

***In vitro* Phosphorylation Assay of Putative Blue-light Receptor Phototropins Using Microsomal and Plasma-membrane Fractions Prepared from *Vallisneria* Leaves**

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[Abstract] An aquatic angiosperm *Vallisneria* (Alismatales: Hydrocharitaceae) has been used as an excellent experimental material over a century to study the light regulation of dynamic intracellular movements including chloroplast redistribution and cytoplasmic streaming (Senn, 1908; Seitz, 1987; Takagi, 1997). However, understanding of the molecular mechanisms lagged behind because of difficulty in applying modern techniques such as gene transformation to this plant. Especially, which kind of photoreceptors function in these intriguing responses has long been an unsolved topic. Recently, genes encoding plant-specific blue-light receptor phototropins were isolated in *Vallisneria*, for the first time from aquatic plants (Sakai *et al.*, 2015). Phototropins were identified first as the photoreceptor for hypocotyl phototropism in *Arabidopsis thaliana*, and now known to regulate many responses including chloroplast photorelocation movements in various plant species (Christie, 2007). Phototropins are localized mainly on the plasma membrane and their auto-phosphorylation induced by blue light is the critical step of signal transduction pathway (Sakamoto and Briggs, 2002; Kong *et al.*, 2006; Kong *et al.*, 2013; Inoue *et al.*, 2010). Here we describe a protocol for *in vitro* protein phosphorylation assay using crude-microsomal and plasma-membrane-enriched fractions of *Vallisneria*, which enabled us to verify the presence of phototropins and characterize their auto-phosphorylation responses. After these analyses, Sakai *et al.* (2015) proposed that *Vallisneria* phototropins mediate the high-intensity-blue-light-induced chloroplast avoidance response.

Materials and Reagents

1. Commonly-used medical cotton gauze
2. 0.2 ml tube
3. *Vallisneria* plants

Note: Young plants of Vallisneria of 20-30 cm long were purchased at a tropical-fish store and cultured to be grown over 100 cm in buckets (20 L) filled with tap water and with a layer of soil at the bottom (Figure 1A). The plants were grown under 12 h light/12 h dark regime at 20-24 °C.

4. Dextran (from *Leuconostoc* ssp., Mr ~500,000) (Fluka, catalog number: 31392)
Note: Currently, it is "Sigma-Aldrich, catalog number: 31392".
5. Polyethylene glycol (Sigma-Aldrich, catalog number: P-3640)
6. SDS-PAGE
7. Deionized water
8. 1% Triton X-100 (Sigma-Aldrich, catalog number: T-9284)
9. 50 mM MOPS-KOH (pH7.6) (DOJINDO, catalog number: 343-01805)
10. 300 mM sucrose (Wako Pure Chemical Industries, Siyaku, catalog number: 196-00015)
11. 10 mM ethylene glycol bis (2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (DOJINDO, catalog number: 342-01314)
12. 5 mM ethylenediaminetetraacetic acid (EDTA) (DOJINDO, catalog number: 345-01865)
13. 10 µg/ml dibutylhydroxytoluene (Tokyo Chemical Industry, catalog number: D0228)
14. 1% casein (Nacalai tesque, catalog number: 073-19)
15. 5 mM K₂S₂O₅ (Wako Pure Chemical Industries, Siyaku, catalog number: 161-03345)
16. 1 mM dithiothreitol (DTT) (Wako Pure Chemical Industries, catalog number: 048-29224)
17. 2.5 mg/ml pepstatin A (Sigma-Aldrich, catalog number: P-4265)
18. 2.5 mg/ml aprotinin (Sigma-Aldrich, catalog number: A-4529)
19. 20 mg/ml EDTA-washed Polyvinylpolypyrrolidone (Sigma-Aldrich, catalog number: P-6755)
20. MOPS-KOH (DOJINDO, catalog number: 345-02225)
21. 25 mM MgCl₂ (Wako Pure Chemical Industries, Siyaku, catalog number: 135-00165)
22. 250 mM KCl (Wako Pure Chemical Industries, Siyaku, catalog number: 163-03545)
23. 40% glycerol (Wako Pure Chemical Industries, Siyaku, catalog number: 075-00616)
24. 20% 2-mercaptoethanol (Sigma-Aldrich, catalog number: A-6365)
25. A small amount of bromophenol blue (Wako Pure Chemical Industries, Siyaku, catalog number: 021-02911)
26. 0.5 M Tris-HCl (pH 6.8) (Sigma-Aldrich, catalog number: 252859)
27. Homogenizing medium (see Recipes)
28. Buffer A (see Recipes)
29. Buffer B (see Recipes)
30. 5x phosphorylation buffer (see Recipes)
31. ATP mixture (see Recipes)
32. 4x SDS buffer (see Recipes)

Equipment

1. Blade (Lion Office Products Corp., model: [L-300](#))

2. Polytron homogenizer (Kinematica AG, model: [PT-35/ 2ST"OD"](#))
3. Centrifuge
4. Ultracentrifuge
5. Teflon homogenizer (10 ml) (Ikemoto Scientific Technology, model: [812-771-04](#))
6. Lighting system (Sugiura Lab, model: [FI-150T](#))
7. Cut-off filter (KenkoTokina Corporation, model: Y-44)
8. Interference filter (KenkoTokina Corporation, model: BP-45)
9. Autoradiography (Fujifilm Corporation)

Procedure

A. Plant material and pretreatment of specimens

Young plants of *Vallisneria* of 20-30 cm long were purchased at a tropical-fish store and cultured to be grown over 100 cm in buckets (20 L) filled with tap water and with a layer of soil at the bottom (Figure 1A). The plants were grown under 12 h light/12 h dark regime at 20-24 °C. The light source was a bank of 20 W fluorescent lamps (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

B. Preparation of crude microsomal (CM) fraction and plasma membrane-enriched (PM) fraction

1. At the end of the light period, 40 g of the healthy fresh leaves were harvested into tap water and kept in complete darkness for 12-16 h.

Note: Before use, snip off unhealthy part from the leaves.

2. The leaves were chopped on ice with a blade (Figure 1B). These small leaf pieces were homogenized with a Polytron homogenizer (Figure 1C) in 120 ml of a homogenizing medium.
3. The homogenate was filtrated through six sheets of gauze (Figure 1D) and centrifuged (8,000 x g, 15 min, 4 °C).
4. The supernatant (Figure 1E) was ultracentrifuged at 156,000 x g for 1 h at 4 °C.
5. The pellet was suspended with a Teflon homogenizer in Buffer B (Figure 1F), followed by another ultracentrifugation at 156,000 x g for 1 h at 4 °C.
6. The pellet (Figure 1G) was washed with buffer A and then re-suspended into a small volume (usually 6 ml) of the fresh buffer A, designated as a CM fraction. When the CM fraction was directly used for phosphorylation assay, it was washed and suspended with buffer B, not buffer A.
7. A highly purified PM fraction was isolated from the CM fraction by applying aqueous polymer two-phase partitioning (Figure 1H-I) using Dextran from *Leuconostoc* ssp. Mr ~500,000 and polyethylene glycol (Yoshida *et al.*, 1983; Takagi *et al.*, 1988; Harada *et al.*, 2002).
8. After the final ultracentrifugation of the polyethylene-glycol-enriched upper phase, the pellet was washed with buffer B and then re-suspended into a small volume of the

fresh buffer, designated as a PM fraction.

Note: All the procedures were carried out on ice at 0-4 °C under dim red light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$). The total protein contents in the CM and PM fractions were determined by Bradford assay. The CM and PM fractions were kept in darkness on ice until use.

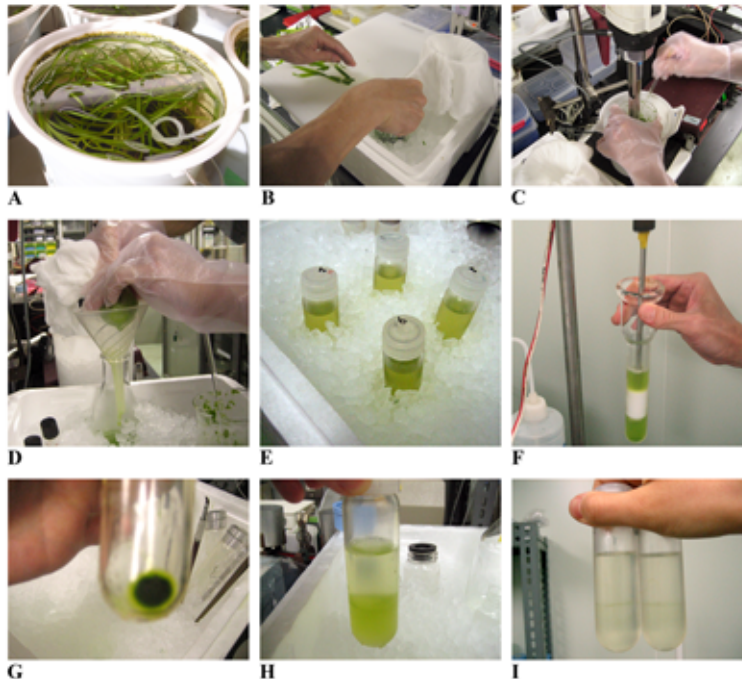


Figure 1. Preparation steps of CM and PM fractions. A. *Vallisneria* plants cultured in buckets. B. Leaves were chopped on ice. C. Leaves were further homogenized with a Polytron homogenizer. D. The homogenate was filtrated through six sheets of gauze. E. The filtrated homogenate before low-speed centrifugation ($8,000 \times g$, 15 min) to remove debris such as cell wall. F. The pellet after the first ultracentrifugation ($156,000 \times g$, 1 h) was suspended with a Teflon homogenizer. G. The pellet after the second ultracentrifugation ($156,000 \times g$, 1 h). H and I. The plasma-membrane-enriched upper phase was separated from the lower phase after the first (H) and second (I) two-phase partitioning.

C. *In vitro* phosphorylation assay

1. The CM and PM fraction were suspended in the reaction mixture as described below.
Reaction mixture (17.5 μl for each reaction)

CM and PM fraction containing 20 μg protein	x μl
5x phosphorylation buffer	4 μl
1% Triton X-100	1 μl
Deionized water	17.5-x μl
2. The reaction mixture put into a 0.2 ml tube was irradiated with blue light (450 nm) for a defined period of time (usually 0-300 sec) using a lighting system equipped with a fiber scope, a cut off filter, and an interference filter (Figure 2A).

3. Immediately after the end of the irradiation, the reaction mixture was mixed with ATP mixture (2.5 μ l) containing radioactive ATP and incubated for 2 min to allow phosphorylation reaction. The reaction was terminated by the addition of 4x SDS buffer (7 μ l).

Note: Steps C1-3 were carried out on ice under dim red light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) in the dark room to avoid any non-specific excitation of photoreceptors by background light.

4. The sample was boiled for 2 min and subjected to SDS-PAGE in a 10.0% (w/v) gel. Separated polypeptides on the gel were stained once with Coomassie Brilliant Blue to visualize the protein profiles (Figure 2B, the left panel).

Note: In each lane, 20 μ g of proteins were loaded.

5. Autoradiography was carried out by exposing the de-stained and dried-up gels to an imaging plate for 0.5-1 h at room temperature (Figure 2B, the right panel).

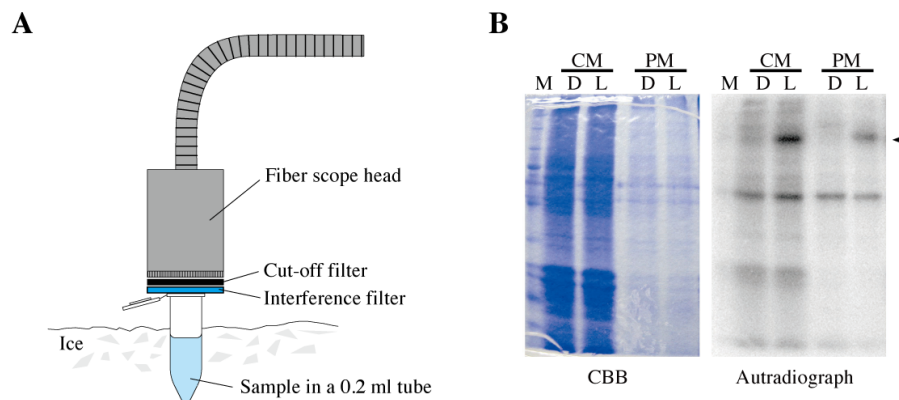


Figure 2. Irradiation and autoradiography. A. Irradiation of a sample in a 0.2-ml tube on ice using a lighting system equipped with a fiber scope, a cut-off filter and an interference filter. B. A dried-up gel stained with Coomassie Brilliant Blue (CBB; the left panel) and its autoradiograph (the right panel). Before (D) and after (L) the actinic irradiation, the protein-phosphorylation levels were examined in a crude microsomal fraction (CM) and a plasma-membrane-enriched fraction (PM). M; molecular size markers, arrowhead; radioactive signal of phosphorylation of *Vallisneria* phototropins.

Recipes

1. Homogenizing medium
 - 50 mM MOPS-KOH (pH 7.6)
 - 300 mM sucrose
 - 10 mM ethylene glycol bis (2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)
 - 5 mM ethylenediaminetetraacetic acid (EDTA)
 - 10 μ g/ml dibutylhydroxytoluene

1% casein
5 mM K₂S₂O₅
1 mM dithiothreitol (DTT)
2.5 mg/ml pepstatin A
2.5 mg/ml aprotinin
20 mg/ml EDTA-washed Polyvinylpyrrolidone

Note: Contents of the medium were mixed with a magnetic stirrer at 4 °C overnight (12-16 h), except K₂S₂O₅, DTT, pepstatin A, aprotinin, and EDTA-washed Polyvinylpyrrolidone, which were added immediately before use. Polyvinylpyrrolidone was previously washed with 5-mM EDTA, filtrated with a filter paper and dried up on the filter paper at room temperature for 1-2 days.

2. Buffer A
10 mM K-phosphate (pH 7.8)
250 mM sucrose
3. Buffer B
5 mM MOPS-KOH (pH7.6)
250 mM sucrose
0.1 mM DTT
4. 5x phosphorylation buffer
250 mM Mops-KOH (pH 7.0)
25 mM MgCl₂
250 mM KCl
5 mM DTT
5. ATP mixture (2.5 µl for each reaction)
10 mCi/ml [³²P] ATP 0.5 µl
20 µM ATP 2 µl
6. 4x SDS buffer
8% sodium dodecyl sulfate (SDS)
40% glycerol
20% 2-mercaptethanol
A small amount of bromophenol blue
0.5 M Tris-HCl (pH 6.8)

Acknowledgments

This protocol was modified from previous work by Takagi *et al.* (1988) and Harada *et al.* (2002). We thank Mr. Motoyuki Iida for taking pictures of culture facilities of *Vallisneria* plants (Figure 1A).

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