Supporting Information

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SI Materials and Methods

Plant Materials and Growth Conditions. The transfer DNA (T-DNA) insertion mutant *akt1* (SALK_071803) was obtained from the Arabidopsis Biological Resource Center. The seeds of *sos4-1* mutant, *pdx3* T-DNA insertion mutant, and *athak5* mutant were kindly provided by Jian-Kang Zhu (Purdue University, West Lafayette, IN), Margaret E. Daub (North Carolina State University, Raleigh, NC), and Weihua Wu (China Agricultural University, Beijing), respectively. *Arabidopsis* plants were grown in soil (rich soil: vermiculite = 2:1, vol/vol) or on half-strength MS media supplemented with indicated chemicals in controlled environmental rooms at 22 °C under white fluorescent light with a 16-h light/8-h dark photoperiod.

Isolation of *sno1* Mutant and Genetic Mapping of the *SNO1* Gene. *Arabidopsis* wild-type seeds were mutated by ethyl methanesulfonate (EMS) according to the standard procedure described previously (1). Seeds of M2 generation were surface-sterilized and plated on half-strength MS media containing 25 μM sodium nitroprusside (SNP; Sigma-Aldrich). After 3-d incubation at 4 °C, the plates were transferred to the growth room for 2 wk. Seedlings with shorter root than wild type were picked as putative mutants. The putative mutants were then transplanted to normal growth conditions to harvest M3 seeds, and the root phenotype of the M3 seedlings was further confirmed using the SNP containing media. The selected mutant (*sno1*) was backcrossed two times, and then was crossed with Col-0 wild type to produce the F1 and F2 generations for genetic analyses. For the genetic mapping, 2,856 individual F2 seedlings derived from the cross *sno1* (in Col-0 background) × Landsberg *erecta* (Ler) were selected as mapping population based on their *sno1*-like responses to SNP. Rough and fine mappings were performed as Lukowitz et al. (2).

Staining of Plant Endogenous NO. Plant endogenous NO was stained using the NO-sensitive fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA; Molecular Probes) as described previously (3). Briefly, seedlings was incubated in a staining buffer [0.1 mM $CaCl_2$, 10 mM KCl, 10 mM Mes·Tris (pH 5.6)] containing DAF-2DA (10 μM) for 30 min, and then washed with the staining buffer three times (5 min each). The fluorescence was examined by a stereomicroscope (Stemi SV 11; Zeiss) equipped with a CCD camera.

Potassium Content Measurement. The 3-d-old well-germinated seedlings were transferred to half-strength MS media supplemented with 0 μM or indicated concentrations of SNP and grown vertically for 15 d. Shoots and roots were harvested respectively and dried at 60 °C for at least 1 wk before being weighed. Samples were then digested with HNO_3 and measured by inductively coupled plasma-MS (Agilent 7500C) for potassium concentration.

Pyridoxal 5′-Phosphate Content Measurement. The 3-d-old seedlings were transferred to half-strength MS media supplemented with 0 μM or indicated concentrations of SNP for 15 d. Vitamin B6 was extracted and quantified by HPLC (Agilent-1100), according to the protocol of González et al. (4). Standard curves were made using commercial pyridoxal 5′-phosphate compound (Sigma-Aldrich).

Real-Time Quantitative PCR Analysis. The 1-wk-old seedlings were transferred to the media containing 0 μM or indicated concentrations of SNP for 2 d and collected for the analysis. Total RNA was extracted using TRIzol reagent (Sigma-Aldrich), and first-strand cDNA was synthesized after DNase treatment using reverse transcriptase (Takara) according to the manufacturer's instructions. Real-time quantitative PCR reactions were performed with SYBR Green master mix (Bio-Rad) on the DNA Engine Opticon 2 System (Bio-Rad) using gene-specific primers as described previously (4). Three technical repeats were performed for each sample, and relative gene expression levels were calculated by normalization to the reference gene *ACTIN2*.

Enzyme Activity Analyses of SNO1 and PDX3. Samples were grown and treated the same as those used for the PLP content measurement. The activity of SNO1 and PDX3 were measured using a colorimetric procedure, according to the protocols described by Zhao and Winkler (5) and González et al. (4), respectively. Briefly, crude extracts were obtained from 500 mg of lyophilized tissue. For the pyridoxal kinase (SNO1) activity analysis, 0.5 mL crude extract (contains 1–7 mg total protein) in a buffer containing 0.2 mM pyridoxal, 0.2 mM ATP, 0.1 mM $ZnCl_2$, and 70 mM K_3PO_4 was used for the reaction. For the pyridoxamine phosphate oxidase activity (PDX3) activity analysis, 0.5 mL crude extract (contains 1–7 mg total protein) in a buffer containing 200 mM Tris·HCl, 200 mM K_3PO_4 (pH 8.5), and 0.2 mM pyridoxaine phosphate was used for the reaction. After 1 h incubation at 37 °C, reactions were stopped by 50 mL chilled 50% TCA (wt/vol). Protein was pelleted by centrifuging (1,500 × g) at 4 °C for 10 min, and then the supernatant was transferred to a clean tube. After the addition of 2% phenyl-hydrazine (in 10 N H_2SO_4) and incubation on ice for 30 min, the PLP formation was measured based on the absorbance at 410 nm. The enzyme activity was calculated by normalizing to the protein amount determined by the Bradford protein assay kit (Bio-Rad). Each reaction was repeated three times.

Electrophysiological Studies in *Xenopus* Oocytes. The expression and voltage-clamp recording in *Xenopus* oocytes was performed as described as Liu and Luan (6). The constructs of *CBL1*, *CIPK23*, and *AKT1* in pGEMHE oocyte expression vector were performed as described previously (7). Briefly, the cRNAs were transcribed in vitro using the mMESSAGE mMACHINE T7 RNA Transcription Kit (Ambion). After the quality check and concentration measurement, cRNAs were coinjected into oocytes. Whole-cell recordings were carried out 2–3 d after the injection, using a two-electrode voltage-clamp technique with a GeneClamp 500B Amplifier (Axon Instruments). The oocytes were bathed with a solution containing 6 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Mes·Tris (pH 5.5), 135 mM mannitol, and 50 mM glutamate, and perfused with the same solution containing 50 μM pyridoxal-5′-phosphate for several minutes. The pipette solution contained 3 M KCl.

Patch-Clamp Recordings in *Arabidopsis* Root Cell Protoplasts. The primary roots of 5- to 7-d-old seedlings were cut into small pieces and kept at 26 °C in an enzyme solution containing 1% cellulysin (Calbiochem), 1.5% (wt/vol) cellulase RS (Yakult Honsha Co.), 0.1% pectolyase (Sigma), 0.1% BSA, 2 mM $MgC1_2$, 10 mM $CaC1_2$, 200 mM mannitol, and 5 mM Mes (pH 5.6) for 40 min to release the protoplasts. The protoplasts were filtered through nylon mesh (80 μm pore size) and washed with standard solution [1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM sucrose, and 2 mM Mes (pH 5.6)]. Protoplast were collected by centrifugation at 150 × *g* for 7 min, resuspended with the standard solution, and kept on ice before use.

Whole-cell patch-clamp recordings were performed as described (8) at room temperature (~20 °C) using the same bath and pipette solutions as before (9). Currents were recorded using an Axopatch 200B amplifier (Axon Instruments), and data were collected using a digitizer Digidata 1200 (Axon Instruments) and software Clampex 9.0 (Axon Instruments). Analyses were carried out using software Clampfit 10 (Molecular Devices), Microsoft Excel, and Origin 8.0 (OriginLab Corp.).

1. Kim Y, Schumaker KS, Zhu JK (2006) EMS mutagenesis of *Arabidopsis*. *Methods Mol Biol* 323:101–103.
2. Lukowitz W, Gillmor CS, Scheible WR (2000) Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. *Plant Physiol* 123(3):795–805.
3. He Y, et al. (2004) Nitric oxide represses the *Arabidopsis* floral transition. *Science* 305(5692):1968–1971.
4. González E, Danehower D, Daub ME (2007) Vitamer levels, stress response, enzyme activity, and gene regulation of *Arabidopsis* lines mutant in the pyridoxine/pyridoxamine 5′-phosphate oxidase (PDX3) and the pyridoxal kinase (SOS4) genes involved in the vitamin B6 salvage pathway. *Plant Physiol* 145(3):985–996.
5. Zhao G, Winkler ME (1995) Kinetic limitation and cellular amount of pyridoxine (pyridoxamine) 5′-phosphate oxidase of *Escherichia coli* K-12. *J Bacteriol* 177(4):883–891.
6. Liu K, Luan S (2001) Internal aluminum block of plant inward K^+ channels. *Plant Cell* 13(6):1453–1465.
7. Li L, Kim BG, Cheong YH, Pandey GK, Luan S (2006) A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in *Arabidopsis*. *Proc Natl Acad Sci USA* 103(33): 12625–12630.
8. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* 391(2):85–100.
9. Ivashikina N, et al. (2001) K^+ channel profile and electrical properties of Arabidopsis root hairs. *FEBS Lett* 508(3):463–469.
10. Vanderschuren H, et al. (2013) Strategies for vitamin B6 biofortification of plants: A dual role as a micronutrient and a stress protectant. *Front Plant Sci* 4:143.

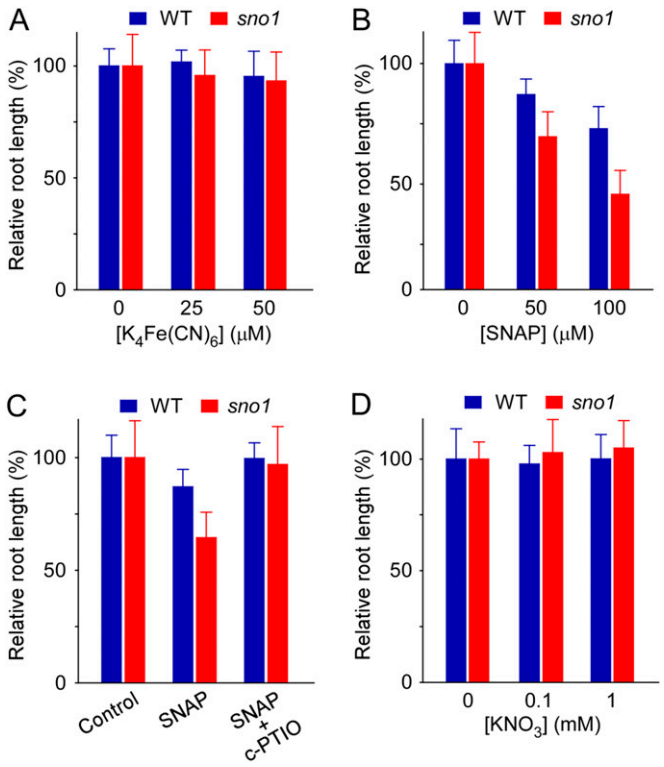

Fig. S1. *sno1* mutant is hypersensitive to NO. (*A*) Relative root length of 15-d-old WT and *sno1* mutant under indicated concentrations of potassium ferrocyanide $[K_4Fe(CN)_6]$. (*B*) The root growth of *sno1* mutant shows hypersensitive response to SNAP treatment under indicated concentrations. (*C*) The SNAP treatment (50 μM)-caused root growth inhibition was rescued with the addition of NO scavenger c-PTIO (50 μM). (*D*) Relative root length of 15-d-old WT and *sno1* mutant under indicated concentrations of potassium nitrate (KNO_3). In *A*–*D*, relative root length shown by percentages is presented as a value relative to the root length on half-strength MS media. Data shown are mean ± SD, $n = 30$ seedlings.

A

Cross	Population	Number of plants WT	*sno1*	Segregation ratio (WT/*sno1*)
WT x *sno1*	F2	2888	917	3.15*

* Data were analyzed by χ^2 test of goodness-of-fit Mendelian trait.

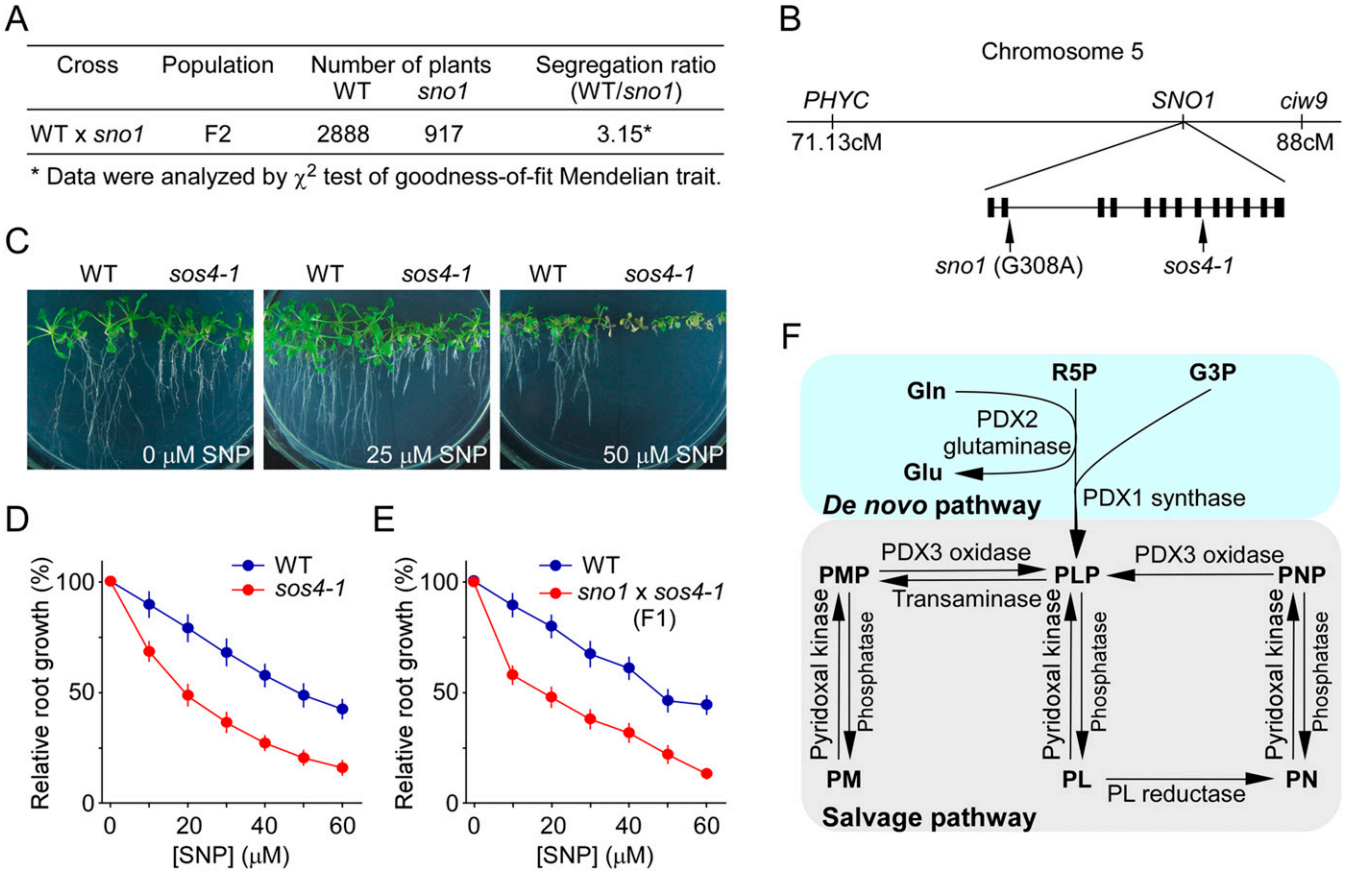

Fig. S2. *SNO1* is identified to be *SOS4*. (*A*) Genetic analysis of F2 seedlings derived from the cross WT (Col-0) × *sno1* showed an ~3:1 (WT vs. *sno1*) segregation ratio under 50-μM SNP treatment. (*B*) *SNO1* was mapped on chromosome 5, and a single nucleotide substitution (G to A) was found at the splicing acceptor site of the second intron in At5G37850 (*SOS4*). Closed boxes indicate exons, and the lines between boxes indicate introns. (*C*) Phenotypes of *sos4-1* seedlings under 0-, 25-, and 50-μM SNP treatments. (*D*) Relative root growth of WT and *sos4-1* mutant in response to indicated concentrations of SNP. (*E*) Relative root growth of F1 seedlings derived from the cross *sno1* × *sos4-1* in response to various concentrations of SNP. (*F*) Vitamin B6 biosynthesis pathways in *Arabidopsis*, based on published papers (4, 10). R5P, ribose 5-phosphate; G3P, glyceraldehyde 3-phosphate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PNP, pyridoxine 5′-phosphate; PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine. In *C*–*E*, germinated seedlings were transferred to the new media for 15 d for the analysis, and data shown in *D* and *E* are means ± SD, $n = 50$ seedlings.

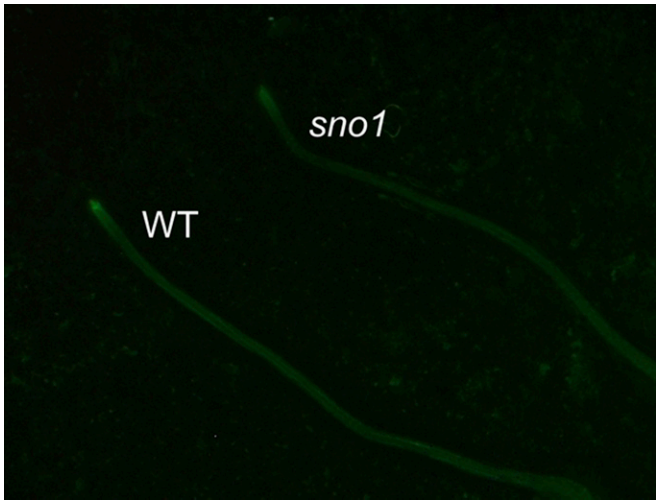

Fig. S3. NO levels in WT and *sno1* mutant. The 15-d-old seedlings were stained with NO-sensitive fluorescent dye DAF-2DA, and primary roots were analyzed. Thirty seedlings of each sample were analyzed in three independent experiments, and representative images are shown.

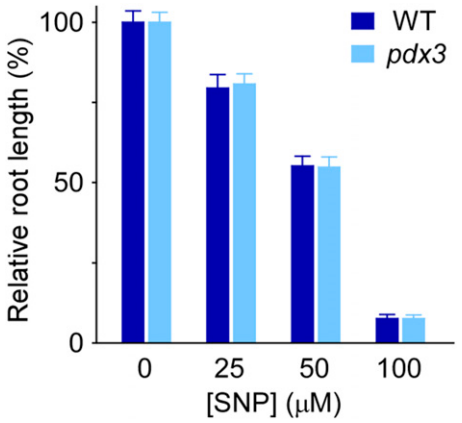

Fig. S4. *pdx3* mutant has similar response to SNP as WT, as shown by relative root length. Seedlings were grown on media containing indicated concentrations of SNP for 15 d for the analysis. Data shown are means ± SD, $n = 50$ seedlings.

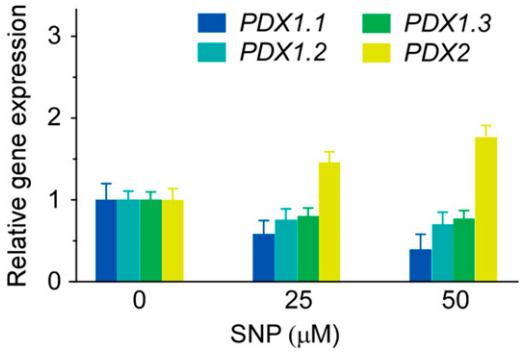

Fig. S5. SNP effect on the expression levels of vitamin B6 de novo biosynthetic pathway genes in *Arabidopsis*. Relative gene expression of all *Arabidopsis PDX1* homologs including *PDX1.1*, *PDX1.2*, and *PDX1.3*, and *PDX2* was tested in response to indicated concentrations of SNP. Data are means ± SD, $n = 3$.

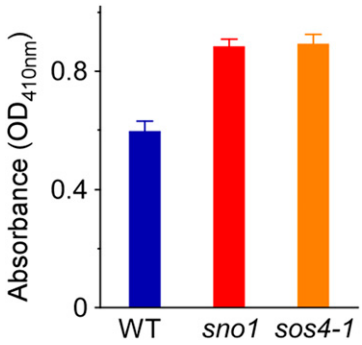

Fig. S6. Analysis of PDX3 enzyme activity in WT, *sno1*, and *sos4-1* plants. Germinated seedlings were transferred to the new plates for 15 d and collected for the analysis. Data shown are means ± SD, $n = 3$.

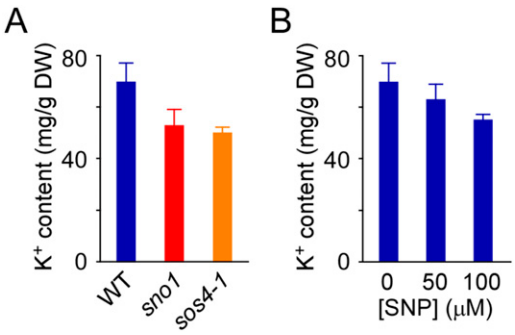

Fig. S7. Shoot K^+ content in *Arabidopsis* seedlings. (*A*) K^+ content in WT, *sno1*, and *sos4-1* plants. (*B*) SNP effect on K^+ content in wild-type seedlings. The shoots of 15-d-old seedlings were collected for inductively coupled plasma–MS analysis. Data shown are means ± SD, $n = 3$.

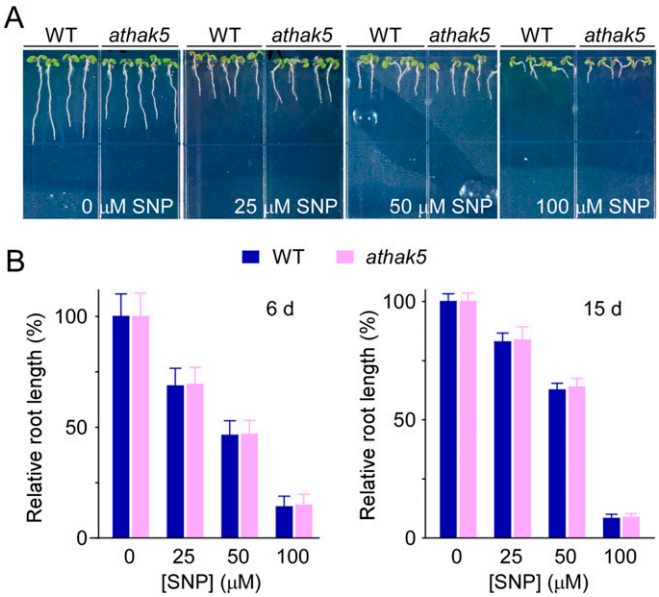

Fig. S8. *athak5* mutant has similar response to SNP as WT. (*A*) Phenotypes of WT and *athak5* mutant grown on the media containing indicated concentrations of SNP for 6 d. (*B*) Quantification of relative root length in WT and *athak5* mutant grown on indicated concentrations of SNP for 6 d and 15 d, respectively. Data shown are means ± SD, $n = 50$ seedlings.

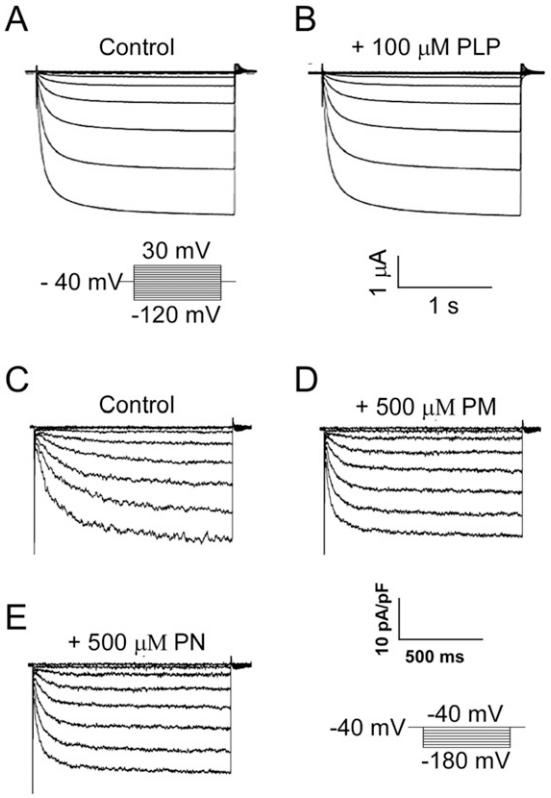

Fig. S9. PLP specifically inhibits the activity of AKT1-mediated currents, as shown by no effect of PLP on KAT1-mediated inward K^+ currents in oocytes (*A* and *B*) and no effects of pyridoxamine (PM) and pyridoxine (PN) on AKT1-mediated currents in *Arabidopsis* root protoplasts (*C–E*). (*A*) Whole-cell recording of KAT1 currents in oocytes. (*B*) Influence of PLP (100 μM) on the whole-cell recording of KAT1-mediated currents in oocytes. (*C–E*) Whole-cell recordings of AKT1-mediated currents under no treatment (control), 500 μM PM, and 500 μM PN, respectively. Representative recording is shown for each treatment.

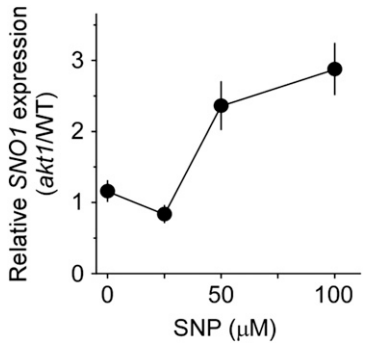

Fig. S10. *akt1* mutant expresses more *SNO1* than WT with the increase of SNP concentration, as shown by the relative *SNO1* expression level in *akt1* vs. WT (*akt1*/WT). The 1-wk-old seedlings were treated with various concentrations of SNP for 2 d and collected for the expression analysis. Data shown are means ± SE, $n = 3$.