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Extraction and Measurement of Abscisic Acid in a Unicellular Red Alga *Cyanidioschyzon merolae*

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[Abstract] Abscisic acid (ABA) has been known as a phytohormone of land plants, which is synthesized in response to abiotic stresses and induces various physiological responses, but is also found from eukaryotic algae. Recently, we reported that a unicellular red alga *Cyanidioschyzon merolae* produced ABA, which prevented cell growth and enhanced salt stress tolerance (Kobayashi *et al.*, 2016). This report describes the detailed method for the extraction and quantification of ABA in the model red alga *C. merolae*.

Keywords: Abscisic acid, Algae, *Cyanidioschyzon merolae,* HPLC, Phytohormone

[Background] The phytohormone ABA has been found in divergent photosynthetic eukaryotes, but the function in unicellular algae remained unclear. In a recent study, we showed that a unicellular red alga *C. melorae* accumulates ABA in response to salt stress by the present protocol. This is the detail of the first published protocol for the extraction and quantification of ABA from *C. merolae*. This protocol is optimized for *C. merolae* based on the land plant protocol.

Materials and Reagents

1. 500 ml centrifuge bottle (Hitachi, model: S305830A)
2. Membrane filters Millex-GV syringe filter unit 0.22 µm (EMD Millipore, catalog number: SLGV033RS)
3. Wild type *C. merolae* 10D cells (National Institute for Environmental Studies, Japan)
4. Abscisic acid (Sigma-Aldrich, catalog number: A4906-250UG)
5. NaCl (Wako Pure Chemical Industries, catalog number: 195-15975)
6. Liquid nitrogen
7. Acetic acid (Wako Pure Chemical Industries, catalog number: 017-00256)
8. Diethyl ether (Wako Pure Chemical Industries, catalog number: 052-01165)
9. Methanol (HPLC grade) (Wako Pure Chemical Industries, catalog number: 132-06471)
10. Boric acid (H_3BO_3) (Wako Pure Chemical Industries, catalog number: 021-15645)
11. Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) (Wako Pure Chemical Industries, catalog number: 133-00725)

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12. Zinc sulfate heptahydrate ($ZnSO_4$) (Wako Pure Chemical Industries, catalog number: 265-00415)
13. Sodium molybdate dehydrate ($Na_2MoO_4 \cdot 2H_2O$) (Wako Pure Chemical Industries, catalog number: 514-30001)
14. Copper(II) sulfate pentahydrate ($CuSO_4$) (Wako Pure Chemical Industries, catalog number: 034-20065)
15. Cobalt(II) nitrate hexahydrate ($Co[NO_3]_2 \cdot 6H_2O$) (Wako Pure Chemical Industries, catalog number: 031-03752)
16. Ammonium sulfate ($[NH_4]_2SO_4$) (Wako Pure Chemical Industries, catalog number: 016-03445)
17. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Wako Pure Chemical Industries, catalog number: 138-00415)
18. Sulfuric acid (H_2SO_4) (Wako Pure Chemical Industries, catalog number: 195-04706)
19. Potassium dihydrogen phosphate (KH_2PO_4) (Wako Pure Chemical Industries, catalog number: 166-04255)
20. Calcium chloride ($CaCl_2$) (Wako Pure Chemical Industries, catalog number: 036-00485)
21. Iron(III) chloride hexahydrate ($FeCl_3$) (Wako Pure Chemical Industries, catalog number: 090-02802)
22. Na_2EDTA (Wako Pure Chemical Industries, catalog number: 345-01865)
23. Polyvinylpyrrolidone K-30 (Nacalai tesque, catalog number: 28314-82)
24. 2,6-di-tert-butyl-p-cresol (Tokyo chemical industry, catalog number: D0228)
25. MA2 medium (see Recipes)
26. Extraction solution (see Recipes)

Equipment

1. Spectrophotometer (Beckman Coulter, model: DU730)
2. Refrigerated centrifuge (Hitachi, model: CF16RXII)
3. Angle rotor (Hitachi, model: R10A3)
4. Mortar and pestle
5. Vortex mixer (M & S Instruments, model: VORTEX-GENIE 2 Mixer)
6. Microcentrifuge (TOMY DIGITAL BIOLOGY, model: MX150)
7. Vacuum centrifugal evaporator with low temperature trapper (TOMY DIGITAL BIOLOGY, model: CC-105 system)
8. pH meter (As One, model: KR5E)
9. HPLC system (Shimadzu, model: X2 HPLC system) equipped with a photodiode array detector (PDA) and column (5 μm, 4.6 x 250 mm) (Senshu Scientific, model: ODS SP100)

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Procedure

A. Extraction

1. The optical density (OD) of *C. merolae* liquid culture is measured by spectrophotometer at 750 nm. When the OD_{750} reaches 10, cells are diluted to yield an OD_{750} of approximately 0.5 in 350 ml MA2 medium (Kobayashi *et al.*, 2010). Grow the cells under illumination with fluorescent white light (50 μM photons m^{-2} s^{-1}) at 42 °C, bubbled with air supplemented with 2% CO_2. After incubation for 16 h, measure the OD_{750}, transfer the culture to 500 ml centrifuge bottle, and collect the cells by–centrifuging with angle rotor at 3,000 *x g* for 3 min at room temperature. Gently resuspend the pellet in 350 ml MA2 medium containing 500 mM NaCl and further cultivate for 3 h under the same condition.
2. Harvest the cells (OD_{750} = 0.8, containing about 2 x 10^7 cells/ml) by centrifugation at 3,000 *x g* for 3 min at 4 °C, discard the medium by decantation.
3. Remove the remaining medium by pipetting. Dissolve the pellet in MA2 (1-2 ml) and flash freeze in liquid nitrogen (Video 1).
4. Grind the frozen cell suspension to powder by a mortar and pestle (Video 1).

Video 1. Video for ABA extraction steps A3 and A4. This video supports the cell harvesting and grinding.

5. Homogenize the powdered sample in 20 ml extraction solution by vortexing for 5 min and centrifuge at 10,000 *x g* for 15 min at 4 °C (Video 2).
6. Filter the supernatant with 0.22 μm membrane filter (Video 2).

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Video 2. Video for ABA extraction steps A5 and A6. This video supports the cell extraction and filtration.

7. Concentrate the aqueous phase by vacuum centrifugal evaporator at room temperature (collect about 4 ml aqueous phase) (Video 3).
8. Measure the pH of aqueous phase by pH meter. Adjust the aqueous phase to pH 2.8 by 0.5 M acetic acid and filter with 0.22 μm membrane (Video 3).

Video 3. Video for ABA extraction steps A7 and A8. This video supports the purification of extract.

9. Extract the ABA from the aqueous phase by three partitions with 5 ml diethyl ether (Video 4).
10. Dry up the collected ether layer by vacuum centrifugal evaporator at room temperature and dissolve the pellet in the 300 μl methanol (Video 4).

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Video 4. Video for ABA extraction steps A9 and A10. This video supports the continuation of purification of extract.

B. HPLC analysis

1. Chromatography is conducted on an HPLC system equipped with a PDA using the full visible spectrum with monitoring employed at 254 nm. Column is a tandem jointed ODS SP100, 250 x 4.6 mm, S = 5 µm.
2. Perform isocratic separation by 50% methanol. Flow rate is 0.5 ml/min at 40 °C, and apply the samples in 10-50 µl. The peak of ABA was detected at retention time 7.1 min (Figure 1).
3. Standard calibration curves were generated at 254 nm with ABA reference.
4. Standard ABAs of concentration of 0, 10, 50, 100, 200, 500, 1000 pmol/µl are prepared and applied to HPLC in 10 µl. HPLC analysis should be performed in triplicates.

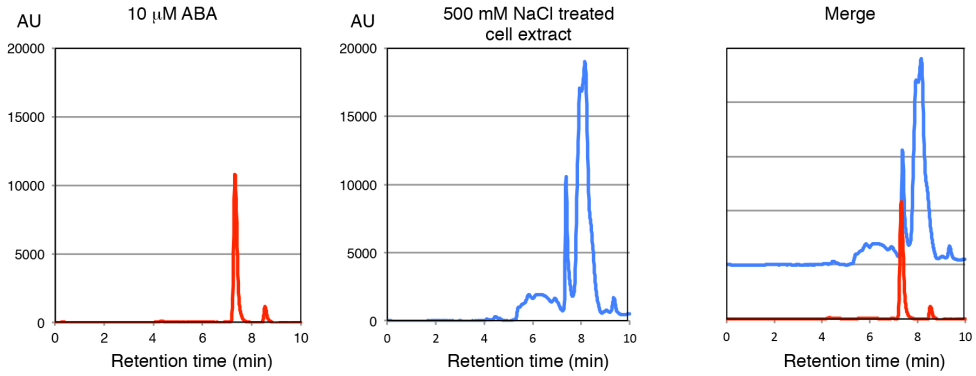

Figure 1. HPLC separation of the *C. merolae* extract with ABA standard reference. Cells were treated with 500 mM NaCl for 3 h. The ABA standard reference and 500 mM NaCl treated cell extract were separated by HPLC with PDA. The absorption spectra of 0.2 mM ABA standard (left panel), 500 mM NaCl treated cell extract (center panel) and merged (right panel) are shown.

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Data analysis

1. The retention time of the single peak areas is recorded and calculate the average of each peak.
2. Create a calibration curve using the average of each peak. Calculate the ABA amount using a specific standard calibration curve (An example is shown in Figure 2).

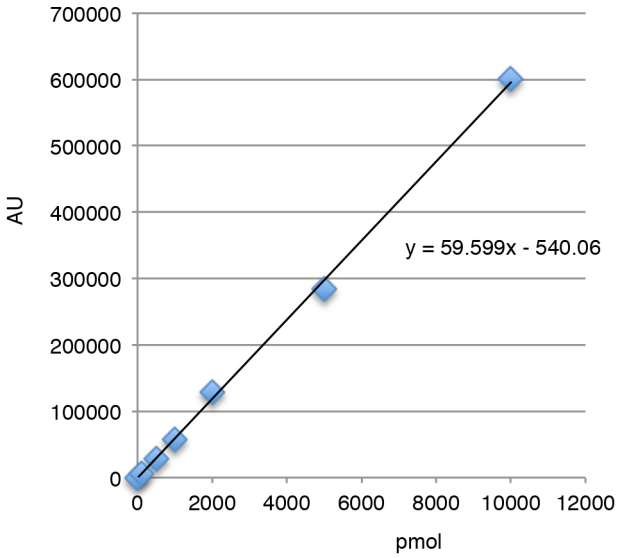

Figure 2. ABA standard curve. Standard curve was made based on peak value in HPLC analysis. Example: 300 µl extract was obtained starting from salt stressed cell culture (350 ml, OD_{750} = 0.8), and 10 µl of the 300 µl was subjected to HPLC analysis, which resulted in the read of 133.533 AU. Based on the calibration curve, the total amount of ABA contained in the starting material was calculated as 68487.5 pmole.

Notes

ABA extraction and HPLC method was modified from Kojima method (Kojima *et al.*, 1995). ABA is detected from *C. merolae* cells only under the salt stressed condition.

Recipes

1. MA2 medium
   Mix solutions I to III and mess up to 1 L. Sterilize by autoclaving. Solution IV should be sterilized by filtration through a 0.22-micron filter. Add solution IV to the mix after autoclave.

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Solution I

Solution I (10x for MA2)	Concentration	For making 1 L of Solution I
$(NH_4)_2SO_4$	400 mM	52.9 g
$MgSO_4·7H_2O$	40 mM	10.0 g
H_2SO_4	54 mM	3 ml
A6 minor salts		40 ml
H_2O		up to 1 L

Solution II

Solution II (100x for MA2)	Concentration	For making 100 ml of Solution II
KH_2PO_4	800 mM	10.88 g
H_2O	40 mM	up to 100 ml

Solution III

Solution III (1,000x for MA2)	Concentration	or making 100 ml of Solution III
$CaCl_2$	1 M	14.7 g
H_2O		up to 100 ml

Solution IV

Solution IV (250x for MA2)	Concentration	
$FeCl_3$	25 mM	0.4 g
Na_2EDTA	20 mM	0.7 g
H_2SO_4		3 drops
H_2O		up to 100 ml

A6 minor salts

A6 minor salts	Concentration	
H_3BO_3	46 mM	2.85 g
$MnCl_2·4H_2O$	9 mM	1.8 g
$ZnSO_4$	0.77 mM	0.105 g
$Na_2MoO_4·2H_2O$	1.6 mM	0.39 g
$CuSO_4$	0.3 mM	0.043 g
$Co(NO_3)_2·6H_2O$	0.17 mM	0.04 g
H_2O		Up to 1 L

2. Extraction solution
   0.6 g/L polyvinylpyrrolidone
   0.22 g/L 2,6-di-tert-butyl-p-cresol
   Dissolve in 80% (v/v) methanol

Acknowledgments

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numbers: 21370015, 23120505, 2424806, 15K14539 to K.T., 13274350, 15621958 to Y.K.)

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