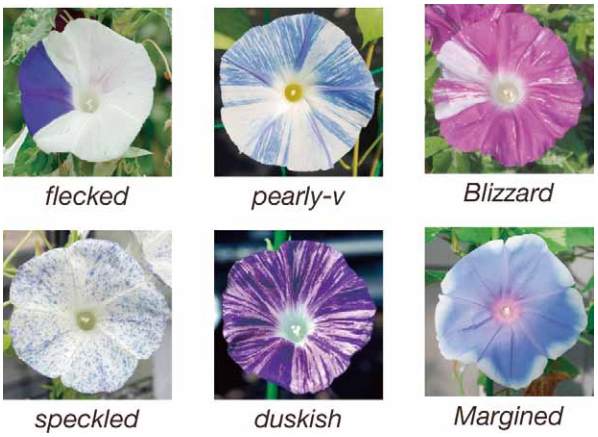

Figure 1. Flower phenotypes of Japanese morning glories.

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 on 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 edges, 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. *de novo* sequencing of Japanese morning glory genome

Although morning glories are studied worldwide, especially in plant physiology and genetics, no whole nuclear genome sequences of any *Ipomoea* species are available. To facilitate the studies of our group as well as all morning glory researchers, we started *de novo* genome sequencing of *I. nil* using high-throughput DNA sequencers. *I. nil* has a genome of about 800 Mbp, and we chose the Tokyo-kokei standard line for genome sequencing. We are collaborating with several laboratories of the National Institute for Genetics and Kyushu University.

IV. 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 117,000 clones by the end of 2010.

Figure 2. Breeding field and greenhouse for morning glory.

LABORATORY OF BIOLOGICAL DIVERSITY

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.

I. Transposition and target preferences of *nDart1* in rice genome

Gene tagging is a powerful tool for elucidating the function of rice genes, and foreign elements, such as T-DNA or maize DNA transposons *Ac*/*Ds* and *En*/*Spm*, and the endogenous retrotransposon *Tos17* have been systematically employed. A potential obstacle to these insertional mutants may be the concomitant occurrence of somaclonal variation associated with tissue cultures because tissue cultures are necessary to either introduce these foreign elements into rice calli or to activate dormant *Tos17* in the genome. While endogenous active DNA transposons, which are free from somaclonal variation because no tissue culture is involved in generating insertion mutants, have been extensively used for gene tagging in maize, snapdragon, petunia, and morning glories, only a few active endogenous DNA transposons, *mPing*, *nDart1*, *dTok*, and *nDaiZ* have been identified in rice. Of these active nonautonomous DNA elements, *nDart1* appears to be more suitable than the others for transposon tagging in rice because their transposition can be controlled under natural growth conditions, *i.e.*, the transposition of *nDart1* can be induced by crossing with a line containing an active autonomous *aDart* element and stabilized by segregating *aDart*. 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.

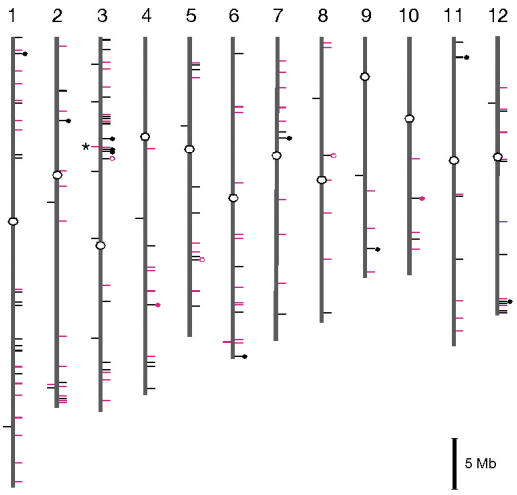

Figure 1. Localization of *nDart1-3* subgroup elements inserted in the genome of the *virescent* mutant and its progeny. The pink and black bars indicate *nDart1-0* and other *nDart1-3* subgroup elements, determined by comparing the results of nDart-TD.

Originally identified *nDart1-0* was found from the mutable *virescent* line as an insertion into the *OsClpP5* gene, *nDart1-0* related elements, and *nDart1-3* subgroups were also identified. In the sequenced Nipponbare genome, most of the *nDart1-3*-related elements reside in GC-rich regions, and no apparent consensus sequence is found in their insertion sites, including 8-bp target site duplications (TSDs). Since the GC content of genes (45%), especially exon (54%), is known to be higher than that of intergenic regions (43%) in rice, we can anticipate that the *nDart1*-related elements are likely to be inserted into genic regions. To assess whether *nDart1* is indeed suitable for gene tagging in rice, we have examined the transposition activities and target specificities of *nDart1* in a rice line containing an active autonomous *aDart1* element. We employed an amplified fragment length polymorphism (AFLP)-based transposon display (TD), by which we were able to visualize approximately 90% of the anticipated bands produced from the *nDart1*-related elements in the Nipponbare genome. Newly transposed *nDart1-3* subgroup elements were found to be integrated into various sites almost all over the genome (Figure1), and more than 60% of these sites were genic regions comprising putative coding and/or intron regions and their 0.5-kb 5'-upstream and 3'-downstream regions. Moreover, approximately two-thirds of these genic insertions were confined in the 0.5-kb 5'-upstream region (Figure2). These results are discussed with respect to the development of an efficient and somaclonal variation-free gene tagging system for rice functional genomics.

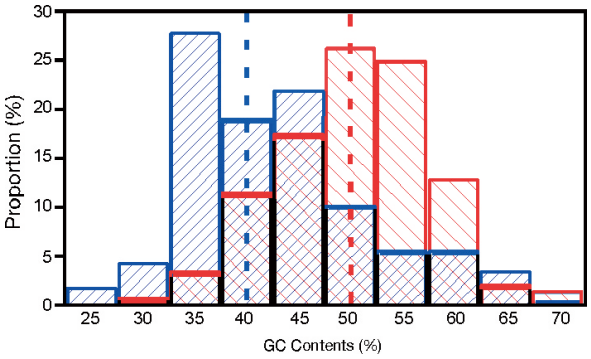

Figure 2. Average GC contents of both flanking 0.5-kb regions of the newly inserted the *nDart1-3* subgroup elements in progeny lines of mutable *virescent* rice. Red and blue histograms represent the GC contents of regions identified by *nDart1* insertions and 240 randomly selected control regions, respectively. The vertical broken lines indicate the intermediated value.

Publication List

〔Original paper〕

● Takagi, K., Maekawa, M., Y., Tsugane, K., and Iida, S. (2010). Transposition and target preferences of an active nonautonomous DNA transposon *nDart1* and its relatives belonging to the *hAT* superfamily in rice. Mol. Gen. Genomics *284*, 343-355.

LABORATORY OF BIOLOGICAL DIVERSITY

YAMAGUCHI Group

Assistant Professor: *YAMAGUCHI, Takahiro*
Postdoctoral Fellow: *NUKAZAWA, Akira*
Technical Assistant: *YAMAGUCHI, Chinami*

Angiosperm leaves generally develop as bifacial structures with distinct adaxial and abaxial identities. However, several monocot species, such as iris and leek, develop "unifacial leaves", in which leaf blades have only an abaxial identity (Figure 1). We are focusing on unifacial leaf development and evolution to understand genetic mechanisms behind diversity and evolution of organismal morphology.

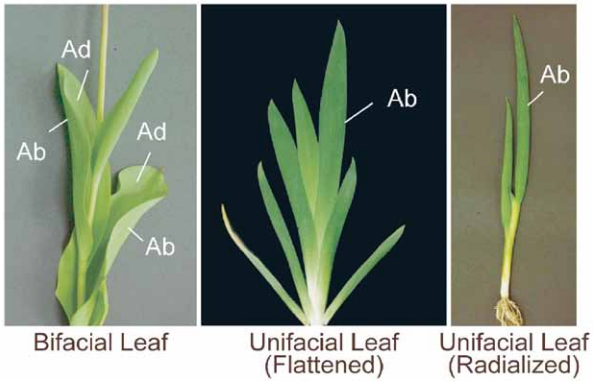

Figure 1. Bifacial and unifacial leaf structures. Ad, Adaxial side; Ab. Abaxial side.

I. Abaxialization of unifacial leaves

The development and evolution of unifacial leaves have long been matters of debate. However, nothing has been studied at the molecular genetic level. We focused on the genus *Juncus* as a model to study the evolution and development of unifacial leaves. *Juncus* contains species with a wide variety of leaf forms and is amenable to molecular genetic studies (Yamaguchi and Tsukaya, 2010). We first characterized unifacial leaf development by investigating gene expression patterns of adaxial and abaxial determinants. As a result, we demonstrated that the unifacial leaf blade is abaxialized at the gene expression level and reveled that dominant abaxial activity leads to the unifacial leaf development (Yamaguchi et al., 2010).

II. Flattening of unifacial leaves

In bifacial leaves, adaxial–abaxial polarity is required for leaf blade flattening, whereas many unifacial leaves become flattened although their leaf blades are abaxialized (Figure1). This indicates independent mechanisms underlying flattened leaf blade formation in bifacial and unifacial leaves.

Using two closely related *Juncus* species, *J. prismatocarpus*, with flattened unifacial leaves, and *J. wallichianus*, with radialized unifacial leaves, we revealed that *DL* expression levels and patterns correlate with the degree of laminar outgrowth. Genetic and expression studies using interspecific hybrids of the two species revealed that the *DL* locus from *J. prismatocarpus* flattens the unifacial leaf blade and expresses higher amounts of *DL* transcripts. Thus, *DL* is a key gene that flattens the unifacial leaf blade. Interestingly, *DL* plays a distinct role in promoting midrib formation during bifacial leaf development. We suggest that morphological convergence of flattened leaf blades in unifacial leaves has occurred via the recruitment of *DL* function, which plays a similar cellular but distinct phenotypic role in monocot bifacial leaves (Figure 2, Yamaguchi et al., 2010).

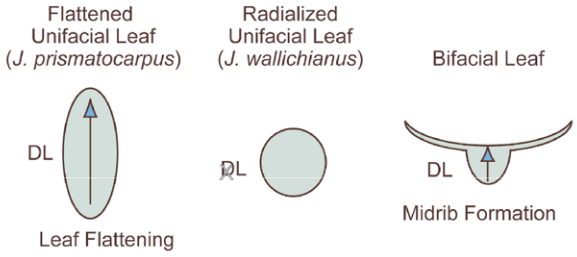

Figure 2. Mechanism of leaf blade flattening in unifacial leaves.

Publication List

〔Original papers〕

- Nakayama, H., Yamaguchi, T., and Tsukaya, H. (2010). Expression patterns of *AaDL*, a *CRABS CLAW* ortholog in *Asparagus asparagoides* (Asparagaceae), demonstrate a stepwise evolution of *CRC/DL* subfamily *YABBY* genes. Amer. J. Bot. *97*, 591–600.
- Nelissen, H., De Groeve, S., Fleury, D., Neyt, P., Bruno, L., Bitonti, M.B., Vandenbussche, F., Van Der Straeten, D., Yamaguchi, T., Tsukaya, H., Witters, E., De Jaeger, G., Houben, A., and Van Lijsebettens, M. (2010). Plant Elongator regulates auxin-related genes during RNA polymerase II transcription elongation. Proc. Natl. Acad. Sci. USA *107*, 1678-1683.
- Toriba, T., Suzaki, T., Yamaguchi, T., Ohmori, Y., Tsukaya, H., and Hirano, H. (2010). Distinct regulation of adaxial-abaxial polarity in anther patterning in rice. Plant Cell *22*, 1452–1462.
- Yamaguchi, T., Yano, S., and Tsukaya, H. (2010). Genetic framework for flattened leaf blade formation in unifacial leaves of *Juncus prismatocarpus*. Plant Cell *22*, 2141–2155.

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

- Ikeuchi, M., Yamaguchi, T., Kazama, T., Ito, T., Horiguchi, G., and Tsukaya, H. ROTUNDIFOLIA4 regulates cell proliferation along the body axis in Arabidopsis shoot. Plant Cell Physiol. 2010 Sep 8.

〔Review article〕

- Yamaguchi, T., and Tsukaya, H. (2010). Evolutionary and developmental studies of unifacial leaves in monocots: *Juncus* as a model system. J. Plant Research. *123*, 35–41.