[Plant Signaling & Behavior 4:7, 663-665; July 2009]; ©2009 Landes Bioscience

Article Addendum

Role of CIPK6 in root growth and auxin transport

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Key words: Arabidopsis, CIPK6, auxin, MDR4/PGP4, root

In our recent publication,[1] we have shown that a T-DNA insertion in Arabidopsis *CIPK6* gene encoding a CBL-interacting protein kinase caused reduction in expression of the gene and emergence of lateral roots. The change in phenotype in the mutant line was likely due to reduction in shoot-to-root acropetal and the root tip basipetal auxin transport. Here we report identification of a homozygous knockout line of *AtCIPK6* (*atcipk6*) with no detectable expression of the gene in normal growth condition. The knockout line exhibited considerable decrease in growth rate of the taproot as well as in emergence of lateral roots. The mutant line also showed reduction in the root tip basipetal and shoot-to-root acropetal auxin transport. Relative rate of auxin transport and the root phenotype of the *atcipk6* closely matched with those of *pgp4-1*, an Arabidopsis line mutated in *PGP4*. This gene encodes an ABC integral membrane transporter, which functions in polar auxin transport. These observations strengthen our earlier proposal that CIPK6 is probably involved in polar auxin transport and indicate that it may function through the PGP4 auxin transporter.

Root cells are first to respond to change in soil conditions. Continuous change in root system architecture is the vital part of the developmental plasticity that allows the plant to adjust in altered environment. Phytohormone auxin is the most important regulator of all the stages of root development. For example, mutation in *Aberrant Lateral Root Formation3* (*ALF3*) gene results in arrest of lateral root development, which can be rescued by exogenous application of auxin.[2] Mutation in *PGP4* that functions in polar auxin transport causes reduction in root length.[3] Recently, an unprecedented role of stress-related phytohormone abscisic acid (ABA) in regulation of lateral root formation is emerging. A gene encoding ABA-biosynthesis enzyme *9-cis-epoxycarotenoid dioxygenase 9* (*NCED9*) was reported to express in the pericycle cells surrounding the lateral root initiation sites.[4] *ABI5*, an ABA-induced transcription factor encoding gene is expressed in the tip of the lateral root[5] and *abi8* mutant showed defects in root meristem maintenance.[6] While lateral root arrest after emergence of primordia in *alf3* mutant can be rescued by exogenous application of auxin; the same treatment cannot rescue ABA-induced arrest of lateral root development.[7] Therefore, it appears that both the phytohormones auxin and ABA play coordinated but distinct roles in lateral root development. Interestingly, in agreement with possible cross talks between the signaling pathways regulated by these two hormones the ABA-responsive mutants, *abi3*, *lrd2* and *aba2-1* show reduced response to polar auxin transport inhibitors.[8] We reported recently that a mutant Arabidopsis line (*atcipk6kd*: SALK_080951) compromised in expression of CBL-interacting protein kinase 6 (CIPK6) showed reduction in polar auxin transport. *CIPK6* gene expression in chickpea is induced by ABA and *atcipk6kd* line is relatively more sensitive to high salinity.[1] This observation reinforces the concept of coordinated function of auxin and ABA signaling in lateral root development and indicates that CIPK6 may be a nodal point of those two signaling pathways.

To further investigate the biological function of *AtCIPK6,* a T-DNA insertion line (GK-448C12-024532) available through the European Arabidopsis stock Center at Nottingham University (NASC: http://arabidopsis.info/) was procured and the seeds obtained were screened for the homozygous T-DNA insertion. In order to confirm the exact position of the T-DNA insertion the junction of *AtCIPK6* and T-DNA was amplified using RP GABI 5'-GAA GAA AGG ATA CGA CGG AGC-3' and 08409 5'-ATA TTG ACC ATC ATA CTC ATT GC-3' (http://arabidopsis.info/) and sequenced. The sequence revealed that the T-DNA insertion in this homozygous line (*atcipk6*) is after the 1088th base from the translation start site of *AtCIPK6* gene (Fig. 1A). Expression of *AtCIPK6* gene was analyzed in this mutant line in normal growth condition. No full-length ORF-specific product was detected after reverse transcription followed by PCR amplification (RT-PCR) up to 30 cycles (Fig. 1C) or by northern analysis with full-length cDNA as probe (Fig. 1B). For morphological phenotype analysis growth rate of primary root was measured in vertically grown seedlings between the period 4-days to 7-days after germination. Growth rate of primary roots of *atcipk6* seedlings was 29%

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Submitted: 05/12/09; Accepted: 05/13/09

Previously published online as a *Plant Signaling & Behavior* E-publication: http://www.landesbioscience.com/journals/psb/article/9002

Addendum to: Tripathi V, Parasuraman B, Laxmi A, Chattopadhyay D. CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plant. Plant J 2009; 58:778-90; PMID: 19187042; DOI: 10.1111/j.1365-313-X.2009.03812.x.

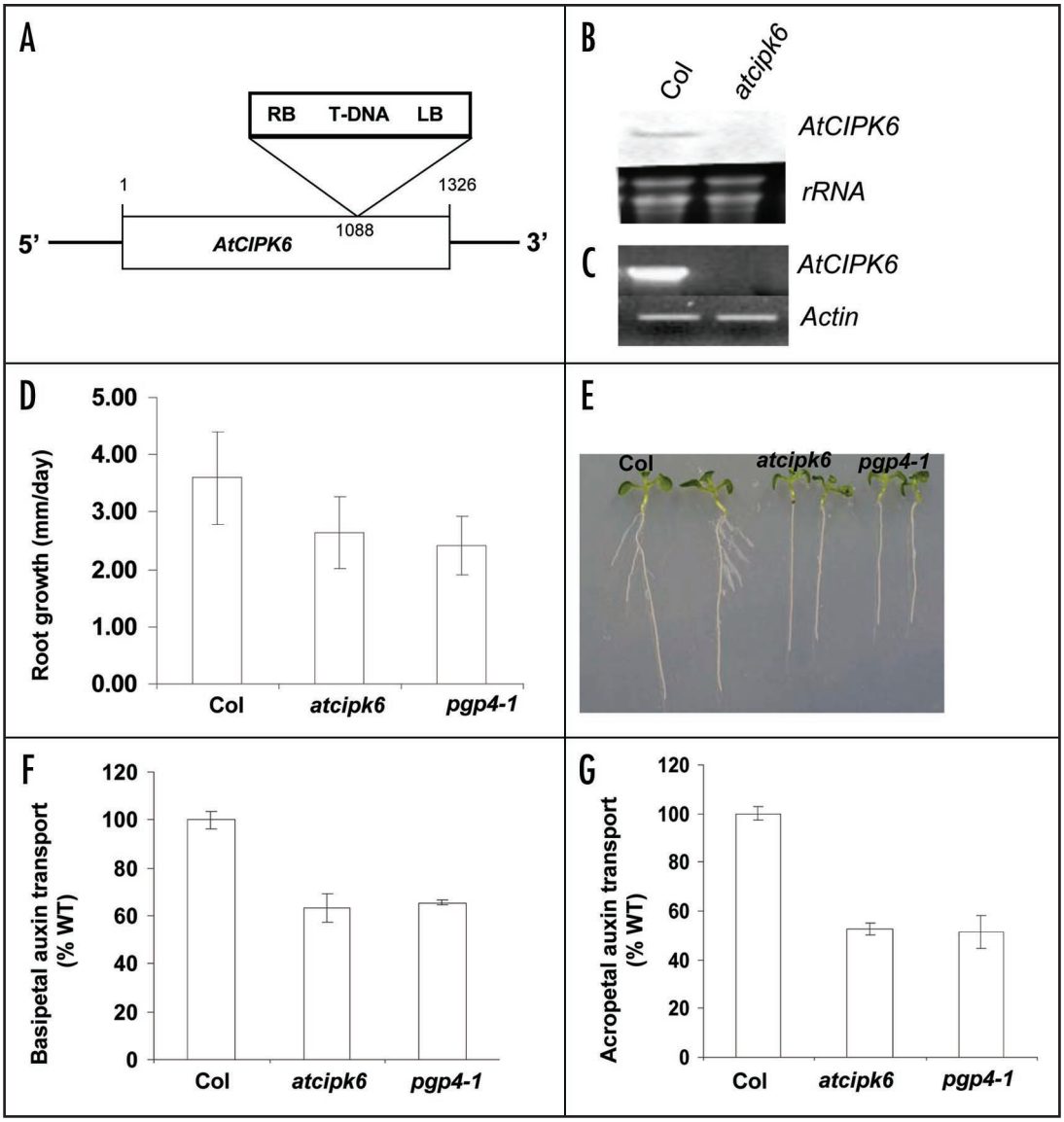

Figure 1. Phenotype characterization of the Arabidopsis *AtCIPK6* knockout mutant. (A) Genomic structure of *AtCIPK6* T-DNA insertion sites in *atcipk6* (GK-448C12-024532). Rectangle represent exon and lines represent untranslated regions of the gene. T-DNA, represented by triangles, is not drawn to scale. RB and LB represent left border and right border of T-DNA. (B) RNA gel blot analysis of *AtCIPK6* gene expression. Ten micrograms of total RNA of wild-type (Col) or mutant (*atcipk6*) seedlings were probed with ^{32}P-labeled full length *AtCIPK6* cDNA (C) RT-PCR (30 cycles) analysis showing At*CIPK6* gene expression in wild-type (Col) and at*cipk6* mutant plants. (D) Comparison of growth rates of the primary root of the seedlings of wild type (Col), *atcipk6* and *pgp4-1* Arabidopsis lines between the period of 4–7 days after germination. The means of three measurements of 20 seedlings each are shown. (E) Morphology of vertically grown 10-day-old (after germination) seedlings of wild type (Col), *atcipk6* and *pgp4-1* Arabidopsis lines. (F) Basipetal root auxin transport in a root segment 2 mm above the site of ^{3}H-IAA application at the root apex in wild type (Col), *atcipk6* and *pgp4-1* mutant lines (G) Acropetal root auxin transport from the root-shoot junction to root measured 2 mm bellow the site of ^{3}H-IAA application. Data are presented as percentage of auxin transport, relative to wild type (Col) (n = 3 group of 7 seedlings each).

($p < 0.005$) less when compared with wild type seedlings (Col) of same stage (Fig. 1D). Unlike the wild type seedlings, the mutant seedlings did not exhibit any lateral root emergence up to 10-day after germination (Fig. 1E). In our previous report, we mentioned that *atcipk6kd* mutant had defects in lateral root emergence and showed reduced polar auxin transport. Based on the observation we proposed a hypothesis that AtCIPK6 may regulate an auxin efflux transporter in Arabidopsis. Two membrane-bound transporter proteins have been identified as substrates of two other CIPKs.[9,10] Exploring the possibility we searched the literature for Arabidopsis lines having mutation in an auxin transporter gene and showing similar phenotypes as *atcipk6*. We found that an Arabidopsis T-DNA insertion line *pgp4-1*, mutated in a gene *MDR4/PGP4*, which functions in the basipetal redirection of auxin transport from the root tip, has almost similar phenotypes like *atcipk6* when 10-day old (after germination) seedlings were compared (Fig. 1E). *pgp4-1* exhibited reduced basipetal auxin transport in roots and a small decrease in shoot-to-root transport.[3] When compared with

atcipk6, almost similar root phenotype was observed for *pgp4-1* mutants showing 34.5% ($p < 0.0003$) reduction in growth rate of primary root in comparison to the wild type (Col) between the period 4-days to 7-days after the germination of the seedlings (Fig. 1E). To further correlate the physiology, root basipetal and shoot to root acropetal auxin transport was measured in *pgp4-1* and *atcipk6 s*eedlings as described previously.[11] Significant decreases of 36.7% and 34.4% from the wild type in root tip basipetal transport of radiolabeled indole acetic acid (IAA) was observed in *atcipk6* and *pgp4-1* seedlings respectively (Fig. 1F). Also, a similar decrease of 47.36% ($p < 0.0002$) and 48.68% ($p < 0.002$) from the wild type (Col) in the root-shoot junction to root acropetal auxin transport was noticed in *atcipk6* and *pgp4-1* seedlings respectively (Fig. 1G) (for experimental methods[11]). RNA gel blot analysis showed that *MDR4/PGP4* expression in stem was relatively less than that in root,[3] while *AtCIPK6* primarily expresses in stem and leaf; and during dehydration and high-salinity also in root. IAA (50 μM) and ABA (100 μM) moderately induced expression of chickpea *CIPK6* gene.[1] *MDR4/PGP4* is a late auxin response gene and ABA treatment caused an oscillatory pattern of expression of this gene.[3] All these correlative data strongly indicate that both CIPK6 and MDR4/PGP4 may operate in the same pathway, functioning together in the polar shoot-to-root auxin transport. Cooperative function of these two proteins in the polar root auxin transport may be relevant for root system plasticity under abiotic stress situations.

Acknowledgements

The project was funded by the Department of Biotechnology, Government of India. V.T. acknowledges fellowship from National Institute of Plant Genome Research. N.S. acknowledges fellowship from Indian Council of Medical Research.

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