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RESEARCH PAPERS

$CaCl_2$ Salt Signaling in Primary Root Architecture and Lateral Root Emergence in *Arabidopsis thaliana*

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Abstract—Plant root architecture modulates during developmental stages and adjusts with the environmental condition. The cytosolic calcium which is a ubiquitous secondary messenger in all eukaryotes strongly affects the root system in *Arabidopsis thaliana* (L.) Heynh. We proposed that calcium chloride gradients affect *PIN2* expression, which in term modulates root architecture and lateral root emergence. In the present study, the root development of *PIN2* overexpressing lines of *A. thaliana* were investigated on different $CaCl_2$ concentrations. This study found that the abundance of PIN2 protein has a direct effect on the root curvature with respect to $CaCl_2$ gradient. In the presence of low concentration of $CaCl_2$, PIN2 protein is stabilized and its subsequent accumulation lead to straight root architecture. However, as the $CaCl_2$ concentration were increased, PIN2 protein destabilized and subsequently degraded which in turn showed a wavy root phenotype. On the other hand, different concentrations of $CaCl_2$ did not show any effect on *PIN2* gene at transcript level.

Keywords: Arabidopsis thaliana, $CaCl_2$, wavy root phenotype, PIN2, salt stress

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INTRODUCTION

Plants being sessile, often experience environmental stresses (biotic and abiotic) that lead to decrease in plant growth and crop productivity [1, 2]. A major feature of plant development is that it is continuously modified by interactions with the environment, thereby enabling plants to cope with the constraints of their sessile lifestyle. A quintessential example is the developmental plasticity of the three-dimensional deployment of the root (the root architecture) in response to the soil environment [3]. This plasticity is critical for a plant's ability to forage the soil for nutrients and water and contributes to a plant's competitive fitness. Root architecture is determined by the pattern of root branching (lateral root formation) and by the rate and direction of growth of individual roots [4]. The formation of lateral roots (LRs) is a reiterative process. LRs form along the length of the primary root and these first order LRs give rise to second order LRs which themselves can form the third order LRs etc. [5, 6]. Several studies have shown that how differences in the composition of the growth medium can alter root architecture [4]. A good example is the LR proliferation and curvature of LRs and primary roots into resource-rich growth media zones such as those enriched in NO_3 or phosphate [7, 8].

On the other hand, less is understood regarding how plant root architecture is modulated in response to different stress conditions, even though this response may be vital for the plant's ability to compete for soil resources. A survey of plant responses to potassium nitrate, phosphate, auxin and oxilipins is characterized by the increase of LRs, inhibition of primary root and formation of wavy roots [9, 10]. The mild chronic stress stimulated stress-induced morphogenic response, which comprises of inhibition of root elongation, localized stimulation of cell division and alteration of cell differentiation. It is suggested that modulation of root architecture is thus typified by growth redistribution rather than by cessation of growth [3]. For example the reduction in root elongation by the osmotic or salinity stress is reported in cotton, maize and bean [2, 11–13]. In most studies using Arabidopsis, osmotic or salt stress severely repressed the formation of LRs [14–17]. Osmotic stress appears to inhibit LR formation after LR initiation and affects the formation of autonomous LRs from LRPs [14].

In the light of these reports regarding the effect of salt stress on *Arabidopsis* root architecture, the effect of $CaCl_2$ salt on *Arabidopsis* root was investigated in the present study. To understand the effect of in vitro calcium stress, we demonstrated morphogenic responses induced in roots of *A. thaliana* transgenic lines overexpessing *PIN2* after application of different concentration of $CaCl_2$ salt. Indeed, we observed visible effect of $CaCl_2$ on primary root architecture and LR emergence in *A. thaliana*.

MATERIALS AND METHODS

Plant material and growth conditions. *Arabidopsis thaliana* (L.) Heynh. (Col-0) homozygous *PIN2* overexpressing lines were provided by the "Cell signalling lab," Division of Plant Sciences, Gyeongsang National University, Korea. They used floral dip method and generated transgenic *Arabidopsis* lines, and MS medium containing cephalosporin and kanamycin were used for select the transgenic plants of T1 and T2 generations. Furthermore, homozygous T3 progenies were confirmed by RT-PCR and were used for these experiments.

Arabidopsis lines overexpressing *PIN2* were tested for root architecture and lateral root emergence. The seeds were sterilized and plated on half the norm of MS [18] with 1.5% sucrose at 4°C in darkness for 48 h of stratification, then transferred to a growth chamber under condition of 20 to 22°C with a 16/8 h light/dark cycle. Five days old seedling with 1.0 to 1.5 cm in length were used for experiments.

$CaCl_2$ treatment. To check the root response on calcium stress, *PIN2* overexpressing plants were grown on media supplemented with different concentration of $CaCl_2$. The five-day-old seedlings were transferred on the calcium deficient media supplemented with various concentrations (0, 0.01, 0.1, 1, 3 and 10 mM) of calcium chloride. The seedling roots were positioned vertically and the initial sited of the root tip were marked. The plates were reoriented by 90° for gravitropic response and the gravitropic response was digitized using (Canon CKI-PowerShot-GII) to observe the root architecture, primary root length and lateral root emergence. All the experiments were conducted with three biological replicates.

***PIN2* expression at protein level.** To study the expression of *PIN2* at potein level, Western Blotting carried out to check the PIN2 abundance after subjecting to different concentrations of $CaCl_2$. The proteins of the *PIN2* overexpressing lines were extracted from ten-day-old plant. Antibodies were diluted as follows: 1 : 1000 for anti-PIN2; 1 : 4000 for anti-rabbit. The picture was developed in dark room using x-ray developing procedure. Each experiment was repeated three times to check the reproducibility.

***PIN2* expression at RNA level.** To study the change in the *PIN2* expression; RT-PCR was carried-out in *Arabidopsis PIN2* overexpressing line after subjecting to different concentrations of $CaCl_2$. The RNA was extracted by RNeasy Plant Mini Kit (Qiagen) from ten-day-old plant (prior to the fact that maximum affect of calcium ion be explored). Two micrograms of DNAse-treated total RNA was used as a template for first-strand cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen) and oligo (dT) primer. The *ACTIN2* gene was used as a reference with a primer 5'-CCTTCGTCTTGATCTTGCGG-3' and 5'-AGCGATGGCTGGAACAGAAC-3' and to detect *PIN2* transcript: 5'-AAGTCACGTACATGCATGTG-3' and 5'-AGATGCCAACGATAATGAGTG-3' primer were used. Twenty-microliter reactions were set-up for each reaction and amplified through 30 PCR cycles and subsequently, 15 μL of the PCR product was run on 1% agrose gel for gel-documentation.

RESULTS

$CaCl_2$ Stress Decreased LRs and Caused Wavy Root Phenotype

Calcium is an essential plant nutrient, involved in various structural roles in the cell wall and membranes differentiation. Calcium is also an intracellular messenger, which coordinates numerous developmental signals and environmental challenges. Here we observed that calcium ion concentration produced wave phenotype as the concentration increases from 0.01 up to 10 mM $CaCl_2$ (Fig. 1a). It was found that the wave root formation prominently observed at 3 and 10 mM of $CaCl_2$ in the calcium deficient media.

Mild $CaCl_2$ Stress Stimulates Root Growth and Lateral Root Proliferation

To examine how $CaCl_2$ stress affects *Arabidopsis* root growth and LRs proliferation, the response of *Arabidopsis* seedlings were tested on increasing $CaCl_2$ concentration. Fig. 1b shows that increasing $CaCl_2$ level up to 0.1 mM stimulate the production of LRs, where seedlings exhibited virtually twofold number of LRs compared with the control plants (with 0 mM $CaCl_2$). However, the numbers of LRs were decreased by transferring the seedlings to 1 mM $CaCl_2$ containing medium, and 10 mM $CaCl_2$ stress caused almost complete inhibition of LRs development. These results propose an acclimation response of LR development to mild levels of $CaCl_2$ stress. Similarly, root length was also increased at lower concentration of $CaCl_2$, i.e., from 0 and 0.01 mM, however its length decreased again after the application of 0.1–10 mM $CaCl_2$ (Fig. 1c).

Phenotype Reverting Assay

With the increase of calcium ion concentration, the *PIN2* overexpressing plants showed a wavy root phenotype. We have found that wavy root phenotype revert to straight phenotype back when transplant from high $CaCl_2$ level to low (Fig. 2). Thus, we confirmed that the wavy root phenotype appearance depends on calcium ion concentration.

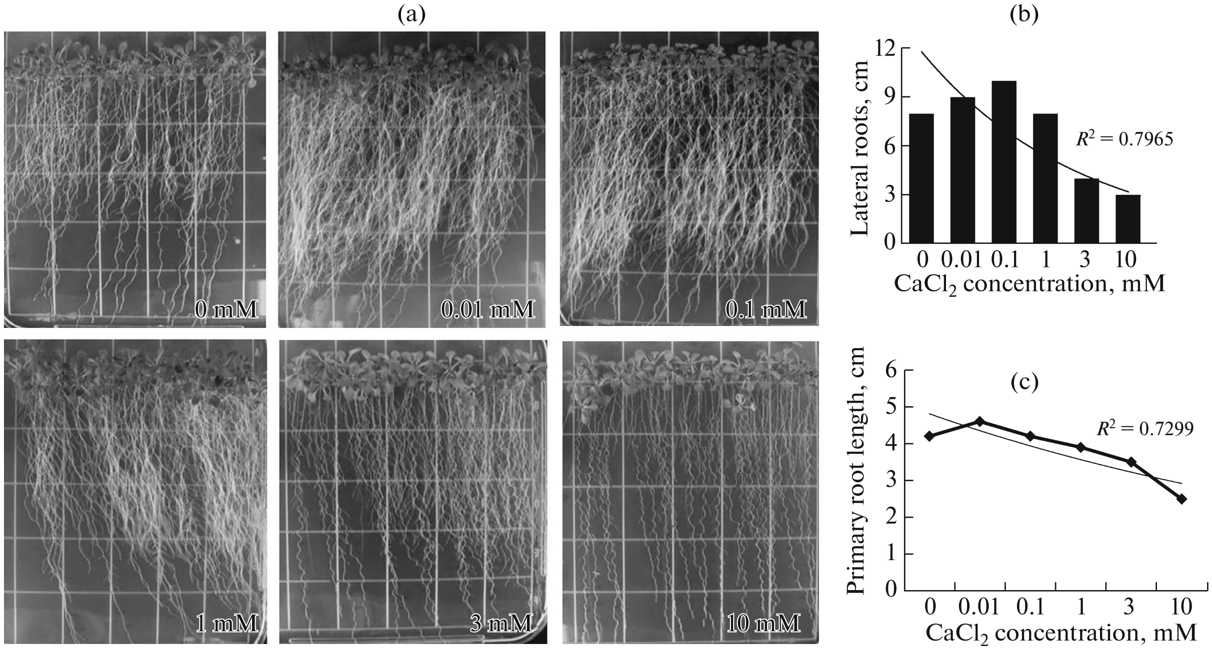

Fig. 1. $CaCl_2$ concentration affects root architecture, primary root length and number of LRs in *Arabidopsis thaliana PIN2* overexpressing plants.

It is previously investigated by many researchers that calcium is fully involved in various structural roles in the cell wall and cell membranes differentiation which ultimately bring changes in the root emergence and the number of LLR emergence. With the increase of $CaCl_2$, the LR emergence/number increased up to 1 mM and decreased with the increase of $CaCl_2$ concentration from 3 mM (Fig. 2).

Ca^{2+} Stress-Induced Gravitropic Responses Are Related to PIN2 Protein Levels

It's known that PIN2 protein play an important role in the redistribution of auxin and subsequently gravitropic responses [19]. It was assumed that the expression of PIN2 protein is modified by calcium concentration. We tested the level of PIN2 protein in the seedling overexpressing *PIN2.* Under low $CaCl_2$ concentration (0 to 0.1 mM) Western Blot showed high intensity/abundance of *PIN2* expression and the plant showed normal gravitropic response with a straight root phenotype. Surprisingly, above 0.1 mM of $CaCl_2$ PIN2 protein abundance was reduced and

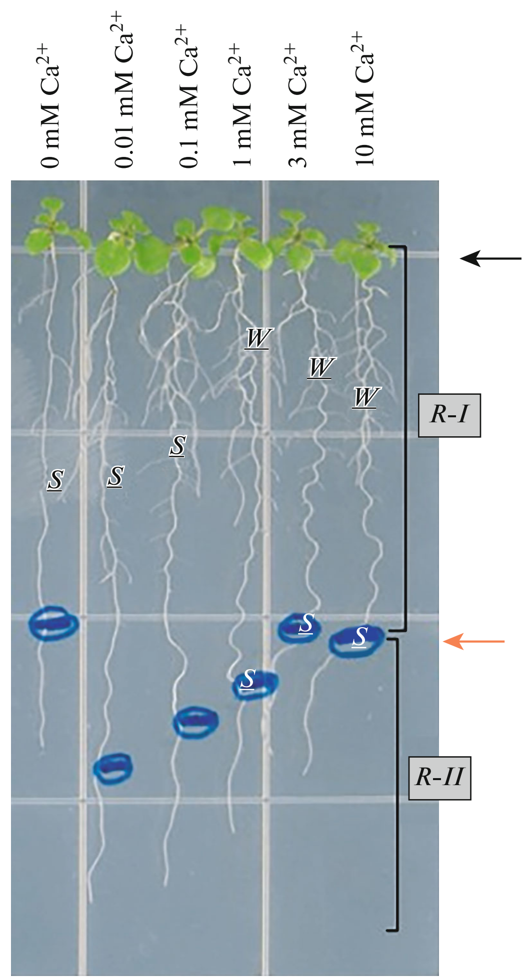

Fig. 2. Calcium-dependent wavy root phenotype got straight at 0 mM $CaCl_2$. R-I—transplant three-day-old plants to a medium containing different concentration of $CaCl_2$; R-II—clustered the plant (from the media having different concentration of $CaCl_2$: R-I) on 0 mM Ca^{2+} containing media; consequently the curvature of the root change to normal, i.e., wavy phenotype (W) reverts into straight (S: R-II).

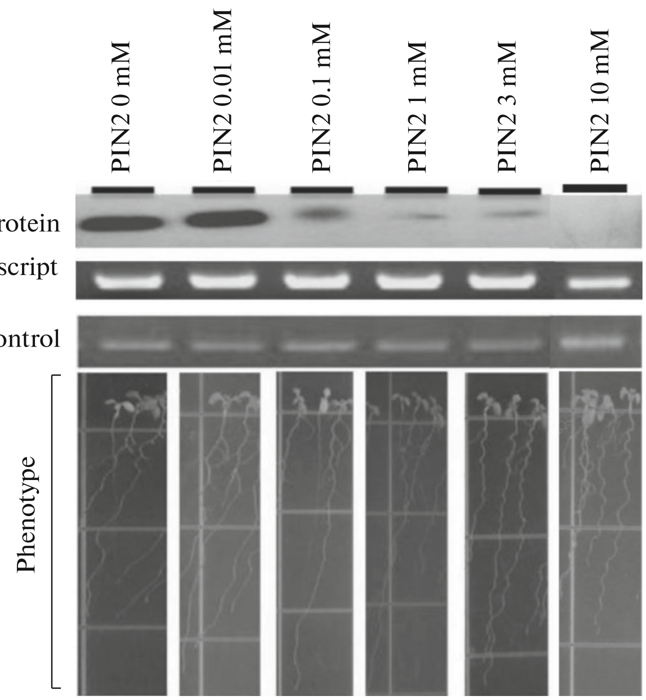

Fig. 3. Response of *Arabidopsis thaliana PIN2* gene expression to differrent concentration of $CaCl_2$.

ultimately defused and plant showed wavy root phenotype. Unlike *PIN2* expression at protein level, the *PIN2* transcript abundance was not affected with the increase or decrease of $CaCl_2$ concentration (Fig. 3). These results suggested that calcium stress represses the abundance and distribution of PIN2 protein influencing the response to gravity.

DISCUSSION

Variation in the supply and distribution of inorganic nutrients affect root development either directly (changes in the external concentration of the nutrient) or indirectly (changes in the internal nutrient status of the plant) resulting trophomorphogenic responses induces in plants [20]. Calcium is one of the major inorganic nutrient elements. In higher plants, it is available in the form of Ca^{2+} with 5000 mg/kg (0.5%) concentration in dry tissue. It is previously investigated by many workers that within the cell Ca^{2+} is fully involved in the formation of middle lamella, essential for cell membrane formation, acts as second messenger in metabolic regulation, help to stabilize the structure of chromosomes and an activator of many enzymes [21]. Similarly, the deficiency of Ca^{2+} within cells causes disintegration of growing meristematic region of the root, stem and leaves and chlorosis along the margins of younger leaves and malformation of younger leaves [22].

Under various concentrations of $CaCl_2$ (0, 0.01, 0.1, 1, 3 and 10 mM), the number of LRs were significantly increased at 0.01 and 0.1 mM $CaCl_2$ than the control seedlings as shown in Fig. 1a. Interestingly, the LRs were reduced and the plant showed slightly negative response to 1 mM $CaCl_2$, but at this stage, the LRs were still significantly more than the control plant. We found that mild $CaCl_2$ concentration (up to 0.1 mM) increase LRs proliferation in PIN2, but increase concentration of $CaCl_2$ decrease LRs proliferation which was further conformed by 3 and 10 mM $CaCl_2$ as shown in Fig. 1b. It was found that the numbers of LRs significantly decreased at 3 and 10 mM of $CaCl_2$, and the primary root was developed almost without lateral root proliferation. These results revealed that mild calcium stress stimulated stress-induced morphogenic response in *Arabidopsis* roots [3], however enhanced $CaCl_2$ concentration cause toxicity to plant and inhibited root growth [23, 24]. It has been elaborated that LR primordium (LRP) initiation phase dependent on a root tip-localized IAA source, and LRP emergence phase dependent on leaf-derived IAA up to 10 days after germination in *A. thaliana* [25, 26]. Our results suggested that uptake of $CaCl_2$ up to 0.1 mM stimulate the developmental stages of lateral root formation resulting more highly branched root system in *A. thaliana PIN2* overexpressing lines but extreme high concentration of $CaCl_2$ inhibit the developmental stages of LRt, assuming that synthesis of tip-localized IAA and leaf-derived IAA is remarkably sensitive to stress of $CaCl_2$. Hence, mild $CalCl_2$ stress stimulates LR proliferation in *A. thaliana*.

For the first time it was hypothesized that wavy root phenotype of *Arabidopsis* is a gravity-induced touch-response to the medium surface and the root tip changed direction in an obstacle-avoidance manoeuvre [27]. Then Simmons et al. [28] proposed that, a combination of gravitropism and circumnutation (natural oscillating growth) act as the major stimuli to induced root waving in *A. thaliana*. Subsequently, Buer et al. [29] demonstrated that the gaseous environment within the Petri dish can override gravitational effects and the medium ion concentrations and gelling polymers modify the wave response. We found that $CaCl_2$ stress-induced wavy root phenotypes are related to PIN2 protein levels. Western blot analysis showed normal *PIN2*-expression in control plant with straight primary root architecture, but when the abundance of *PIN2*-expression was reduced and ultimately diffused with high $CaCl_2$ concentration [25], the plants showed wavy primary root consequently. The wavy root formation was prominently observed at 3 and 10 mM of $CaCl_2$ in the calcium deficient media. These results suggested that high concentration of $CaCl_2$ limits the abundance of PIN2 protein which have direct effect on the wavy root phenotype development.

To conclude, low concentration of $CaCl_2$ brings morphogenic responses inducing root length and LRt emergence in *A. thaliana*. However, higher concentration of $CaCl_2$ inhibit root growth, LR emergence and causes wavy root phenotype. LR emerge after several developmental stages occur in plants and depend on growth regulator therefore we suggested that $CaCl_2$ interfere in main physiological events which have direct effect on LRs proliferation. We have also concluded that abundance of PIN2 protein directly affect wavy root phenotype. High concentration of $CaCl_2$ represses the abundance of PIN2 protein in response to gravity resulting the plant showed wavy root phenotype.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants as objects of research.

AUTHORS CONTRIBUTIONS

M. Nisar designed the project, conducted experiments and wrote the paper. A. Ullah and H. Park modified and reviewed the paper. Z. Ali, A. Ali, R. Aman and S.F. Wadood helped M. Nisar in the experiments.

REFERENCES

1. Ullah, A., Sun, H., Yang, X., and Zhang, X., Drought coping strategies in cotton: increased crop per drop, *Plant Biotechnol. J.*, 2017, vol. 15, p. 271.
2. Ullah, A., Sun, H., Yang, X., and Zhang, X., A novel cotton WRKY gene, *GhWRKY6-like*, improves salt tolerance by activating the ABA signaling pathway and scavenging of reactive oxygen species, *Physiol. Plant.*, 2018, vol. 162, p. 439.
3. Zolla, G., Yair, M., Heimer, and Barak, S., Mild salinity stimulates a stress-induced morphogenic response in *Arabidopsis thaliana* roots, *J. Exp. Bot.*, 2010, vol. 61, p. 211.
4. Malamy, J.E., Intrinsic and environmental response pathways that regulate root system architecture, *Plant Cell Environ.*, 2005, vol. 28, p. 67.
5. Ullah, A., Manghwar, H., Shaban, M., Khan, A.H., Akbar, A., Ali, U., Ali, E., and Fahad, S., Phytohormones enhanced drought tolerance in plants: a coping strategy, *Environ. Sci. Pollut. Res.*, 2019, vol. 25, p. 33103.
6. Chen, Q., Liu, Y., Maere, S., Lee, E., van Isterdael, G., Xie, Z., Xuan, W., Lucas, J., Vassilileva, V., Kitakura, S., Marhavý, P., Wabnik, K., Geldner, N., Benková, E., Le, J., et al., A coherent transcriptional feed-forward motif model for mediating auxin-sensitive *PIN3* expression during lateral root development, *Nat. Commun.*, 2015, vol. 6: 8821.
7. Linkohr, B.I., Williamson, L.C., Fitter, A.H., and Leyser, H.M.O., Nitrate and phosphate availability and distribution have different effects on root system architecture of *Arabidopsis, Plant J.*, 2002, vol. 29, p. 751.
8. Malamy, J.E. and Ryan, K.S., Environmental regulation of lateral root initiation in *Arabidopsis, Plant Physiol.*, 2001, vol. 127, p. 899.
9. Potters, G., Pasternak, T.P., Guisez, Y., Palme, K.J., and Jansen, M.A., Stress-induced morphogenic responses: growing out of trouble? *Trends Plant Sci.*, 2007, vol. 12, p. 98.
10. Potters, G., Pasternak, T.P., Guisez, Y., and Jansen, M.A.K., Different stresses, similar morphogenic responses: integrating a plethora of pathways, *Plant Cell Environ.*, 2009, vol. 32, p. 158.
11. Croser, C., Renault, S., Franklin, J., and Zwiazek, J., The effect of salinity on the emergence and seedling growth of *Picea mariana, Picea glauca*, and *Pinus banksiana, Environ. Pollut.*, 2001, vol. 115, p. 9.
12. Akhtar, S.S., Andersen, M.N., Naveed, M., Zahir, Z.A., and Liu, F., Interactive effect of biochar and plant growth-promoting bacterial endophytes on ameliorating salinity stress in maize, *Funct. Plant Biol.*, 2015, vol. 42, p. 770.
13. Talaat, N.B., Ghoniem, A.E., Abdelhamid, M.T., and Shawky, B.T., Effective microorganisms improve growth performance, alter nutrients acquisition and induce compatible solutes accumulation in common bean (*Phaseolus vulgaris* L.) plants subjected to salinity stress, *Plant Growth Regul.*, 2015, vol. 75, p. 281.
14. Deak, K.I. and Malamy, J., Osmotic regulation of root system architecture, *Plant J.*, 2005, vol. 43, p. 17.
15. Ding, Z.J., Yan, J.Y., Li, C.X., Li, G.X., Wu, Y.R., and Zheng, S.J., Transcription factor WRKY 46 modulates the development of Arabidopsis lateral roots in osmotic/salt stress conditions via regulation of ABA signaling and auxin homeostasis, *Plant J.,* 2015, vol. 84, p. 56.

16. Xiong, L., Wang, R.G., Mao, G., and Koczan, J.M., Identification of drought tolerance determinants by genetic analysis of root response to drought stress and abscisic acid, *Plant Physiol.*, 2006, vol. 142, p. 1065.

17. Kim, S., Kang, J.Y., Cho, D.I., Park, J.H., and Kim, S.Y., ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance, *Plant J.,* 2004, vol. 40, p. 75.

18. Murashige, T. and Skoog, F., A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiol. Plant.*, 1962, vol. 15, p. 473.

19. Rahman, A., Takahashi, M., Shibasaki, K., Wu, S., Inaba, T., Tsurumi, S., and Baskin, T.I., Gravitropism of *Arabidopsis thaliana* roots requires the polarization of PIN2 toward the root tip in meristematic cortical cells, *Plant Cell*, 2010, vol. 22, p. 1762.

20. Prinzenberg, A.E., Barbier, H., Stich, B., Salt, D.E., and Reymond, M., Relationships between growth, growth response to nutrient supply, and ion content using a recombinant inbred line population in Arabidopsis, *Plant Physiol.*, 2010, vol. 154, p. 1361.

21. Hepler, P.K., Calcium: a central regulator of plant growth and development, *Plant Cell*, 2005, vol. 17, p. 2142.

22. Karthika, K.S., Rashmi, I., and Parvathi, M.S., Biological functions, uptake and transport of essential nutrients in relation to plant growth, in *Plant Nutrients and Abiotic Stress Tolerance*, Hasanuzzaman, M., Fujita, M., Oku, H., Nahar, K., and Hawrylak-Nowak, B., Eds., Singapore: Springer, 2018, p. 1.

23. Ullah, A., Mushtaq, H., Fahad, S., Shah, A., and Chaudhary, H.J., Plant growth promoting potential of bacterial endophytes in novel association with *Olea ferruginea* and *Withania coagulans, Microbiology*, 2017, vol. 86, p. 119.

24. Julkowska, M.M. and Testerink, C., Tuning plant signaling and growth to survive salt, *Trends Plant Sci.*, 2015, vol. 20, p. 586.

25. Ditengou, F.A., Teale, W.D., Kochersperger, P., Flittner, K.A., Kneuper, I., van der Graaff, E., Nziengui, H., Pinosa, F., Li, X., Nitschke, R., Laux, T., and Palme, K., Mechanical induction of lateral root initiation in *Arabidopsis thaliana, Proc. Natl. Acad. Sci. USA*, 2008, vol. 105, p. 18818.

26. Ullah, A., Akbar, A., Luo, Q., Khan, A.H., Manghwar, H., Shaban, M., and Yang, X., Microbiome diversity in cotton rhizosphere under normal and drought conditions, *Microb. Ecol.*, 2019, vol. 77, p. 429.

27. Okada, K. and Shimura, Y., Reversible root tip rotation in Arabidopsis seedlings induced by obstacle-touching stimulus, *Science*, 1990, vol. 250, p. 274.

28. Simmons, C., Söll, D., and Migliaccio, F., Circumnutation and gravitropism cause root waving in *Arabidopsis thaliana, J. Exp. Bot.*, 1995, vol. 46, p. 143.

29. Buer, C.S., Masle, J., and Wasteneys, G.O., Growth conditions modulate root-wave phenotypes in Arabidopsis, *Plant Cell Physiol.*, 2000, vol. 41, p. 1164.