

# Hydrogen peroxide mediates plant root cell response to nutrient deprivation

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**Potassium ( $K^+$ ) is an essential nutrient required by plants in large quantities, but changes in soil concentrations may limit  $K^+$  acquisition by roots. It is not known how plant root cells sense or signal the changes that occur after the onset of  $K^+$  deficiency. Changes in the kinetics of  $Rb^+$  uptake in *Arabidopsis* roots occur within 6 h after  $K^+$  deprivation. Reactive oxygen species (ROS) and ethylene increased when the plants were deprived of  $K^+$ . ROS accumulated in a discrete region of roots that has been shown to be active in  $K^+$  uptake and translocation. Suppression of an NADPH oxidase in *Arabidopsis* (*rhd2*), which is involved in ROS production, prevented the up-regulation of genes that are normally induced by  $K^+$  deficiency, but the induction of high-affinity  $K^+$  transport activity was unchanged. Application of  $H_2O_2$  restored the expression of genes induced by  $K^+$  deficiency in *rhd2* and was also sufficient to induce high-affinity  $K^+$  transport activity in roots grown under  $K^+$ -sufficient conditions. ROS production is an early root response to  $K^+$  deficiency that modulates gene expression and physiological changes in the kinetics of  $K^+$  uptake.**

Potassium ( $K^+$ ) is essential to and required in large quantities by plants. To ensure the adequate supply, plants have a number of redundant mechanisms for  $K^+$  acquisition and translocation (1–3). Whereas much is known about  $K^+$  transport mechanisms, little is known about how plant cells sense and respond to changes in the  $K^+$  concentrations encountered in their environment. At the whole-plant level,  $K^+$  deficiency is known to enhance root respiration (4). This response suggests a shift in plant metabolism required for mineral acquisition or compensation for increased stress. External  $K^+$  concentrations alter the kinetics of uptake, which may be due to allosteric regulation by internal changes in  $K^+$  concentrations (5). Specific proteins in the plasma membrane and tonoplast were shown to be produced in response to  $K^+$  deficiency, indicating the synthesis of new transport proteins (6–8). Whereas changes in respiration and membrane transport occur in response to  $K^+$  deficiency, the cell biological events that trigger these changes have not been elucidated.

In *Escherichia coli*, some of the molecules involved in the sensing and signaling of  $K^+$  deficiency have been elucidated (9). KdpD is a sensor kinase that undergoes autophosphorylation and transfers a phosphoryl group to a response regulator KdpE (10). The response regulator controls the expression of an operon coding for the high-affinity  $K^+$  uptake system in *E. coli*. The sensor kinase is thought to transduce changes in turgor caused by low  $K^+$  (11). It is not clear whether similar mechanisms are involved in transducing  $K^+$  deprivation in plants.

Roots are the primary organs involved in mineral acquisition for plants and function at the interface with the rhizosphere. Although genes whose expression is related to external changes in nutrient composition have been identified, the cascade of cellular responses involved in sensing and signaling nutrient deficiency has not been elucidated (12–18). To identify the initial cellular responses to  $K^+$  deprivation, we performed preliminary microarray experiments on *Arabidopsis* roots and developed the working hypothesis that reactive oxygen species (ROS) production and ethylene may be involved in root response to  $K^+$  deprivation. In this study, we used uptake kinetics, biochemical

analyses, an NADPH oxidase mutant, and a set of  $K^+$ -responsive genes to identify ROS as one component of the signal transduction pathway in roots deprived of  $K^+$ . To our knowledge, no comparable study on nutrient deprivation has implicated a link between ROS and nutrient deficiency.

## Materials and Methods

**Plant Material and Growth Conditions.** Plants were grown in nutrient solutions by using a rock wool system (19) for 6–8 weeks at 22°C with 8 h daylight at 200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Maize seeds (*Zea mays* L. cv. FR27  $\times$  FRM017) were also planted in rock wool plugs in the same nutrient solution as *Arabidopsis* and grown at 22°C, humidity 50%, 16 h light/8 h dark, and 200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Two-week-old maize plants were used for the  $H_2O_2$  measurement.

**Root Growth Assay and  $Rb^+$  Uptake Assay.** For the root growth assay, *Arabidopsis* was planted on nutrient containing (19) 1.2% Seakem LE Agarose (BMA, Rockland, ME) and 2% sucrose. Ten seedlings were transferred to full nutrient or  $K^+$ -deprived plates, and their growth was measured every day for 10 days. The result of the root growth assays is the average of three experiments.

On the day of each  $Rb^+$  uptake experiment, control solutions were changed in the morning to solutions with and without  $K^+$  as described (20). After 6 h, plants were moved to beakers to equilibrate with the  $Rb^+$  at the different concentrations used for 5 min. Trace amounts of  $^{86}\text{Rb}^+$  were added to start a 10-min uptake period. After the uptake period, roots were desorbed for 10 min in 0.5 mM  $\text{CaSO}_4$  at 22°C. Roots were then blotted, weighed, and placed in scintillation vials containing scintillant. Radioactivity in the roots was counted by using a Beckman LS6500 scintillation counter. The experiments were repeated on two different days with plants from four different hydroponic tanks ( $n = 4$ ). From each tank subsamples of two to three plants were used at each concentration at which uptake was measured. Data were fitted with a one-site binding model (hyperbola) by using the equation  $Y = (V_{\text{max}} \cdot X) / (K_m + X)$ ; where  $X$  was the  $Rb^+$  concentration and  $Y$  was the uptake rate. The nonlinear regression was performed by using PRISM software (GraphPad, San Diego), which finds the values of  $V_{\text{max}}$  and  $K_m$  at which the sum of squares are the smallest and calculates the SE for those kinetic parameters.

**RT-PCR Analysis and  $H_2O_2$  Treatment.** RNA was extracted from the roots of plants that were deprived of  $K^+$  or supplied with sufficient  $K^+$  for 6 and 30 h by using RNawiz. cDNAs were synthesized by using the SuperScript first-strand synthesis system (Invitrogen) and an oligo(dT)<sub>18</sub> primer. For the  $H_2O_2$  treatments, 1 mM (Sigma) was added to the nutrient medium for 6

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Abbreviations: ROS, reactive oxygen species; DPI, diphenylene iodonium; ACC, 1-aminocyclopropane- $\alpha$ -carboxylic acid.

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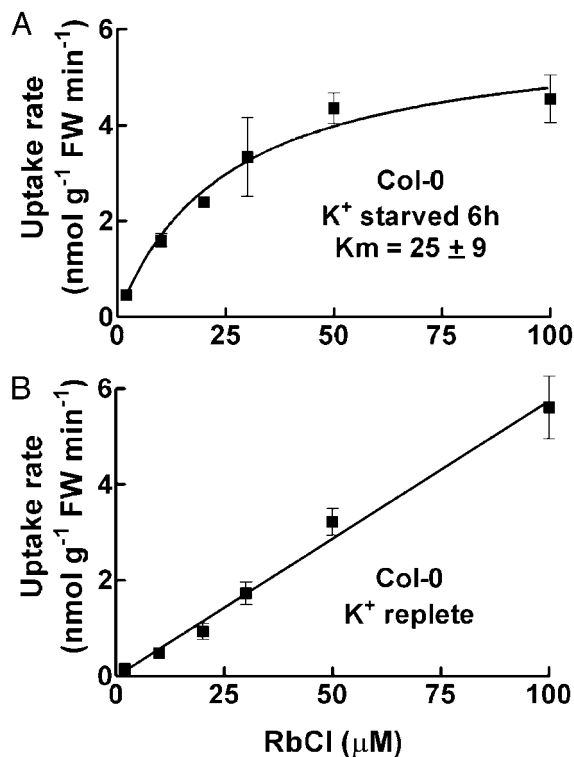
**Table 1. Root growth under K<sup>+</sup>-deficient and -sufficient conditions**

Condition	Final primary root length, cm	Lateral root length, cm per cm of primary root	Total no. lateral roots
+K <sup>+</sup>	3.23 ± 0.06	1.04 ± 0.02	15 ± 0.4
-K <sup>+</sup>	3.21 ± 0.09	0.46 ± 0.03	7 ± 0.5
	NS	P < 0.001	P < 0.001

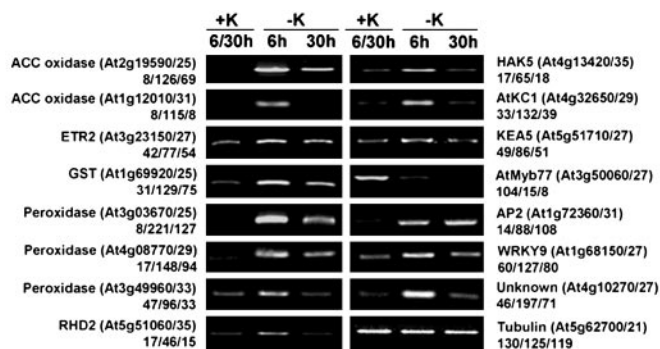
Means ± SE are shown. Results are the average of three experiments (n = 4 plates, 5 plants per plate). Comparison between roots grown with and without K<sup>+</sup> were performed by one-way ANOVA. NS, no significant differences. P values shown indicate highly significant differences between treatments in lateral roots.

or 30 h. For the diphenylene iodonium (DPI) experiment (Sigma), 100 μM DPI was supplied in the nutrient medium for 2 h before K<sup>+</sup> deprivation. Plants were then transferred to K<sup>+</sup>-deficient or -sufficient nutrient solution for 6 h before harvest. Each PCR/experiment was performed three times with different cDNA sets from independent biological replicates. All PCR products were sequenced to ensure that the correct transcript was detected. PHOTOSHOP 7.0 (Adobe Systems, San Jose, CA) was used to determine the mean relative intensity of each amplification product.

**Ethylene Measurement.** Six-week-old hydroponic cultured *Arabidopsis* was transferred to 40-ml vials containing K<sup>+</sup>-sufficient or -deficient nutrient medium for 6 and 30 h. One milliliter of the headspace was taken from the vials, and ethylene concentration



**Fig. 1.** Kinetics of Rb<sup>+</sup> uptake in *Arabidopsis* roots. (A) Rb<sup>+</sup> uptake rates in Col-0 roots starved of K<sup>+</sup> for 6 h showed a saturable kinetic component; K<sub>m</sub> = 25 ± 9 μM. (B) Rb<sup>+</sup> uptake rates in Col-0 roots grown under K<sup>+</sup>-sufficient conditions do not show saturable kinetics over the concentration range tested. Mean uptake rates at each concentration are shown ± SE; n = 4 for each concentration.



**Fig. 2.** Changes in gene expression upon K<sup>+</sup> deprivation monitored by RT-PCR. RNA was extracted from *Arabidopsis* roots grown under K<sup>+</sup>-sufficient conditions (+K), 6 h after K<sup>+</sup> deprivation (6h), and 30 h after K<sup>+</sup> deprivation (30h). β-Tubulin was used for cDNA normalization. The gene number follows a description of the gene function, and the number of PCR cycles used for amplification follows the gene number. Mean relative intensity for each RT-PCR amplification product is shown below the gene number or description.

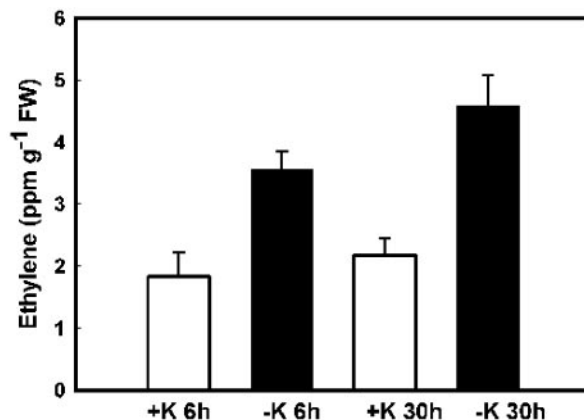
was measured with a Shimadzu (Kyoto) GC-8A gas chromatograph. The data were analyzed by ANOVA, and means were compared by using Student's *t* test. Experiments were repeated three times.

**H<sub>2</sub>O<sub>2</sub> Measurement and Localization.** Six-week-old plants were used to measure H<sub>2</sub>O<sub>2</sub> production in controls and after K<sup>+</sup> deficiency by using an Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes). The data were analyzed by ANOVA, and means were compared by using Student's *t* test. Experiments were performed three times for *Arabidopsis* and maize.

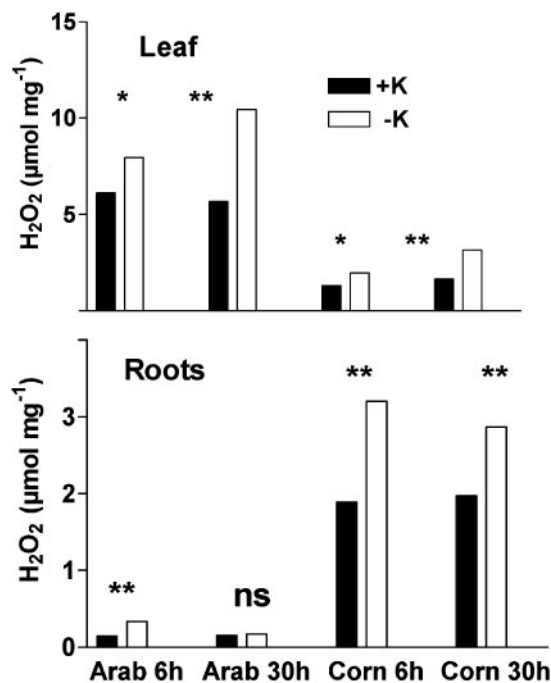
Five-day-old plants were used to localize the generation of ROS in the root. For experiments with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; (Molecular Probes), plants were incubated on K<sup>+</sup>-deficient or -sufficient plates for 30 h (21). All fluorescence images were obtained with a Nikon SMZ1500 microscope and a QImaging (Burnaby, BC, Canada) Retiga cooled mono 12-bit camera.

## Results

**K<sup>+</sup> Deficiency Affects Lateral Root Growth and Alters the Kinetics of Uptake.** *Arabidopsis* seedlings were transferred to plates with and without K<sup>+</sup> 3 days after germination. Final primary root length



**Fig. 3.** Ethylene production in *Arabidopsis* plants grown under hydroponic conditions with K<sup>+</sup> or without K<sup>+</sup> for 6 and 30 h. Experiments were performed three times, and the mean (n = 6 plants ± SE) is shown from one experiment. Mean comparison showed that ethylene levels were significantly higher than control levels at 6 (P < 0.01) and 30 h (P < 0.001) after deprivation.

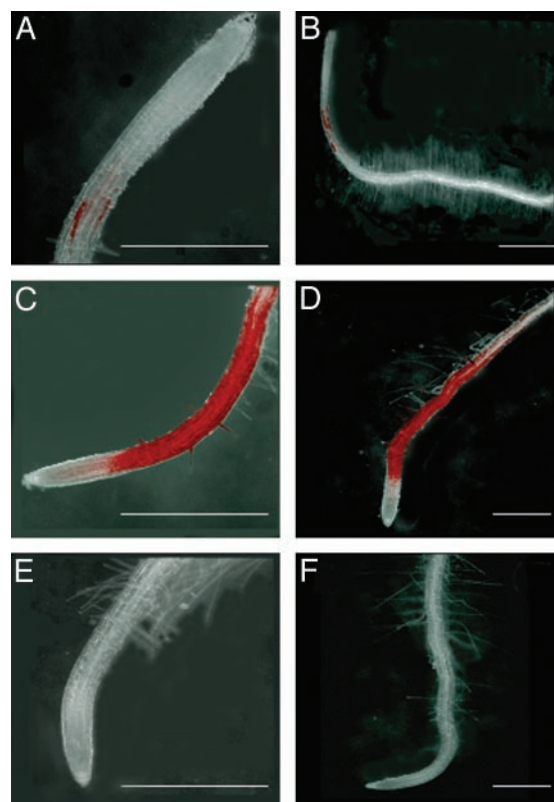


**Fig. 4.** H<sub>2</sub>O<sub>2</sub> production after K<sup>+</sup> deprivation in *Arabidopsis* and maize. Experiments were performed three times for *Arabidopsis* and maize, and one representative set of results are shown. +K, Plants grown with 1.75 mM KCl; -K, plants that were deprived of K<sup>+</sup>. The data were analyzed by ANOVA. \*, *P* < 0.05 and \*\*, *P* < 0.01 between treatments.

was the same 10 days after transfer to plates containing low K<sup>+</sup> and sufficient K<sup>+</sup> (Table 1). However, lateral root length and the numbers of lateral roots decreased in plants on low K<sup>+</sup> medium (Table 1). These data show that in the long term, root growth is affected by the lack of K<sup>+</sup>.

To demonstrate that after 6 h, K<sup>+</sup> deprivation alters root function, we measured the kinetics of K<sup>+</sup> uptake by using Rb<sup>+</sup> as an analog for K<sup>+</sup> (22). Classical studies (2) have shown that large differences in kinetic parameters are induced in many plant species by changes in the composition of the growth medium. In *Arabidopsis*, kinetic uptake studies of K<sup>+</sup>-deprived plants have not been published. Our studies using Rb<sup>+</sup> uptake show that a component of high-affinity uptake is induced after 6 h of K<sup>+</sup> deprivation. Fig. 1A shows that the kinetics of uptake after deprivation were saturable at low concentrations of Rb<sup>+</sup> with a *K<sub>m</sub>* of 25 ± 9 μM. In contrast, the plants grown with sufficient amounts of K<sup>+</sup> showed linear rates of K<sup>+</sup> uptake (Fig. 1B) over the same concentration range.

**Identification of Genes Whose Expression Changes upon K<sup>+</sup> Deprivation.** Preliminary microarray experiments of changes in *Arabidopsis* root gene expression identified genes that were related to ROS production, ethylene synthesis, K<sup>+</sup> transport, and transcription. From these experiments, we identified up-regulated genes related to ROS metabolism and ethylene synthesis (Fig. 2). The expression of a gene encoding the K<sup>+</sup> transporter *HAK5* increased, which confirms previous results (20). Two other genes related to K<sup>+</sup> transport *KEA5* and *AtKC1* were also up-regulated (Fig. 2). An ethylene-responsive AP2 transcription factor, a WRKY-type transcription factor (*WRKY9*), and a *Myb*-type transcription factor (*AtMyb77*), were up- or down-regulated (Fig. 2). One unknown gene *At4g10270* was strongly up-regulated by K<sup>+</sup> deficiency and is homologous to a tomato wound-inducible gene (Fig. 2). We also measured the changes in gene expression at early time points for the genes in Fig. 2. We found no change



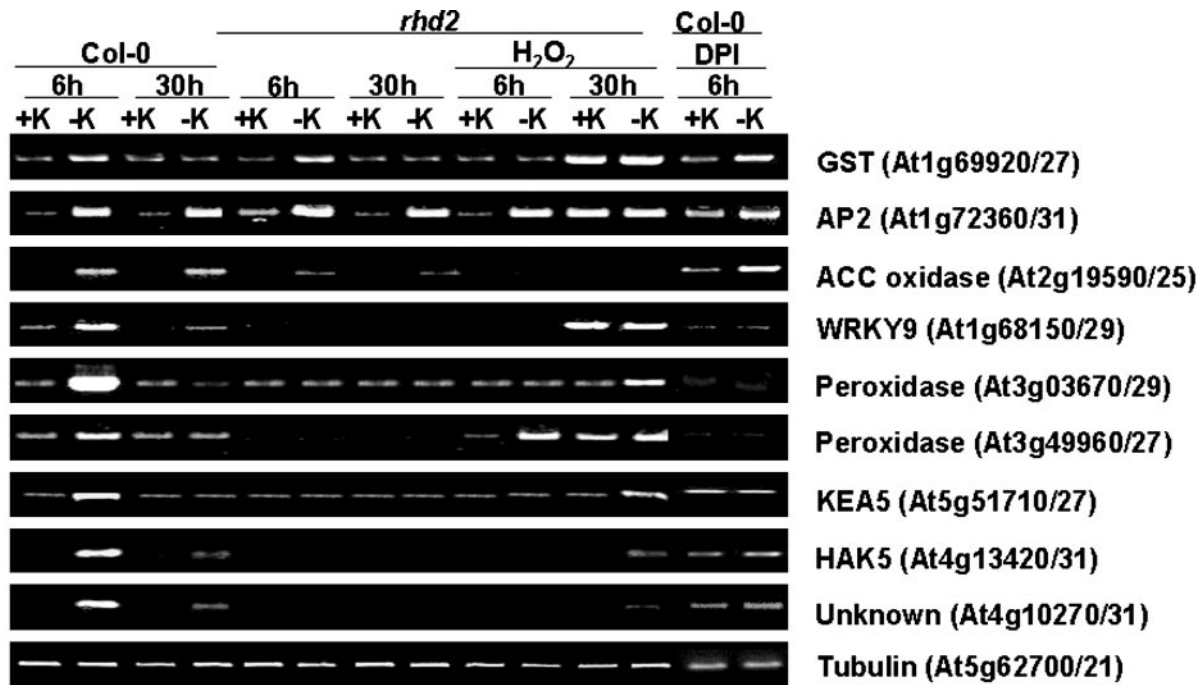
**Fig. 5.** Localization of ROS in *Arabidopsis* roots during K<sup>+</sup> deprivation. Pseudocolor images of bright field (white) and fluorescence (red) are shown for K<sup>+</sup>-sufficient (A and B) roots and for K<sup>+</sup>-deficient roots with 50 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (C and D) and without it (E and F). Fluorescence indicates the presence of ROS. (Scale bar, 5 mm.) Ten control and 10 K<sup>+</sup>-deprived roots showed similar results.

in gene expression in this group of genes (Fig. 2) after 30 min. After 2 h, the expression of many of these genes was up-regulated, including *At2g19590*, *At3g03670*, *At3g23150*, *At1g72360*, *At1g68150*, *At4g32650*, and a single gene, *At3g50060*, was down-regulated. Plants used in these experiments were analyzed by using inductively coupled plasma-MS, which showed that the mineral composition (except for K<sup>+</sup>) was not altered by the short-term treatments.

**K<sup>+</sup> Deficiency Alters *In Vivo* Ethylene Level.** Because several genes encoding enzymes that are involved in ethylene production were up-regulated by K<sup>+</sup> deprivation (Fig. 2), we determined that ethylene production increased after K<sup>+</sup> starvation by measuring ethylene production in plants 6 and 30 h after K<sup>+</sup> deprivation. The result of three experiments showed that ethylene production increased by a factor of 1.5–2 after K<sup>+</sup> starvation (Fig. 3).

**H<sub>2</sub>O<sub>2</sub> Production Increases After K<sup>+</sup> Deficiency.** Several genes related to ROS metabolism were significantly up-regulated by K<sup>+</sup> starvation (Fig. 2), and therefore H<sub>2</sub>O<sub>2</sub> levels were measured in roots after K<sup>+</sup> starvation by using multiple plants in three sets of independent biological replicates. Leaves and roots of *Arabidopsis* and maize that were deprived of K<sup>+</sup> were analyzed with Amplex red reagent (10-acetyl-3,7-dihydrophenoxazine). Maize was used to determine whether a monocot with large roots and seed stores responds in a way similar to *Arabidopsis*. The production of H<sub>2</sub>O<sub>2</sub> in roots was higher at 6 h than at 30 h. The level of H<sub>2</sub>O<sub>2</sub> in *Arabidopsis* leaves increased only slightly at 6 h, but increased by a factor of two 30 h after deprivation (Fig. 4).





**Fig. 6.** Differential gene expression by  $K^+$  deprivation and the NADPH oxidase inhibitor DPI and the NADPH oxidase mutant *rhd2*. RT-PCR analysis of selected  $K^+$ -responsive genes in *rhd2* and Col-0 plants 6 and 30 h after  $K^+$  deprivation with and without  $H_2O_2$  or added DPI. The roots of hydroponic cultured plants were treated with 1 mM  $H_2O_2$  for 6 or 30 h, and 100  $\mu$ M DPI for 2 h before  $K^+$  starvation.  $\beta$ -tubulin was used for cDNA normalization. The gene number follows a description of the gene function and the number of PCR cycles used for amplification. +K, Plants grown with sufficient  $K^+$  (1.75 mM); -K, plants deprived of  $K^+$ . Mean relative intensity for each RT-PCR product is shown in Table 2, which is published as supporting information on the PNAS web site.

The trends in  $H_2O_2$  production in *Arabidopsis* and maize were similar, but *Arabidopsis* had a higher amount of  $H_2O_2$  in leaves than maize, and *Arabidopsis*  $H_2O_2$  levels in the roots were lower (Fig. 4). The increase in  $H_2O_2$  after  $K^+$  deprivation suggested that it might play a role in cellular signaling of  $K^+$  deprivation.

To investigate whether ROS are localized to a specific region of the root, an ROS-sensitive dye was loaded into root cells. Roots were deprived of  $K^+$  for 30 h. ROS increased after  $K^+$  deprivation in a discrete region of the root just behind the elongation zone (Fig. 5 C and D). In contrast, only small amounts of ROS accumulated in  $K^+$ -sufficient roots (Fig. 5 A and B) in the same region as in  $K^+$ -deficient plants, just behind the zone of elongation. There was a significant increase in ROS accumulation in plants grown under  $K^+$ -deficient conditions as compared with  $K^+$ -sufficient conditions.

**$H_2O_2$  Modulates Gene Expression After  $K^+$  Deprivation.** Biochemical assays confirmed that ROS levels increased in response to  $K^+$  deprivation in a specific location in roots, just behind the zone of elongation. To test whether  $H_2O_2$  might regulate gene expression in response to low  $K^+$ , we deprived plants of  $K^+$  and then studied the effects on the expression of a selected number of  $K^+$ -responsive genes in wild-type *Arabidopsis* Col-0 with an NADPH oxidase inhibitor and in the *Arabidopsis* mutant *rhd2*, in which a single NADPH oxidase (At5g51060) is not functional (21). The expression of this NADPH oxidase increases upon  $K^+$  deprivation (Fig. 2).

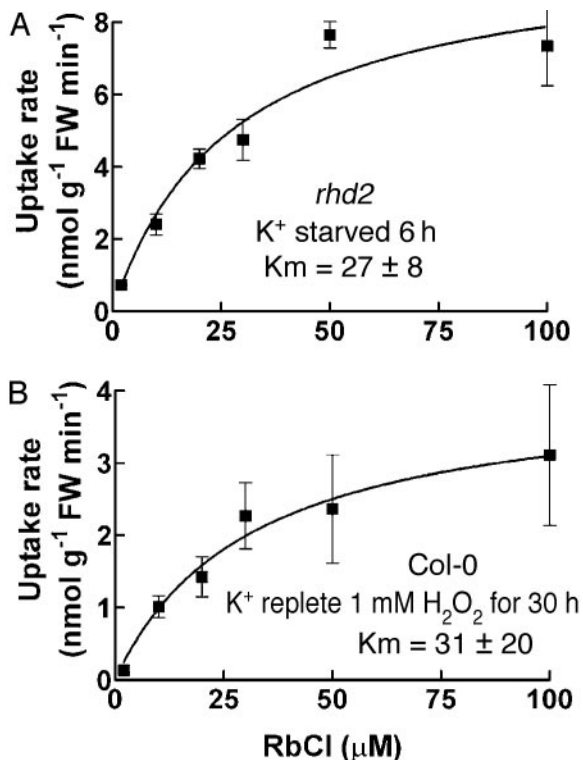
Treatment with DPI caused a higher basal level of expression of most genes tested as compared with the non-DPI-treated controls, but abolished or greatly reduced the up-regulation of six of the nine genes tested (Fig. 6). In contrast to the wild type, in *rhd2* plants we found that two peroxidases, the *WRKY9* transcription factor, the  $K^+$  transporters *KEA5* and *Hak5*, and an unknown gene, were not up-regulated in response to  $K^+$  deprivation. This result suggests that  $H_2O_2$  produced by the RHD2

NADPH oxidase regulates the expression of these genes in response to  $K^+$  deficiency. We also found that the regulation of certain genes including a GST, an AP2 transcription factor, and an 1-aminocyclopropane-a-carboxylic acid (ACC) oxidase were the same in *rhd2* mutant as compared to the wild type (Fig. 6), and therefore may not be directly modulated by  $H_2O_2$ .

To further test the effects of  $H_2O_2$  on the expression of selected genes,  $H_2O_2$  was applied to roots for 6 and 30 h in  $K^+$ -sufficient and -deficient conditions. In some cases, application of  $H_2O_2$  induced the expression of these  $K^+$ -responsive genes in the *rhd2* mutant even under  $K^+$ -sufficient conditions (Fig. 6). In other cases,  $H_2O_2$  was required, but was not sufficient, for the induction of a peroxidase, *Hak5*, and an unknown gene at 6 and 30 h after deprivation. For these three genes,  $K^+$  deficiency and  $H_2O_2$  are necessary for increased gene expression. These results show that  $H_2O_2$  and  $K^+$  deprivation play an important role in the regulation of gene expression in response to  $K^+$  deficiency.

**Relationship Between the Kinetics of  $Rb^+$  Uptake and  $H_2O_2$ .** In response to  $K^+$  deprivation, the induction of some genes was altered in the *rhd2* mutant. To test whether these changes in gene expression led to changes in the induction of high-affinity  $K^+$  uptake, we measured the kinetics of  $Rb^+$  uptake, which is commonly used as an analog for  $K^+$ . After 6 h of  $K^+$  deprivation, we found that a component of high-affinity uptake was induced in *rhd2* with  $K_m = 27 \pm 8 \mu$ M (Fig. 7A), which was similar to the  $K_m$  for  $Rb^+$  uptake in Col-0 after 6 h of  $K^+$  deprivation (Fig. 1A).

To determine whether the application of  $H_2O_2$  altered the kinetics of  $K^+$  uptake, we added 1 mM  $H_2O_2$  to the nutrient solutions containing sufficient (1.75 mM  $K^+$ )  $K^+$  in which Col-0 plants were growing. After 30 h of exposure to  $H_2O_2$ , the kinetics of  $Rb^+$  uptake by the roots of Col-0 plants were measured. We observed that the addition of  $H_2O_2$  induced a high-affinity component of  $Rb^+$  uptake ( $K_m = 31 \pm 20 \mu$ M) even though the



**Fig. 7.** Kinetics of  $\text{Rb}^+$  uptake in *Arabidopsis* roots. (A)  $\text{Rb}^+$  uptake rates in *rhd2* roots starved of  $\text{K}^+$  for 6 h showed a saturable kinetic component;  $K_m = 27 \pm 8 \mu\text{M}$ . (B)  $\text{Rb}^+$  uptake rates in *Col-0* roots grown under  $\text{K}^+$ -sufficient conditions and treated with 1 mM  $\text{H}_2\text{O}_2$  for 30 h showed a saturable kinetic component;  $K_m = 31 \pm 20 \mu\text{M}$ . Mean uptake rates at each concentration are shown  $\pm$  SE;  $n = 4$  for each concentration.

plants were growing in  $\text{K}^+$ -sufficient conditions (Fig. 7B). The  $K_m$  for  $\text{Rb}^+$  uptake over the concentration range tested was similar in the  $\text{H}_2\text{O}_2$ -treated plants (Fig. 1C) and in plants that were deprived of  $\text{K}^+$  (Fig. 1A). These results suggest that  $\text{H}_2\text{O}_2$  is sufficient for the induction of a component of high-affinity  $\text{Rb}^+$  uptake. Variation in  $V_{\text{max}}$  of uptake between experiments could be attributed to the differences in the age of plants used in the different experiments (6–8 weeks old).

## Discussion

Overall, the short-term effects of  $\text{K}^+$  deprivation are a subtle stress, because plant cells may have mechanisms in place to ensure that the cytoplasmic  $\text{K}^+$  concentration is maintained homeostatically (23, 24). The removal of  $\text{K}^+$  from the growth medium does not impose a large osmotic stress, but it leads to changes in the membrane polarization (25). To confirm that cellular changes occur after a brief period of  $\text{K}^+$  deprivation, we conducted classical kinetic uptake studies 6 h after deprivation. These studies on  $\text{K}^+$ -deprived plants have been performed on other plant species, but there are no available results for such experiments on *Arabidopsis*. After 6 h of  $\text{K}^+$  deprivation, a high-affinity uptake component was induced in *Arabidopsis* roots; this finding confirmed that cellular changes occurred after a brief period of deprivation.

To understand how  $\text{K}^+$  deficiency alters root development over a longer period of deprivation, we measured root growth and lateral development. Our data showed that lateral root growth and number were reduced (Table 1). In contrast, low phosphate availability increased lateral root growth more than primary root growth (26, 27). Lateral root initiation was repressed after transfer to low-nitrogen plates by a high sucrose-

to-nitrogen ratio in areas of new root growth (28, 29). This response was not due to nitrogen starvation alone, because lowering the sucrose concentration restored lateral root initiation even under low-nitrogen conditions. However, lateral root number was reduced in older regions of the root under nitrogen starvation (28).

Root development is affected by plant hormones as well as nutrients. Several studies have reported that increased ethylene levels inhibit root growth (30–33). Ethylene and auxin are essential for the development of root hairs in response to iron deficiency but are apparently not required for root hair development induced by phosphate deficiency (34). ACC is a precursor of ethylene that has been shown to inhibit lateral root formation (27). This precursor increased elongation in high phosphorus, but decreased elongation in low phosphorus (35). Nothing is known about the role that hormones play in root growth responses to  $\text{K}^+$  deficiency. Our results showed that lateral root growth was inhibited after  $\text{K}^+$  deprivation, and in the short term the expression of genes related to ethylene production and ethylene production increased. Because the ethylene precursor ACC inhibits lateral root number (27), it is also possible that the higher levels of ethylene under  $\text{K}^+$  deprivation led to reduced lateral root initiation in the response to  $\text{K}^+$  deprivation.

Changes in the expression of genes related to  $\text{K}^+$  transport were evident after  $\text{K}^+$  deprivation. The up-regulation of *Hak5*, which encodes a  $\text{K}^+$  ion transporter upon  $\text{K}^+$  deprivation was confirmed (20). The homologue of *Hak5* in tomato was also regulated by nitrate, phosphate, and iron deprivation (12). The function of this transporter is unknown, but has been shown to transport  $\text{K}^+$  with high affinity (36). In addition, *AtKCl1*, which is a  $\text{K}^+$  channel (37, 38) that is expressed in roots, and *KEA5*, which is suggested to be a  $\text{K}^+/\text{H}^+$  antiporter (1), were also up-regulated, highlighting the changes in  $\text{K}^+$  transport across various membranes that occurs when plants are deprived of  $\text{K}^+$ . Previous reports (38) have shown that *AtKCl1* is regulated by salt stress, but not strongly by  $\text{K}^+$  deprivation.

$\text{H}_2\text{O}_2$  has been shown to play a role in many different signaling pathways, including: ABA-mediated stomatal closure (39), auxin-regulated root gravitropism (40), responses to wounding, systemin, and methyl jasmonate (41, 42), and programmed cell death (43). Because recent studies confirm that ROS, notably  $\text{H}_2\text{O}_2$ , is a signaling molecule in plants, we tested whether ROS plays a role in signaling after  $\text{K}^+$  deprivation. Our studies show that  $\text{H}_2\text{O}_2$  production increases after  $\text{K}^+$  deprivation and that the increase in ROS production is localized to a specific region of the root. The region of ROS accumulation in  $\text{K}^+$ -starved roots was similar to the region where antioxidants such as ascorbate and dehydroascorbate accumulated in onion roots under control conditions (44). Increased ascorbate free radicals in this specific region were demonstrated to increase the uptake of sucrose, glucose, and nitrate in onion roots (45). In another study, (46) oxygen-free radicals in *Arabidopsis* roots were shown to cause a transient increase in  $\text{K}^+$  efflux. Although ROS may play different roles in ion transport, it is interesting to note that the region just behind the elongation zone (where we observed high concentrations of ROS) may be the most active in uptake and translocation to the shoots (47). The addition of 1 mM  $\text{H}_2\text{O}_2$  to plants growing in  $\text{K}^+$ -sufficient medium led to the induction of a component of high-affinity  $\text{K}^+$  uptake, which supports the conclusion that  $\text{H}_2\text{O}_2$  is involved in  $\text{K}^+$  signaling after deprivation and leads to changes in transport processes.

$\text{H}_2\text{O}_2$  may play an important role in modulating the induction of some of the genes in *Arabidopsis* in response to  $\text{K}^+$  deficiency. The NADPH oxidase inhibitor DPI abolished or greatly reduced the increased expression of  $\text{K}^+$  ion transporter genes (*Hak5* and *KEA5*), *WRKY9*, two peroxidases, and an unknown protein that were normally up-regulated after  $\text{K}^+$  deprivation. Recently Foreman *et al.* (21) showed that the basis for a root-hair-deficient

mutant in *Arabidopsis* was the inactivation of NADPH oxidase (At5g51060, *rhd2*). In *rhd2*, the increased expression of several genes after K<sup>+</sup> deprivation was abolished 6 and 30 h after deprivation. Those genes include: two K<sup>+</sup> transporters, the *WRKY9* transcription factor, two peroxidases, and an unknown protein. The expression of these genes in *rhd2* could be restored by H<sub>2</sub>O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> in combination with K<sup>+</sup> deprivation. The results with DPI and the *rhd2* mutant were similar, and both results indicate that H<sub>2</sub>O<sub>2</sub> plays a role in controlling the expression of certain genes in response to K<sup>+</sup> deprivation. We also found that a component of high-affinity K<sup>+</sup> uptake was induced in the *rhd2* mutant by K<sup>+</sup> deficiency. Therefore, the subset of genes that are controlled by the H<sub>2</sub>O<sub>2</sub> may not be required for the induction of high-affinity K<sup>+</sup> uptake. In the *rhd2* mutant, many genes are still induced by K<sup>+</sup> deficiency, and therefore the genes that are still induced by K<sup>+</sup> deprivation may be involved in high-affinity K<sup>+</sup> uptake. Alternatively, the component of high-affinity K<sup>+</sup> uptake induced by K<sup>+</sup> deprivation in the *rhd2* mutant after 6 h of K<sup>+</sup> deprivation may be due to the posttranslational modification of an existing transporter.

The cellular events that occur when plant root cells are deprived of K<sup>+</sup> are poorly understood. We showed that preliminary gene expression data could lead to testable biochemical and physiological hypotheses related to cellular signaling in response to K<sup>+</sup> deprivation. From these biochemical and physiological studies, we found that certain cellular responses to K<sup>+</sup> deficiency in *Arabidopsis* roots are activated by ROS and perhaps ethylene. At this time, the specificity of the ROS-induced responses are not known, in part because little is known about nutrient signaling cascades in plants. We propose that these signals regulate changes in root function and growth. In the future, it will be important to understand how protein function is modulated by factors other than gene expression when plants are deprived of K<sup>+</sup>.

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