ウェブサイト公開用

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海 外 特 別 研 究 員 最 終 報 告 書

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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：カーネギー研究所（国名：アメリカ合衆国）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
植物の形態形成における EPFL シグナルネットワーク転用仮説の検証

3. 派遣期間：2018 年 4 月 1 日　～　2020 年 3 月 31 日

4. 受入機関名及び部局名
Department of Plant Biology, Carnegie Institution for Science

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

[Background and Research Objectives]
In my previous study, I detected 3 genes regulating awn elongation in rice which named *Regulator of Awn Elongation 1* (*RAE1*), *RAE2*, and *RAE3*. *RAE1* and *RAE2* work independently but always work with *RAE3*. It suggested that *RAE3* is a hub gene for signal transduction of awn elongation.
I would like to know how these 3 genes (or each pair) regulates awn elongation. According to the cellular localization, *RAE1-RAE3* and *RAE2-RAE3* seem that they do not interact physically. It means that there are other genes/proteins exist between *RAE1-RAE3* or *RAE2-RAE3*. To detect the intervene genes in these pathways, the orthologue genes of *RAE2* and *RAE3* in *Arabidopsis thaliana* are used. There are several reasons; (1) *A. thaliana* grows for short life cycle (~2 months), (2) leaf is the serial homologous organ with rice flower (including awn). (3) Further, *RAE2* encodes *Epidermal Patterning Factor Like protein 1* (*EPFL1*) which is involved in secreted peptide family. Tameshige *et al* (2016) reported *EPFL2* which is sister gene of *EPFL1* regulates leaf serration in *Arabidopsis thaliana*. According to this result and since floral organ is originally derived from leaf (Tsukaya, 2002), I hypothesized *RAEs* could regulate leaf formation not only awn elongation. In this study, I tried to reveal the molecular function of *EPFL1* and *RAE3* orthologue for the leaf morphogenesis in *Arabidopsis thaliana* (Fig. 1).

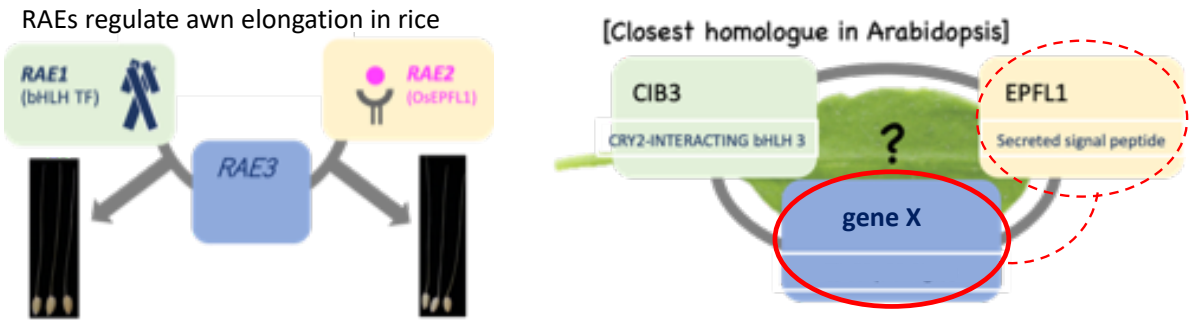

Fig. 1 Research objective in my research; to reveal the molecular function of the orthologues of *RAE2* and *RAE3* in *Arabidopsis thaliana*.

[Results]

1. Analyzing the *EPFL1* function

In research plan, firstly I tried to identify expression pattern (specific organ and time periods) of *EPFL1* and its sister genes *EPFL2* and *EPFL3* because their detail expression pattern has not been reported. If I detected the high expression of *EPFLs* at specific time point and specific tissues, I would perform RNA-seq using the tissues where *EPFLs* highly expressed part to detect the downstream genes. In addition to performing RNA-seq, I planed to execute the phospho-proteomic analysis using EPFL mutants. It is because the receptor of EPFLs called ERACTA which is classified receptor like kinase and transduces the downstream signal by phosphorylation on its substrates. I hypothesized that phosphorylation of downstream genes should change at the tissues where EPFLs highly expressed.

To examine the effect of *EPFL1* and *EPFL2* on *Arabidopsis* growth, the overexpression constructs of both genes were transduced into Col-0 line, however I could not observe obvious phenotypic change at leaf or any other organs. To examine *EPFLs* expression pattern, I made the constructs containing each promoter fused with GUS or GFP, and got T2 plants. Since there are no *epfl1* and *epfl2* mutants in ABRC, I also tried to create CRISPR-Cas9 lines of *EPFL1* and *EPFL2*. However, at that moment when I started observation of the gene expression or CRISPR lines, unfortunately, one paper was published and it reported *EPFL1* function in *A. thaliana* (Kosentka et al 2018). In this paper, the authors mentioned that *EPFL1, EPFL2, EPFL4*, and *EPFL6* function redundantly in the SAM and these genes are expressed at the SAM-leaf boundary and in the peripheral zone. This result suggested that *EPFL1* works redundantly with the other EPFLs and multiple crossed mutant line is needed for its function analysis.

2. Low temperature effect on leaf serration

There are several reports that leaf serration became deeper under the low temperature such as related species of holly (Chitwood and Sinha, 2016). Moreover, awn elongates under low temperature in rice strains without functional *RAE1* and *RAE2*. Taken together, I made the hypothesis which low temperature might promote the gene expression of *RAE1*, *RAE2* or *RAE3* or its downstream genes to promote the leaf serration or awn elongation.

To examine the effect of low temperature, I used Col-0 and Ler as material which are the different ecotypes but used as wild type of *A. thaliana*. Ler carries spontaneous mutation in *ERECTA* and it does not show obvious leaf serration compared with Col-0. That's why it can be considered as a negative control if when Col-0 showed phenotypic change under the low temperature condition. I grow both strains in 10°C and 22°C growth chamber. However, the plant growth was excessively suppressed at 10°C which might not be suitable for checking the temperature effect on leaf morphogenesis. I grow them at 16°C instead of 10°C. The serration depth of Col-0 did not change, while the serration depth increased in Ler leaf at 16°C (data not shown). This suggests that the change of serration under low temperature condition is controlled by the non-ERECTA-mediated pathway. Furthermore, when rice was grown under a low temperature condition of 20°C, elongation was observed, but no increase in expression of *RAE1* or *RAE2* was observed. According to these results, it was concluded that whether the signal pathway of leaf serration alteration in *A. thaliana* under low temperature and awn elongation in rice under low temperature are caused by unknown signal excluded EPFL1 or ERECTA-mediating pathway.

3. Understand the *geneX* function which is *RAE3* orthologue gene. To reveal geneX molecular function which is RAE3 orthologue, I examined *geneX* expression pattern, cellar localization and find the substrates of *geneX*. The member in my current laboratory established the techniques which can identify proximal proteins which are the potential interactor of own target proteins, this technique calls TurboID.

3-1. Expression pattern of *geneX*

I made *geneX*promoter:GUS line to examine *geneX* expression pattern (Fig.2A), and found that geneX expressed in SAM, young rosette leaf, young flower organ but not in older organs. Semi RT-PCR indicates that *geneX* highly expressed in young inflorescence (Fig. 2B). The numbers under PCR bands indicate relative intensity of *geneX* expression to internal control, UBQ expression.

3-2. Cellular localization of *geneX*

I made 35S:geneX-YFP construct to examine its cell localization (Fig. 2C), and found that geneX localizes plasma membrane and cytoplasm (geneX-YFP can be observed as dots in cytoplasm region in epidermal cells of *Arabidopsis* root tip, so it means it is on the way to plasma membrane though.). geneX-YFP in onion epidermal cells also supports this result. SUBA4 (Subcellular localization Database for Arabidopsis) indicates GENEX localizes plasma membrane (Fig. 2D).

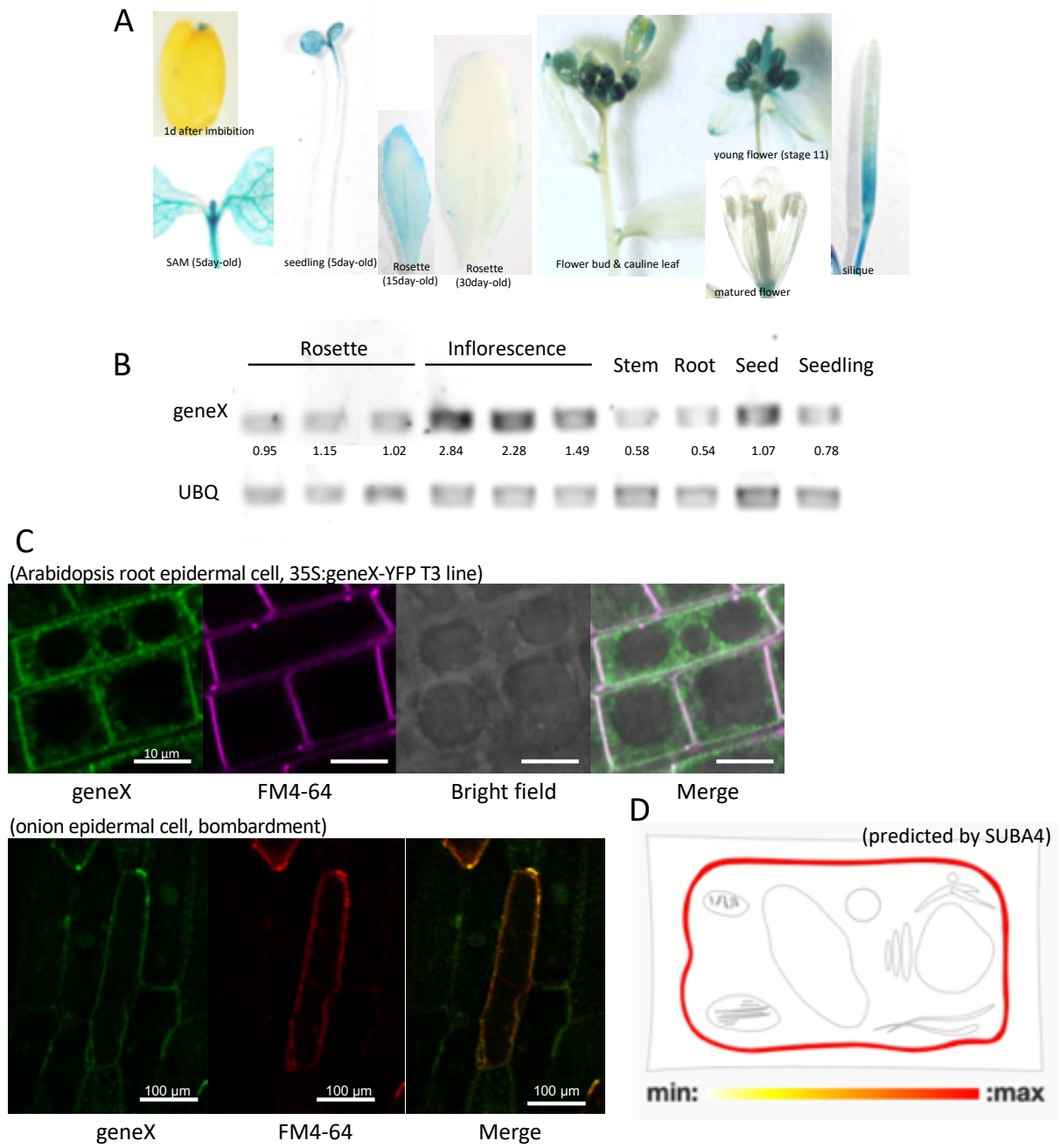

Fig. 2 Expression pattern and cell localization of geneX. (A) GUS staining result in geneXpromoter:GUS line. (B) RT-PCR result using several stage tissues. (C) cell localization of geneX. (D) Prediction of geneX localization by SUBA4.

3-3. <u>GENEX overexpression line's phenotype.</u>

GeneX contains RING-H2 domain which is conserved domain for interacting its interactors. I made 35S:geneX(full length; FL) and 35S:geneX(ΔRING) transgenic lines. The purpose for making geneX(ΔRING) is to see the effect of the deletion of functional domain. 35S:geneX(FL) line showed dwarf, round leaf, short petiole phenotype, on the other hand, 35S:geneX(ΔRING) showed normal phenotype as WT (Fig.3A). This growth inhibition of geneX(FL)ox was appeared to correlate with geneX expression level (Fig.3B).

3-4. <u>Cell shape and size alteration in GENEX overexpression lines</u>

To examine why the morphological alteration can be observed in geneX(FL)ox, I observed epidermal cell by SEM. I used 7th true rosette leaf from 30-day old plant and observed distal part (yellow square) which is matured compared with proximal part (near the petiole) (panel A). SEM observation revealed that the cell shape becomes simpler in geneX(FL)ox than WT and geneX(ΔRING)ox (panel B). The ratio of small cell (~200 μm^2) was over 60% in geneX(FL)ox but it was 40~50% in WT and geneX(ΔRING)ox (panel C). I also observed the epidermal cells in proximal part of silique (yellow square in panel D). Originally, cell shape in silique is simple, so there are not big difference among 3 lines (panel E). The ratio of small cell (~200 μm^2) was over 80% in GENEX(FL)ox but it was ~20% in WT (panel F). Interestingly, silique phenotype of GENEX(ΔRING)ox is similar with WT (page7, panel C), however the ratio of small cell (~200 μm^2) was over 80% in GENEX(ΔRING)ox (panel F).

In *A. thaliana*, cell expansion happened depends on endoreduplication which is skip M phase during cell division (Vanhaeren et al. 2015) even though a causal relation between endoreduplication levels and cell size is still unknown (Tsukaya, 2013b). So I examined expression level of the genes reported as a endoreduplication marker. According to the result (panel G), CycA2;1 and CycB1;1 showed higher expression in geneX(FL)ox compared to others. Further, HisH4 which is cell division marker significantly highly expressed in geneX(FL)ox. These results suggested that endoreduplication was suppressed in geneX(FL)ox, on the contrary, cell division was promoted.

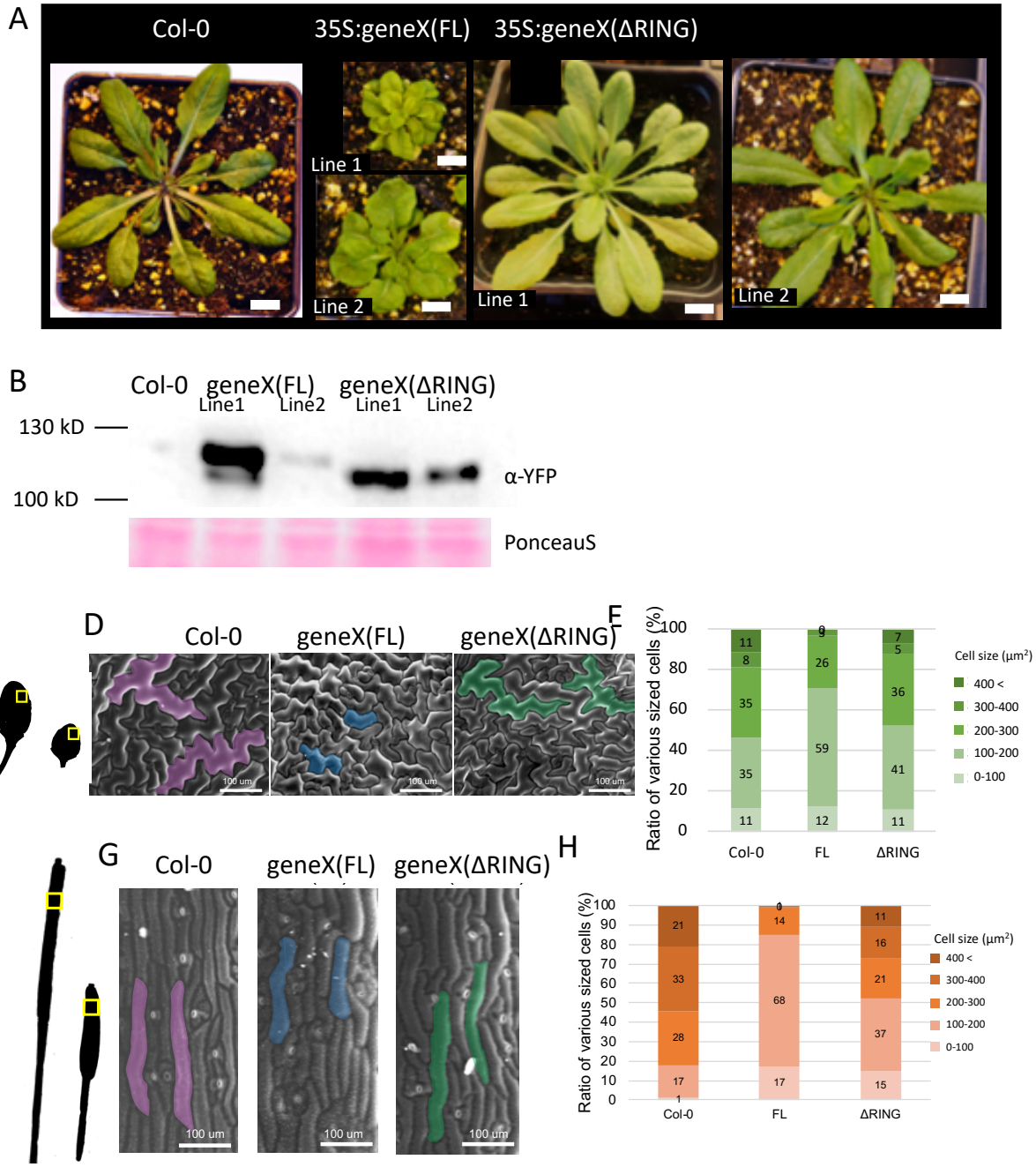

Fig. 3 Phenotype of overexpression lines of geneX(FL) and geneX(ΔRING). (A) Rosetta leaf phenotype of Col-0 (WT), geneX(FL)ox and geneX(ΔRING)ox. (B) Expression level of transduced protein detected by YFP antibody. Internal control is indicated by PonceauS staining. (C) Single rosetta leaf phenotype. Left is WT and right is geneX(FL)ox. (D) Epidermal cells of rosetta leaf observed by SEM. (E) Cell size variation in rosetta leaves. (F) Single silique phenotype. Left is WT and right is geneX(FL)ox. (D) Epidermal cells of silique observed by SEM. (E) Cell size variation in silique.

<u>Cell shape and cell size in petal of geneX overexpression lines.</u>

Cells in petal were reported that no endoreduplication happened. I checked cell size and cell shape in petals in WT, geneX(FL)ox, geneX(RING)ox, there are no significant difference (data not shown).

3-5. Define the candidate of interactor with GENEX using TurboID

To understand the molecular function of geneX, I carried out proximity labeling using TurboID which is byotynilation enzyme *in vivo*. I tried 2 times of this analysis. First time I used T2 generation of 35S:geneX-TbID, second time I used T3 generation. I can detect ~160 genes in first analysis, and ~500 genes in second analysis. As for the expression level of YFP-YFP-TbID bait protein which showed significantly low in first time but as much as geneX(FL) in second time, thus I decide to use the data from second time analysis. GeneX(FL) might be auto-ubiquitinated and degraded. That's why the peptide intensity is lower than that of GeneX(Δ RING). I detected over 500 proteins which are the possible candidates of interactor of geneX. I omit the proteins detected in YFP-YFP-TbID and produce Venn diagram using the proteins detected in geneX(FL)-TbID and geneX(RING)-TbID lines by two biological replicates (Fig.4A). I focused on pink part of this Venn diagram. There are 2 reasons; Overlap part between geneX(FL)-TbID and geneX(Δ RING)-TbID suggests the interactors which would be degraded by 26S proteasome but remaining, or which would make complex with geneX (391 proteins indicated in (i)). The proteins detected only in GENEX(RING) would be completely degraded in geneX(FL) and cannot be detected in it (55 proteins indicated in (ii)). Comparison of the peptide intensity in the group classified in (i) indicated the difference of the mean value between geneX(FL) and geneX(Δ RING) (Fig.4B). It might be caused by protein degradation in geneX(FL)-TbID.

Gene ontology enrichment was carried out using total 446 proteins in pink part. Subcellular localization of the detected peptide in geneX(Δ RING)-TbID was analyzed by SUBA toolbox. The result showed that most of the proteins localized on plasma membrane (19%) and second is cytosol (10%) (Fig.4C) . This is reasonable based on geneX is the plasma membrane localized protein. Functional classification and visualization using ReviGO indicated that the interactor candidates are related to protein transport or localization, protein phosphorylation, and response to biotic stimulus (Fig.4D).

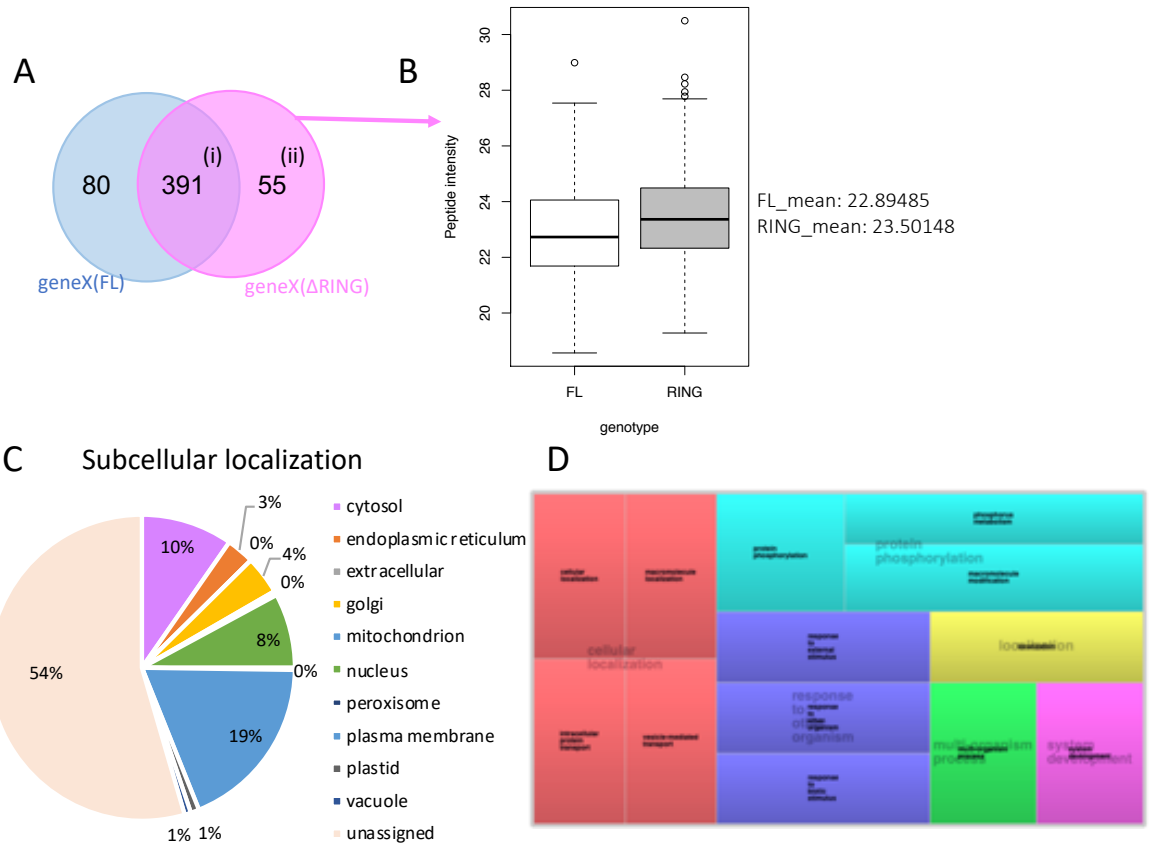

Fig. 4 Detection of interactors of geneX by using TurboID. (A) Venn diagram representing the proteins detected in geneX(FL)-TbID and geneX(ΔRING)-TbID. (B) Average of protein intensity detected in geneX(ΔRING)-TbID line. (C) Subcellular localization of the interactors of geneX detected in both of geneX(FL)-TbID and geneX(ΔRING)-TbID lines. (D) Functional classification of the interactors of geneX detected in both of geneX(FL)-TbID and geneX(ΔRING)-TbID lines.

In the candidate list, there are several MAPK proteins which are known interactor of ERECTA pathway. ERECTA is the receptor of EPF/EPFLs. This result suggested there is a link between *EPFL1* which is *RAE2* orthologue and *geneX* which is *RAE3* can be connected mediating MAPK proteins. To understand these proteins relationship, further research are needed.

[Presentation and Publication]

(Publication)

1. Reuscher S, Furuta T, Bessho-Uehara K, Cosi M, Jena K, Toyoda A, Fujiyama A, Kurata N, Ashikari M. Assembling the genome of the African wild rice Oryza longistaminata by exploiting synteny in closely related Oryza species. Commun Biol. 1:162. doi: 10.1038/s42003-018-0171-y. (2018)
2. Bessho-Uehara K, Nugroho J, Kondo H, Shim R and Ashikari M. Sucrose affects the developmental transition of rhizomes in Oryza longistaminata. J. Plant Res. 131(4): 693–707. (2018) ☆**2019 Best Paper Award**

(Presentation)

1. Third Japan-US Science Forum, **Bessho-Uehara K**, Omori T, Nugroho JE, Kojima M, Sakakibara H, Ashikari M. The mechanism of plant stem growth regulated by small molecules. Poster, Boston, USA, Nov 10, 2018.
2. American Society of Plant Biology, **Bessho-Uehara K**, Hobo T, Omori T, Reucher S, Sakakibara H, Yamaguchi S, Ashikari M. Identification of rhizome-specific hormone accumulation and gene expression by various tissue analysis in *Oryza longistaminata*. Poster, San Jose, USA, Aug 3 – 7, 2019.
3. 第 83 回日本植物学会, **Bessho-Uehara K**, Hsu CC, Wang Z, 口頭, 仙台, 9/15 – 17, 2019.
4. Fourth Japan-US Science Forum, **Bessho-Uehara K**, Hsu CC, Park CH, Wang Z. Alteration of phosphorylation status regulated by Brassinosteroid affects gluconeogenesis. Poster, Boston, USA, Nov 3, 2019.
5. Plant and Animal Genome XXVIII, **Bessho-Uehara K**, Masuda K, Wang DR, McCouch S, Ashikari M. Oral, San Diego, USA, Jan 11-15, 2020.