(Format No. 13)

SUMMARY OF DOCTORAL THESIS

Name: SULTANA MST MOMTAZ

Title: Development of binary vector systems for promoter assay and expression analysis of AtMLLR genes in *Arabidopsis thaliana*

(プロモーターアッセイのためのバイナリベクターシステム開発とシロイヌナズナにおける AtMLLR 遺伝子の発現解析)

The research described in this thesis has a specific goal to develop gateway compatible binary vector construction and their strategies in detail for the study of organ-, tissue-, or cell-specific expression pattern of genes in *Arabidopsis thaliana* through promoter:reporter assay. In this section the results of the study are summarized below:

In Chapter 1, the author presents a general introduction to the work. Chapter 2 describes development of new vectors and expression analysis of genes in *Arabidopsis thaliana*. A new series of Gateway cloning technology-compatible binary vectors, pGWBs (attR1-attR2 acceptor sites) and R4L1pGWB (attR4-attL1 acceptor sites), carrying organelle-targeted sGFP (ER-, nucleus-, peroxisome-, and mitochondria-targeted sGFP) and organelle-targeted TagRFP (nucleus-, peroxisome-, and mitochondria-targeted TagRFP) has been developed to facilitate promoter:reporter assays in *A. thaliana*. Both pGWB and R4L1pGWB were designated as 4xx, 5xx, 6xx and 7xx, and 4, 5, 6, and 7 represent four kinds of plant selection markers where 4xx refers to neomycin phosphotransferase II (NPTII) conferring kanamycin resistance (Km^r), 5xx indicates hygromycin phosphotransferase (HPT) conferring hygromycin resistance (Hyg^r), 6xx refers to bialaphos resistance gene (bar) conferring BASTA resistance ($BASTA^r$), and 7xx indicates UDP-N-acetylglucosamine: dolichol phosphate N-acetylglucosamine-1-P transferase (GPT) conferring tunicamycin resistance ($Tunica^r$). These marker genes were placed in reverse orientation under regulation of the nopaline synthase (nos) promoter and followed by nos terminator. The last two digits represent the reporter type of organelle-targeted fluorescent proteins; 62 for ER-sGFP, 65 for NLS-sGFP, 68 for Px-sGFP, 71 for Mt-sGFP, 85 for NLS-TagRFP, 88 for Px-TagRFP and 91 for Mt-TagRFP. To confirm the intracellular localization of newly developed organelle-targeted TagRFP constructs, co-localization analysis by transient expression were done using Pro35S:NLS-sGFP (established nucleus localization marker), Pro35S:NLS-TagRFP, Pro35S:Px-sGFP (established peroxisome localization marker), Pro35S:Px-TagRFP, Pro35S:Mt-sGFP (established mitochondria localization marker), Pro35S:Mt-TagRFP constructs by particle bombardment into Japanese leek epidermal cells. Confocal microscopic analysis revealed the correct co-localization of newly constructed TagRFPs in plant cells. In order to analyze the performance of newly constructed vectors for highly sensitive detection of gene expression, ProPl-PKβ1:Px-sGFP for pGWB565 and ProMYB21:MYB21-NLS-sGFP and ProMYB21:MYB21-Px-sGFP for pGWB565, and pGWB468, respectively were used. For testing the performance of these vectors and reported the highly sensitive detection of fluorescence signals with nucleus- and peroxisome-targeted sGFP compared with normal sGFP (no organelle targeted type) in promoter:reporter analysis using transgenic *A. thaliana*, ProPl-PKβ1:sGFP and ProMYB21:MYB21-sGFP constructs were used as control. Bright peroxisome-localized GFP fluorescence was observed in ovary, stigma, style, petal, sepal, pedicel, stamen, and maturing seeds of ProPl-PKβ1:Px-sGFP transgenic *A. thaliana* but in ProPl-PKβ1:sGFP transgenic *A. thaliana,* a faint GFP fluorescence was observed only in stigma. In ProMYB21:MYB21-Px-sGFP transgenic *A. thaliana,* strong GFP fluorescence expression was clearly observed in the ovary and the vascular system of petals, sepals, leaf, pedicel, and root. In the Pro-MYB21:MYB21-NLS-sGFP transgenic *A. thaliana,* nucleus-localized GFP signal was observed in leaf, ovary, the vascular system of sepals and petals, pedicel, and root. On the other hand, very little or no GFP fluorescence was observed

in all examined organs of ProMYB21:MYB21-sGFP transgenic *A. thaliana.* Therefore, Confocal microscopic results indicated that promoter activity was monitored typical localization pattern more sensitively with organelle targeted sGFP than normal sGFP by accumulating expressed fluorescent protein in peroxisomes and nucleus. Expression of *A. thaliana* DALL2 and KAT2 increases when induced by wounding and dark respectively. In the leaves of ProDALL2: NLSsGFP and ProKAT2:NLSsGFP transgenic *A. thaliana,* nucleus-localized GFP signals were detected even under non-induced conditions, with clear increasing of the frequency and intensity of nucleus-localized GFP fluorescence after wounding and dark treatment respectively. On the other hand, ProDALL2:sGFP and ProKAT2:sGFP exhibited almost no GFP signals under non-induced conditions and only faint GFP fluorescence was observed after wounding. Thus, wound induction of ProDALL2:sGFP, ProDALL2:NLS-sGFP construct and dark induction of ProKAT2:sGFP, ProKAT2:NLS-sGFP constructs revealed that fluorescence signals of organelle-targeted GFP were enhanced and made possible clear observation of gene expression.

MLD was found in extracellular domain of some plant RLK and presumed to be a sensor of extracellular environment. In Chapter 3 four new proteins of *A. thaliana* containing leucine-rich repeat (LRR) in addition to MLD were identified and were named AtMLLR1 (AT1G25570), AtMLLR2 (AT1G28340), AtMLLR3 (AT3G05990), and AtMLLR4 (AT3G19230). AtMLLR1 and 2 were predicted to be membrane-localizing receptor-like proteins composed of signal peptide (SP), MLD, LRR, and transmembrane domain (TMD). AtMLLR3 and 4 were predicted to be extracellular proteins composed of SP, MLD, and LRR. To understand physiological functions of these proteins, AtMLLR2 and AtMLLR3 were initially examined in detailed using a promoter:GFP+promoter:GUS assay. Dual-promoter:reporter Gateway cloning system (GWB3450-ProAtMLLR2: G3GFP+ProAtMLLR2:GUS and GWB3450-ProAtMLLR3:G3GFP+ProAtMLLR3:GUS) were then constructed and used for transformation of *A. thaliana* for organ-, tissue-, or cell-specific expression analysis of these genes. Regarding GUS expression, seedlings (1, 4, 8, and 14-days after germination (DAG)) and dissected organs (40-DAG) from T3 lines of each construct were observed. In AtMLLR2, GUS staining was observed in the cotyledons, hypocotyl, and root of seedlings, specifically in guard cells, trichomes, pollen, and seeds. In AtMLLR3, GUS staining was observed in the root vascular bundle and root apical meristem of seedling, growing plants vein, vascular bundle of the stem, and trichome of young leaves and at a very specific region in the flower, namely, the junction between the filament and anther. The expression of GFP was observed in the trichome and root apical meristem of AtMLLR2 and AtMLLR3 respectively. The pGWB565 vector described in chapter 2 was also used to confirm specific expression and bright GFP fluorescence in the nuclei of the trichome and guard cells were detected from ProAtMLLR2:NLS-sGFP construct.

In the Chapter 4, the author provides proposed conclusion remarks and discusses the findings presented in this study.

Overall, total 56 of Gateway cloning technology-compatible binary vectors carrying organelle localization strategies described in this thesis, provides an effective approach to robustly enhance the expression, have been considered to meet the demands of fundamental research. For the first step toward understanding physiological function of AtMLLR gene, expression of AtMLLR2 and 3 were analyzed in detail with dual-promoter:reporter Gateway cloning system and organelle targeting fluorescent protein. Moreover, AtMLLR genes provide an invaluable resource to the plant community, allowing for fast generation of a variety of tissue-specific GUS and GFP expression constructs. Expression patterns in organs and cells revealed that expression of AtMLLR3 will be a unique marker gene that specifies the tissue connecting the filament and anther. So, the organs and cells expressing AtMLLRs using Gateway cloning technology in the present study will be good targets to search for and analyze phenotypes in corresponding T-DNA insertion lines. In conclusion, a set of Gateway-compatible vectors provide a reliable cloning method for organ-, tissue-, or cell-specific gene expression by quick formation of expression constructs will be a beneficial tool for many biological researches.