SHORT COMMUNICATION

Visualization of Phosphoinositides via the Development of the Transient Expression System of a Cyan Fluorescent Protein in the Red Alga *Porphyra yezoensis*

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Running title Expression of PH domain-CFP fusions in a red alga

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Abstract

Phosphoinositides (PIs) play important roles in signal transduction pathways and the regulation of cytoskeleton and membrane functions in eukaryotes. Subcellular localization of individual PI derivative is successfully visualized in yeast, animal and green plant cells using PI derivative-specific pleckstrin homology (PH) domains fused with a variety of fluorescent proteins, however expression of fluorescent proteins has not yet been reported in any red algal cells. In the present study, we developed the system to visualize these PIs using human PH domains fused with a humanized cyan fluorescent protein (AmCFP) in the red alga *Porphyra yezoensis*. Plasma membrane-localization of AmCFP fused with the PH domain from phospholipase Cδ1 and Akt1, but not Bruton's tyrosine kinase, was observed in cell wall-free monospores, demonstrating the presence of phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-3,4-bisphosphate in *P. yezoensis* cells. This is the first report of the successful expression of fluorescent protein and the monitoring of PI derivatives in red algal cells. Our system based on transient expression of AmCFP could be applicable for the analysis of subcellular localization of other proteins in *P. yezoensis* and other red algal cells.

Inrtoduction

Phosphoinositides (PIs) are derivatives of phosphatidylinositol (PtdIns) and involved in a wide variety of physiological regulation of cytoskeleton, vesicle trafficking, ion channels and ion pumps (Zonia and Munnik 2006). Our understanding of physiological importance of PIs has been advanced mostly by the progress toward visualizing the subcellular localization of PI-binding protein domains fused to fluorescent proteins. For instances, phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] was visualized by the pleckstrin homology (PH) domain (Helling et al. 2006; van Leeuwen et al. 2007), while the FYVE (Fab1, YOTB, Vac1 and EEA1) domain was employed for monitoring phosphatidylinositol-3-phosphate (Helling et al. 2006; Vermeer et al. 2006). Moreover, according to the differences in PI-specificity, distribution of individual PI derivative has been analyzed using the different PH domains. For instance, the PH domains from human phospholipase Cδ1 (PLCδ1) and Bruton's tyrosine kinase (Btk) are PtdIns(4,5)P_2- and phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3]-specific, respectively, whereas that from v-akt murine thymoma viral oncogene homolog 1 (Akt1) has a specificity to both phosphatidylinositol-3,4-bisphosphate [PtdIns(3,4)P_2] and PtdIns(3,4,5)P_3 (Salim et al. 1996; Frech et al. 1997; van Leeuwen et al. 2007). Despite these progresses, there is no report on the expression of the PH domains from Akt and Btk in plant cells to date.

Our goal is to elucidate the physiological role of the PI signaling pathway in plant cells using the marine red alga *Porphyra yezoensis*, a model of marine plants (Saga and Kitade, 2002). However, the presence and functions of PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are poorly understood in *P. yezoensis* cells. Therefore,

we here analyzed these PI derivatives using the PH domains from human proteins. Because there is no system available for the expression of fluorescent proteins in red algae, the present study also addressed the establishment of an expression system of a fluorescent protein in the cells of *P. yezoensis*.

Materials and Methods

Molecular cloning of the 5' upstream region of the *actin 1* gene from *P. yezoensis*

Isolation of the 5' upstream region of the *actin 1* (*PyAct1*) gene from *P. yezoensis* was performed based on an inverse polymerase chain reaction (IPCR) as described previously (Fukuda et al. 2008), except for using *Sac*I, *Sal*I and *Xho*I for digestion of genomic DNA and primers PyAct1-IPCR-F1 and PyAct1-IPCR-R1 (Table 1). Since a 3.1 kb IPCR products from *Sac*I-digested DNA does not contain a translation start codon, this fragment was used for PCR with primers PyAct1-PCR-F1 and PyAct1-PCR-R1 (Table 1), which carried out with an initial incubation at 94^{o}C for 1 min followed by 30 cycles of 98^{o}C for 10 s and 68^{o}C for 4 min, and final incubation at 72^{o}C for 15 min with LA *Taq*. The nucleotide sequence of the amplified PCR fragment has been submitted to the GenBank under accession no. AB455256.

Construction of plasmids for expression of the PH domain-CFP fusion proteins

We used p35S-PyGUS (Fukuda et al. 2008) for the basic plasmid using construction of plasmids for fluorescent protein expression in *P. yezoensis* cells. At first, the 5' upstream

region of the *PyAct1* gene (3.0 kb) was amplified again using primers HindIII-PyAct1-F1 and BamHI-PyAct1-R1 (Table 1) with the following program: 98°C for 10 s followed by 30 cycles of 94°C for 10 s and 60°C for 30 s with Prime STAR HS DNA polymerase (TaKaRa). To replace the CaMV 35S promoter with the 5' upstream region of the *PyAct1* gene, the amplified DNA fragment digested with *Hin*dIII and *Bam*HI was inserted into *Hin*dIII-*Bam*HI-digested p35S-PyGUS. Next, a complete ORF for a humanized cyan fluorescent protein from a sea anemone *Anemonia majano* (AmCFP) in pAmCyan1-C1 (Clontech) was amplified using primers, BamHI-CFP-F1 and SacI-CFP-R1 (Table 1), with the following program: 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and final incubation at 72°C for 7 min with LA *Taq* polymerase (TaKaRa). The AmCFP coding sequence was then replaced with PyGUS after digestion of pPyAct1-PyGUS and the PCR fragment with *Bam*HI and *Sac*I, which resulted in the production of pPyAct1-AmCFP. Although this construct contains a PyAct1 coding sequence corresponding to the first 3 amino acids, in-frame fusion occurred between the small PyAct1 and AmCFP coding sequences.

We employed the Gateway Technology (Invitrogen) to construct AmCFP fused with the PH domains as follows. To make entry plasmids, PH domains of PLCδ1 and Btk were amplified with sets of primers, PLCδ1PH-U and PLCδ1PH-L and BtkPH-U and BtkPH-L (Table 1) from plasmids containing PH domain-GFP fusion genes (Várnai and Balla 1998) and that of Akt1 from a TrueClone SC116883 containing a full-length cDNA encoding Akt1 from OriGene Technologies, Inc. (USA) using primers Akt1PH-U and Akt1PH-L (Table 1). The conditions of the PCR reactions were 30 cycles of 98°C for 10 s, 60°C for 5 s and 72°C for 1 min with pyrobest (TaKaRa). Each amplified DNA was directly subcloned into pENTR/D-TOPO (Invitrogen). To produce

the destination vector, pPyACT1-AmCFP was digested with *Bam*HI located at the junction of the PyAct1 and AmCFP coding sequences, filled with Klenow enzyme and ligated with a Rf cassette which was amplified using primers Rfb-F1 and Rfc'-R1 (Table 1). The resultant plasmid was designated pActC-AmCFP-DES. Then, the LR recombination reactions were performed with entry plasmids and pActC-AmCFP-DES according to the manufacture's instructions of the above system, producing plasmids designated pPyAct1C-PLCδ1PH-AmCFP, pPyAct1C-Akt1PH-AmCFP and pPyAct1C-BtkPH-AmCFP.

Transient gene expression and observation of CFP fluorescence

Materials for transient transformation was leafy gametophytes in which monosporangia had been developed, which allowed us to obtain monospores transformed with desired expression plasmids. Protocols of transient transformation of *P. yezoensis* were essentially the same as described previously (Fukuda et al. 2008), except for using a constant condition as 700 mmHg of vacuum pressure, 0.4 MPa of helium pressure, 6 cm of target distance, and 20 μg of plasmids. After bombardment, gametophytes were replaced in medium plates and incubated for 48 h prior to observation of a fluorescent signal from AmCFP. Observations of the AmCFP fusion proteins were performed by using a fluorescence microscope (DM5000B; Leica, Germany) equipped with fluorescence filter CFP (Leica) for CFP detection. Images were captured with Leica DFC 300FX camera running on Leica Application Suite.

Results and Discussion

The critical importance of optimization of the codon usage for highly expression of the foreign gene in *P. yezoensis* cells was demonstrated by our previous experiments (Fukuda et al. 2008). We extended this observation to develop a system for the efficient expression of a humanized cyan fluorescent protein AmCFP whose codon usage is fit to that in genes from *P. yezoensis*. When a plasmid for the expression of AmCFP was constructed with a promoter of the *P. yezoensis Actin 1* (*PyAct1*) gene and the terminator of the nopaline synthase gene (*nos*) derive from *Agrobacterium tumefaciens*, AmCFP signal was observed in cells after transient transformation of gametophytic cells by particle bombardment (Fig. 1a and b). Thus, humanized AmCFP is successfully expressed in gametophytic cells under the direction of the *PyAct1* promoter.

Since AmCFP was able to express in the cells of *P. yezoensis*, we then examined the subcellular localization of PH domain-AmCFP fusions. In these experiments, we employed the PH domains from PLCδ1, Akt1 and Btk, because these domains can bind cognate PtdIns *in vivo* as a fusion protein with certain fluorescent ptotein (Salim et al. 1996; Frech et al. 1997; van Leeuwen et al. 2007). When expression plasmids had been introduced into gametophytic cells of *P. yezoensis* by particle bombardment, PLCδ1PH-AmCFP and Akt1PH-AmCFP localized at edges of cells with a number of bright lines (Fig. 1c-f), although no fluorescent signal from the BtkPH-AmCFP was observed (data not shown). The observation by scanning electron microscope showed that the surface of gametophytic cells is not smooth with upheavals and sinkings and covered with numerous pleats of mucilagious cell wall (Uppalapati and Fujita 2000; Fan et al. 2008), resulting in overlaps of plasma membranes at various

positions on cell surface. The bright fluorescent lines were probably caused by overlapping plasma membranes. Therefore, our system using wide-field epifluorescent microscope seems to have difficulties to define the subcellular localization of PLCδ1PH-AmCFP and Akt1PH-AmCFP in the vegetative cells of the developed gametophytic thalli.

We next examine the subcellular localization of AmCFP, PLCδ1PH-AmCFP and Akt1PH-AmCFP in monospores, spherical single-cell without cell wall released from the gametophytic thalli (Miura 1985). As shown in Fig. 2a-f, AmCFP fluorescent signal was observed in the monospores and the germinating thalli of two-cell stage, indicating the successful expression of AmCFP during the early development of monospores. Moreover, the fluorescent signals from PLCδ1PH-AmCFP and Akt1PH-AmCFP were limited at the cell surface of monospores (Fig. 2g-j), suggesting that these markers localized at plasma membrane. Large vacuoles in plant cells prevent from defining the locality of fluorescent markers at plasma membrane, so an additional biochemical confirmation is generally required to rule out the possibility of the presence at the cytoplasmic or vacuolar membrane. However, the vacuole is not well developed in monospores and fluorescent images of PH domain-AmCFP fusions were clearly different from those of AmCFP without PH domains (Fig. 2). Therefore, we concluded that the makers with the PH domains from human PLCδ1 and Akt1 are localized at plasma membrane of the monospores.

Above results let us to propose plasma membrane localization of PH domains from PLCδ1 and Akt1 in gametophytic cells. To test this possibility, gametophytic thalli were treated with the medium containing 300 mM NaCl for 30 min that generally induces plasmolysis, a separation of plasma membrane from cell wall by cell shrinkage.

When gametophytic cells were plasmolysed (compare Fig. 3a and c), fluorescence from PLCδ1PH-AmCFP fusion was still observed at the surface of shrank cells without changes in the intensity (compare Fig. 3b and d). The same results were obtained for Akt1PH-AmCFP (data not shown). These results strongly suggested the plasma membrane localization of PH domain-AmCFP fusions in gametophytic cells as in monospores.

In the present study, we developed the system for the expression of fluorescent protein in red algal cells and demonstrated plasma membrane localization of the PH domains from PLCδ1 and Akt1 in *P. yezoensis* cells (Fig. 1, 2 and 3). This is the first for the efficient expression of fluorescent protein in red algal cells and the successful expression of the Akt1PH domain in plant cells. Our results indicate the presence of PtdIns(3,4)P_2 and PtdIns(4,5)P_2 in *P. yezoensis* cells. However, since the expression of BtkPH-AmCFP was not observed in both gametophytic cells and monospores (data not shown), the presence of PtdIns(3,4,5)P_3 in *P. yezoensis* cells is still unclear.

The newly established system in this study could be highly useful for analysis of subcellular localization of proteins desired in *P. yezoensis* cells. In our observations, however, most of monospores showing AmCFP fluorescent signal kept their round shape but were able to remain viable, in which 25% of AmCFP expressing monospores developed into two cell stage (data not shown), suggesting that overexpression of AmCFP seems to effect negatively on development of monospores. Moreover, although we observed plasma membrane localization of PLCδ1PH-AmCFP and Akt1PH-AmCFP in monospores (Fig. 2), development of these cells was inhibited completely (data not shown). Since AnCFP itself has a negative effect on monospore development as mentioned above, further experiments are needed to conclude the inhibitory effects of

overexpressed PH domains on developmental regulation of monospores. Taken together, to provide further application of our system on monitoring of subcellular localization of AmCFP fusion proteins during the development of monospores, it is necessary to reduce the inhibitory effects of AmCFP and find other fluorescent proteins lacking negative effects on development of monospores.

The most obvious feature of the plant PI signaling pathway is the lack of PtdIns(3,4,5)P_3 under normal growth conditions as in yeasts (Zonia and Munnik 2006; Michell 2008), which is thought to be due to the lack of the type I PI 3-kinase (PI3K) in these organisms (Michell 2008). In consistent, fluorescent signal was not observed from PtdIns(3,4,5)P_3-specific BtkPH-AmCFP under the normal growth conditions in our experiments (data not shown). However, an ability of the production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 has recently been demonstrated in a type III PI3K (Vsp43)-dependent manner in an yeast *Schizosaccharomyces pombe* (Mitra et al. 2004). As we have already found *P. yezoensis* ESTs encoding the type III PI3K (data not shown), further experiments are necessary to resolve if *P. yezoensis* also synthesizes PtdIns(3,4,5)P_3 under appropriate growth conditions via the type III PI3K-dependent system.

At present, an important question still remains as to what are roles of PtdIns(3,4)P_2 and PtdIns(4,5)P_2 in *P. yezoensis* cells. Moreover, physiological, biochemical and molecular biological studies are highly scarce for the PI signaling system in *P. yezoensis* and other red algae. Therefore, further modification of our system for the expression of the PH domains fused to AmCFP could extend our understanding of the functional importance of the PI signaling system on development and environmental stress responses in red algal cells.

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Table 1 List of primers used for PCR reactions in this study

Primers	Sequence
PyACT1-IPCR-F1	5’-ATCTACGACCGCACTGTGGG-3’
PyACT1-IPCR-R1	5’-GAAGCAATCGGTGCACGTG-3’
PyACT1-PCR-F1	5'-CCACGCTCAGAGGGTTGAAG-3'
PyACT1-PCR-R1	5'-CCATGATCGACTTCGCCTTTG-3'
HindIII-PyACT1-F1	5'-CCCAAGCTTCCACGCTCAGAGGGTTGAAG-3'
BamHI-PyACT1-R1	5'-CGCGGATCCGGGCTTGCTCATGGTGGC-3'
BamHI-CFP-F1	5'-CGCGGATCCATGGCCCTGTCCAACAAGTTC-3'
SacI-CFP-R1	5'-CGAGCTCTTAAGCTCGAGATCTGAGTCCG-3'
Rfb-F1	5'-ATCAACAAGTTTGTACAAAAAA-3'
Rfc'-R1	5'-TTCGAACCACTTTGTACAAGAAA-3'
PLCδ1PH-U	5'-CACCATGGACTCGCGGGCCGGGAC-3'
PLCδ1PH-L	5'-CTTCAGGAAGTTCTGCAGC-3'
Akt1PH-U	5'-CACCATGAAGCGACGTGGCTATTG-3'
Akt1PH-L	5'-GGACACCTCCATCTCTTC-3'
BtkPH-U	5'-CACCATGGCCGCAGTGATTCTG-3'
BtkPH-L	5'-GCTTCCATTCCTGTTCTCC-3'

*Hin*dIII, *Bam*HI and *Sac*I sites are underlined.

Legends to figures

Fig. 1 Expression of AmCFP, PLCδ1PH-AmCFP and Akt1PH-AmCFP in gametophytic cells of *P. yezoensis*. Expression plasmids such as pPyAct1-AmCFP (a and b), pPyAct1C-PLCδ1PH-AmCFP (c and d), pPyAct1C-Akt1PH-AmCFP (e and f) and pPyAct1C-Btk1PH-AmCFP (data not shown) were introduced into gametophytic cells of *P. yezoensis* by particle bombardment. Gametophytic thalli were examined by fluorescent microscopy after 48 h of transient transformation. Fluorescent images (b, d and f) are shown together with the bright field images (a, c and e). Scale bar corresponds to 5 μm.

Fig. 2 Expression of AmCFP, PLCδ1PH-AmCFP and Akt1PH-AmCFP in monospores of *P. yezoensis*. By particle bombardment, plasmids such as pPyAct1-AmCFP (a to f), pPyAct1C-PLCδ1PH-AmCFP (g and h), pPyAct1C-Akt1PH-AmCFP (i and j) and pPyAct1C-Btk1PH-AmCFP (data not shown) were introduced into apical parts of gametophytic thalli in which monosporangia had been produced. Monospores released from blades were examined by fluorescent microscopy during their development after transient transformation. Fluorescent images (b, d, f, h and j) are shown together with the bright field images (a, c, e , g and i). Scale bar corresponds to 5 μm.

Fig. 3 Expression of PLCδ1PH-AmCFP in plasmolysed gametophytic cells of *P. yezoensis*. The pPyAct1C-PLCδ1PH-AmCFP was introduced into gametophytic cells by particle bombardment. Then, gametophytes were examined by microscopy after 48 h

(a and b) or after 48 h plus further treatment for plasmolysis with the medium containing 300 mM NaCl for 30 min (c and d). Fluorescent images (b and d) are shown together with the bright field images (a and c). Scale bar corresponds to 5 μm.

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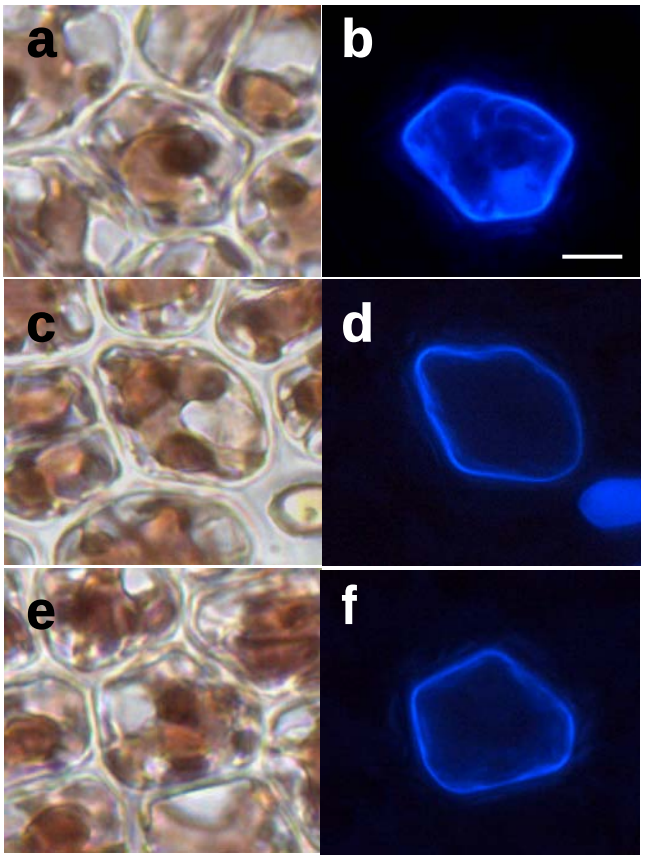

Fig. 1

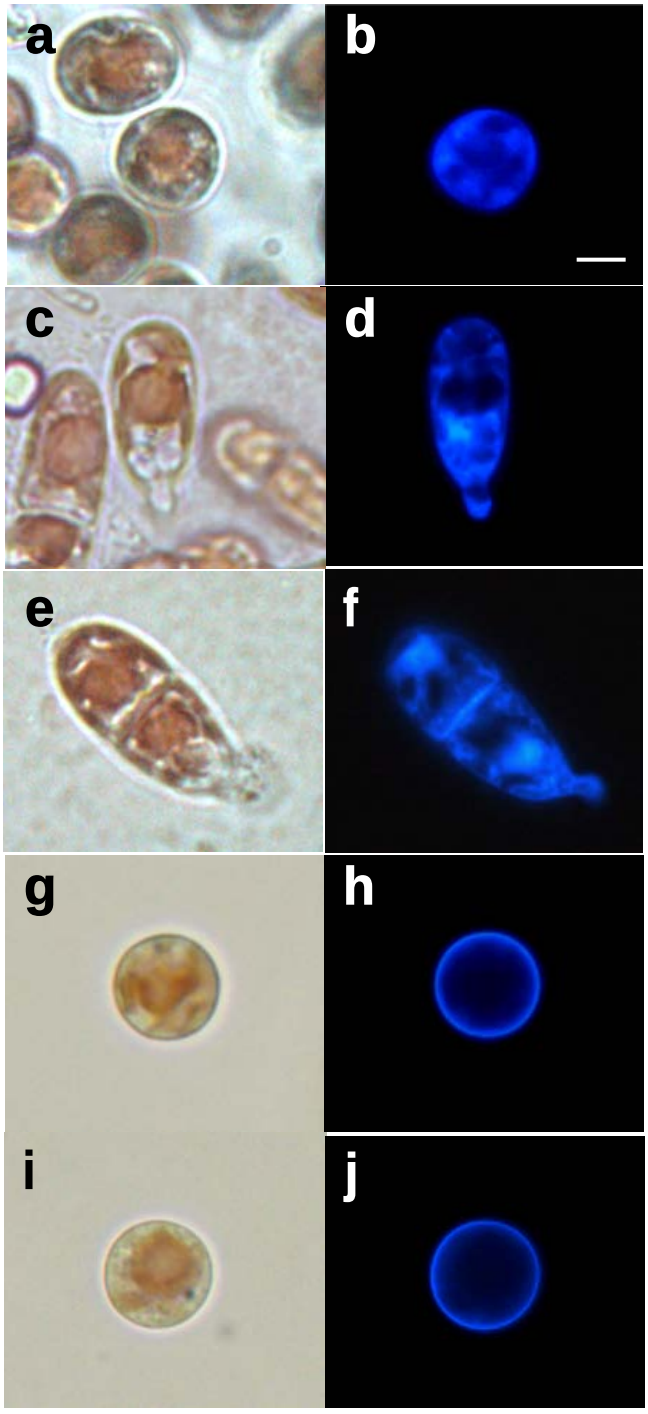

Fig. 2

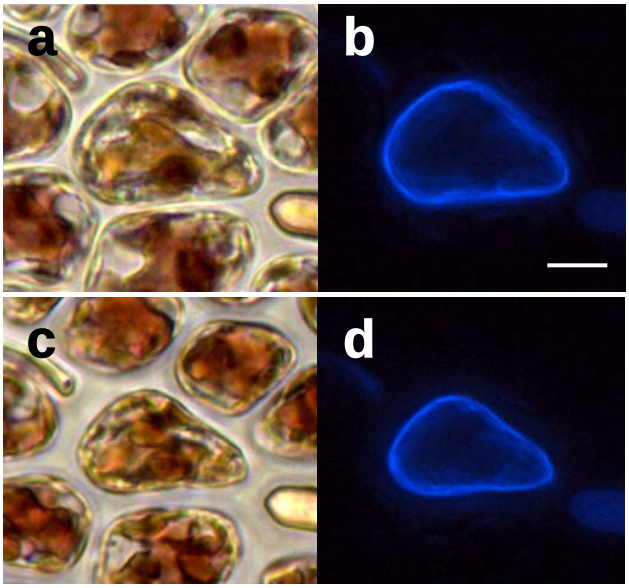

Fig. 3