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Detection of Alternative Oxidase Expression in *Arabidopsis thaliana* Protoplasts Treated with Aluminium

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[Abstract] Aluminium (Al), a non-essential metal widespread in the environment that is known to be toxic to humans as well as to plants, can cause damage not only to the roots but also to the aerial parts of plants. Its toxicity has been recognized as one of the major factors that limit crop production on acid soil. Alternative oxidase, the respiratory terminal oxidase in plants, which contributes to maintain the electron flux and reduce mitochondrial ROS levels, is often dramatically induced to make plants to adapt better to stress conditions like Al stress. In this protocol, the expression of alternative oxidase induced by Al treatment was detected in *Arabidopsis* protoplasts using an adaptation of previous methods (Yamamoto *et al*., 2002; Li *et al*., 2011; Liu *et al*., 2014), which contribute to research on the mechanism of alternative oxidase in Al treatment.

Materials and Reagents

1. Rosette leaves of *Arabidopsis* (Columbia, 3 weeks)
2. $AlCl_3$ (Sigma-Aldrich, catalog number: 237051)
3. $CaCl_2$ (Sigma-Aldrich, catalog number: C7902)
4. HCl (Sigma-Aldrich, catalog number: 258148)
5. FDA (Sigma-Aldrich, catalog number: 596-09-8)
6. TRI reagent (Sigma-Aldrich, catalog number: 93289)
7. Cellulase R10 (Yakult Honsha, catalog number: C6260)
8. Macerozyme R10 (Yakult Honsha, catalog number: 8032-75-1)
9. Mannitol (Sigma-Aldrich, catalog number: M4125)
10. MES (Sigma-Aldrich, catalog number: M8250)
11. KCl (Sigma-Aldrich, catalog number: P3911)
12. Bovine serum albumin (Sigma-Aldrich, catalog number: A-6793)
13. NaCl (Sigma-Aldrich, catalog number: S6150)
14. Glucose (Sigma-Aldrich, catalog number: G7528)
15. Trition X-100 (Sigma-Aldrich, catalog number: T-8787)
16. Phenylmethylsulfonyl fluoride (Sigma-Aldrich, catalog number: P7626)
17. Tris (Sigma-Aldrich, catalog number: T1378)
18. SDS (Sigma-Aldrich, catalog number: L6026)

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19. $(NH_4)_2S_2O_8$ (Sigma-Aldrich, catalog number: A3426)
20. TEMED (Sigma-Aldrich, catalog number: T9281)
21. Tween-20 (Sigma-Aldrich, catalog number: P9416)
22. SuperScript II first-strand synthesis system (Life Technologies, Invitrogen™, catalog number: 11904-018)
23. SYBR Premix Ex Taq (Takara, catalog number: RR420A)
24. Bio-Rad protein assay kit (Bio-Rad Laboratories, catalog number: 500-0001)
25. Anti-AOX antibody (Agrisera, catalog number: AS10699)
26. Anti-Rubisco antibody (Agrisera, catalog number: AS03037)
27. Anti-rabbit IgG (DylightTM800 4x PEG Conjugate) secondary antibody (CST, catalog number: 5151)
28. Anti-mouse IgG (Dylight™ 800 Conjugate) secondary antibody (CST, catalog number: 5257)
29. Enzyme solution (see Recipes)
30. W5 solution (see Recipes)
31. 5 mM $AlCl_3$ solution in Ca medium
32. Real time PCR reaction solution (see Recipes)
33. Lysis buffer (see Recipes)
34. 10 ml separating gel (see Recipes)
35. 6 ml stacking gels (see Recipes)
36. TBST (see Recipes)

Equipment

1. pH meter
2. Thermal Cycler (Roche, model: Light cycler 2.0)
3. Confocal laser-scanning microscope (Carl-Zeiss, model: LSM510/ConfoCor2)
4. Auto microplate reader (Tecan Trading AG, model: infinite M200)
5. Two-color infrared imaging system (Odyssey, model: 9120)
6. Centrifuge
7. Orbital shaker
8. PVDF membranes (Bio-Rad Laboratories, catalog number: 162-0177)
9. Nylon mesh
10. 96-well plates
11. 1.5 ml microcentrifuge tube
12. Generic razor blade
13. Vacuum pump (Vacuubrand, model: MZ 2C)
14. BioPhotometer (Eppendorf, model: AG 22331)

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Software

1. Image J 1.43 software

Procedure

A. Prepare for *Arabidopsis* protoplasts
   1. Healthy rosette leaves from *Arabidopsis thaliana* (3 weeks) were sliced with a razor blade into small leaf strips (0.5-1 mm).
   2. The leaves were covered with enzyme solution in a petri dish and placed in a vacuum chamber connected to a vacuum pump, and approx. 100 to 400 mmHg of vacuum was applied for 30 min. The leaves were then incubated in the dark for 3 h without shaking at room temperature.
   3. The digested sample was filtrated through a 75 µm nylon mesh, the crude protoplast filtrates were sedimented by centrifugation for 3 min at 100 *x g* at room temperature.
   4. The purified protoplasts were suspended in W5 solution.

B. Al treatment for *Arabidopsis* protoplasts
   1. 10 µl $AlCl_3$ solution in Ca medium was added to 90 µl of protoplast solution in 96-well plates.
   2. The protoplasts were incubated for 1 h at room temperature in darkness.
   3. The protoplasts were incubated with 50 µM FDA for 5 min and Al-induced protoplast death was observed by confocal microscopy (Figure 1).

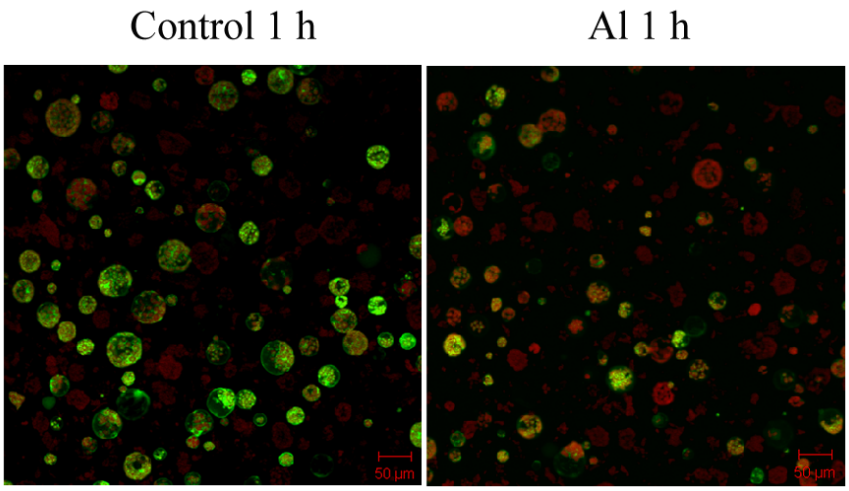

Figure 1. Al-induced protoplast death. Viability of wild-type (WT) *Arabidopsis* protoplasts after 0.5 mM Al treatment. Protoplasts (2 x 10^5/ml) were incubated with 50 µM FDA and observed with a confocal laser-scanning microscope.

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C. RNA extraction and quantitative RT-PCR

1. Total RNA was extracted from *Arabidopsis* protoplasts according to the manufacturer's instructions using TRI reagent.
2. The concentration of RNA was determined by measuring A_{260} using BioPhotometer. 4 μg RNA was used for reverse transcription PCR.
3. First strand cDNA was synthesized with SuperScript II First strand synthesis system for qRT-PCR. Eppendorf BioPhotometer was used to determine the concentrations of cDNA.
4. The transcript of *AOX* genes were analyzed by quantitative RT-PCR using *ACTIN2* as an endogenous control. The *AOX1a* gene was amplified with the primers 5'-ATGATGATAACTCGCGGTGGAGC-3' and 5'-GCAACATTCAAAGAAAG CCGAATC-3'. PCR was carried out using 50 ng of cDNA and SYBR PCR Master Mix following the manufacturer's protocol. For quantitative RT-PCR analyses, the Light Cycler 2.0 instrument was used to run the two-step program. PCR cycling conditions for amplification were 95 °C for 30 sec followed by 40 cycles of 95 °C for 5 sec, 55 °C for 30 sec.
5. Relative expression levels were calculated using the $2^{(-\Delta\Delta Ct)}$ analysis method.

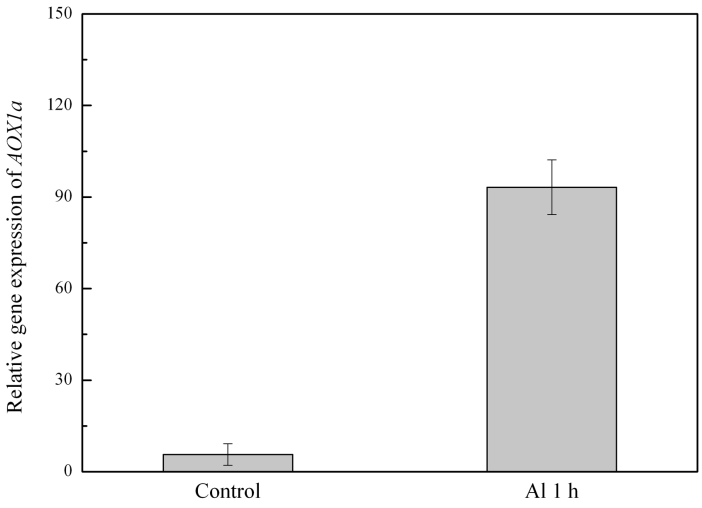

Figure 2. qRT-PCR analysis of the gene expression levels of *AOX1a* gene in Al-treated WT protoplasts

D. Protein extraction and western blot

1. The treated protoplasts were re-suspended in lysis buffer and incubated on ice with gentle shaking on a level shaker for 30 min.
2. The samples were centrifuged for 5 min at 1,2000 *x g* at 4 °C, and the supernatants were transferred to new 1.5 ml tubes.
3. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bio-Rad protein assay kit).

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4. Proteins extracts were separated by 12% sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE).
5. The gel was transferred to PVDF membranes and then electrophoresis for 45 min.
6. The membrane was blocked with TBST containing 5% non-fat milk for 1 h.
7. The membrane was incubated with anti-AOX antibody or anti-Rubisco antibody at 4 °C overnight.
8. The membrane was washed with TBST three times (about 10 min each time), and then incubated with secondary antibody [AOX: Anti-rabbit IgG (Dylight^{TM}800 4x PEG Conjugate) secondary antibody; Rubisco: Anti-mouse IgG (Dylight^{TM}800 Conjugate) secondary antibody] at room temperature for 2 h.
9. When the membrane was dried, detected by using Odyssey two-color infrared imaging system.

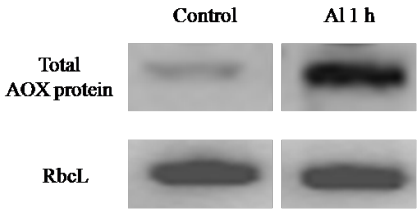

Figure 3. Western blot analysis of the level of total AOX proteins in Al-treated WT protoplasts. Blots were probed with monoclonal anti-AOX antibody.

Quantitative analysis was carried out using Image J 1.43 software (Figure 4). A step-by-step guide on how to use Image J for this purpose is shown in Supplementary data 1.

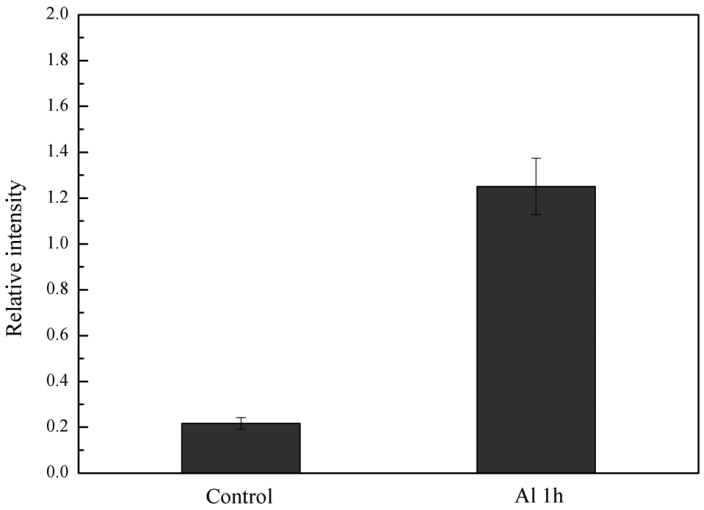

Figure 4. Quantitative analysis of western blot. Quantitative analysis was carried out using Image J software.

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Recipes

1. Enzyme solution
   1%-1.5% (w/v) cellulose R10
   0.2%-0.4% (w/v) macerozyme R10
   0.4 M mannitol
   20 mM MES
   20 mM KCl
   10 mM $CaCl_2$
   0.1% (w/v) bovine serum albumin
   pH 5.7
2. W5 solution
   154 mM NaCl
   125 mM $CaCl_2$
   5 mM KCl
   5 mM Glc
   15 mM MES-KOH (pH 5.6)
3. 5 mM $AlCl_3$ solution in Ca medium
   5 mM $AlCl_3$
   30 mM $CaCl_2$
   pH 4.5
4. Real time PCR reaction solution

SYBR premix Ex Taq	10.0 µl
PCR forward primer (10 µM)	0.4 µl
PCR reverse primer (10 µM)	0.4 µl
cDNA	2.0 µl
ddH_2O	7.2 µl

5. Lysis buffer
   15 mM NaCl
   1% Triton X-100
   100 mg/ml phenylmethylsulfonyl fluoride
   50 mM Tris-HCl (pH 8.0)
6. 10 ml separating gel

30% polyacrylamide	4.0 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml
10% SDS	100 µl
10% $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl
ddH_2O	3.29 ml

7. 6 ml stacking gels

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30% polyacrylamide	1.0 ml
1 M Tris-HCl (pH 6.8)	0.75 ml
10% SDS	60 µl
10% $(NH_4)_2S_2O_8$	60 µl
TEMED	6 µl
ddH_2O	4.124 ml

8. TBST
   10 mM Tris-HCl (pH 7.4)
   150 mM NaCl
   0.1% Tween-20

Acknowledgments

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