様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　４ 年　 ６ 月 ２１ 日現在

機関番号：１０１０１
研究種目：基盤研究(C)（一般）
研究期間：2019～2021
課題番号：１９Ｋ０６７０１

研究課題名（和文）How proteoglycans mediate auxin gradient sensing in planar cell polarity?

研究課題名（英文）How do proteoglycans mediate auxin gradient sensing in planar cell polarity?

研究代表者
Ｔｅｈ　Ｏｏｉｋｏｃｋ（Teh, Ooi Kock）

北海道大学・高等教育推進機構・助教

研究者番号：８０７９１２７７

交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：SBの過剰発現は、SBとその機能的パラログ、SBのような、加速された配偶子母細胞の開始をノックアウトしながら細胞増殖を阻害し、細胞壁が緩んでいることを示します。 SBの変異分析は、SB機能にはグリコシル化が必要であるが、SB細胞内局在には影響を与えないことを示しました。 さらに、SBがARFCの発現レベルを正に調節することを示すことにより、SBとC型オーキシン応答因子（AFRC）との間に関連性を確立しました。 さらに、arfc機能喪失変異体はsbを表現型コピーし、SB過剰発現表現型を抑制し、ARFCがSBの下流で作用していることを示しています。

研究成果の学術的意義や社会的意義
Our finding points to a previously unknown role of proteoglycan in sensing the cell wall changes. This knowledge has a potential to be applied in the agricultural sector to manipulate cell wall compositions for efficient biofuel production.

研究成果の概要（英文）：We found that SB plays an inhibitory role in cell wall loosening. SB overexpression inhibits cell expansion while knocking out SB and its functional paralog, SB-like, accelerated gametophore initiation, an indication of loosen cell walls. Mutations analysis on the SB indicated that glycosylations are required for SB functions but has no effect on the SB subcellular localization. We further established a link between SB and a C-type auxin response factor (AFRC) by showing that SB positively regulated the expression level of ARFC. Furthermore, arfc loss-of-function mutants phenocopied sb and repressed the SB overexpressor phenotype, demonstrating that ARFC is acting downstream of SB. We performed RNAseq analyses using an ARFC-inducible line to identify ARFC transcriptional targets. Gene ontology (GO) analyses showed that cell wall-related GO terms were highly enriched. We propose that AGP may act as a cell wall structural protein that senses changes in cell wall compositions.

研究分野：Developmental biology

キーワード：Physcomitrium patens　Proteoglycans　Cell wall

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Our unpublished data shows that a glycoprotein named Sleeping Beauty (SB) in the moss *Physcomitrium patens* may be involved in planar cell polarity.

２．研究の目的

We want to characterize the role of SB in planar cell polarity.

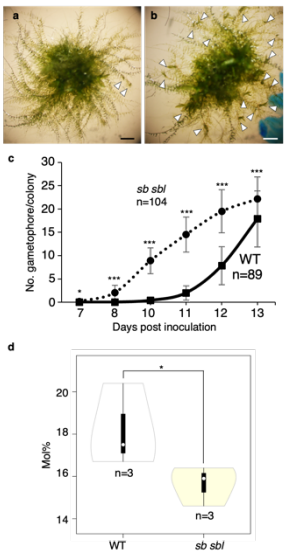
３．研究の方法

We propose to use genetics and cell biology approaches to unravel the function of SB.

４．研究成果

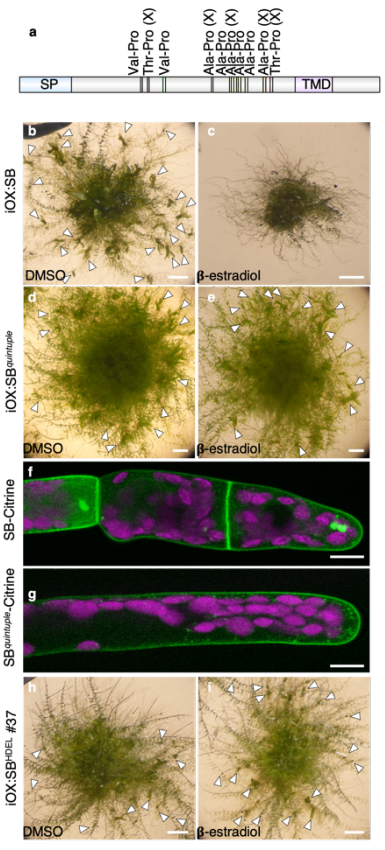
The initial hypothesis of this proposal was that SB as a proteoglycan plays essential roles in planar cell polarity. However, careful examinations on the *sb sbl* knock-out mutant (*sb sbl*) were unable to support this claim as the protonemal branching remains unaffected (not shown). Instead we noticed that the gametophore formation was significantly accelerated in the *sb sbl* (Figure 1a-c), suggesting that SB negatively controls the gametophore formation. We therefore switched our attention to investigate the inhibitory effects of SB on gametophore formation. As SB is a putative arabinogalactan protein (AGP) in the cell wall, we therefore investigated possible changes in the cell wall compositions of *sb sbl*. We found that the galactose in *sb sbl* cell wall was significantly reduced (Figure 1d), raising a scenario in which the pectin biosynthesis/modelling was affected in the *sb sbl* as galactose is a predominant monosaccharides in the side chains of pectin domain Rhamnogalacturonan I.

Figure 1



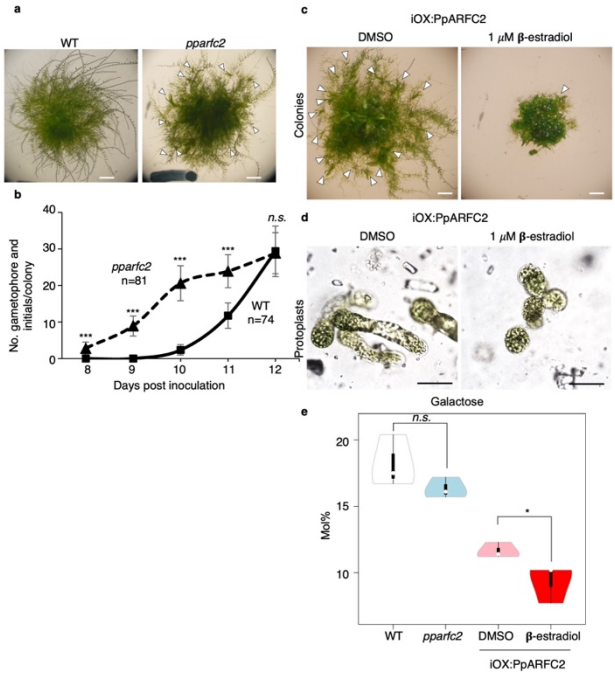
Despite this change of research direction, we also investigated the importance of glycosylation on SB as we have set out in our original proposal. A key feature of the AGP backbone is the presence of repetitive Ala-Pro, Ser-Pro, Thr-Pro, and Val-Pro dipeptide motifs. The Pro residues in these motifs are hydroxylated by P4H before O-link glycosylation by glycosyltransferases and subsequent additions of type II arabinogalactan to the Hyp residues can take place. The SB protein backbone contains 6 Ala-Pro (a.a. 85th, 93rd, 95th, 97th, 100th, 108th), 2 Thr-Pro (57th, 111th) and 2 Val-Pro (54th, 64th) dipeptide motifs (Fig. 2a). In order to investigate the functional role of these putative O-linked glycosylation sites, we replaced the Proline residues with a nonpolar amino acid Alanine to abolish the glycosylation sites (crosses in Fig. 2a) and generated transgenic lines, iOX:SB^{quintuple} that inducibly expressed the SB^{Pro58,86,96,109,112Ala} variant. Using the phenotype in iOX:SB overexpressor as a readout of SB function, we examined the gametophore formation in iOX:SB^{quintuple}. Overexpression of the wild-type SB upon 1 µM β-estradiol treatment completely inhibited the gametophore formation (Fig. 2b,c). However this inhibitory effect on gametophore formation was lost in the iOX:SB^{quintuple} variant overexpressor (Fig. 7d,e), indicating that these glycosylation sites and the O-linked glycosylations thereof are required for SB to exert this inhibitory effect. Interestingly SB^{quintuple} remained correctly targeted as revealed by the plasma membrane-localisation of SB^{quintuple}-Citrine (Fig. 2f,g), suggesting that carbohydrate moieties in the SB are not required for its secretion but instead may play some functional roles on the plasma membrane. Next we attempt to validate the functionality of SB on plasma membrane by fusing an endoplasmic reticulum (ER)-retrieval signal HDEL to the C-terminal of SB. Addition of the HDEL signal is known to effectively retain secreted proteins in the ER. In transgenic line that inducibly overexpressed SB^{HDEL}, iOX: SB^{HDEL}, the inhibitory effect of gametophore formation was diminished (Fig. 2h,i), confirming the necessity of plasma membrane targeting for SB to

Figure 2



exert any functional roles. Taken together, our results imply that glycosylations on SB and its secretion to the plasma membrane are required for SB functionality.

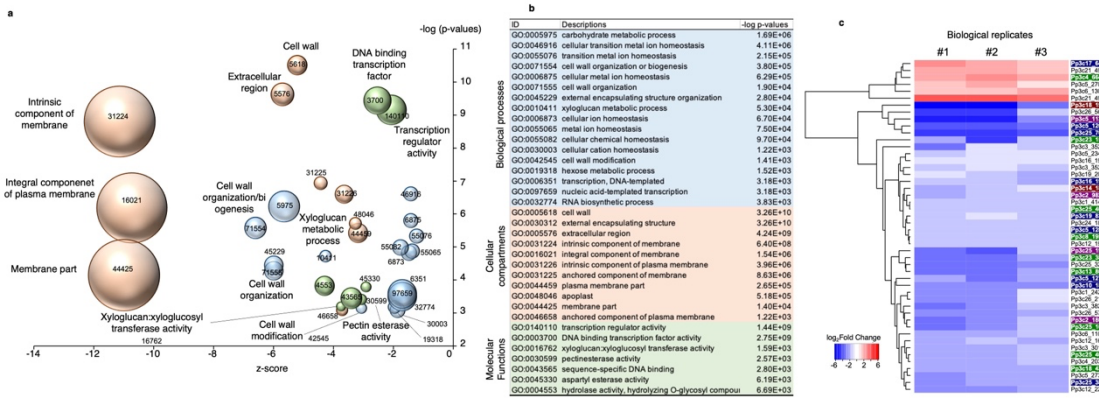
Figure 3



Our preliminary data suggest that SB positively regulates the expression of an auxin-dependent transcription factor Auxin Response Factor C2 (PpARFC2). To establish a genetic link between SB and PpARFC2, we generated a *pparfc2* knock-out mutant using homologous recombination approach. The *pparfc2* mutant phenocopied *sb sbl* and exhibited accelerated gametophore formation by at least 3 days when compared to WT (Fig. 3a,b). Notably, overexpression of PpARFC2 also led to the inhibition of gametophore formation and apical growth during protoplast regeneration in iOX: PpARFC2 (Fig. 3c,d), greatly resembled the overexpressor phenotype of iOX:SB. Failure of gametophore formation in iOX: PpARFC2 may reflect an increase in cell wall stiffness which inhibits gametophore morphogenesis. Indeed, galactose levels in the cell wall compositions was also reduced in the *pparfc2* (16.33 ± 0.776 mol %, Fig. 3e) and iOX:PpARFC2 (DMSO: 11.63 ± 0.586 mol %; EST: 9.36 ± 1.443 mol %). Collectively, phenotypic similarities between knock-out and overexpressor mutants of SB and PpARFC2 provide a genetic basis for their connection in playing a common role in the same genetic pathway to regulate cell wall remodeling and subsequently gametophore morphogenesis.

Since PpARFC2 is an auxin-dependent transcription factor, we further performed RNA sequencing (RNASeq) analyses using iOX:PpARFC2 to unravel the transcriptional targets of PpARFC2. Upon a 16 h β -estradiol induction, a time point before the overexpressor phenotype became apparent, a total of 2,284 differentially expressed genes (DEG) were identified between the DMSO-treated and β -estradiol-induced samples. Gene ontology (GO) analyses of these DEG showed that cell wall modification-related GO terms are highly enriched (FDR adjusted P values ≤0.005) in all three biological replicates (Fig. 4a,b). Gene ontology (GO) analyses of these DEG showed that cell wall modification-related GO terms are highly enriched (FDR adjusted P values ≤0.005) in all three biological replicates (Fig. 4a,b). For example, GO terms of Biological Process in “cell wall organization/biogenesis” (GO:0071554, GO:0071555, GO:0042545, GO:0071669), “pectin metabolic/catabolic processes” (GO:0045490, GO:0045488) and “xyloglucan metabolic process” (GO:0010411) were amongst the most significantly enriched (Fig. 4a,b). In line with the PpARFC2 role being a transcription factor, Molecular Functions of “DNA binding transcription factor activity” (GO:0003700) and “transcription regulator activity” (GO:0140110) were also enriched, in addition to “pectin esterase activity” (GO:0030599) and “xyloglucan:xyloglucasyl transferase activity” (GO:0016762) (Fig. 4a,b). In summary, a large-scale downregulation of cell wall loosening and modification-associated genes such as EXPANSIN, PECTIN

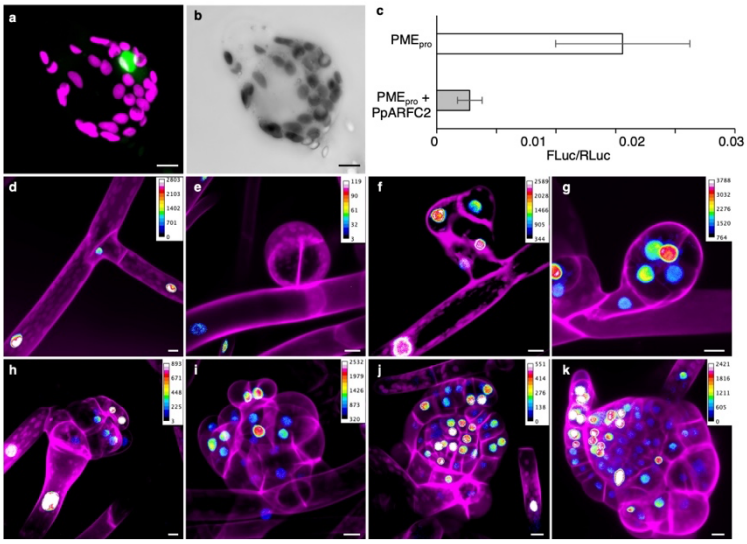
Figure 4



METHYLESTERASES (PME), COBRA and xyloglucan-modifying enzymes was observed (Fig. 4c), suggesting that PpARFC2 functions as a repressor ARF in cell wall remodeling. Particularly of interest is PMEs, which are encoded by at least 42 loci in the P. patens genome. A short induction (16h) of PpARFC2 overexpression led to at least 9 PMEs being repressed in all 3 biological replicates (Fig. 4c), with an additional 3 PMEs were down-regulated in one of the biological replicates (not shown). Mis-regulation of multiple PMEs may account for the iOX:PpARFC2 phenotype in which the gametophore morphogenesis and apical growth was affected, likely to arise from cell wall stiffening.

Molecular, genetic, and biochemical studies have revealed binding motifs on promoter region on which ARFs bind to. These DNA target sequences are termed auxin response cis-elements (AuxREs) and are typified by a hexanucleotide motif 5'-TGTCNN-3', with different ARFs showing variable affinities. Our RNASeq experiment indicates that PpARFC2 may suppress the transcription of cell wall remodeling-related genes by binding to their promoter regions. We explore this possibility by examining the presence of hexanucleotide AuxREs 5'-TGTCGG-3' and 5'-TGTCTC-3' in the 2-kb promoter regions of all putative transcriptional targets. Of the 48 putative transcriptional targets that were mis-regulated in iOX:PpARFC2, one PME (Pp3c5_12660) promoter region contains 5 AuxREs-like motifs, fitting the required presence of multiple AuxREs for ARF effective binding. In order to validate the binding between PpARFC2 and the promoter region of this targeted PME (PMEpro), we generated C- and N-termini yellow fluorescent protein (YFP)-tagged fusion proteins for PpARFC2, driven by an Arabidopsis UBQ10 promoter. While the PpARFC2-YFP yielded no fluorescence, YFP-PpARFC2 was localized to the nucleus when transiently expressed in the *P. patens* protoplasts (Fig. 5a, b), supporting the notion that PpARFC2 is a transcription factor. By co-expressing the YFP-PpARFC2 and a PMEpro: firefly luciferase (FLuc) fusion constructs, we performed promoter transactivation assay in tobacco leaves. FLuc activity was normalized using a co-infiltrated Renilla luciferase (RLuc) construct. FLuc activity was detected when the PMEpro:FLuc was expressed alone, likely to reflect a basal expression that was driven by endogenous ARFs in the tobacco leaves (Fig. 5c). When co-expressed with the YFP-PpARFC2, we observed a significant reduction of the FLuc activity (Fig. 5c), implying that PpARFC2 represses the FLuc expression through competing with endogenous ARFs in tobacco and may directly bind to the PME promoter region. This supports the RNAseq data that demonstrated PME downregulation by the PpARFC2 (Fig. 4c). Hence PpARFC2 is likely to bind the regulatory sequences of PME to suppress its expression during cell wall remodeling to affect gametophore morphogenesis.

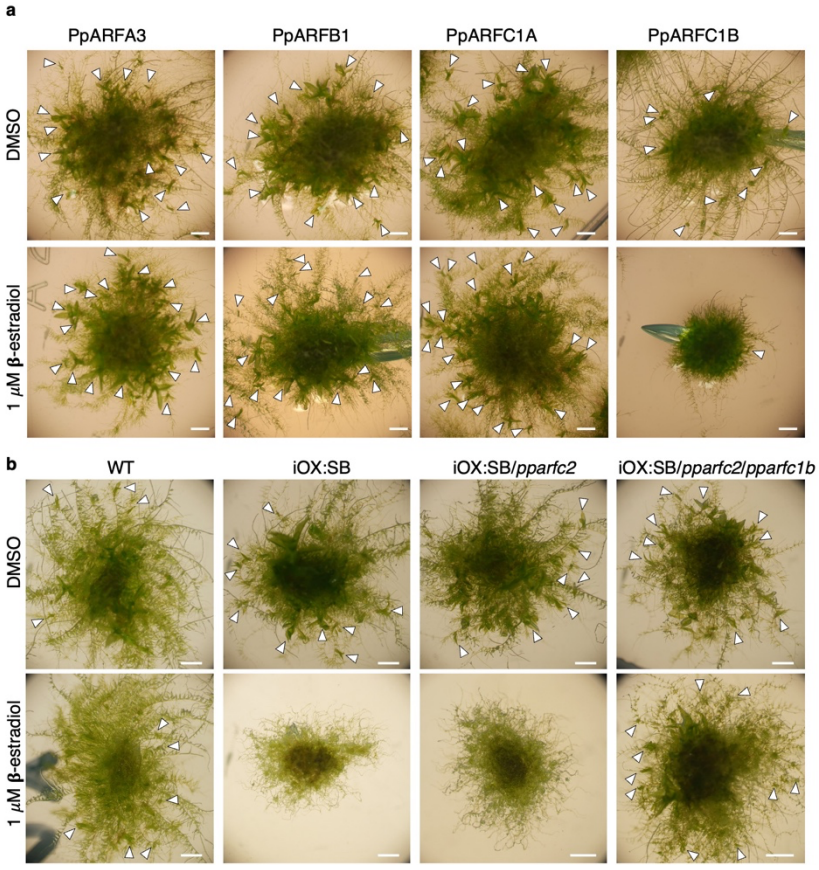
Figure 5



To gain insights into the PpARFC2 expression pattern and how this contributes to gametophore morphogenesis, we generated a PpARFC2 promoter fusion reporter, PpARFC2pro:nls-GFP that encompasses 3980 bp regulatory sequences upstream to the PpARFC2 coding sequence. Overall, the PpARFC2 expressed ubiquitously at protonema stage with insignificant differences (Fig. 5d). At the 3-cell stage of gametophore initials in which the gametophore cell fate can be ascertained, PpARFC2 expression was not detected (Fig. 5e), suggesting that PpARFC2 was not directly involved in the gametophore bud initiation. Thereafter, PpARFC2 exhibited differential spatial expressions in the gametophore bud tissues throughout its development, often showing highest expression levels at the apical stem cells (Fig. 5f-k), reflecting its role in regulating the gametophore morphogenesis.

Phenotypic similarities between *sb sbl* and *pparfc2* suggests that *SB* and *PpARFC2* may share a genetic pathway that regulates cell wall remodelling during gametophore morphogenesis. To test if there is epistasis between *SB* and *PpARFC2*, and if PpARFC2 is functioning downstream of SB, we generated a *pparfc2* disruptant in the iOX:SB background and asked if the iOX:SB overexpressor phenotype is rescued. On the contrary to our expectation, gametophore formation was not restored in iOX:SB/*pparfc2* when SB overexpression was induced (not shown), implying that PpARFC2 may function redundantly with an additional class C ARF, namely PpARFC1A and/or PpARFC1B, downstream of SB. We therefore overexpressed PpARFC1A and PpARFC1B to look for a similar gametophore inhibition phenotype. While overexpression of PpARFC1B inhibited gametophore formation, similar inhibitory effects were not observed in PpARC1A, an A-clade ARF PpARFA3 and a B-clade ARF PpARFB1 overexpressors (Fig. 6a), suggesting a paralogous function between PpARFC1B and PpARFC2. Consistently, when PpARFC1B was disrupted in the iOX:SB/pparfc2 background, gametophore inhibition phenotype was rescued (Fig. 6b). We ruled out the possibility that phenotype reversal observed in iOX:SB/*pparfc2*/*pparfc1b* was a consequence of reduced SB overexpression as SB transcript abundance in the iOX:SB/*pparfc2*/*pparfc1b* was comparable to that in the iOX:SB parental line (Fig. 6c).

Figure 6



A manuscript that reports the data from this study is currently under revision at Development (IF=6.868)

５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　4件／うち国際共著　1件／うちオープンアクセス　0件）

１．著者名 Do Thi Huong、Pongthai Prapaporn、Ariyarathne Menaka、Teh Ooi-Kock、Fujita Tomomichi	４．巻 133
２．論文標題 AP2/ERF transcription factors regulate salt-induced chloroplast division in the moss Physcomitrella patens	５．発行年 2020年
３．雑誌名 Journal of Plant Research	６．最初と最後の頁 537～548
掲載論文のDOI（デジタルオブジェクト識別子） 10.1007/s10265-020-01195-y	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Brillada Carla、Teh Ooi-Kock、Ditengou Franck Anicet、Lee Chil-Woo、Klecker Till、Saeed Bushra、Furlan Giulia、Zietz Marco、Hause Gerd、Eschen-Lippold Lennart、Hoehenwarter Wolfgang、Lee Justin、Ott Thomas、Trujillo Marco	４．巻 17
２．論文標題 Exocyst subunit Exo70B2 is linked to immune signaling and autophagy	５．発行年 2020年
３．雑誌名 The Plant Cell	６．最初と最後の頁 404~419
掲載論文のDOI（デジタルオブジェクト識別子） 10.1093/plcell/koaa022	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Ginanjar Eggie Febrianto、Teh Ooi-kock、Fujita Tomomichi	４．巻 233
２．論文標題 Characterisation of rapid alkalinisation factors in Physcomitrium patens reveals functional conservation in tip growth	５．発行年 2022年
３．雑誌名 New Phytologist	６．最初と最後の頁 2442～2457
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/nph.17942	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Bao Liang、Inoue Natsumi、Ishikawa Masaki、Gotoh Eiji、Teh Ooi-Kock、Higa Takeshi、Morimoto Tomoro、Ginanjar Eggie Febrianto、Harashima Hirofumi、Noda Natsumi、Watahiki Masaaki、Hiwatashi Yuji、Sekine Masami、Hasebe Mitsuyasu、Wada Masamitsu、Fujita Tomomichi	４．巻 8
２．論文標題 A PSTAIRE-type cyclin-dependent kinase controls light responses in land plants	５．発行年 2022年
３．雑誌名 Science Advances	６．最初と最後の頁 1~12
掲載論文のDOI（デジタルオブジェクト識別子） 10.1126/sciadv.abk2116	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計2件（うち招待講演　0件／うち国際学会　1件）

1．発表者名 Teh Ooi Kock, Ren Junling, Tomomichi Fujita
2．発表標題 Arabinogalactan proteis modulate auxin signalling in Physcomitrium patens to control gametophore formation
3．学会等名 Annual meeting of the Japanese Society of Plant Physiologist
4．発表年 2021年

1．発表者名 Ooi-kock Teh, Junling Ren, Mitsuyasu Hasebe, Tomomichi Fujita
2．発表標題 Ancient Arabinogalactans Modulate Auxin Signaling In Physcomitrella patens To Regulate Polarity
3．学会等名 The 61st Annual Meeting of the Japanese Society of Plant Physiologists（国際学会）
4．発表年 2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

―

6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究分担者	藤田　知道 (Fujita Tomomichi) (50322631)	北海道大学・理学研究院・教授 (10101)	

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関