

Histochemical Detection of Zn in Plant Tissues

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[Abstract] Accumulation of metals in plant tissues, and occasionally, different cells of the same tissue, may be highly non-uniform (Seregin and Kozhevnikova, 2008). Easy-to-use histochemical methods may greatly help to investigate the distribution and accumulation of metals within and among plant tissues, and also provide information on their subcellular localization (Seregin and Kozhevnikova, 2011). The histochemical techniques of zinc (Zn) visualization are based on the formation of the blue-colored complex of Zn with the metallochrome indicator Zincon ($C_{20}H_{15}N_4NaO_6S$), or the green-fluorescent complex with Zinpyr-1 ($C_{46}H_{36}Cl_2N_6O_5$) (Seregin *et al.*, 2011; Seregin and Kozhevnikova, 2011). A method for histochemical Zn detection in plant tissues using Zinpyr-1 was first proposed by Sinclair *et al.* (2007), and later modified by Seregin *et al.* (2011), and Seregin and Kozhevnikova (2011). Histochemical data supplement the results of quantitative analysis, thus allowing a detailed study of the distribution, accumulation, and translocation pathways of Zn within the plant, which are important topics in modern plant physiology. These histochemical techniques have been successfully applied in different plant species, for example *Zea mays* (Seregin *et al.*, 2011), *Noccaea caerulescens* and *Thlaspi arvense* (Kozhevnikova *et al.*, 2014a), *Capsella bursa-pastoris* and *Lepidium ruderale* (Kozhevnikova *et al.*, 2014b), in which Zn was detected in different root and shoot tissues. Here, we present the full staining protocols for these methods, developed or modified in our lab (Seregin and Kozhevnikova, 2011; Kozhevnikova *et al.*, 2014a; Kozhevnikova *et al.*, 2014b).

Materials and Reagents

A. Staining with Zinpyr-1 (see Recipes)

1. Zinpyr-1 or 4', 5'-Bis[bis(2-pyridylmethyl)aminomethyl]-2',7'-dichlorofluorescein ($C_{46}H_{36}Cl_2N_6O_5$, Mr= 823.72) (Sigma-Aldrich, catalog number: 40667 or Millitech, catalog number: ZP1)
2. Dimethyl sulfoxide or DMSO (C_2H_6OS)
3. Ethylenediaminetetraacetic acid (disodium salt) (Na_2EDTA , $C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$, Mr = 372.24)
4. Super demineralized water

B. Staining with Zincon (see Recipes)

1. Zincon sodium salt ($C_{20}H_{15}N_4NaO_6S$, Mr = 462.4) (Sigma-Aldrich, catalog number: 201332)
2. Borax ($Na_2B_4O_7 \cdot 10 H_2O$)
3. Sodium hydroxide (NaOH)
4. Ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA , $C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$, Mr = 372.24)
5. Super demineralized water

Equipment

1. Light microscope with a color digital camera attachment (staining with Zincon); confocal microscope or fluorescence microscope with appropriate filters and digital camera attachment (staining with Zinpyr-1; see below for spectral characteristics of the dye)
2. Micro pipettes (100-1,000 μ l) and pipette tips
3. Vortex
4. Precision balances
5. Razor blades
6. 50 ml, 100 ml and 1 L flasks
7. 2 ml microtubes
8. 5 ml or 15 ml centrifuge tubes
9. Heat-resistant 20 ml flask
10. Microscope slides and cover glasses
11. Tweezers
12. Dissecting needles
13. Magnetic stirrer with heating
14. Axio Imager Z2 microscope (ZEISS)

Procedure

A. Preparation of plant materials

To get rid of the metal absorbed on the root surface, the roots should be incubated in Na_2 -EDTA (20 mM) for 10 min and then rinsed in demineralized water prior to the analysis. To prepare 20 mM Na_2 -EDTA solution, dissolve 7.44 g of Na_2 -EDTA in 1 L of demineralized water.

B. Staining procedure

1. Make series of sections of the examined plant material on a glass slide using a safety razor blade. The optimal thickness is about 1-2 intact cells, but it depends on plant material. Leaf epidermis can be peeled with tweezers. It is important to use fresh living plant material. It is not useful to analyze the zinc distribution in fixed plant tissue owing to potential redistribution or wash-out of part of the cellular Zn during the procedure of chemical fixation. For example, large losses (up to 75-80%) of loosely bound Zn from roots to the fixative and dehydrating solutions during tissue preparation by conventional fixation for electron microscopy have been documented by Davies *et al.* (1991).
2. Add 3-4 drops of analytical reagent.
3. Cover the sections or peels with a cover glass. If needed, remove excessive reagent with filter paper. If during the examination the reagent dries out on the slide, it should be added under the cover glass.

C. Microscopy

Staining with Zinpyr-1

During the incubation keep the preparations in the dark. Preparations can be examined under a confocal scanning fluorescent light microscope, or fluorescent light microscope after 15-60 min (depending on the size and amount of plant material). The excitation and emission maxima of Zinpyr-1 are both within the visible spectrum: 490 and 525 nm, respectively. We used filter set 38 for the Axio Imager Z2 microscope, with excitation wavelength range 450-490 nm and emission wavelength range 500-550 nm. In order to avoid background fluorescence, it is important to remove the Zinpyr-1 solution from the slides after the treatment using filter paper and substitute it with superdemineralized water. The location of Zn in plant tissues is indicated by the green fluorescence of the Zn-Zinpyr-1 complex (Figure 1 A-C).

Staining with Zincon

Preparations can be examined under the light microscope after 5-15 min (depending on the size and amount of plant material). Preparations cannot be stored longer than a couple of hours. The location of Zn in plant tissues is indicated by the blue color of the Zn-Zincon complex (Figure 1 D-G).

Representative data

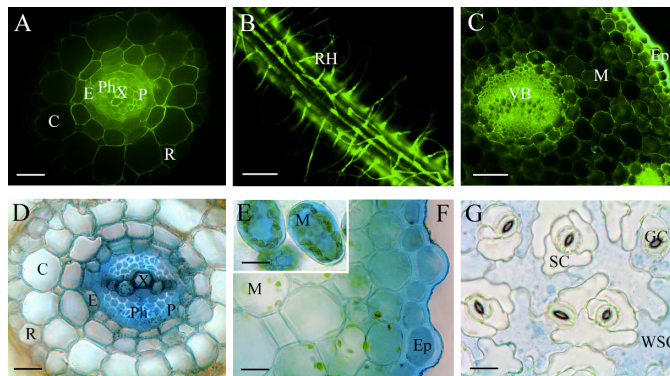


Figure 1. Zn localization in plant tissues using Zinpyr-1 (A-C) and Zincon (D-G). A. Root section, B. Root, C. Leaf petiole section of *Capsella bursa-pastoris* exposed to 20 μ M Zn for 8 weeks; D. Root section, E-F. Leaf sections, G. Leaf epidermal peel of *Noccaea caerulescens* exposed to 800 μ M Zn for 8 weeks. Green fluorescence indicates the location of the Zn-Zinpyr-1 complex, blue colour indicates the location of the Zn-Zincon complex. Designations: C-cortex; E-endodermis; Ep-epidermis; GC-stomata guard cells; M-mesophyll; P-pericycle; Ph-phloem; R-rhizodermis; RH-root hairs; SC-subsidiary cell; VB-vascular bundle; WSC-water-storage epidermal cell; X-xylem. Bar: 10 μ m (A, D-G); 50 μ m (B, C)

Video 1. The procedure of sample preparation and staining with Zincon dye



Notes

1. To prove that green fluorescence observed after staining with Zinpyr-1 or blue coloring observed after staining with Zincon are Zn-dependent indeed, tissue sections can be incubated in a 1mM-solution of TPEN [N, N, N', N'-tetrakis (2-pyridylmethyl)ethane-1, 2-diamine] for 2 h at room temperature prior to staining with Zinpyr-1 or Zincon. TPEN is a chelator capable of penetrating cell membranes and showing high affinity and

specificity toward Zn ions. Treatment of samples with TPEN should lead to almost complete absence of Zn-dependent green fluorescence after subsequent treatment with Zinpyr-1 or absence of blue staining after subsequent treatment with Zincon.

2. The intensity of staining corresponds with the level of Zn accumulation in cells and tissues. Therefore, it is possible to semi-quantitatively estimate and compare the Zn contents within and among sections on a per unit area basis. The Zn contents of cells of different tissues can be compared only if the cell sizes in these tissues are similar. In particular, when Zn distribution in the growing parts of plants is analyzed, one should take into consideration that the reduced staining intensity of elongating cells, as compared with meristematic cells, may point to a decrease in the Zn content in elongating cells only on a per unit volume basis. The Zn content per cell may be the same or even higher as a result of ongoing Zn uptake during the elongation phase.
3. In our hands, Zincon staining was visible at Zn concentrations of 10 μ M or higher. The threshold Zn concentration of the fluorescence method (Zinpyr-1), as determined by analysis of the emission spectrum of Zinpyr-1 at various Zn concentrations, was 1 nM. One should bear in mind that the lack of coloration of certain tissues or organs only means that the Zn content in the tissues is below the detection limit of the histochemical method. However, plants can accumulate Zn at concentrations that are one or more orders of magnitude higher than in their nutrient or soil solution. Therefore, even when the Zn concentration in the medium is identical to or below the detection limit of these histochemical methods, the Zn content within the plants will most likely be sufficient for detection.

Recipes

1. Staining with Zinpyr-1
 - a. To prepare 1 mM Zinpyr-1 stock solution dissolve 1 mg of Zinpyr-1 in 500 μ l of DMSO, stir it well using a vortex mixer, pipette the solution into a 2 ml microtube and add 714 μ l of DMSO. Vortex it well again. Make 50 μ l aliquots of Zinpyr-1 stock solution and store them in a freezer at -20 $^{\circ}$ C.
 - b. Directly prior to the analysis, dilute an aliquot of Zinpyr-1 DMSO stock solution (1 mM) to a final concentration of 5 or 10 μ M with superdemineralized water. To prepare 5 ml of 10 μ M Zinpyr-1 working solution dissolve 50 μ l of Zinpyr-1 DMSO stock solution in 4.95 ml of superdemineralized water. Alternatively, to prepare 5 ml of 5 μ M Zinpyr-1 working solution dissolve 25 μ l of Zinpyr-1 DMSO stock solution in 4.975 ml of superdemineralized water.
2. Staining with Zincon
 - a. To prepare 50 ml of 1 M NaOH stock solution dissolve 2 g of NaOH in 50 ml of superdemineralized water.

- b. To prepare 10 ml of working solution dissolve 0.0065 g of Zincon and 0.1906 g of borax in 9 ml of superdemineralized water. Add 0.2 ml of 1 M NaOH and adjust the volume with superdemineralized water to 10 ml.
- c. Heat up the solution to 80-90 °C on a magnetic stirrer with heating and then cool down to room temperature. The solution may be stored for a week in darkness at room temperature.

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