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学位論文題目	A study on *KNOX* gene functions for shoot apical meristem development and maintenance in rice
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The body plan is one of the most remarkable differences between animals and plants. During embryogenesis, in contrast to animals that usually complete their organogenesis, plants form only a simple structure consisted of two distinctive regions at opposite poles. One is the shoot apical meristem (SAM) and the other is the root apical meristem. Both meristems are organized groups of undifferentiated cells responsible for post-embryogenic organ formation. The SAM maintains itself through replenishment of the indeterminate cell population by stem cell activity, and produces all above ground organs throughout the life cycle. Therefore, the unique body plan of plants relies on the dynamic but sustainable activity of the SAM, and the elucidation of the mechanisms to maintain the SAM is a fundamental issue in developmental biology.

From previous studies, it has been revealed that Class I *Knotted1*-like homeobox (*KNOX*) genes play indispensable roles in the SAM. *KNOX* genes are expressed in shoot apex specifically, and down-regulated in differentiating organs. Loss-of-function mutants of *KNOX* genes show shoot meristemless phenotype in *Arabidopsis* and maize, and conversely their ectopic expression in differentiating organs disturbs normal differentiation, and disrupts proper development in various plant species. Thus, *KNOX* genes are required for the establishment and maintenance of the SAM through the regulation of indeterminate cell state. In spite of importance of the SAM-specific expression of *KNOX* genes, virtually nothing is known about the regulatory system that activates and/or maintains the expression of *KNOX* genes in the SAM in any plant species. In addition, understanding of *KNOX* gene function and SAM maintenance mechanism in rice was limited because of lack of loss-of-function mutants except for *OSH15*.

In order to gain the understanding of *KNOX* gene function in rice, I identified a loss-of-function mutant of another member of rice *KNOX* gene, *OSH1* using TILLING sytem. Expression analysis of mRNA and protein showed that this mutant was a null allele of *OSH1*. *osh1* mutant once formed the SAM during embryogenesis, but lost the SAM just after germination. *osh1 osh15* double mutant failed SAM formation completely during embryogenesis. These results revealed that *OSH1* is required for both formation and maintenance of the SAM, and *OSH15* can compensate requirement of *OSH1* for SAM formation, but not for SAM maintenance.

Surprisingly, regenerated shoot from *osh1* callus could survive until reproductive phase, but no shoot was regenerated from *osh1 osh15* double mutant callus. This result implied different contributions of rice *KNOX* genes to SAM formation and maintenance between germination and regeneration. Indeed, expression analysis revealed that *osh1* mutant underwent the *KNOX*-scarce state temporally during germination, whereas induction of *OSH6*, *OSH15* and *OSH71* by phytohormone cytokinin (CK) could bypass the state during regeneration. Interestingly, the expression level of *OSH43* was severely reduced in *osh1* mutant both during germination and regeneration, and *OSH6* and *OSH15* were also slightly down-regulated even with CK treatment. In *osh1 osh15* double mutant embryo and regenerating callus, the expression level of all *KNOX* genes were severely

reduced. Thus, these data indicated that the expression of *KNOX* genes depended not only on CK but also on the function of *KNOX* gene themselves.

In consistent with this idea, ChIP assay using anti-OSH1 antibody showed that OSH1 protein directly bound to all *KNOX* loci in vivo. Sequence comparison analysis among *KNOX* gene orthologs in poaceae revealed that there were evolutionally conserved putative OSH1 binding sites, TGAC/GTCA in enriched region by ChIP assay. Given that all *KNOX* genes have redundant functions in the SAM, it is conceivable that the expression of *KNOX* genes is regulated by a direct positive autoregulation.

Previous reports have shown that *KNOX* genes induce the expression of CK biosynthetic emzyme adenosine phosphate isopentenyltransferase (IPT), and promote the accumulation of CK in the SAM in *Arabidopsis* and rice. In this study, it was revealed that the expression of *KNOX* genes was induced by CK. This indicates that the functions of *KNOX* genes and CK mutually facilitate each other, and suggested the presence of another positive autoregulatory loop of *KNOX* genes via phytohormone CK. A previous study, which has shown that the expression of *OSH1* eventually disappears in the loss-of-function mutants of LONELY GUY, an enzyme catalizing the final step of CK biosynthesis, also supports this notion.

Taken together, I propose two positive autoregulatory loops of *KNOX* genes. In this model, all cells in the SAM express *KNOX* genes through direct and indirect autoregulation to maintain their indeterminate state during replenishment of cell population prior to lateral organ initiation. In these cells, KNOX proteins directly bind to evolutionally conserved TGAC/GTCA sequences on all *KNOX* loci, and activate and/or maintain the expression of themselves. In addition, *KNOX* genes also induce the expression of CK biosynthetic emzyme IPT, and promote the accumulation of CK in the SAM. In turn, CK also activates the expression of *KNOX* genes. Based on this hypothesis, the positive autoregulations of *KNOX* genes emerges as a novel self-regulatory system of the SAM that ensures the unique body plan of plants.

博士論文の審査結果の要旨

植物の茎頂分裂組織（Shoot Apical Meristem :SAM）は、地上部のすべての器官を生成するとともに、一定数の細胞が分裂後も維持される。SAM形成に影響するトウモロコシやシロイヌナズナの突然変異体の解析からクラスI Knotted-like homeobox（KNOX)遺伝子群が同定されている。KNOX遺伝子群は、SAMで特異的に発現し、SAMの維持に必要である。しかしながら、KNOX遺伝子の発現が特異的に活性化され維持される機構については、ほとんどわかっていなかった。

津田勝利君は、KNOX遺伝子とSAMの制御機構に興味を持ち、イネを用いた研究を行った。まず、TILLING法を用いた逆遺伝学的アプローチで、イネのKNOX遺伝子の一つOSH1（Oryza Sativa Homeobox 1）の突然変異体を同定した。この突然変異体ともう一つのKNOX遺伝子であるOSH15の突然変異体を用いた解析の結果、これらの遺伝子がSAM形成や維持に関与することがわかった。突然変異体はSAM欠損表現型を示すが、この表現型は、発生ステージによっては、組織培養でサイトカイニンを補うことで救済できた。また、この際、サイトカイニンの有無がKNOX遺伝子の多くのメンバーの発現に影響した。サイトカイニン生成にKNOX遺伝子が影響することが知られていたが、逆方向の制御の存在が示唆された。

また、OSH1遺伝子の突然変異体では、他のKNOX遺伝子の一つの発現が低下し、OSH1とOSH15の二重変異体では他の全てのKNOX遺伝子の発現が低下した。さらに、OSH1蛋白質に対するクロマチン免疫沈降実験の結果、OSH1蛋白質が細胞内で、自身および他のKNOX遺伝子に結合していることがわかった。これらの結果にもとづき、津田君は、KNOX遺伝子の発現誘導とその維持において、正の自己制御機構が働いているという仮説を提案した。この正のフィードバックループには、サイトカイニン生産を介したものと、KNOXが自身の転写を直接活性化する機構の両方が関与していると考えられる。

これらの研究は、植物発生の重要なステップであるSAM形成機構とそれに関与するKNOX遺伝子群の発現制御機構に新たな知見をもたらすものである。津田君は、複数のアプローチを組合わせることで、この大きな問題の解決に向けて分野を大きく前進させた。審査員全員で審査した結果、本大学院における学位授与の水準を十分に満たす論文であると判断した。