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NKS1, Na^+- and K^+-sensitive 1, regulates ion homeostasis in an *SOS*-independent pathway in *Arabidopsis*

Wonkyun Choi [a,b,1], Dongwon Baek [a,b,1], Dong-Ha Oh [a,b], Jiyoung Park [a,b], Hyewon Hong [a,b], Woe-Yeon Kim [a,b], Hans J. Bohnert [a,b,c,d], Ray A. Bressan [a,b,e,f], Hyeong Cheol Park [a,b,*], Dae-Jin Yun [a,b,*]

[a] Division of Applied Life Science (BK21 Program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea
[b] Division of Applied Life Science (BK21 Program), Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea
[c] Department of Plant Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[d] Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[e] Center for Plant Environmental Stress Physiology, Purdue University, West Lafayette, IN 47907, USA
[f] King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia

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ABSTRACT

An *Arabidopsis thaliana* mutant, *nks1-1*, exhibiting enhanced sensitivity to NaCl was identified in a screen of a T-DNA insertion population in the genetic background of Col-0 *gl1 sos3-1*. Analysis of the genome sequence in the region flanking the T-DNA left border indicated two closely linked mutations in the gene encoded at locus At4g30996. A second allele, *nks1-2*, was obtained from the *Arabidopsis* Biological Resource Center. *NKS1* mRNA was detected in all parts of wild-type plants but was not detected in plants of either mutant, indicating inactivation by the mutations. Both mutations in *NKS1* were associated with increased sensitivity to NaCl and KCl, but not to LiCl or mannitol. NaCl sensitivity was associated with *nks1* mutations in *Arabidopsis* lines expressing either wild type or null alleles of *SOS1*, *SOS2* or *SOS3*. The NaCl-sensitive phenotype of the *nks1-2* mutant was complemented by expression of a full-length *NKS1* allele from the *CaMV35S* promoter. When grown in medium containing NaCl, *nks1* mutants accumulated more Na^+ than wild type and K^+/Na^+ homeostasis was perturbed. It is proposed NKS1, a plant-specific gene encoding a 19 kDa endomembrane-localized protein of unknown function, is part of an ion homeostasis regulation pathway that is independent of the SOS pathway.

1. Introduction

Salinization of cultivated land represents a significant factor impacting crop yield reduction throughout the world (Pardo and Quintero, 2002; Ward et al., 2003). High salinity causes Na^+ or Cl^- ion toxicity, ion imbalance, and generates dehydration stress due to increased osmolarity in plant cells, particularly in the roots. Na^+ accumulation initiates damages subsequent to inhibition of enzyme activities and cytoplasmic stress that is based on disruption of K^+ acquisition (Manabe et al., 2008; Tester and Davenport, 2003). Therefore, in addition to the necessity of maintaining low intracellular Na^+ concentrations, balancing cellular ratios of Na^+ and K^+ is critical for survival of plants and successful adaptation to high NaCl environments (Hasegawa et al., 2000).

Our understanding of the molecular mechanisms by which plants resist NaCl toxicity has been greatly advanced by studies in the model plant *Arabidopsis thaliana*. The first genes identified in a screen for plants hypersensitive to NaCl were the *Arabidopsis SOS* genes. At *SOS1* encodes a plasma membrane Na^+/H^+ antiporter (Shi et al., 2000, 2002). At *SOS2/CIPK24* encodes a Ser/Thr protein kinase with a regulatory C-terminal domain (Guo et al., 2001) that interacts with At *SOS3/CBL4* (Guo et al., 2001). At *SOS3* encodes a Ca^{2+}-binding protein which resembles the regulatory B subunit of calcineurin and related proteins of the neuronal Ca^{2+} sensor family (Zhu, 2000). Significant advances have been made in unraveling mechanisms by which proteins of the SOS pathway enable plants to maintain a high intracellular K^+/Na^+ ratio (Apse et al., 2003; Shi et al., 2000). While it is clear that *SOS* genes play major roles in ameliorating a plant's sensitivity to NaCl, there is evidence that additional genes are involved in controlling sensitivity to NaCl and that at least some of these genes function independent of the SOS pathway (Quintero et al., 2002; Shabala et al., 2005; Zhu, 2003; Zhu et al., 2007).

Under salt stress, plants must maintain a high level of K^+ and low level of Na^+ in the cytosol, and regulating cellular ion

Abbreviations: T-DNA, transfer DNA; SOS, salt overly sensitive; UTR, untranslated region; ER, endoplasmic reticulum; GFP, green fluorescent protein; RFP, red fluorescent protein; GUS, beta-glucuronidase.

* Corresponding authors. Tel.: +82 55 751 6256; fax: +82 55 759 9363 (H.C. Park).
E-mail addresses: hcpark@gnu.ac.kr (H.C. Park), djyun@gnu.ac.kr (D.-J. Yun).

[1] These authors contributed equally to this work.

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concentration is crucial for plant survival. Regulation occurs by adjusting activities of membrane Na^+ and K^+ transporters, channels and co-transporters mediated by H^+-ATPase (Zhu, 2003). The maintenance of K^+/Na^+ homeostasis is crucial under various environmental stresses. To cope with salt stress, the vacuolar compartmentation of toxic ion contributes to regulation of the cytosolic ion level. In *Arabidopsis*, AtNHX1 and AtNHX2, which are localized in the tonoplast membrane, are working in Na^+ compartmentation to the vacuole (Blumwald, 2000; Apse et al., 1999). Regulatory mechanism of these membrane transporters has been intensively studied. However, little is known about the relationship between these ion homeostasis regulators and endomembrane proteins.

We describe herein the identification and characterization of *Arabidopsis NKS1*. The *NKS1* encodes a 19 KDa endomembrane-localized protein of unknown function that is expressed ubiquitously in *Arabidopsis* tissues. We show that *NKS1* functions independent of the *SOS* pathway in the control of K^+ and Na^+ ion homeostasis under salt stress.

2. Results and discussion

2.1. Isolation of nks1 as an enhancer of the NaCl sensitivity of sos3-1

The *sos3-1 nks1-1* (Na^+- and K^+-sensitive 1) mutant was isolated during a screen of 65,000 T2 lines from a T-DNA insertional mutant population in the *Arabidopsis* Col-0 *gl1 sos3-1* genetic background for enhanced sensitivity to NaCl. In the absence of added NaCl, root and shoot growth of the *sos3-1 nks1-1* double mutant were not significantly different from the phenotypic characteristics of Col-0 *gl1* and/or *sos3-1* mutants (Fig. 1A, left). In medium containing 50 mM NaCl, the *nks1-1* mutation enhanced root growth retardation phenotype of the *sos3-1* mutant (Fig. 1A, right and Fig. 1B).

High concentrations of NaCl reduce plant growth by two types of stresses, ion toxicity and osmotic imbalance (Blumwald et al., 2000). To distinguish between these two mechanisms, root growth phenotypes of the isogenic Col-0 *gl1*, *sos3-1* and *sos3-1 nks1-1* lines were compared in media supplemented with NaCl, KCl, LiCl and mannitol. Root growth of the *sos3-1* mutant was hypersensitive to elevated levels of NaCl and LiCl, but not to KCl or mannitol in the growth medium (Fig. 1B–E), as reported before (Liu and Zhu, 1997). Root growth of the *sos3-1 nks1-1* double mutant was more sensitive to NaCl and KCl stress than the response shown by the *sos3-1* mutant (Fig. 1B and C). However, root growth inhibition of the *sos3-1 nks1-1* double mutant in media supplemented with LiCl remained comparable to inhibition observed with the *sos3-1* mutant (Fig. 1D). As noted for the *sos3-1* single mutant, root growth of the *sos3-1 nks1-1* double mutant showed an insensitivity to mannitol supplements to the medium (Fig. 1E). These phenotypes pointed to an association of *nks1-1* with increased sensitivity to ionic imbalance but not to osmotic stress. Specifically, *nks1-1* established an association with increased sensitivity to Na^+ and K^+ ions.

Analyses of the F1 and F2 progenies obtained from a backcross of the *sos3-1 nks1-1* mutant with *sos3-1* showed that the NaCl-hypersensitive root growth phenotype of the double mutant was the result of a single recessive mutation that co-segregated with the T-DNA insertion, i.e., following the dominant BASTA resistance marker. F1 progeny were all BASTA resistant and root growth of F1 seedlings on 50 mM NaCl medium was comparable to that of the *sos3-1* parent ($n = 179$). For the F2 progeny seedlings, the segregation ratio for enhanced sensitivity to 50 mM NaCl was approximately 1:3 (132 NaCl super-sensitive:402 NaCl sensitive; χ^2 test $P = 0.88$) and the segregation ratio for the dominant BASTA resistance marker was close to 3:1 (103 BASTA resistant: 37 BASTA sensitive). Thus, *nks1-1* behaved as a recessive allele resulting from the single T-DNA insertion. Together, these results suggested that *AtNKS1* encodes a positive regulator of Na^+ and K^+ tolerance.

2.2. NKS1 is a positive regulator of Na^+ and K^+ tolerance in the presence or absence of SOS genes

Thermal asymmetric interlaced PCR analysis (Liu et al., 1995) of *sos3-1 nks1-1* plants and subsequent diagnostic PCR showed that

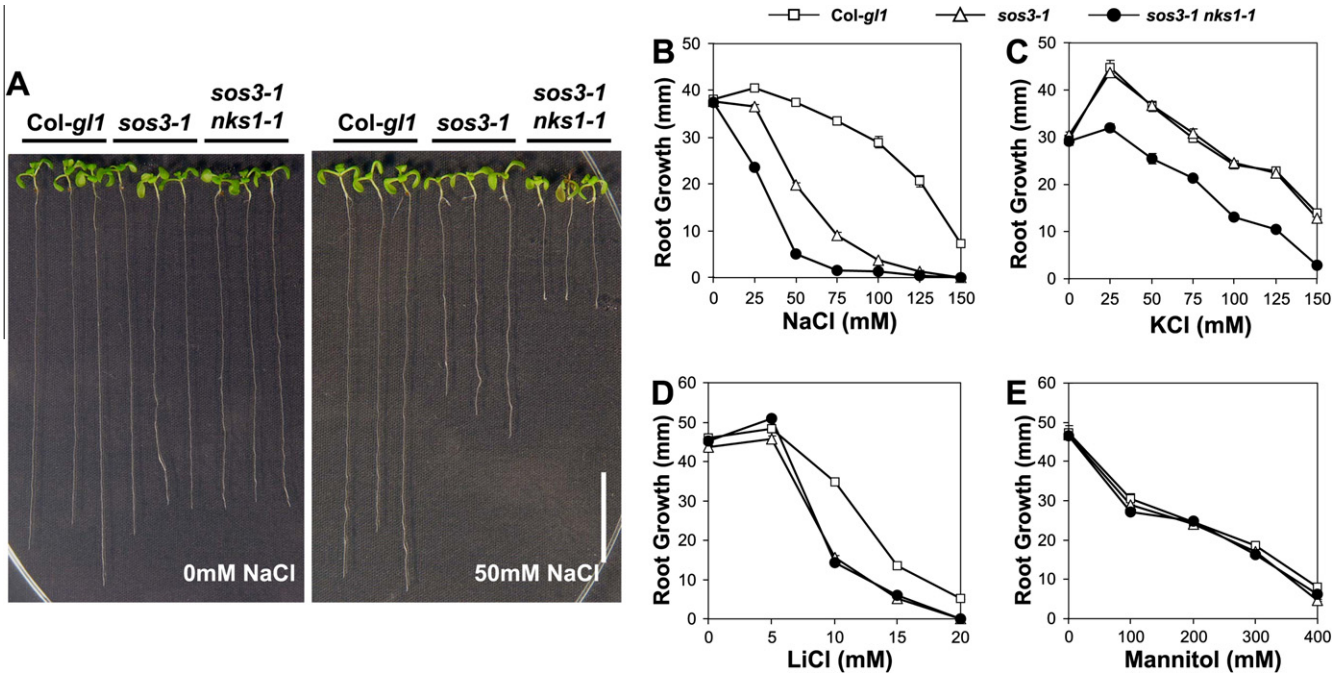

Fig. 1. Isolation of *nks1-1* as a *sos3-1* enhancer. (A) Root growth of Col-0 *gl1*, *sos3-1* and *sos3-1 nks1-1* plants with or without 50 mM NaCl. Four-day-old Col-0 *gl1*, *sos3-1* and *sos3-1 nks1-1* seedlings were transferred to medium without or with 50 mM NaCl. Plates were photographed after 11 days (scale bar = 1 cm). (B–E) Dose responses of plants to NaCl (B), KCl (C), LiCl (D), and mannitol (E). Four-day-old seedlings were transferred to media without or with supplements as indicated. Shown are the increases in root length (root growth) after 11 days. Error bars represent standard deviations ($n = 15$).