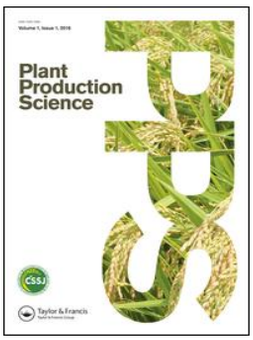

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Localization of Casparian Bands and Crystal Cells in Relation to Aluminum Distribution in the Primary Root of Eddo under Aluminum Treatment

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Abstract : The primary roots of eddo (*Colocasia esculenta* (L.) Schott var. *antiquorum* Hubbard & Rehde) were treated with aluminum, and the localization of Casparian bands and crystal cells in relation to aluminum was investigated. The calcium oxalate crystal idioblasts (crystal cells) were localized at the peripheral parts of cortex between about 0.5 mm and 6 mm from the tip of primary roots. The crystal cells were arranged in tubular form, which is referred to as 'tubular arrangement of crystal cells'. The Casparian band was apparently detected in the endodermis at 3 mm from the root tip of primary roots treated with aluminum, under a fluoresence microscope with berberin hemi-sulfate. Aluminum was significantly stained with eriochrome cyanine in the root apical region of eddo after aluminum treatment. Not only in the periphery part of stele, but also the external part of the tubular arrangement of crystal cells was significantly stained. In addition, marked accumulation of aluminum stained with lumogallion was observed inside the crystal cells by z-staking observation with a confocal laser scanning microscope. The results suggested that Casparian bands in endodermis and the tubular arrangement of crystal cells obstructed the influx of aluminum into the inside of stele.

Key words : Aluminum, Calcium oxalate crystal, Casparian band, *Colocasia esculenta*, Eddo, Oxalate, Taro.

Eddo (*Colocasia esculenta* (L.) Schott var. *antiquorum* Hubbard & Rehder), called 'satoimo' in Japanese, belongs to the group of taros. Eddo is naturally tolerant to acid soil. In acid soil regions of the world, aluminum (Al) is a major factor limiting crop growth (Ma et al., 1997). It was reported that taro tolerates excess Al (Miyasaka et al., 1993a).

The ability of organic acids to chelate and render Al nonphytotoxic is well established, and Al-tolerant plants have been suggested to use organic acids to detoxify Al either internally or in the rhizosphere (Delhaize and Ryan, 1995). In taro, roots exuded an increasing amount of oxalate with increasing Al stress (Ma and Miyasaka, 1998). Thus, it was proposed that the secretion of oxalate is a mechanism by which taro avoids Al toxicity. Internal oxalate has also been reported to detoxify cytosolic Al by chelation in buckwheat (Ma et al., 1997).

The Casparian band is a wall modification that contains lignin and suberin (Schreiber et al., 1999) and localizes in the endodermis in virtually all vascular plants and in the exodermis in many angiosperms (Clarkson and Robards, 1975; Peterson and Perumalla, 1990). In most vascular plants, the Casparian band in the endodermis proved to have at least one essential function to prevent the apoplastic passage of ions from the cortex to the stele (Enstone et al., 2003). However, the function of the band under Al stress has not been identified. In this study, we found that calcium oxalate crystal idioblasts (crystal cells) were localized and arrayed like barrier at a peripheral part in the cortex of tip parts of primary roots in eddo. The aim of this study is to elucidate whether the Casparian band and the crystal cells are related with the mechanism of Al tolerance in eddo. Here we report the Al distribution in the roots by staining Al with eriochrome cyanine and observing under a bright-field microscope, and by staining Al with lumogallion and observing under a confocal laser scanning microscope. The localization of Casparian bands in the roots was observed by fluorescence microscopy after treatment with berberin hemi-sulfate.

Materials and Methods

1. Plant material

Eddo (*Colocasia esculenta* (L.) Schott var. *antiquorum* Hubbard & Rehder) cv. Aichiwase was used. Seed corms were planted in pots filled with vermiculite and plants were grown with a modified Stein-berg culture solution (Ma and Miyasaka, 1998) in a growth chamber (25ºC in a light period, 19ºC in a dark period, natural light and 55% RH). The macronutrient concentrations

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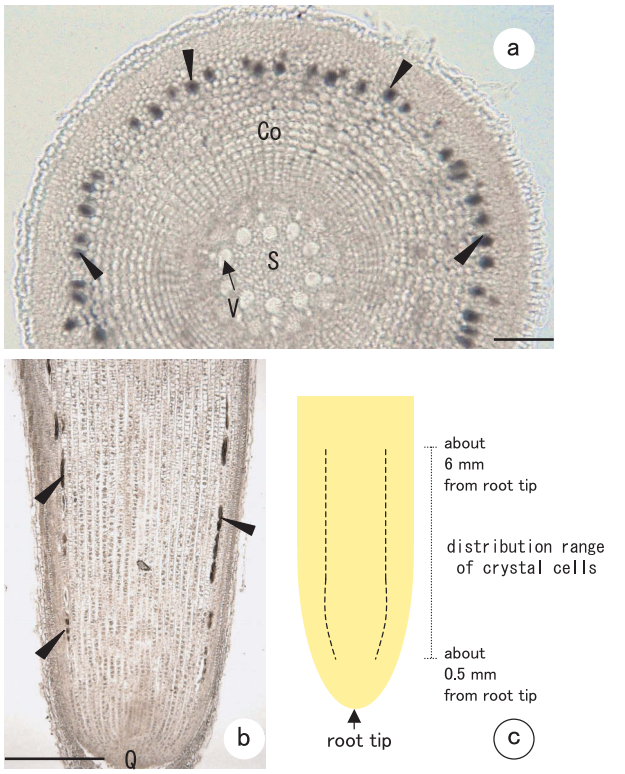

Fig. 1. Crystal cell distribution in a primary root. (a) Cross-section at 3 mm from the root tip. (b) Longitudinal section of apical region of the root.
(c) Diagram of crystal cell distribution pattern in longitudinal section. Co, cortex; Q, quiescent center; S, stele; V, vessel. Arrowheads indicate crystal cells.
(a) Bar=100 μm. (b) Bar=500 μm.

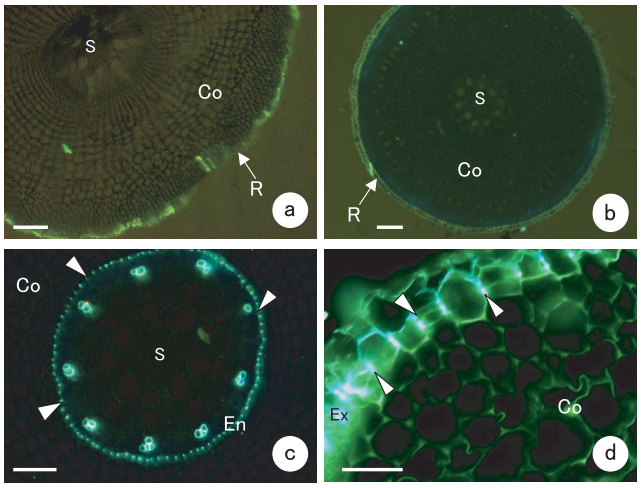

Fig. 2. Casparian bands in primary roots, observed under a fluorescence microscope. Cross-sections were cut from the roots not treated (a, b) or treated (c, d) with 9000 μM Al for 7 days. (a) Section at 10 mm from the root tip without staining. Autofluorecence was detected in the root cap. (b, c) Sections at 3 mm from the root tip stained with berberin. Casparian band was detected in endodermis of Al-treated root. (d) Section at 10 mm from root tip stained with berberine. Casparian band was detected in exodermis. Co, cortex; En, endodermis; Ex, exodermis; R, root cap; S, stele. Arrowheads indicate Casparian bands. Bars=100 μm.

in the culture solution were (in mM): NH_4-N, 1.2; NO_3-N, 3.6; P, 0.1; K, 1.2; Ca, 1.0; Mg, 0.4; and S; 0.7. The micronutrients were (in μM): Mn, 4; B, 12; Zn, 2; Cu, 1; Mo, 0.2 and Fe, 10. Plants were treated with Al when they were about 20 cm tall and their primary roots were approximately 15 cm long. In eddo, primary root is the root which is directly generated from the corm. Primary roots were used for the following investigations.

2. Al staining with eriochrome cyanine

Plants were treated with an ascending series of Al, 180, 450, 540, 900 and 1800 μM Al, each for seven days, or 900, 1350, 1800, 2700 and 9000 μM Al (pH 4), each for seven days, using a modified Stein-berg culture solution in pots filled with vermiculite in a growth chamber (25°C in a light period, 19°C in a dark period, natural light in May and June, and 55% RH). The solution was exchanged every day.

Al-treated primary roots were sampled and stained with 0.2%(v/w) eriochrome cyanine to investigate Al distribution. Root segments were fixed in 0.05 M sodium phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 3% paraformaldehyde at 20°C for 5 hr. The segments were dehydrated with an ethyl alcohol series and then immersed in t-butyl alcohol. They were then embedded in paraffin (Paraplast plus™, Oxford, U.S.A.). Ten μm transverse sections were stained with 0.2% (v/w) eriochrome cyanine for observation under a bright-field microscope (BX51; Olympus, Japan).

Sections of twelve roots per each treatment embedded in paraffin were also used for observation of basic structure of primary roots.

3. Al staining with lumogallion

Plants were treated with 900 μM Al in a modified Stein-berg solution (pH 4) for seven days in a growth chamber (23–27°C in a 12-hr light period, 19°C in a 12-hr dark period, a light intensity of 230 μmol m^{-2} s^{-1} and 75% RH). The solution was bubbled with an air compressor. Primary roots treated with Al for seven days were excised and washed with 10 mM citrate for 30 min. The roots were then embedded in 5% agarose and sectioned in 100 μm slices using a slicer (DTK-1500; D.S.K., Japan). Sections were washed in acetate buffer (pH 5.2) at 25°C, and treated with the Al-indicator lumogallion in darkness for 60 min at 50°C in an incubator-shaker. Root sections were then rinsed in acetate buffer twice each for 15 min and mounted on slide glasses. The Al distribution in the roots was observed by z-staking under a confocal laser scanning microscope (LSM 510; ZEISS, Germany). The excitation laser wavelength was 488 nm and the emission filter was 580 LP. The fluorescence image

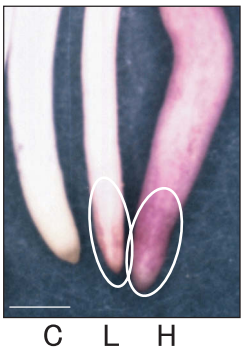

Fig. 3. Al distribution in primary root treated with eriochrome cyanine. Root was treated with (L) an ascending series of Al (180, 450, 540, 900, 1800 μM Al) or (H) (900, 1350, 1800, 2700, 9000 μM Al) solution for 7 days at each concentration, and (C) was not treated with Al. Apical regions surrounding the circles were significantly stained. Bar = 4 mm.

was taken through a computer and pseudo-color was added according to the intensity of fluorescence.

4. Staining Casparian band with berberine

Plants were treated with 9000 μM Al in a modified Stein-berg solution (pH 4) for five weeks in pots filled with vermiculite in a growth chamber (23–27°C in a 12-hr light period, 19°C in a 12-hr dark period, a light intensity of 230 μmol m^{-2} s^{-1} and 75% RH). In this culture solution, a phosphorous concentration was adjusted to 0.5 mM. The treatment solution was exchanged each day. Primary roots treated with Al and then nine primary roots were embedded in 5% agarose and sectioned in 100 μm slices using a slicer. For Casparian band staining, the sections were treated with 0.1% berberine hemi-sulfate for 1 hr, and rinsed in distilled water. They were then treated with 0.1% (w/v) $FeCl_3$ in 50% (v/v) glycerin, and mounted in the same solution. Slides were examined with a fluorescence microscope fitted with a mercury burner (IX-FLA; Olympus, Japan) and a violet filter assembly (U-MWBV; Olympus, Japan). Autofluorescence was monitored by comparison with unstained sections.

Results

1. Distribution of crystal cells

The elongation of primary roots treated with Al was inhibited, in comparison with the roots not treated with Al. Crystal cells were distributed in the periphery of cortex at about 0.5 to 6 mm from the tip of the primary roots which were treated or not treated with Al. Crystal cells were arranged circularly in the transverse section and formed a line in the vertical section (Fig. 1a, b and c). From these observations, it was shown that crystal cells were arranged in tubular form. Thus, we refer to this structure as 'tubular arrangement of crystal cells'hereafter.

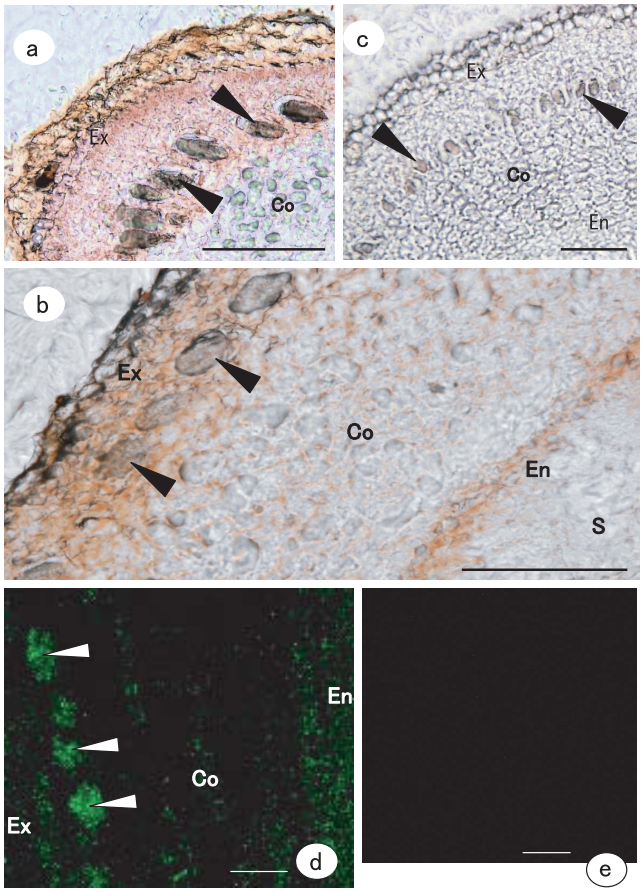

Fig. 4. Al distribution in the sections of primary root. (a-c) Cross-sections at apical region of the roots were stained with eriochrome cyanine under a bright-field microscope. (a, b) Roots treated with an ascending series of Al (900, 1350, 1800, 2700, 9000 μM Al) solution for 7 days at each concentration. (c) Root not treated with Al. (d, e) Longitudinal sections at apical region of Al-treated roots were stained with lumogallion under a confocal laser scanning microscope. Sections stained (d) or not sainted (e) with limogallion. Co, cortex; En, endodermis; Ex, exodermis; S, stele. Arrowheads indicate crystal cells. (a-c) Bars = 100 μm. (d, e) Bars = 25 μm.

2. Localization of Casparian band

Autofluorescence was detected in the root cap (Fig. 2a and b). In the roots not treated with Al, Casparian bands in endodermis were detected at 10–20 mm from the root tip, but not at 3 mm from the root tip (Fig. 2b). In the roots treated with Al, the Casparian band was apparently detected in endodermis at 3 mm from the root tip (Fig. 2c). Casparian bands were also detected in exodermis at 30–40 mm from the tip of the roots not treated with Al, although the bands were not clearer than the bands in endodermis. In the Al-treated roots, Casparian bands were detected in the exodermis at about 10–20 mm from the root tip (Fig. 2d).

3. Aluminum distribution

The apical region of primary roots treated with Al

was exclusively stained with eriochrome cyanine (Fig. 3). In the transverse sections of Al-treated root tips, not only the periphery of stele but also the external part of the tubular arrangement of crystal cells was significantly stained with eriochrome cyanine (Fig. 4a, b and c) and with lumogallion (Fig. 4d and e). In addition, crystal cells themselves were significantly stained with eriochrome cyanine (Fig. 4a, b and c). The inside of these crystal cells was markedly stained with lumogallion by z-staking observation with a confocal laser scanning microscope (Fig. 4d and e).

Discussion

The apical region of primary roots after Al treatment was exclusively stained with eriochrome cyanine. This result showed that Al was greatly accumulated in the root apical region of eddo. In primary roots of eddo, crystal cells arrayed like a barrier in the apical region where Al was markedly accumulated. In the sections of the roots, Al was observed in the external part of the tubular arrangement of crystal cells and in individual crystal cells. These results suggested that the tubular arrangement of crystal cells obstructed the influx of Al into the inside of the tubular arrangement.

In taro, the secretion of oxalate was proposed to be the mechanism to avoid Al toxicity (Ma and Miyasaka, 1998). Oxalic acid was found to be secreted in the region 0 to 10 mm from the root tip in buckwheat (Zheng et al., 1998). In crystal cells forming calcium oxalate crystals, oxalate was biosynthesized (Kostman et al., 2001). Whether oxalate was secreted from the crystal cells by Al or not has not been identified. In this study, however, Al stained with lumoallion in crystal cells were clearly observed by z-staking observation with a confocal laser scanning microscope. It is quite likely that oxalate in crystal cells binds Al because Al easily binds to oxalate in substance. Further studies are needed on the oxalate release from crystal cells and Al transport into the cells caused by Al.

The Casparian band formed by wall modification contains lignin and suberin. In most plants, Casparian bands have at least one essential function, that is, to prevent the apoplastic passage of ions from the cortex to the stele (Enstone et al., 2003). The Casparian band was obviously detected in the endodermis adjacent to the stele in the apical region of the primary roots of eddo. In this experiment, Al was significantly detected at the periphery of stele at root apical region. In barley seedlings, the application of spermidine decreased Na^+ in roots and shoots and improved compartmentation of Na^+ in leaf sheaths under saline conditions; and, such beneficial effects may be partially due to strengthened barrier of Casparian bands induced by spermidine treatment (Zhu et al., 2006). In the primary roots of maize, the radial width of the lignified region and the tightly adhering region of the plasma membrane both increased under salt stress (Karahara et al., 2004). These observations suggested that the function of the band as the barrier to apoplastic transport is enhanced under salt stress. Taro does not accumulate high concentrations of Al in its shoot (Miyasaka and Webster, 1993b). In this study, the Al content of the shoot was much less than that of the root (data not shown). The Casparian band in the endodermis may suppress the movement of Al from the cortex into the stele, which contained vascular tissues. In the Al-treated roots, Casparian bands were detected in the exodermis at about 10–20 mm from the root tip. This region and the basal region were not exclusively stained with eriochrome cyanine, in comparison with the apical region of the roots. Casparian bands in the exodermis may also obstruct the influx of Al into the inside of the roots.

The distance between the lowermost position of Casparian band and root tip was decreased by Al treatment in eddo roots. In cotton roots under salt stress (Reinhardt and Rost, 1995) and in maize roots under osmotic stress (Perumalla and Peterson, 1986), the distance has been reported to decrease. The formation of the band at a position closer to the root tip under salt stress was due to a decrease in the number and length of the cells in the endodermis between the root tip and the lowest position of the band. In the primary roots of maize, the estimated time required for a cell to complete formation of the band under salt stress was not very different from the time in distilled water or was only slightly longer (Karahara et al., 2004). It was suggested that a decrease in the distance between the lowermost position of the band and the root tip in Al-treated roots was not caused by acceleration of Casparian band formation under Al treatment.

To our knowledge, this is the first report on the localization of Casparian bands and crystal cells in the primary root of eddo in relation to aluminum distribution under aluminum treatment. The results suggested that Casparian bands in the endodermis and the tubular arrangement of crystal cells obstructed the influx of aluminum into the inside of stele. The extent of the contribution of these structures to Al tolerance of eddo needs to be studied to further clarify the mechanism of Al tolerance in eddo.

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