Morphological Changes and Function of Calcium Oxalate Crystals in Eddo Roots in Hydroponic Solution Containing Calcium at Various Concentrations

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Abstract: Morphological changes and function of calcium oxalate crystals in eddo roots in hydroponic solution containing calcium at various concentrations were investigated. Bundles of needle-shaped crystals in crystal idioblasts were tubularly arranged in the peripheral part of cortex in the apical zone of primary roots. Under scanning electron microscopy and optical microscopy, crystals in the idioblasts of roots cultured in 1 mM calcium nitrate solution were larger than those in 0 mM calcium solution but smaller than those in the solutions containing either 15 mM calcium nitrate or 15 mM calcium chloride. The number and area of crystal bundles in the sections of the apical zone in 1 mM calcium nitrate solution were significantly larger than those in 0 mM calcium solution and smaller than those in the solutions containing 15 mM calcium. The calcium mapping image obtained by energy dispersive X-ray spectrometry showed that the amount of calcium in crystal idioblasts was increased with increasing calcium concentration in the solutions. However, the weight percentage of calcium per cortex parenchyma cell in root apical zone did not vary significantly with the concentration of calcium in the solutions. In the root zone apart from the root apex having no crystals, the weight percentage of calcium per cortex parenchyma cell in the solutions containing 15 mM calcium was significantly higher than that in either 0 mM calcium or 1 mM calcium nitrate solution. These results suggested that the crystals in the tubular arrangement participated in the regulation of calcium levels in the apical zone of primary roots.

Key words: Calcium, Calcium oxalate crystal, Calcium regulation, Eddo, Morphology, Root.

The calcium oxalate crystal is the most abundant insoluble biomineral in plants. The crystal occurs in different plant tissues, including leaves, stems, roots, seeds and flora of over 215 plant families (Franceschi and Horner, 1980; Lersten and Horner, 2000; Monje and Baran, 2002; Franceschi and Nakata, 2005; Korth et al., 2006; Demiray, 2007). The crystals are formed in calcium oxalate crystal idioblasts. In plants, the crystals have been proposed to have various functions (Franceschi and Nakata, 2005; Nakata, 2012). These functions include regulation of calcium levels in plant tissues and organs, protection against herbivory, detoxification of heavy metals, strengthening of the tissues and the gathering and reflecting light for photosynthesis (Prychid and Rudall, 1999; Franceschi, 2001; Molano-Flores, 2001; Nakata, 2003; Franceschi and Nakata, 2005; Nakata, 2012).

Previous studies showed that there is a positive correlation between the crystal formation and calcium concentration in growth medium. The number and size of calcium oxalate crystal in leaves of Pistia stratiotes (Volk et al., 2002), Corchorus olitorius and Malva parviflora (Faheed et al., 2013) were larger when the growth medium contained calcium in excessive amounts and smaller when calcium is limited in the growth medium. Wu et al. (2006) also reported that the average distribution density of calcium oxalate crystals was higher in the leaves of Morus australis Poir grown in a solution containing a high concentration of calcium and no calcium oxalate crystals were found in the leaves of plants grown in a low calcium solution. In many cases, calcium oxalate crystals have been proposed to regulate calcium levels in the aerial plant organs and tissues. In plant roots of Lemma minor, crystal bundle formation could be induced by increasing the exogenous calcium concentration and the crystals dissolved under calcium-deficient condition (Franceschi, 1989). These results supported the hypothesis that crystal-forming cells participate in the regulation of calcium levels in plant organs and tissues (Franceschi, 1989). However,
there is little information related to calcium regulation of the crystals in roots compared with the aerial part of plants.

Kawasaki et al. (2008) reported the tubular arrangement of idioblasts containing calcium oxalate crystals localized in the peripheral part of the cortex in the apical zone of the primary roots in eddo. According to the observation of Storey et al. (2003), the apical zone of roots of grapevine has a structure similar to the tubular arrangement of crystal idioblasts in eddo roots. Storey et al. (2003) suggested that the crystals could initially act as a calcium sink involved in the regulation of calcium levels in root apexes of grapevine. However, the variability of crystals in the root apical zone of grapevine under different concentrations of calcium in growth medium has not been reported. The relationship between the tubular arrangement of crystal idioblasts and the regulation of calcium levels in the root apical zone is not clear.

Calcium is an essential macronutrient for all living plants. However excess calcium can be cytotoxic because it precipitates with inorganic phosphate (Marschner, 2012). Plants are susceptible to calcium deficiency because aluminum is usually the dominant cation at soil cation exchange sites under acid soil conditions (Schroder et al., 1988). Meanwhile in non-acid soils, excess calcium may become a problem because calcium is usually the dominant cation at soil cation exchange sites. Therefore, the cytoplasmic concentration of calcium ion in plants must be maintained at suitable levels to avoid deficiency and the toxicity for normal plant growth. Calcium mainly enters into the xylem thorough the apical part of roots (White, 2001). Identification of the mechanism of calcium regulation in tissues and organs of crop roots would be beneficial for developing strategies to improve the adaptation to soil environment and the growth of crops. Therefore, it is important from the aspect of crop science to understand the mechanism of calcium regulation in crop roots.

In this study, we investigated whether and how the crystals in the crystal idioblasts tubularly arranged in the apical zone of roots in eddo participated in calcium regulation using the roots in hydroponic solutions containing different concentrations of calcium. By scanning electron microscopy and optical microscopy, morphological characteristics of crystals were investigated and the variability in the number and size of crystals was statistically analyzed. Calcium mapping and the measurement of the weight percentage of calcium per cortex parenchyma cell and per crystal idioblast in roots were performed by energy dispersive X-ray spectrometry (EDS).

Materials and Methods

1. Plant materials and treatments

   Eddo (Colocasia esculenta (L.) Schott var. antiquorum Hubbard & Rehder) cv. Aichiwase was used in this experiment. Seed corms were planted in plastic pots filled with vermiculite in May 2012 and the plants were sprouted by watering under natural temperature, light and humidity conditions in the green house at Hirosaki University (40°59’N, 140°47’E, 53 m above sea level), Hirosaki, Japan. After the plants were 15 – 18 cm tall under the condition for 2 mo, they were transferred to a water culture solution (Kawasaki et al., 2008) with continuous aeration. The plants were then grown in a growth chamber (MLR-351H, SANYO) kept at 24°C, with 18 hr light at about 150 µM m² s⁻¹ (measured by MES-136, KOITO), 6 hr dark and 60% humidity. After the plants were grown for 1 wk to about 20 cm tall and with primary roots about 15 cm, they were transferred to tall beakers containing culture solution. Different concentrations of calcium were supplied in the culture solutions as 0 mM calcium, 1 mM calcium nitrate [Ca(NO₃)₂] (normal concentration of calcium as the control), 15 mM calcium nitrate [Ca(NO₃)₂] and 15 mM calcium chloride [CaCl₂] in the same water culture solution (Kawasaki et al., 2008). None of the plants used for this treatment showed abnormal growth, stress disorder or a transition to the reproductive growth, such as flower-bud formation. The treatment solutions were maintained at about pH 5.2. Plants were treated with these treatment solutions for 7 d in the growth chamber under the same temperature, light and humidity conditions as described above. The treatment solutions in the tall beakers were changed every day to prevent lack of oxygen. After the treatment, the roots were sampled and washed with deionized water. The roots were then used for the following measurements.

2. Calcium determination

   Roots treated with different concentrations of calcium solutions were sampled, dried at 60°C for 3 d, ground and stored in a desiccator. Samples were digested with 1% hydrochloric acid for 4 d and diluted with distilled water. To determine the amount of calcium, each sample was analyzed by flame atomic absorption spectrophotometer (Z-2000, HITACHI). The extraction solution contained 1000 ppm lanthanum chloride to prevent disturbances at the time of the spectrophotometry. Roots of six plants per treatment were used for calcium determination.

3. SEM observation

   The treated roots were fixed in 0.05 M sodium phosphate buffer (pH 7.2) containing 1% glutaraldehyde and 2% paraformaldehyde at 20°C for 5 hr. Roots were then rinsed in 0.05 M sodium phosphate buffer and soaked in 1% osmium solution at 4°C for overnight. Then the roots were dehydrated in a graded ethanol series, immersed in 3-methylbutyl acetate at 20°C and dried using a critical point dryer (JCPD-5, JEOL). The cracked cross

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sections of dried roots at 1 mm from the root apex were mounted on stubs with conductive carbon tape, coated with platinum using an auto fine coater (JFC-1600, JEOL) and observed by SEM (JSM-7000F, JEOL) at 5 kV to identify the structure of the calcium oxalate crystal. One root per plant and five roots per treatment were observed.

4. Bright-field optical microscopy and measurement of the number and size of crystal bundles

After the treatment, the roots were sampled and immersed in formalin-acetic acid-alcohol solution (formalin : acetic acid : 70% ethanol = 1 : 1 : 18) at 20ºC under reduced pressure r/hr and under normal pressure for 3 d. The roots were dehydrated in an ethanol series and then immersed in t-butil alcohol. They were then embedded in paraffin. Longitudinal sections (4 µm in thickness) were made by using a microtome (RV-240, YAMATO). The sections were placed on glass slides and stained by hematoxylin and eosin stain after deparaffinization using a xylene-ethanol series. Stained sections were observed under a bright-field optical microscope (BX51, Olympus) equipped with a digital camera. The number and size of crystals in the area between 0.25 and 2.16 mm from the root apexes were determined using optical microscopic images. The crystal size was calculated by measuring the area of crystals using Image J. One root per plant, five roots per treatment, and two longitudinal sections at the center position in each root were investigated and statistically analyzed to compare the number and size of crystals in the apical zones of the roots.

5. Calcium mapping by EDS

After the treatment, the roots were sampled and frozen quickly in liquid nitrogen. Then the roots were freeze-dried under a pressure of 11 Pa at –20ºC for 50 hr by using a vacuum freeze dryer (FDU-1200, EYELA). The cracked cross sections of dried roots at 3 and 55 mm from the root apexes were mounted on stubs with conductive carbon tape and coated with platinum using an auto fine coater (JFC-1600, JEOL). Calcium localization on the cross sections at 3 and 55 mm from the root apexes were investigated at 20 kV using SEM equipped with an energy dispersive X-ray spectrometer (JED-2300F, JEOL). In this experiment, one root per plant was used and three roots per treatment were investigated.

6. Measurement of weight percentage of calcium per cortex parenchyma cell and per crystal idioblast by EDS

Root samples prepared for calcium mapping were also used for this measurement. The percentage of calcium weight per total weight of the major constituent elements (boron, carbon, nitrogen, oxygen, calcium, sodium, magnesium, aluminum, silicon, phosphorus, sulphur, chlorine, potassium, manganese, iron, zinc, molybdenum and copper) in each cortex parenchyma cell and each crystal idioblast on the cross section of roots was measured at 20 kV using SEM equipped with an energy dispersive X-ray spectrometer. One root per plant was used and three roots per treatment were investigated. Two cortex parenchyma cells at the intermediate area between epidermis and pericycle on the cross sections at 3 and 55 mm from the root apexes per root were measured. Two crystal idioblasts on the cross section at 3 mm from the root apexes per root were also measured.

7. Statistical analysis

Analysis of variance (ANOVA) for the data of the number and area of crystal bundle and the weight percentage of calcium per cortex parenchyma cell and per crystal idioblast was performed by Tukey’s test.

Results

1. Calcium content of roots

The calcium contents of the treated roots were measured to examine whether the treatments could change the calcium concentration in roots. Calcium contents of the roots varied with the concentration of calcium (Fig. 1). Calcium content of roots in 0 mM calcium solution was lower than that in 1 mM calcium nitrate solution, but the difference was not statistically significant (Fig. 1). Calcium contents of roots grown in 15 mM calcium nitrate and 15 mM calcium chloride solutions were significantly (P < 0.01) higher than the contents in roots in 0 mM calcium and 1 mM calcium nitrate solutions (Fig. 1). There was no significant difference between 15 mM calcium nitrate and 15 mM calcium chloride treatments.

2. Structure of the calcium oxalate crystals

The bundles of needle-shaped crystals in crystal idioblasts arranged tubularly in the apical zones of primary

![Fig. 1. Calcium content of roots. Different letters indicate significant difference at 1% level (n = 6, Tukey's test).](image-url)
roots were observed by using SEM (Fig. 2). The crystals in the roots in 1 mM calcium nitrate solution were larger than those in 0 mM calcium solution but smaller than those in both 15 mM calcium nitrate and 15 mM calcium chloride solutions (Fig. 2). Optical microscopy was used to examine the difference in morphological characteristics of crystals among the treatments. In the roots treated with 0 mM calcium, crystal idioblasts contained few if any crystals (Fig. 3). The crystal bundles increased in number and enlarged in size as the calcium concentration in treatment solution was increased (Fig. 3). We also investigated the morphological changes in the root zone between 10 and 20 mm from the root apexes. In this zone, no crystal was observed in all treatments (Fig. 4).

3. Number and size of the crystal bundle

According to the statistical analysis of the optical microscopic images, the number of crystal bundles in the apical zone of roots in 15 mM calcium nitrate and 15 mM calcium chloride solutions was significantly \((P < 0.01)\) larger than that in 0 mM calcium and 1 mM calcium nitrate solutions (Fig. 5A). The bundle number in 1 mM calcium nitrate treatment was significantly \((P < 0.01)\) higher than that in 0 mM calcium treatment (Fig. 5A). There was no significant difference in the bundle number between 15 mM calcium nitrate and 15 mM calcium chloride treatments (Fig. 5A). The size of crystal bundle was significantly \((P < 0.01)\) higher in the roots in 15 mM calcium nitrate and 15 mM calcium chloride solutions than that in 0 mM calcium and 1 mM calcium nitrate solutions (Fig. 5B). The crystal size in the roots in 0 mM calcium treatment was significantly \((P < 0.01)\) lower than that in 1 mM calcium nitrate solution (Fig. 5B). There was no significant difference in the crystal size between 15 mM calcium nitrate and 15 mM calcium chloride treatments (Fig. 5B).

4. Calcium localization in roots

Fig. 6 shows the images of SEM and calcium mapping by EDS of the cross sections at 3 and 55 mm from the apexes of primary roots. In the cross sections at 3 mm, red dots indicating the presence of calcium was intense in the crystal idioblasts. In the two 15 mM calcium treatments, the dots were more intense than that in 1 mM calcium nitrate and 0 mM calcium treatments (Fig. 6). In the cross sections at 3 mm in 0 mM calcium treatment, the red dots in crystal idioblasts were the smallest in number among the treatments. In the cross sections at 55 mm from the root
apexes, the red dots were present as uniformly distributed (Fig. 6). The density of red dots in the cortex of the cross sections at 55 mm from the apexes of roots in the two 15 mM calcium solutions were higher than that in 1 mM calcium nitrate and 0 mM calcium solutions. It was also higher than that in the cross sections at 3 mm from the apexes of roots in all treatments.

5. Weight percentage of calcium per cortex parenchyma cell and per crystal idioblast in roots

Weight percentage of calcium per cortex parenchyma cell and per crystal idioblast in the cross sections of roots was measured by EDS. In the cross sections at 3 mm from the root apexes, there was no significant difference in the weight percentage of calcium per cortex parenchyma cell among the treatments (Fig. 7A). In the cross sections at 55 mm, the weight percentage of calcium per cortex parenchyma cell of roots in the two 15 mM calcium solutions were significantly ($P < 0.01$) higher than that in 0 mM calcium and 1 mM calcium nitrate solutions (Fig. 7A). In 15 mM calcium nitrate and 15 mM calcium chloride solutions, the weight percentage of calcium per cortex parenchyma cell at 55 mm from the root apexes was higher than that at 3 mm. There was no significant difference in the weight percentage of calcium per crystal idioblast at 3 mm from the root apexes among the treatments (Fig. 7B). However, the weight percentage of calcium per crystal idioblast was noticeably higher than that per cortex parenchyma cell in all treatments (Fig. 7).

Discussion

The calcium content of roots was determined to...
confirm whether the treatment could change the calcium content of roots. The calcium content of the roots increased with the increase of calcium concentration in the culture solution (Fig. 1). These results showed that the treatment could affect the calcium content of the roots. These treated roots were used for investigation of crystal structure, the variability of the number and size of crystals, calcium mapping in roots and the weight percentage of calcium per cortex parenchyma cell and per crystal idioblast in the roots to elucidate whether and how the crystals in the crystal idioblasts tubularly arranged in eddo roots participated in the calcium regulation.

Previous studies showed a positive correlation between the calcium concentration in growth medium and crystal formation in the aerial parts of the plants such as leaves of *Pistia stratiotes* (Volk et al., 2002), *Morus australis* Poir (Wu et al., 2006), *Conchorus olitorius* (Faheed et al., 2013) and *Malva parviflora* (Faheed et al., 2013). In the roots of *Lemna minor*, crystal bundle formation could be induced by increasing the exogenous calcium concentration and the crystals dissolved under a calcium-deficient condition (Franceschi, 1989). These results supported the hypothesis that crystal-forming cells participate in the regulation of calcium levels in plant organs and tissues (Franceschi, 1989). In this study, scanning electron microscopy and optical microscopy showed that the crystals were larger in the roots in 1 mM calcium nitrate solution than that in 0 mM calcium solution but smaller than that in the 15 mM calcium solutions (Fig. 2, 3). In addition, it was confirmed by statistical analysis that both the number and size of crystal bundles in the roots in 1 mM calcium nitrate solution were significantly higher than that in 0 mM calcium solution and lower than that in the 15 mM calcium solutions (Fig. 5). According to calcium mapping, the presence of calcium in crystal idioblasts at 3 mm from the apexes of roots in 1 mM calcium nitrate treatment were more intensive than in 0 mM calcium and less intensive than that in 15 mM calcium treatments (Fig. 6). These results showed that the crystal variability in the apical zone of primary roots is directly responsive to calcium concentration in the culture solutions.

Crystals in idioblasts were tubularly arranged in the peripheral part of the cortex in the apical zone of roots (Fig. 3). These crystals were not observed in the root zones apart from the root apex in eddo (Fig. 4, 6). In grapevine, the apical zone of roots has a structure similar to that of eddo roots (Storey et al., 2003), i.e., the tubular arrangement of crystal idioblasts. Crystal idioblasts appeared to form a discontinuous cone in the outer region of the root meristem and the raphide crystals within these idioblasts were less apparent in older regions of the root, 10 – 12 mm basipetal from the root apex in grapevine. Storey et al. (2003) also reported that the disappearance of the raphides may indicate remobilization of calcium to the elongation zone where cell wall synthesis occurs and calcium demand is high. In general, dividing cells and very young cells have little or no vacuolar compartments, therefore they would not have a large capacity to deal with calcium by sequestration in apoplastic space such as vacuolar (Franceschi and Nakata, 2005). In the parts at 55 mm from the root apexes which were older than the parts at 3 mm, there were no crystals and calcium was uniformly distributed within the tissues (Fig. 6). At 55 mm from the root apexes, the weight percentage of calcium per cortex parenchyma cell at 3 mm from the apexes was significantly higher than that per cortex parenchyma cell at 3 mm from the root apexes in all treatments (Fig. 7A). From the information and the results, it is likely that crystals in the idioblasts dissolve and release calcium to the surrounding tissues in the root zone apart from the apex of roots.

In the root apical zone containing the crystals, the
weight percentage of calcium per cortex parenchyma cell did not significantly change in response to the calcium concentration in the culture solutions (Fig. 7A). These results indicated that the calcium concentration in the cortex parenchyma cells in root apical zone was maintained at a constant level under different concentrations of calcium in the culture solutions compared with the cells in the zone apart from the root apex. The amount of crystals in crystal idioblasts increased when the calcium concentration in the culture solution was high (Fig. 2, 3, 5). Additionally, it was confirmed by EDS that crystal idioblasts containing the crystals had a markedly higher content of calcium than cortex parenchyma cells in the root apical zones (Fig. 7) and calcium content in crystal idioblasts increased with increasing calcium concentration in the culture solution (Fig. 6). Therefore, it was suggested that calcium was accumulated in crystals under calcium-deficient conditions and released from crystals under a calcium-deficient condition for the stabilization of calcium concentration level in cortex parenchyma cells in the root apical zones.

This study indicated a strong possibility that the crystals in crystal idioblasts tubularly arranged in the apical zone of roots participated in the regulation of calcium levels in the cortex parenchyma cells of root apical zones in eddo. The findings obtained in this study will be beneficial for understanding the overall mechanism related with the regulation of calcium levels in eddo roots and for developing strategies to improve the adaptation to soil environment, the growth and eating quality of eddo.

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