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Localization of Aluminum in Root Tip Tissues of Wheat Varieties Differing in Aluminum Tolerance

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The purpose of this study was to investigate the mechanisms of Al tolerance in wheat varieties by the direct observation of Al distribution in root cells using the hematoxylin stain method. Five-day-old seedlings of Al-tolerant varieties, Atlas 66 and Shirosanjyaku, and an Al-sensitive variety, Chikushikomugi, were treated with 150 μM Al in 0.4 mM $CaCl_2$, pH 4.5 for 24 hours under aseptic conditions; afterwards the roots were stained with hematoxylin. Comparison of hematoxylin stain of root tips among the varieties showed that the root cap of Al-sensitive seedlings was stained intensively, reflecting high accumulation of Al in this region. The roots developed symptoms of Al toxicity such as increased vacuolation, and swelling and rapture of the cells of the epidermis. On the other hand, in Al-tolerant varieties the central cap cells and the cortical region of the meristem and elongation zone were not stained. The site of the earliest Al accumulation in Al-tolerant varieties seemed to be the edge cells of the root cap. In the elongation zone, Al accumulated mainly in the cell wall and nuclei. Those differences in the staining pattern were probably due to immobilization of Al in the cytosol that could not react with hematoxylin and/or due to the ability of Al-tolerant varieties to better exclude Al from cytoplasm.

INTRODUCTION

To elucidate the mechanisms of Al toxicity in plants, the characterization of the primary site for Al uptake in the roots is of primary importance. In order to localize Al in root tissues, various methods have been used, from the use of Al dyes (hematoxylin, aluminon, morin) to such sophisticated techniques as X-ray microanalysis (Ownby, 1993; Naidoo et al ., 1978) or secondary-ion mass specrtrometry (Lazof *et al.*, 1994). The hematoxylin stain method (Polle et *al.*, 1978) is of very popular use in evaluation of Al tolerance in plants (Takagi et al., 1981; Wallace and Anderson, 1984). The method is based on the colorimetric property of hematoxylin to give a blue-purple stain when it is complexed with Al (Baker, 1962; Rincon and Gonzalez, 1992). With this technique it has been possible to indicate the differences in Al tolerance among wheat varieties (Takagi et al., 1981; Wheeler *et al.*, 1992a) and histochemical localization of Al in root surface and the cell wall and nuclei of root cells.

The aim of the present study was to investigate the mechanisms of Al tolerance in wheat varieties differing in Al tolerance by the direct observation of distribution of Al in root tissues and cells by using the hematoxylin method.

MATERIALS AND METHODS

Seedlings of Al-tolerant varieties, Atlas 66 (AT) and Shirosanjyaku (SH), and an Al-

sensitive variety, Chikushikomugi (CK), of wheat (*Triticum aestivum* L.) were grown aseptically. The seeds were disinfected by soaking in NaClO solution (1.5% active chlorine) for 90 minutes, rinsed several times with sterile water, and then germinated on moistened paper in sterile Petri dishes in the dark for 48 hours. Twenty seedlings were transferred onto a stainless-steel screen in a test tube (20 cm in length, 4 cm in diameter) over 50 mℓ of sterile nutrient solution (pH 4.5) containing macronutrients (mM): 0.50 NH,', 1.0 NO_3^-, 0.50 Ca^{2+}, 0.50 K^+, 0.30 Mg^{2+}, 0.40 Cl-, 0.40 SO_4^{2-}, 0.30 Na^+, 0.0025 PO_4^{3-}, and micronutrients (μM): 0.91 Mn^{2+}, 0.03 Cu^{2+}, 4.63 BO_3^{3-}, 0.08 Zn^{2+}, 3.2 Fe^{3+} (Na-EDTA). The tubes were placed on a rotary shaker (120 rpm) at 20 °C for 4 days in a growth chamber with light intensity of 150 μmol $m^{-2}s^{-1}$ and a photoperiod of 12 hours. On the fifth day, after the solution was aspirated from the tubes the seedlings were treated with 150 μM Al ($AlCl_3$ or $Al_2(SO_4)_3$ + 0.4 mM $CaCl_2$) and without Al (0.4 mM $CaCl_2$) at pH 4.5 for 24 hours. Each tube contained 40 mℓ of the treatment solution. Al was added after Al stock solution was passed through 0.45 μm membrane filter.

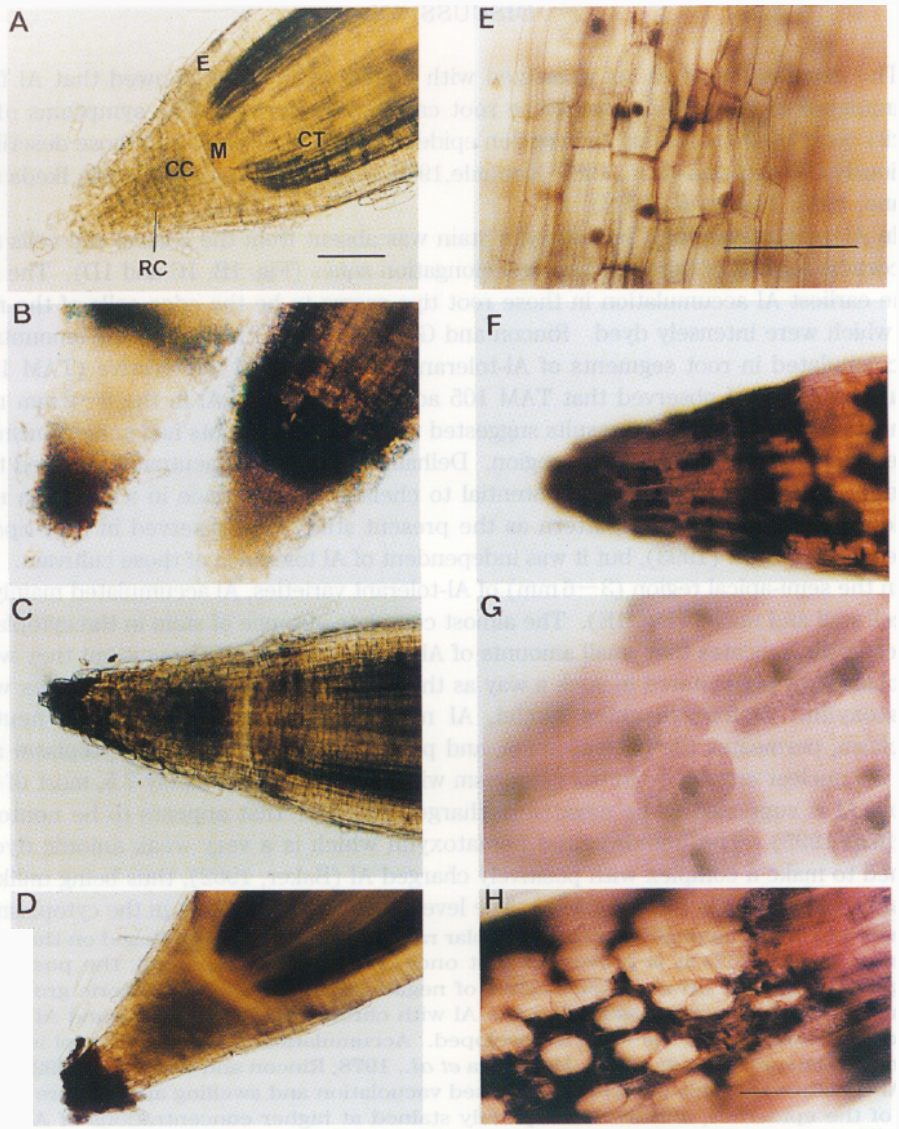
After exposure to Al, the seedlings were stained in 50 mℓ of hematoxylin solution (2 g hematoxylin (Nacalai Tesque, Kyoto) and 0.2 g $NaIO_3$ in 1 liter of deionized water) for 20 minutes according to the method of Polle *et al. (1978).* Thereafter, the seedlings were rinsed in running water for 1 minute and soaked in 50 mℓ of deionized water, which was changed 3 times at each interval of 20 minutes. The terminal 2 — 3 cm segments of roots were excised and root apices were mounted on glass slides and examined with a Nikon Optiphoto light microscope. Photographs were taken with a Nikon FX-35 WA camera by using a Nikon HFX-IIA system automatic exposure unit.

RESULTS

A root tip of AT that was not exposed to Al but stained with hematoxylin is shown in Fig. 1A. The root showed lack of staining on the root cap and epidermis. The pattern of the hematoxylin staining of the roots treated with Al showed that Al accumulated mainly in the apical region (root cap and meristem) in both Al-tolerant AT and Al-sensitive CK. Nevertheless, clear differences in the staining intensity were observed between tolerant and sensitive root tips (Fig. 1B), which is in agreement with results of other varieties in previous reports (Polle *et al.*, 1978; Wheeler *et al.*, 1992a; Rincon and Gonzalez, 1992; Ownby, 1993).

The primary site for accumulation in roots of Al-tolerant SH and AT appeared to be the edge cells (sloughed-off cells) of the root cap that were intensely dyed (Fig. 1C and 1D). No staining was observed in the central cells of the root cap and the cells of meristem zone were only slightly stained. In the cortical and epidermal cells of semi-apical region of root tips (4 — 6 mm), Al accumulated mainly in the cell walls, intercellular spaces, and nuclei (Fig. 1E).

In Al-sensitive CK, root tips was intensively stained in all the experiments, showing a deeper staining pattern in the cells of the root cap (Fig. 1F). The meristematic cells were swollen and extensively vacuolated (Fig. 1G and 1H).



Photographs of hematoxylin staining of Al-treated and Al-untreated roots of wheat varieties differ

Fig. 1. ing in aluminum tolerance **Plate A** An **apex** of Atlas 66 root unexposed to Al. Bar.0 lmm Symbols: CC:central cap cells, CT:cortex, E:epidermis, M:meristem, RC:root cap. **Plate B** Apices of Shirosanjyaku (left) and Chikushikomugi (right) roots treated with 75 μM Al (SO,)$_3$ for 24 hours. Magnification is the same as in Plate A. **Plate C An** apex of Shirosanjyaku root treated with 75 μM $Al_2(SO_4)_3$ for 24 hours. Magnification is the same as in Plate A **Plate D An** apex of Atlas 66 root treated with 75 μM Al_2(SO,) for 24 hours. Magnification is the same as in Plate A. **Plate E** Semi-apical region of the tip of Shirosanjyaku root treated with 150 μM $AlCl_3$ for 24 hours. Bar:50 μm. **Plate F** An apex of Chikushikomugi root treated with 150 μM $AlCl_3$ for 24 hours. Magnification is the same as in Plate A. **Plate G** Cells of the root cap of Chikushikomugi root treated with 150 μM $AlCl_3$ for 24 hours. Magnification is the same as in Plate E. **Plate H Cells** of the meristematic region of Chikushikomugi root treated with 150 μM $AlCl_3$ for 24 hours Bar:100 μm.

DISCUSSION

The distribution of Al, as visualized with hematoxylin stain, showed that Al first accumulated in the outer cells of the root cap and epidermis. The symptoms of Al toxicity such as tissue lesions and swollen epidermal cells were similar to those described previously (Wagatsuma *et al.*, 1987; Kinraide, 1988; Rincon and Gonzalez, 1992; Ikeda and Tadano, 1993; Ryan *et al.*, 1993).

In Al-tolerant varieties, hematoxylin stain was absent from the central cap cells and the cortical regions of the meristem and elongation zones (Fig. 1B, 1C and ID). The site of the earliest Al accumulation in those root tips seems to be the edge cells of the root cap, which were intensely dyed. Rincon and Gonzalez (1992) measured the amounts of Al accumulated in root segments of Al-tolerant (Atlas 66) and Al-sensitive (TAM 105) wheat varieties and observed that TAM 105 accumulated more Al in the 0 – 2 mm root segments than Atlas 66. The results suggested that Al-tolerant roots had a mechanism to prevent accumulation of Al in this region. Delhaize *et al.* (1993) acutually reported that excretion of organic acids having potential to chelate Al took place in a l-3 mm root portion. The same staining pattern as the present study was observed in root tips of maize by Ryan *et al.* (1993), but it was independent of Al tolerance of those cultivars.

In the semi-apical region (3 – 6 mm) of Al-tolerant varieties, Al accumulated mainly in the cell wall and nuclei (Fig. 1E). The almost complete absence of stain in the cytoplasm of those cells indicates that small amounts of Al reached this compartment but they were immobilized or precipitated in such a way as they were not able to make a complex with hematoxylin. Before reaching nuclei, Al necessarily passes through the neutral cytoplasm, permeates the plasmalemma, and passes through the neutral cytoplasm and then the nuclear envelope. In the cytoplasm with a pH of approximately 7.5, most of the soluble Al is supposed to be negatively charged $Al(OH)_4^-$ that appears to be nontoxic (Kochian 1995). The unmordanted hematoxylin which is a very weak anionic dye is inclined to make a complex with positively charged Al (Baker, 1962), thus being unlikely to stain the $Al(OH)_4^-$ in the cytoplasm. The level of Al^{3+} that may exist in the cytoplasm is estimated to be in the picomolar to nanomolar ranges (Kochian 1995). Based on the work of Baker (1960;1962) it is presumed that once Al enters into the cell, the positively charged Al might be driven towards sites of negatively charges of phosphoric groups of the nucleic acid molecule, associating the Al with chromatin. Then, the bound Al might attract hematoxylin and the color is developed. Accumulation of Al in the nuclei is often reported (Matsumoto *et al.*, 1976; Morimura et al., 1978; Rincon and Gonzalez, 1992).

Al-sensitive root tips presented increased vacuolation and swelling and rupture of the cells of the epidermis, and were completely stained at higher concentrations of Al (Fig. 1F, 1G and 1H). These symptoms are associated with Al toxicity (Wheeler *et al.*, 1992b; de Lima and Copeland, 1994; Marienfeld et *al.*, 1995). Although vacuolation is commonly observed in cells intoxicated by Al, the reason for the increased vacuolation was probably not a direct effect of Al but an effect of growth retardation.

The complete staining of the root apices of Al-sensitive CK reflects high accumulation of Al in this portion. This pattern of hematoxylin staining could result from solid phase precipitation of Al at high pH's or due to Al fixed in root tissues as $AlPO_4$ as suggested by Ownby (1993). In fact the Al-hematoxylin complex precipitates at pH's higher than 4.8.

Ownby (1993) demonstrated by using X-ray microanalysis that Al and P were co-localized in the cell wall regions of the outer cortex of Al-stressed roots which is in accordance with early observations of Clarkson (1967) and Naidoo *et al.* (1978). This solid phase Al-P might be able to react with hematoxylin. Based on those results, Ownby (1993) postulated that cultivars whose root cells were damaged by Al allowed cellular phosphate to leak into the cell wall region and precipitate as $AlPO_4$ and consequently were intensely stained with hematoxylin. This hypothesis seems to be plausible because it is unlikely that Al-hematoxylin complex would be formed under the neutral cytoplasmic pH conditions as described above. Moreover, Al-sensitive CK generally showed greater damage in the tissue and it would facilitate the leakage of P from the cells.

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