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Paper-based plant supporting media affect salt-tolerance in the primary root of rice by releasing calcium ion

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ABSTRACT

The rice primary root was very sensitive to environmental stimuli and their elongation was strongly inhibited in the NaCl solution. However, when rice seedlings were grown on the supporting rolls based on Korean calligraphy paper, the primary root growth was less sensitive to the same concentrations of NaCl solution. The effects of the paper rolls were not due to better aeration from the support material, because the stimulatory effect of the paper rolls was observed also under the hypoxic conditions. When paper discs were put on the NaCl solution, roots of submerged seedlings gained salt-tolerance, indicating that certain substance(s) were diffused from the discs and enhanced salt tolerance. Addition of a calcium chelator, EGTA completely abolished the restoring effect of the paper. Calcium ion is an well-known factor enhancing salt tolerance of plants. However, the stimulatory effect of Ca^{2+} on the primary root growth under salt stress conditions did not appear when rice seedlings were grown on the paper rolls. ICP mass-spectrometry analysis showed that the concentration of Ca^{2+} released from the paper discs into the solution reached 0.7-0.8 mM, which is high enough to endow salt-tolerance to diverse plants.

Key words : Paper-based supporting medium, salt stress, Ca^{2+}, Root, Rice (*Oryza sativa*)

Introduction

Observation of root phenotypes is basic and indispensable work for plant biologists. It looks very simple and easy to observe the principle morphology with naked eyes without further magnification. However, in practice, it is nearly impossible to obtain the intact root system that is grown in the soil. The physical hindrance destroys fine structures, or even the main axis, of the roots during the recovery of the plant. Even after the digging out the whole plant, the soil particles attached to the root system are hardly washable because of slimy substances that are secreted by the roots (for reviews to see 1, 2, 3, 4). To overcome these problems, scientists have developed diverse supporting media where the roots can be easily grown and harvested for observation. Agar medium is suitable for easy observation, but is unfavorable for oxygen supply during root growth and inadequate for the harvest of the roots (5). For long-term observations, hydroponic culture system is frequently utilized (6). However, the submerged root phenotypes generally look different even with careful aeration. A popular way for short-term growth is using paper-based media, e.g. paper roll system for maize roots (7) and even simpler wet filter paper put on the bottom of growing vessels for small dicot plants. Because the paper roll system is quite simple and effective, the rolls have been extensively used for the screening of

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root developmental mutants of maize (3, 7, 8) and rice (5).

Because the root development is quite plastic depending on the given environmental conditions, it is very important to select a suitable support medium that offers good watering and air supply required for root growth (5). Therefore, it is highly possible that the nature of the supporting media could mask or amplify certain interesting aspects of the root development. If the purpose of the work is physiological, choosing a plant supporting medium could be far more critical than that for mutant screening.

We have used a modified paper roll system with the traditional Korean calligraphy paper for rice growth in the purpose of mutant screening (5), because it is suitable for the supply of water and air. Recently, we have applied the calligraphy paper roll system for the study of salt stress in rice roots. During the course of the study, we realized that the paper-based plant supporting medium was affecting our experiments. We tried to understand the reasons by tracing the primary root growth as the marker of salt stress. Finally we found that calcium ions released from the paper were the cause of the observed salt-tolerance in rice roots grown in the paper roll system.

Materials and Methods

Seeds of rice (*Oryza sativa*) were surface sterilized in 80% ethanol for 2 min and then in 4% sodium hypochlorite for 5 min. The sterilized seeds were washed in distilled water 5 times and soaked in distilled water in a vessel set in a growth chamber at 28°C for 2 d in darkness. The germinated seedlings still with barely visible radicles were then put on a sheet of wet calligraphy paper (470 mm × 90 mm × 0.2 mm; 3.15 g) in a row 1 cm below the edge of the wide side. Then the whole sheet containing the seedings was rolled and set in a 100 ml-beaker filled with water and/or other materials as similar to the "paper roll system" for maize root growth (7). We have optimized the paper roll system for rice plants, which have thinner and sensitive roots compared to maize, using the Korean calligraphy paper instead of normal paper towels. In some experiments, seeds were germinated in petridishes containing 20 ml growth solution in the presence or absence of discs (diameter = 12 cm) of the calligraphy paper. The prepared seedings in paper rolls or petridishes were grown in a growth chamber at 28°C for 3 d in darkness. The elongation of the primary root was selected as the maker for the salt stress and the root length was measured to monitor the stress responses in the presence or absence of the paper-based plant supporting media. All the chemicals included in the growth solutions were purchased from commercial sources, e.g., Sigma-Aldrich.

To test the effects of plant-supporting paper media on the salt tolerance under the hypoxic conditions, we aspirated the air from a vacuum desiccator with a pump and filled it again with nitrogen gas. Before the aspiration, paper rolls and petridishes containing rice seedlings were set in the desiccators. In our test conditions oxygen level was decreased to one-third of the normal air.

The ions contained in the growth solutions were analyzed by ICP mass-spetrometry at the Joint Machinery Center of Dankook University, Korea.

Results and Discussion

Salt stress evoked by NaCl inhibited the primary root growth of rice seedlings germinated in the absence of supporting paper medium (**Fig 1**, closed circles) as known for many other plants (9, 10). When the rice seedlings were grown on the supporting rolls of Korean calligraphy paper, the rice root growth was also inhibited by salt stress (Fig 1, open circles). However, the sensitivity of the roots to the salt stress was much less on the paper rolls, i.e., at 100 mM NaCl the length of the roots grown in the paper rolls was 37.4 ± 7.8 mm in contrast that the length in the absence of the paper was not measurably short.

To understand the difference, we hypothesized that the better aeration in the paper rolls could improve the rice root growth and lessen the symptoms of salt stresses. Because the seedlings were submerged in the absence of supporting paper, the roots could suffer from complex influences of salt and hypoxic stresses (11). However, when we reduced the oxygen level by replacing the air with nitrogen gas, the effect of paper rolls on salt stress was not affected (**Fig 2**). Under

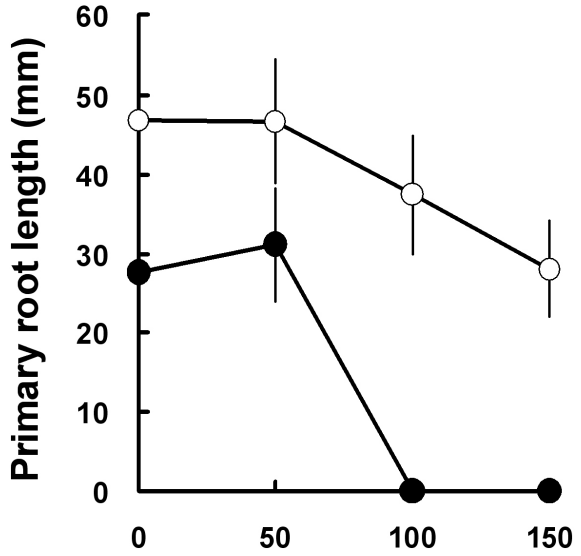

Fig 1. Effects of supporting paper rolls for rice growth under the salt-stressed conditions. The primary root growth of rice was measured in the presence (open circles) or absence (closed circles) of the supporting paper. Data are presented as mean ± standard deviations.

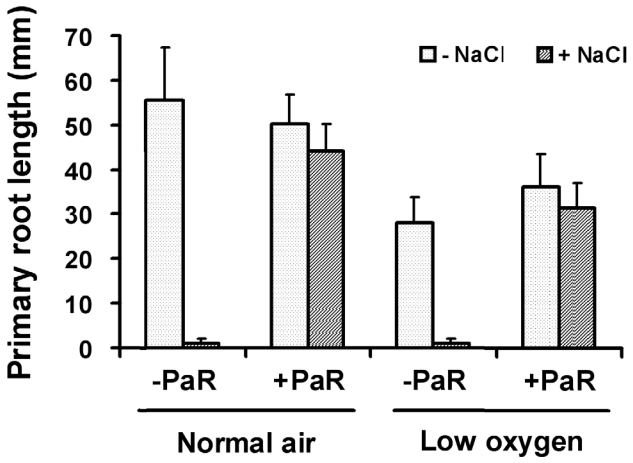

Fig 2. Influence of paper rolls (PaR) on the rice roots grown with or without salt stresses under the normal and low oxygen conditions. Salt stress was induced by 150 mM NaCl. Measurements are presented as mean ± standard deviations.

hypoxic conditions the root length was generally decreased and this observation may explain the length difference caused by paper rolls in the absence of salt stress as appeared in Fig 1. However, the air supply model did not support the hypothesis, because the restoration of the root growth by the calligraphy paper rolls appeared just same under the hypoxic conditions.

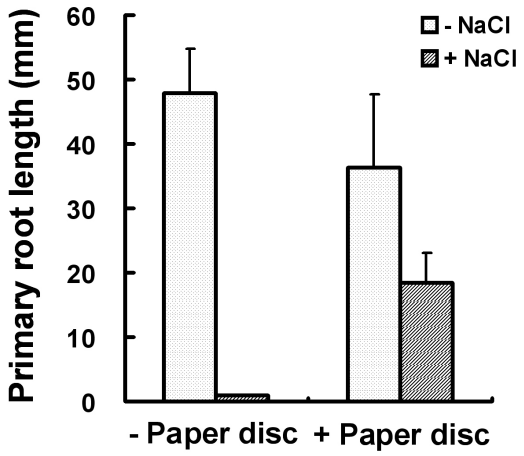

Fig 3. Impacts of paper discs that were put into the growth solutions on the salt stress. The concentration of NaCl was 150 mM. Values are presented as mean ± standard deviations.

Then, we examine the possibility that some substances released from the paper may affect the salt tolerance of rice roots. To test this hypothesis, two discs (diameter = 12 cm) of calligraphy papers were put on the bottom of petridishes before pouring the growth solutions with or without NaCl. By using this method we could give the same hypoxic conditions to the seedings, whether the paper was included or not, and solely evaluate the effects of the paper discs. The paper discs submerged in the growth solution surprisingly endowed salt tolerance to the growing rice roots (**Fig 3**). Filter paper submerged in the growth solutions revealed also similar restoring effects (Yoon, unpublished).

Although the way of the paper effects remained only illusive, we assumed that the influence may be due to certain substance(s) that was (were) water soluble and easily released from the paper into the growth solutions. Literature searches indicated that salt stress can be overcame by applying some ions especially Ca^{2+} (12). Because the effects of Ca^{2+} will be canceled by application of a specific chelator EDTA (ethylene glycol tetraacetic acid), we tested the restoration effect of the discs of calligraphy paper on the salt-stressed rice root growth in the presence of 10 mM EGTA. As expected, the stress tolerance by the paper discs disappeared in the presence of EGTA (data not shown). However, the conclusion was not so concrete because the root growth of unstressed plants were also inhibited by

Table 1. Ion contents in the growth medium after the incubation for 3 days in the presence or absence of supporting media based on the Korean paper for calligraphy. Two discs (diameter = 12 cm) of calligraphy paper were put on the bottom of a petridish containing 20 ml of distilled water. The ion contents are presented as ppm ± standard deviations. ND stands for "not detected".

Ions	Without paper discs	With paper discs
Ca^{2+}	8.198 ± 0.53	29.413 ± 0.48
Mg^{2+}	0.457 ± 0.01	1.919 ± 0.06
Mn^{2+}	0.087 ± 0.00	0.187 ± 0.00
Na^{+}	2.580 ± 0.04	3.206 ± 0.04
K^{+}	ND	ND

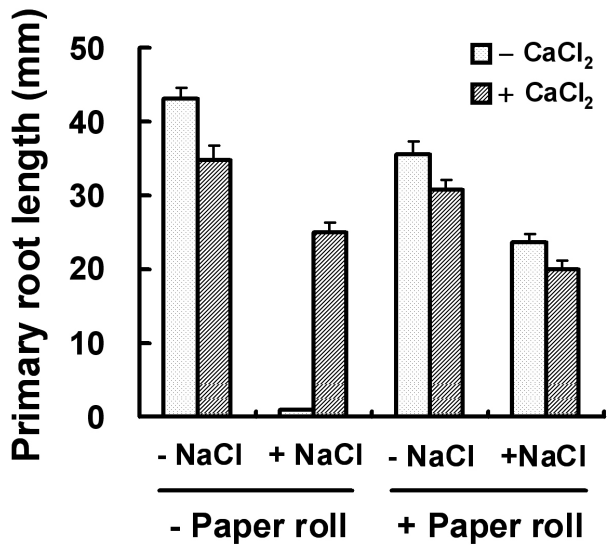

Fig 4. Effects of $CaCl_2$ on the salt stress in the absence of presence of supporting paper rolls. Concentrations were 150 mM and 10 mM for NaCl and $CaCl_2$, respectively. Results were presented as mean ± standard errors.

EGTA (data not shown), indicating that Ca^{2+} plays many other important roles besides stress-tolerance.

When we directly tested the effects of $CaCl_2$ on the salt stress in the presence or absence of the paper rolls, 10 mM $CaCl_2$ restored the salt-suppressed root growth in the absence of the supporting paper rolls. However, on the paper rolls $CaCl_2$ did not affect the root growth any further regardless the salt stresses, indicating that the effective Ca^{2+} level was already saturated in the presence of the calligraphy paper.

Finally, we examined whether the calligraphy paper really released Ca^{2+} ions to the growth solutions. For this work we collected the growth solutions that were used for the stress tests, which were presented in Fig 3, in the presence or absence of the paper discs (**Table 1**) and determined the ion contents by ICP mass-spectrometry. After the incubation period for plant growth with dipped two discs of calligraphy paper (diameter = 12 cm), the concentrations of several ions including Ca^{2+}, Mg^{2+} and Mn^{2+} were increased. Especially, the concentration of Ca^{2+} reached 2.58 ± 0.04 ppm. In terms of molarity, it was about 0.7 – 0.8 mM which has been reported to give slat-tolerance in many plants (10).

We understand that the unexpected restoring effect of the Korean calligraphy paper was due to the released Ca^{2+} in the growth solutions. This finding indicates that the paper roll system itself that has been used for many years for the genetic research of crop root development could affect the phenotypes especially related to some physiologies concerning Ca^{2+}. Although germination of plant seeds on wet paper has been a very popular method for growing plants for diverse physiological studies for nearly a century, research workers should carefully consider that the traditional way for plant germination on a paper is really suitable for their own study purposes.

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