Exogenously applied RbCl revealed the role of potassium in the regulation of directional cell growth in the primary root of maize (*Zea mays*)

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Abstract

Rubidium was applied to the maize root system as a potassium antagonist. Exogenously applied RbCl dramatically decreased primary root length and caused radial expansion. However, the decrease in fresh weight was not prominent. The endogenous level of potassium decreased accompanying accumulation of rubidium following RbCl treatment. The altered root growth caused by RbCl was restored by KCl, suggesting that potassium is required for directional regulation of root cell growth. Microscopic observations of cells in the elongation zone of control and RbCl-treated roots supported this idea. Gravitropic curvature, which is dependent on fine regulation of differential cell elongation, was also impaired in RbCl-treated roots, indicating that potassium is not simply involved in cell elongation itself, but is necessary for directional regulation of cell growth.

Keywords: root elongation, radial expansion, potassium, rubidium, maize (*Zea mays*)

Introduction

Rubidium and potassium are located in the same column of the periodic table of elements and their size and chemical properties are similar. $^{86}Rb^+$ has been used to trace cellular uptake of K^+ in plants (El-Sheikh et al, 1971; Hong and Sucoff, 1976; Drobner and Tyler, 1998; Amrutha et al, 2007), because Rb^+ and K^+ have similar membrane transport kinetic properties and the half life (18.6 d) of $^{86}Rb^+$ is longer than that (12.5 h) of $^{42}K^+$ (Läuchli and Epstein, 1970).

The very similar membrane transport characteristics of Rb^+ and K^+ suggest similar binding activities of Rb^+ to K^+ binding sites of any potassium-binding protein. Because potassium binding to proteins is important not only for ion transport, but also for physiological function, whether rubidium can replace potassium is an interesting question.

A few case studies have reported the effects of rubidium on plant growth. Rubidium is slightly beneficial for sugar beet plant growth when supplied in small doses (El-Sheikh et al, 1967; El-Sheikh and Ulrich, 1970). However, applying rubidium in a plant nutrient solution results in reduced dry matter accumulation and inhibits internode elongation and epinasty in tomato (Maynard and Baker, 1965; Berry and Smith, 1969). Loss of chlorophyll and a decrease in dry weight, particularly in leaves, have been observed in bean plants (Valdez-Aguilar and Reed, 2008). Applying rubidium to the monocot barley changes leaf color to dark green and characteristically stunts and thickens crown roots (Hurd-Karrer, 1939).

Because K^+ and Rb^+ uptake is mutually competitive (El-Sheikh et al, 1971; Drobner and Tyler, 1998), apparent rubidium toxicity is likely to be due to potassium deficiency. Restoration of the stunted and thickened root morphology of RbCl-treated barley roots (Hurd-Karrer, 1939) supports this idea, suggesting that rubidium can be used as an inhibitor of potassium-related physiology.

The maize root system consists of a primary-, two or more lateral-seminal-, and many crown roots (Hochholdinger et al, 2004) and secondary lateral roots develop on each root type. The role of potassium in the regulation of early root growth within several days after imbibition remains unknown. Omitting nutrients from the growth medium during such a short period does not result in any deleterious effects on early root growth. This is because the maize kernel probably contains sufficient nutrients including potassium to support plant growth for several days. Therefore, applying a potassium antagonist could reveal the role of potassium in the regulation of early root growth.

In this study, RbCl was used as an inhibitor of potassium-related physiological activities to investigate the role of potassium in the regulation of early growth in the primary roots of maize (*Zea mays*). First, the effects of RbCl on maize primary root growth and its restoration by KCl were investigated to evaluate using rubidium as a potassium antagonist. Then, detailed changes were scrutinized and their implications on the biological role of potassium in the directional regulation of cell growth in the elongation zone of maize primary roots are discussed.

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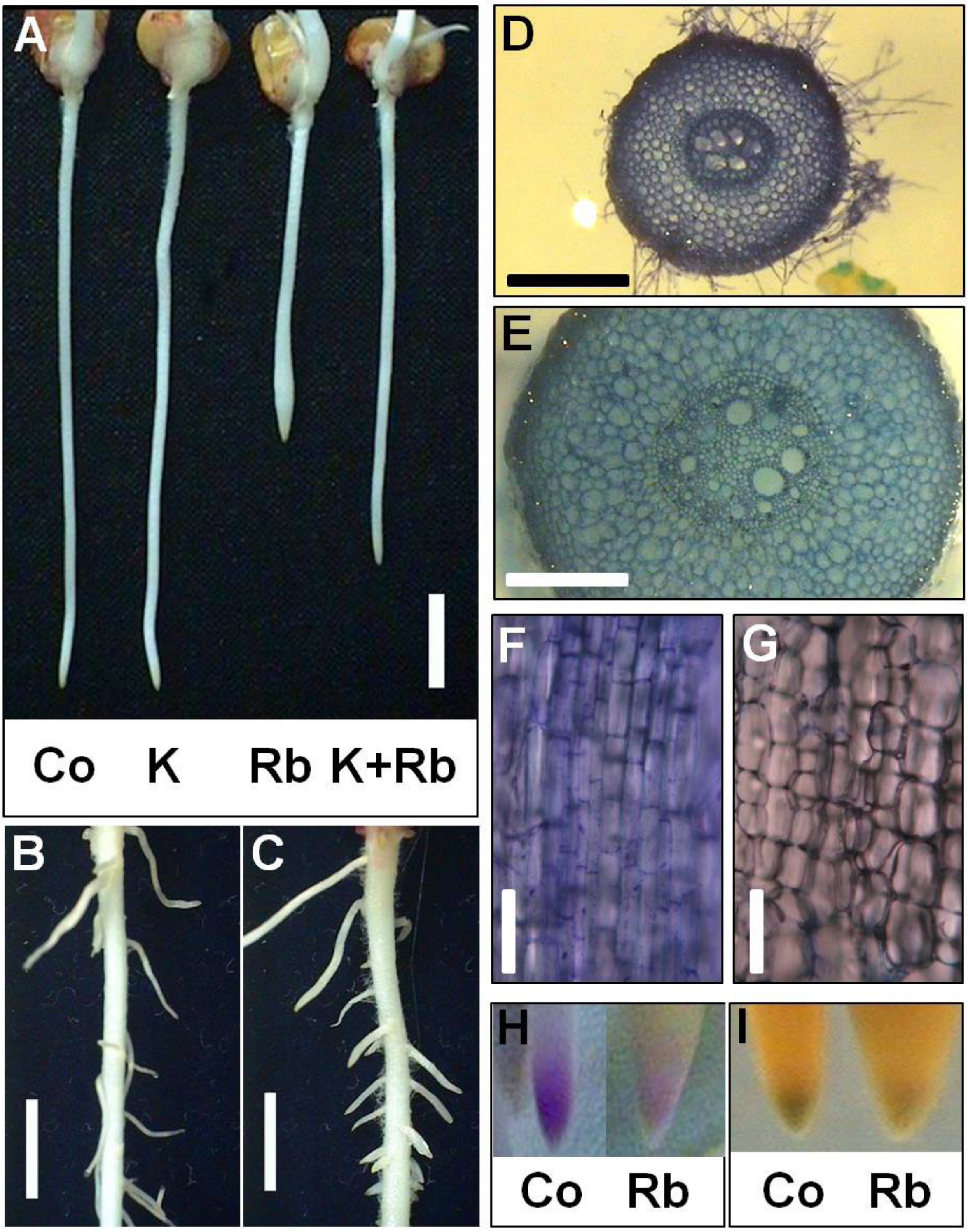

Figure 1 - Effects of RbCl and KCl on maize root growth. (A) Shape of control roots (Co), roots treated with KCl (K), with RbCl (Rb) and with both KCl and RbCl (K+Rb). (B) Lateral roots of control plants. (C) Lateral roots of RbCl-treated plants. (D) Cross-section of the elongation zone of a control root. (E) Cross section of a RbCl-treated root. (F) Longitudinal section of a control root. (G) Longitudinal section of an RbCl-treated root. (H) Visualized apical meristem by Feulgen staining in control (Co) and RbCl-treated (Rb) roots. (I) Starch granules in the root caps revealed by Lugol's iodine staining in control and RbCl-treated roots. The RbCl and KCl concentrations were 20 mM and 60 mM, respectively. Bars indicate 1 mm (A), 5 mm (B), 0.5 mm (D and E) or 0.1 mm (F and G). Plants were 2-d- (A, H, I) or 3-d-old (B - G). Co: control; K: 60 mM KCl; Rb: 20 mM RbCl.

Materials and Methods

Plant materials

Maize (*Zea mays* cv. Golden Cross Bantam 70) seeds were washed in distilled water and soaked for 24 h on a shaker (30 cycles/min). The imbibed seeds were placed on a double layered sheet of wet paper towels (79 cm x 7.5 cm) in a row 1 cm below the edge of the wide side. Then the sheet was rolled up and set in a 100 ml-beaker filled with water and/or other solutions as described in the "paper roll system" for maize root growth (Hetz et al, 1996). The plants prepared in paper rolls were grown in a growth chamber at 26°C for 2, 3, or 5 d under a 16 h day: 8 h night photoperiod.

Preparation of chemicals

RbCl, KCl, and NaCl were prepared as 2 M stock solutions in distilled water and diluted to final concentrations. Silver thiosulfate (STS) was prepared by mixing 100 mM $AgNO_3$ with 400 mM NaS_2O_3 (Cramer, 1992).

Observations and root growth measurement

Primary root length was measured in mm with a ruler, and diameter was measured with the ITPro image analyzing program (Sometech, Seoul, Korea) after capturing digital photos using an image-capturing system with a magnifying lens (icamscope, Sometech). The meristem was observed with the image analyzing system after Feulgen staining (Hoecker et al, 2006). Isolated primary root tips were fixed in 70% ethanol for 2 h and then incubated in 1 N HCl at 60°C for 15 min. The samples were then incubated in Schiff's reagent (Merck, Summit, NJ, USA) in the dark for about 30 min until a reddish color appeared. Starch granules at the root cap were stained with Lugol's iodine solution, which was a mixture of 5% iodine and 10% potassium iodide (Vandenbussche et al, 2011).

Freehand sectioning and microscopic observation

Cross-sections and longitudinal sections of the maize primary root elongation zone were obtained by freehand sectioning with a sharp razor blade. The sections were stained with 0.1% Toluidine Blue O for 1 min and washed with distilled water. Then, the cut surface was observed with an image capturing system (icamscope, Sometech) and with a light microscope (Axiostar Plus, Zeiss, Oberkochen, Germany).

Measurement of gravitropic curvature

Maize seedlings were grown for 2 d in the presence or absence of substances to be tested, e.g., RbCl. Then the seedlings were fixed between the bottom and cover plates of square dishes. Discs of Styrofoam cushions were set on both the bottom- and cover sides to provide mild pressure and effectively fix the kernels. The seedlings were then fixed between the Styrofoam cushions in the vertical direction in which the roots were located. A sheet of paper towel was rolled up, wet with distilled water, and placed on the side of the square dish facing the roots

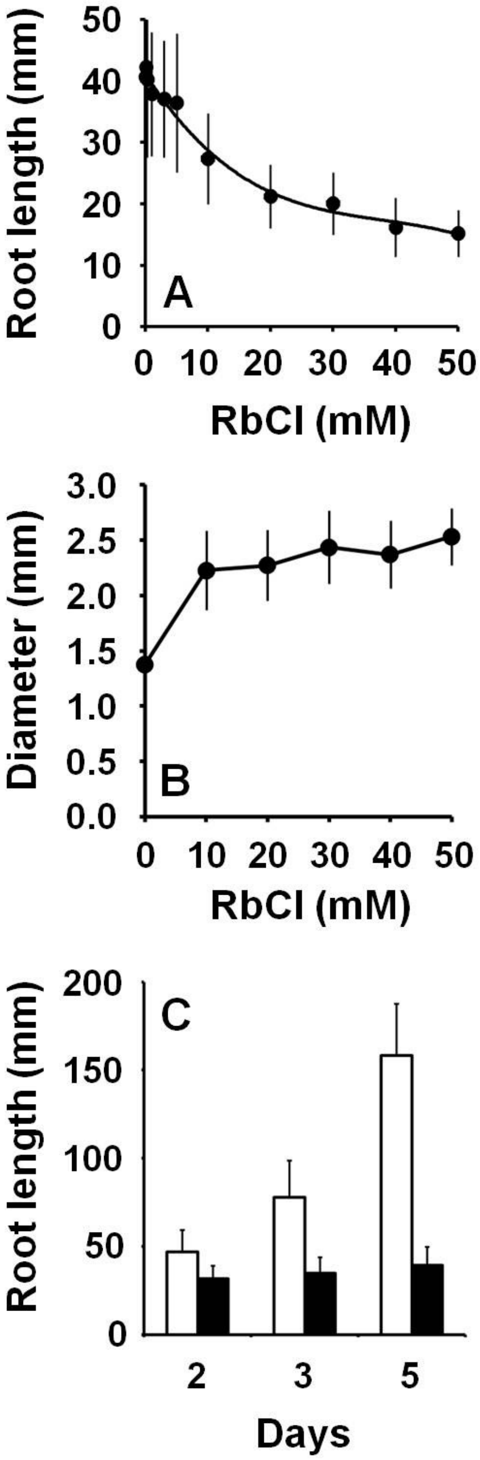

Figure 2 - Effects of RbCl on primary root growth in maize. Changes in elongation (A) and diameter (B) following the RbCl concentration gradient in 2-d-old plants and root growth (C) at 2, 3, and 5 d in the absence (white column) and presence of 20 mM RbCl (black column) are presented. Diameter was measured under an image analyzing system with digital records. Vertical bars indicate standard deviations.

to saturate relative humidity and neutralize the effect of hydrotropism (Miyazawa and Takahashi, 2007). After the square dishes were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA), the dishes were turned to a horizontal direction of the maize root, and images were captured with a digital image-recording system (icamscope, Sometech). Gravitropic curvature was recorded every 2 h, and the angle was measured with image analysis software (ITPro, Sometech).

Ion quantification

Whole primary roots were ground in de-ionized water (50 ml/ g fresh weight) and filtered through Miracloth (Calbiochem, La Jolla, CA, USA). The flowthrough was then cleared by centrifugation (29,900 g x 30 min, at 4°C) and the supernatant was collected. A 5 ml aliquot of the supernatant was mixed with 5 ml of concentrated nitric acid and incubated overnight. Then, 15 ml of 2% nitric acid was added. K^+, Rb^+, Na^+, Mg^{2+}, Mn^{2+}, and Ca^{2+} contents were determined by inductively coupled mass spectrometry (ICP-MS; ELAN DRE-e, Perkin-Elmer, Waltham, MA, USA) at the Joint-Machinery Center of Dankook University.

Results

RbCl reduced cell elongation but increased radial expansion in maize primary root

The length of the primary root decreased when maize seedlings were grown with RbCl (Figure 1A). The growth inhibition was concentration-dependant (Figure 2A) and reduced to 40% (15.2 ± 3.8 mm) of the control (40.7 ± 10.9 mm) in the presence of 50 mM RbCl. Radial expansion was observed in the same samples and was most prominent in the region above the root apical meristem (Figure 1A), which corresponds to the elongation zone (Ishikawa and Evans, 1995) in control roots. RbCl increased primary root diameter up to 184% (2.532 ± 0.255 mm) of that of the control (1.376 ± 0.231 mm) in the presence of 50 mM RbCl (Figure 2B). Elongation of RbCl-treated roots was strongly inhibited (Figure 2C); on day 2, lengths of control and RbCl-treated roots were 46.9 ± 12.5 mm and 31.7 ± 7.3 mm, respectively, and they were 158.4 ± 29.2 and 39.3 ± 10.4 on day 5, respectively.

Cross-sections showed more radial expansion in the RbCl-treated root cells (Figure 1E) compared to that in control roots (Figure 1D). Radial expansion was prominent in the cortex, and such cell expansion was also observed in the stele. Longitudinal sectioning revealed that cortical cells in the elongation zone of RbCl-treated roots were expanded radially (Figure 1G). In contrast, the corresponding cells in control roots started to elongate rather than expand radially (Figure 1F).

No clear differences were observed except the direction of cell growth. The size of the root apical meristem (Figure 1H) and the pattern of starch staining in the root cap were similar (Figure 1I). The direction of the lateral roots in the control and RbCl-treated plants appeared similarly plagiogravitropic (Figure 1B and 1C).

RbCl was not affected by the ethylene action inhibitor STS

Treatment with 4 mM STS did not restore RbCl-inhibited root growth (Figure 3), although stunted root growth and increased radial expansion were very similar to typical ethylene responses (Taiz and Zeiger, 2006). The length of control roots decreased from 58.0 ± 9.1 mm to 28.9 ± 6.8 mm following treatment with 20 mM RbCl. Root length was 30.2 ± 8.2 mm when 4 mM STS was applied with 20 mM RbCl, but no difference (p = 0.64) was observed from roots treated with RbCl alone.

RbCl decreased K+ content in the maize primary root

Water content in the control and 20 mM RbCl-treated roots was measured by comparing fresh and dry weights after drying for 3 days at 60°C to estimate ion concentrations. Water contents were 92.0 ± 0.4% and 91.7 ± 0.3%, respectively, of the control and 20 mM RbCl-treated roots (P = 0.17, n = 9, each measurement contained 8 - 10 roots).

The K^+ concentration in the maize primary root was determined by ICP-MS in the absence and presence of 20 mM RbCl (Table 1). K^+ level in the roots treated with 20 mM RbCl was only 40% (12.0 ± 3.2 mM) of that of the control (30.1 ± 3.2 mM). Rb^+ concentration in the 20 mM RbCl-treated sample reached 39.5 ± 2.6 mM, indicating a 97% increase in Rb^+ accumulation over the applied concentration. NaCl level also decreased to some extent, although with marginal significance (p = 0.0493). The levels of Mg^{2+}, Mn^{2+}, and Ca^{2+} were unchanged by RbCl.

KCl specifically counteracted the RbCl effects

The effect of KCl on RbCl-inhibited root growth

Table 1 - Concentrations (mM) of ion species in the primary root of control and 20 mM RbCl-treated maize seedlings. Data are from replicated tests including 8 - 10 roots each. Data are mean ± standard deviation.

Ions	Control	+RbCl	t-test
K^+	30.1 ± 3.2	12.0 ± 3.2	P = 0.000002 (n = 6)
Rb^+	0.3 ± 0.1	39.5 ± 2.6	P = 0.000055 (n = 4)
Na^+	4.8 ± 2.4	2.2 ± 1.3	P = 0.0493 (n = 6)
Mg^{2+}	1.7 ± 0.3	2.1 ± 0.4	P = 0.0705 (n = 6)
Mn^{2+}	0.0 ± 0.0	0.0 ± 0.0	P = 0.0876 (n = 6)
Ca^{2+}	4.3 ± 4.9	6.7 ± 7.6	P = 0.5207 (n = 6)

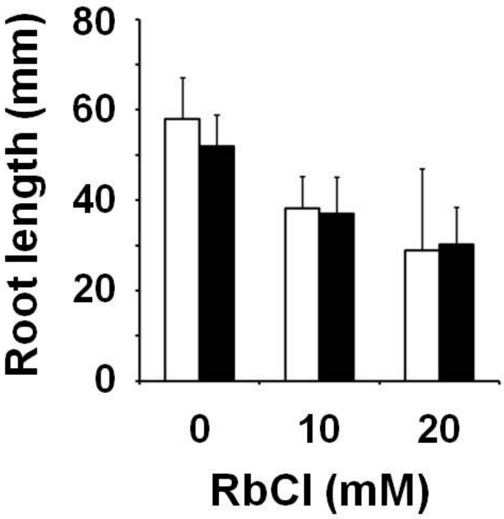

Figure 3 - RbCl-inhibited root growth after 2 d in the absence (white columns) and presence (black columns) of 4 mM silver thiosulfate (STS), an ethylene action inhibitor. STS was applied to the growth medium. Vertical bars indicate standard deviations.

was examined, because KCl tissue level decreased in the presence of RbCl. When KCl was included in the growth medium, the inhibited growth of the primary root treated with 20 mM RbCl was restored based on the KCl concentrations (10 - 60 mM) and reached 77% of the control level (Figure 4A). The increased diameter of RbCl-treated roots was also restored with increasing KCl concentration (Figure 4B). The diameter of RbCl-treated roots was 178% of the control level, but it decreased to 113.5% in the presence of 60 mM KCl.

Such a restoration of RbCl-affected root growth was not observed with NaCl (Figure 5D). Both KCl (60 mM) and NaCl (60 mM) slightly inhibited root growth when they were applied to control roots (Figure 5A and 5D).

RbCl did not affect primary root fresh weight

RbCl (20 mM) reduced root length to 43% of that in control roots. (Figure 5A). However, primary root fresh weight in the presence of 20 mM RbCl was 83% of the control level (Figure 5B); the fresh weight of the roots treated with 20 mM RbCl was 65.9 ± 13.6 and that of the control roots was 76.6 ± 25.4 mg (p = 0.04).

Growth was not inhibited in the shoot system by RbCl under the test conditions used (Figure 5C).

RbCl-treated roots showed reduced gravitropic curvature.

The effects of KCl and RbCl on gravitropic curvature were tested to assess the hindrance in regulated cell elongation by RbCl. When plants were horizontally positioned, the primary root direction was defined as 180°, and the direction of gravity was marked as 90° (Figure 6). Root direction was recorded at 2, 4 and 6 h. The control root curved continuously to the ground and reached 122.7 ± 14.0° after 6 h (Figure 6). The final direction was 137.5 ± 17.3° when the roots were treated with 20 mM RbCl. This reduced gravitropic curvature was restored to 116.8 ± 13.2° by 60 mM KCl. KCl itself did not affect gravitropism (119.6 ± 11.1; p = 0.271).

Discussion

Data on the interaction between RbCl and KCl indicated that the RbCl effects observed were due to inhibiting potassium-related physiological activities in the regulation of early growth in maize. Exogenously applied RbCl inhibited primary root growth in maize (Figure 1 and 2), and the RbCl-inhibited growth was restored by KCl (Figure 4A and 5A), but not by NaCl (Figure 5D), indicating that the restoring effect was

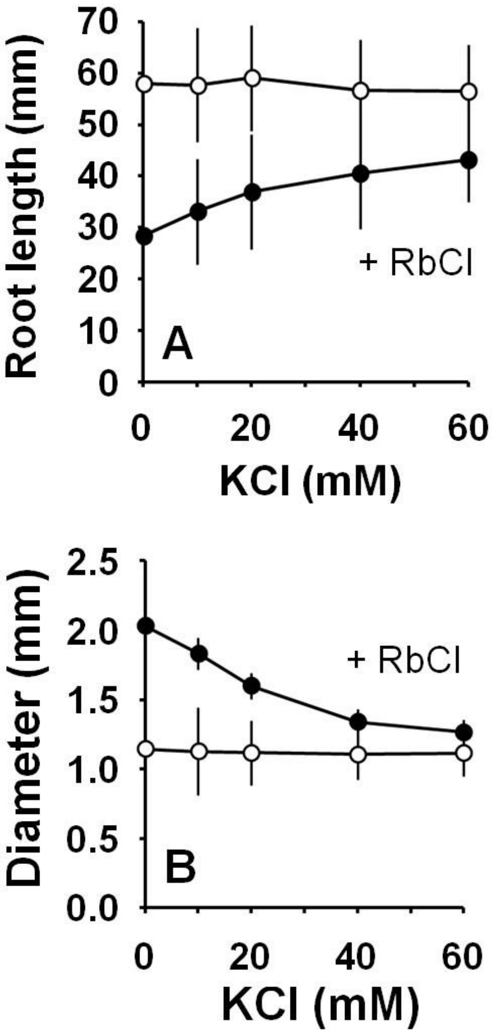

Figure 4 - Restoration of 20 mM RbCl-affected root growth of 2-d-old plants by KCl. Effects of KCl gradient on root length (A) and diameter (B) are shown. Vertical bars indicate standard deviations.

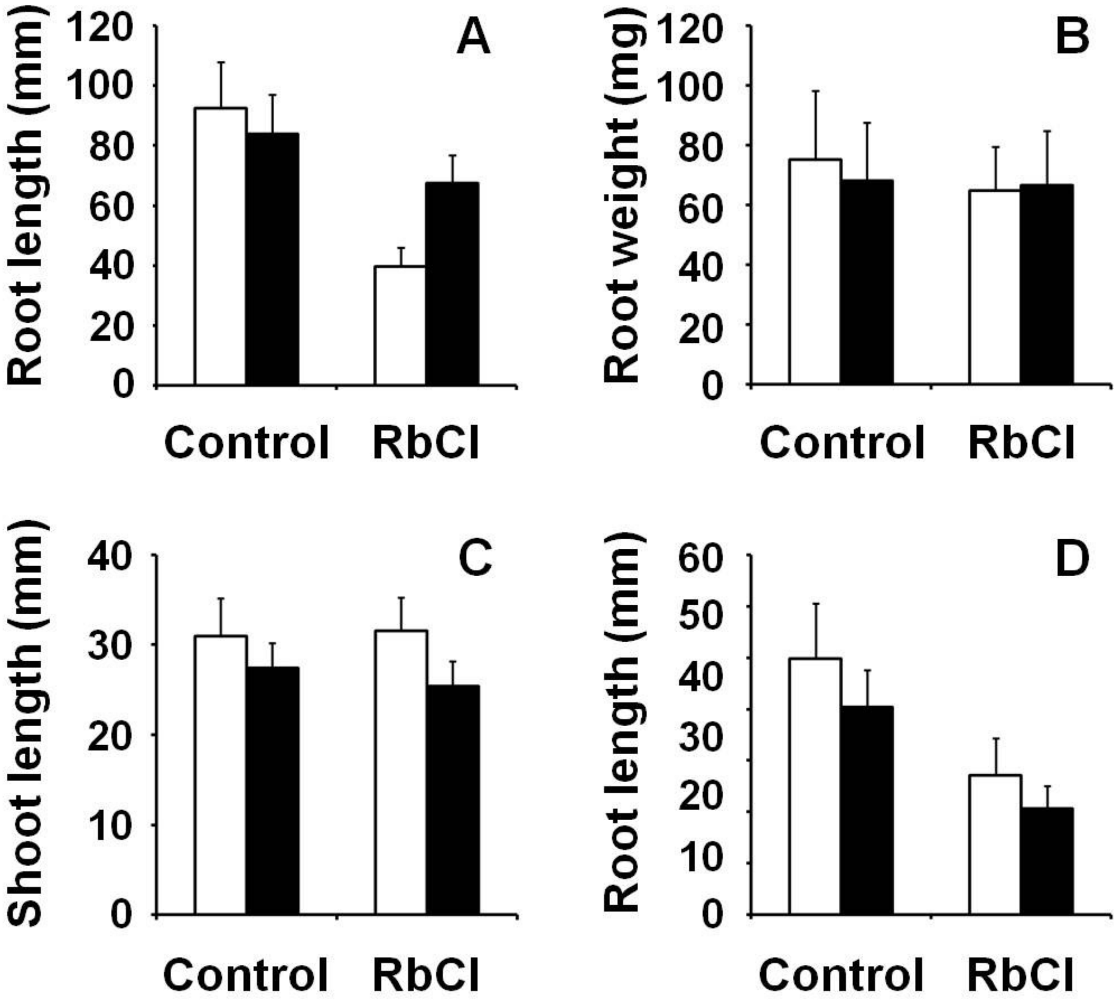

Figure 5 - Influence of KCl and NaCl on the effect of RbCl in 3-d-old plants. Effects of 20 mM RbCl and 60 mM KCl on the length (A) and fresh weight (B) of the maize primary root and on shoot length (C) are shown. Effects of 20 mM RbCl and 60 mM NaCl on root growth are presented in D. Vertical bars indicate standard deviations. Black columns indicate the results obtained with 60 mM KCl (A, B, C) or 60 mM NaCl (D).

potassium specific. This observation suggests that the inhibitory effect of Rb^+ was due to hindrance of action or uptake of K^+.

Quantification of ions by ICP-MS analysis showed a reduced level of K^+ in RbCl-treated roots (Table 1) and supported the idea that inhibited root growth was the result of a potassium deficiency, at least in part. This observation does not necessarily mean that Rb^+ does not affect K^+ action. The level of Rb^+ in the RbCl-treated root was 197.5% of that in the growth medium, indicating considerable Rb^+ accumulation. Therefore, Rb^+ seemed not only to disturb K^+ uptake, but also to replace it in the maize primary root.

The K^+ concentration in the control root was lower than that of an earlier report (see Table I in Mengel and Pflüger, 1972). They reported that K^+ content is about 100 mM, whereas I estimated it to be 30.1 ± 3.2 mM. One possibility for the discrepancy is that only a portion of K^+ was measured in this study due to insufficient yield. Another possibility is that the different growth conditions may have caused differences in K^+ contents. No nutrients were applied to the growth medium in this study, whereas Mengel and Pflüger (1972) used a nutrient solution to grow maize.

The level of Na^+ decreased, although with only marginal significance ($P = 0.0493$). The reason for this change is unclear. However, secondary effects of shifted equilibria of certain ions on other ions could occur (Hopkins and Hüner, 2009)

Because Rb^+ and K^+ have very similar membrane transport properties, Rb^+ has been suggested to compete with K^+ for cellular uptake (El-Sheikh et al, 1971). When the growth inhibitory effects of RbCl in the presence and absence of KCl were expressed as relative values to the control (% inhibition) and analyzed by applying double-reciprocal analysis (Supple-

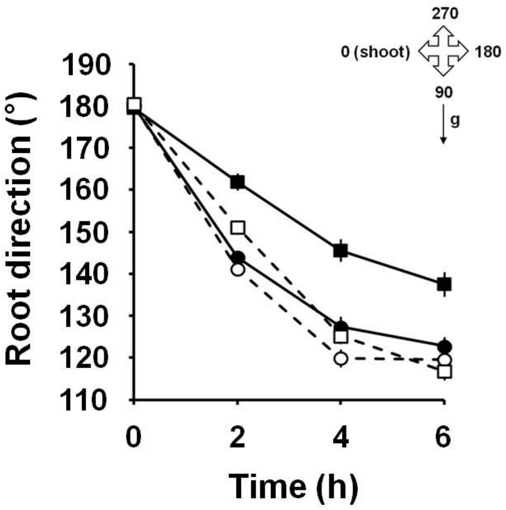

Figure 6 - Gravitropic curvature of the primary root of 2-d-old maize. Directions of the roots without treatment (black circles), in the presence of 20 mM RbCl (black squares), 60 mM KCl (white circles) and both RbCl and KCl (white squares) are presented. Initial horizontal direction is defined as 180° and the direction of gravity (g) is marked as 90°. Time-lapse images were used to measure root angle with an Image analyzing system. Vertical bars indicate standard deviations.

mentary Figure 1), the shape of the plot was mixed-type but was very close to the competitive inhibition shape, suggesting that the observed reversibility of RbCl and KCl depended on a competitive mechanism such as K^+ uptake at least in part. The slight inhibitory effect of 60 mM KCl (Figure 5A) on primary root growth might change the shape of the plot from the classical competitive type. Although KCl showed a slight growth inhibitory effect at 60 mM due to weak salt stress, the 60 mM concentration was the best compromised level for restoring the Rb^+-inhibited growth, but with minimal stress under the experimental conditions.

The effects of RbCl were concentrated in the maize root system (Figure 5A) and did not affect leaves (Figure 5C) in our test system with a relatively short-term incubation period (2 - 3 d), showing some differences with previous reports (Maynard and Baker, 1965; Berry and Smith, 1969; Valdez-Aguilar and Reed, 2008). This specificity, together with the reversibility of KCl as described above, provided a good test system to investigate the immediate effect of RbCl on root development.

The RbCl treatment induced morphological changes expected in ethylene-treated plants (Whalen and Feldman, 1986; Taiz and Zeiger, 2006). Ethylene inhibits root elongation in maize (Whalen and Feldman, 1986). However, STS, an ethylene action inhibitor, did not restore hindered root growth caused by RbCl (Figure 3), suggesting that the effect of RbCl is rather more direct than secondary via the action of ethylene.

K^+ is unlikely to be simply involved in cell elongation itself but to be engaged in directional regulation of cell growth, although K^+ is a necessary factor for auxin and fusicoccin-induced cell elongation in maize coleoptiles (Claussen et al, 1997; Tode and Lüthen, 2001). The idea of directional regulation is supported by the observation that maize roots, whose elongation was inhibited by RbCl (Figure 5A), had almost the same fresh weight as that of control roots (Figure 5B). Considering that RbCl decreased root length (Figure 2A and 5A) and increased diameter (Figure 2B) and marginally changed fresh root weight (Figure 5B), it could be concluded that the direction of cell expansion changed from longitudinal to lateral by the action of RbCl. Microscopic observations showed lateral expansion of root cells in the maize primary root elongation zone (Figure 1D - 1G). This lateral expansion was most prominent in the cortex but was also observed in the stele.

Gravitropic curvature, which is dependent on delicately controlled cell elongation, was also affected by RbCl and restored by KCl (Figure 6), as expected based on the failure of directional cell growth control by RbCl. Gravity-sensing seemed to be normal in RbCl-treated plants, because the lateral roots were plagiogravitropic (Figure 1C), as in the lateral roots of the control plants (Figure 1B). Lateral roots may have a different directional mechanism or sensitivity to gravity from those of the primary root. Because differential cell elongation is the final step in the control of gravitropic curvature (Boonsirichai et al, 2002), the RbCl-disturbed primary root showed aberrant gravitropism even though it sensed the direction of gravity.

The root cells elongated longitudinally in the presence of K^+. When the K^+ level was dropped or K^+-requiring physiological activity was hindered by RbCl, the root cells expanded radially, indicating that the default direction of cell growth in the maize primary root is lateral in the absence of K^+. The K^+-requiring mechanism in the directional regulation of cell growth is still uncertain. It is believed that the change in cell growth direction is related to microtubule reorientation (Kropf et al, 1998). However, microtubules interact with actin to control directional cell expansion in plants (Bannigan and Baskin, 2005; Thomas et al, 2009; Higaki et al, 2010). The actin assembly was induced by potassium in a test with purified actin from amoeba and rabbit muscle (Pardee and Spudich, 1982). Root growth is stunted in Arabidopsis (Nishimura et al, 2003) when actin polymerization is disturbed. Furthermore, latrunculin B, an actin disrupting chemical, causes radial swelling in the maize primary root (Blancaflor, 2000), as RbCl did in this study (Figure 1A and 2B). Therefore, correlations between K^+-regulated directional cell expansion and actin polymerization may warrant further study.

The results also suggest that RbCl is a useful K^+ antagonist and could be used to specifically disturb

K^+ physiology and induce artificial K^+ deficiency, even in young seedlings.

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