Adenosine Kinase Modulates Root Gravitropism and Cap Morphogenesis in Arabidopsis

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Adenosine kinase (ADK) is a key enzyme that regulates intra- and extracellular levels of adenosine, thereby modulating methylation reactions, production of polyamines and secondary compounds, and cell signaling in animals. Unfortunately, little is known about ADK’s contribution to the regulation of plant growth and development. Here, we show that ADK is a modulator of root cap morphogenesis and gravitropism. Upon gravistimulation, soluble ADK levels and activity increase in the root tip. Mutation in one of two Arabidopsis (Arabidopsis thaliana) ADK genes, ADK1, results in cap morphogenesis defects, along with alterations in root sensitivity to gravistimulation and slower kinetics of root gravitropic curvature. The kinetics defect can be partially rescued by adding spermine to the growth medium, whereas the defects in cap morphogenesis and gravitropic sensitivity cannot. The root morphogenesis and gravitropism defects of adk1-1 are accompanied by altered expression of the PIN3 auxin efflux facilitator in the cap and decreased expression of the auxin-responsive DR5-GUS reporter. Furthermore, PIN3 fails to relocalize to the bottom membrane of statocytes upon gravistimulation. Consequently, adk1-1 roots cannot develop a lateral auxin gradient across the cap, necessary for the curvature response. Interestingly, adk1-1 does not affect gravity-induced cytoplasmic alkalinization of the root statocytes, suggesting either that ADK1 functions between cytoplasmic alkalinization and PIN3 relocalization in a linear pathway or that the pH and PIN3-relocalization responses to gravistimulation belong to distinct branches of the pathway. Our data are consistent with a role for ADK and the S-adenosyl-L-methionine pathway in the control of root gravitropism and cap morphogenesis.

The primary roots of most plants perceive their orientation within the gravity field mainly through the sedimentation of starch-filled plastids within the columella cells of the root cap (Blancaflor and Masson, 2003). There, a largely unknown transduction pathway is activated upon root reorientation, resulting in fast and transient alkalinization of the cytoplasm (Scott and Allen, 1999; Fasano et al., 2001), acidification of the apoplast (Scott and Allen, 1999; Fasano et al., 2001), and induction of lateral cell polarity with accumulation of the PIN3 auxin efflux facilitator at the new lower side of the cells (Friml et al., 2002). Consequently, a lateral gradient of auxin forms across the root cap, which can be observed indirectly by the asymmetrical activation of expression of auxin-responsive reporters, such as DR5-GUS or DR5-GFP (Boonsirichai et al., 2003; Ottenschläger et al., 2003), at the bottom flank of the cap. This gradient of auxin is then transmitted basipetally through cell files using a transport system that involves auxin influx and efflux carriers, such as the AUX1, PGP4, and AGR1/EIR1/PIN2/WAV6 proteins, respectively (Bennett et al., 1996; Chen et al., 1999; Terasaka et al., 2005). At the elongation zones, the gravity-induced auxin gradient, probably coupled with other signals, promotes a complex differential cellular elongation between upper and lower flanks, resulting in gravitropic curvature (Blancaflor and Masson, 2003). Other than those that affect starch synthesis or accumulation in columella amyloplasts, only a few genes have thus far been uncovered through genetic screens for their involvement in gravity signal transduction within the root statocytes (Blancaflor and Masson, 2003). Mutations in the ARG1 gene affect root and hypocotyl gravitropism without altering root-growth responses to phytohormones and polar auxin transport inhibitors, or phototropism. The ARG1 gene encodes a J-domain peripheral membrane protein that is associated with components of the vesicular transport system.
Adenosine Kinase Modulates Root Gravitropism

Trafficking pathway and is needed for lateral auxin transport across the root cap and for gravity-induced cytoplasmic alkalinization (Boonsirichai et al., 2003). One of its paralogs, ARL2, also contributes to root and hypocotyl gravitropism and appears to function in the same genetic pathway (Guan et al., 2003).

In this article, we show that adenosine kinase (ADK; EC2.7.1.20; ATP), an enzyme that converts adenosine (Ado) into AMP using one molecule of ATP (Moffatt et al., 2000), also modulates root gravitropism and cap morphogenesis in Arabidopsis (Arabidopsis thaliana). ADK is a key player in the S-adenosyl-l-Met (AdoMet) cycle, which provides methyl groups for a variety of transmethylation reactions involved in the metabolism of molecules as diverse as pectin, lignin, flavonoids, phosphatidyl choline, indole-3-acetic acid (IAA), cytokinins, salicylic acid, jasmonate, etc., as well as in DNA methylation and mRNA capping (Moffatt et al., 2000). AdoMet is also a precursor in the biosynthesis of ethylene and polyamines. Ado, on the other hand, is a by-product of the AdoMet cycle and a feedback inhibitor of AdoMet regeneration. Therefore, ADK’s function in converting Ado into AMP is essential to avoid feedback inhibition of the pathway and its derived branches (Moffatt et al., 2000; Schoor and Moffatt, 2004).

Here, we show that soluble ADK levels and ADK activity increase in the root tip within 12 min of gravistimulation. Mutation in one of two Arabidopsis ADK genes, ADK1, results in altered gravisensitivity, abnormal cap morphology, and delayed kinetics of the root curvature response to gravistimulation. These defects are associated with altered expression and distribution of PIN3 in the root cap, an inability for PIN3 to realign in the statocytes upon gravistimulation, and a lack of asymmetrical activation of DR5-GUS expression in the lower flank of the root cap upon gravistimulation. Spermine, an AdoMet-derived product of the cycle, can partially rescue the delay in kinetics of root gravitropism when added to the growth medium. However, this polyamine cannot rescue the cap morphological defect and altered gravisensitivity of adk1-1 roots. Our results are discussed in view of ADK’s contribution to the modulation of root gravitropism and cap morphogenesis in plants.

RESULTS AND DISCUSSION

A Proteomic Approach Identifies ADK as Potential Modulator of Root Gravitropism

To gain further insights into the molecular mechanisms that modulate gravity signal transduction in roots, we used a proteomic approach based on comparative two-dimensional gel electrophoresis to identify root tip proteins whose abundance fluctuates early in response to gravistimulation (N. Murthy U.M., L.-S. Young, G. Sabat, and P.H. Masson, unpublished data). One of the differentially represented Tris buffer-soluble protein spots identified in this screen was found to increase in staining intensity 1.8-fold during the first 12 min of a graviresponse (Fig. 1), and this increase was reproducible (three repeats). This protein spot did not change in intensity when seedlings were subjected to similar rotation for 30 s before return to the vertical for the remaining time, indicating that the change in spot intensity observed upon 12 min of gravistimulation did not derive from the mechanostimulus that typically accompanies plate rotation (Kimborugh et al., 2004).

Mass spectrometric analysis of this protein revealed a sequence tag that matches Arabidopsis ATP: adenosine 5’-phosphotransferase (EC2.7.1.20), also referred to as ADK in this article (Supplemental Fig. S1, A and B). Western-blot analysis of total protein extracts using an anti-ADK antibody (Moffatt et al., 2000) confirmed the increase in ADK protein abundance relative to a histone H3 loading control upon gravistimulation (Supplemental Fig. S2). Furthermore, the gravity-induced changes in ADK protein abundance in the Tris-soluble fraction correlated with changes in ADK enzymatic activity in total root-tip extracts, which increased from 21.4 ± 1.2 units in control root tips to 26.5 ± 1.4 units after 12 min of gravistimulation (P = 4.5 × 10−6, t test).

Reverse Genetics Reveals a Role for ADK1 in the Modulation of Root Gravitropism

Arabidopsis encodes two ADK isoforms: ADK1 (AAG45246) and ADK2 (AAG45249). The corresponding genes are expressed ubiquitously, although ADK1 is expressed more highly than ADK2 in the root tip, including the cap columella cells (Schoor and Moffatt, 2004; Nawy et al., 2005). The steady-state levels of their transcripts are not altered in the root tip in response to gravistimulation (Kimborugh et al., 2004), suggesting the gravity-induced changes observed in our proteomic studies are posttranscriptional.

ADK1 and ADK2 are 92% identical in amino acid sequence and 56% identical to human ADK (Mathews et al., 1998; Supplemental Fig. S1C). The sequence tag discrimination accuracy was increased from 88% to 95% by systematic analysis of related species and sequences.

Figure 1. ADK protein responses to gravistimulation. Silver-stained 2-DE gels showing several Tris-soluble protein spots, including ADK (white arrowhead), from control (left 2-DE image) and 12-min gravistimulated (right 2-DE image) Arabidopsis root tips. On the right of the 2-DE images, a bar graph generated by PDQuest (Bio-Rad) shows the normalized ADK spot intensity (n = 3; 3 ± 0.17-fold the average value) in unstimulated controls (left bar) and 12-min (right bar) gravistimulated samples. Quantification of the maximum bar is represented on the right of the graph, in parts per million as a normalization unit (PPM). The control bar is drawn in proportion of the highest bar. A standard spot number provided by the software is indicated at the bottom of the graph.

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identified by mass spectrometric analysis of the differentially represented protein spot did not allow distinction between the two isoforms (Supplemental Fig. S1, B and C). To investigate the role of these proteins in gravity signal transduction, we characterized the gravitropic phenotypes of plants homozygous for either \textit{adk1-1} or \textit{adk2-1}, two recessive alleles that carry T-DNA insertions in the last exon of the corresponding genes. In both cases, the insertion is upstream of codons encoding highly conserved amino acids that are essential for substrate binding (Mathews et al., 1998), and reverse transcription-PCR did not detect transcripts 3' of the T-DNA insertion site in \textit{adk1-1} (A. Sundøs-Larsson, personal communication). Furthermore, ADK activities were low in \textit{adk1-1} and \textit{adk2-1} mutant leaves, reaching 46.9% ± 3.5% and 56.7% ± 6.4% of wild-type levels, respectively. These differences were statistically significant (\(P < 0.05\), \(t\) test). \textit{adk1-1} \textit{adk2-1} double mutants could not be tested because they are embryonic lethal (B. Moffatt, unpublished data).

\textit{adk1-1} displays altered root gravitropism either in light or darkness (Fig. 2, A and B), while its hypocotyl retains wild-type gravitropism kinetics (Fig. 2C). The root gravitropism defect of \textit{adk1-1} is rescued by expression of a wild-type \textit{ADK1} transgene (Fig. 2D, lines F3-A and F3-N), demonstrating that it is a consequence of the \textit{adk1-1} mutation. \textit{adk2-1}, on the other hand, displays wild-type kinetics of root curvature in response to 90° gravistimulation. This lack of a gravitropic phenotype for \textit{adk2-1} could reflect a lack of involvement of ADK2 in root gravitropism, indicate that the ADK activity associated with the more highly expressed ADK1 isoform is sufficient for full graviresponsiveness in \textit{adk2-1}, or result from ecotype differences.

To determine if \textit{ADK1} is required for the differential growth that drives the root graviresponse, we examined root-growth rate and root-growth response to exogenous auxin. Results shown in Figure 3A indicate that the roots of light-exposed 4-d-old \textit{adk1-1} seedlings grow slowly but eventually reach near wild-type rate after 36 h. On the other hand, under our growth conditions, the roots and hypocotyls of etiolated mutant seedlings grow at wild-type rates (Fig. 3B). Hence, reduced growth rate does not seem to be responsible for the gravitropic defect of \textit{adk1-1} roots. Similarly, \textit{adk1-1} roots display almost wild-type root-growth response to the exogenous auxin IAA (Fig. 3C), with transformants carrying the \textit{pKWG-ADK1} complementation construct (F3-A and F3-N, respectively; \(n = 90–107\)). E, Presentation time of \textit{Ler} and \textit{adk1-1} (\(n = 86–98\)). The thick lines correspond to the best-fit logarithmic functions associated with the observed data. The x-intercepts of these lines correspond to the predicted presentation time. Correlation coefficients are 0.93 for \textit{Ler} and 0.97 for \textit{adk1-1}. In A through D, asterisks indicate statistically significant differences (\(P < 0.05\)), and error bars represent SEs. The data shown here are derived from one representative experiment, which was repeated once in A, C, and D and twice in B and E, with similar results.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{\textit{adk1-1} roots display altered kinetics of gravitropic curvature and decreased sensitivity to gravistimulation. A, Root reorientation kinetics in light of wild-type \textit{Ler}, Columbia, \textit{adk1-1}, and \textit{adk2-1} mutant seedlings after vertically oriented plates were rotated 90° at time zero (\(n = 88–92\)). B, Root reorientation kinetics in darkness of \textit{Ler} and \textit{adk1-1} (\(n = 90–108\)). C, Hypocotyl reorientation in darkness of \textit{Ler} and \textit{adk1-1} (\(n = 70–90\)). D, Functional complementation of the \textit{adk1-1} gravitropic phenotype by a wild-type \textit{ADK1} transgene. Root gravitropism of wild-type \textit{Ler}, \textit{adk1-1}, and progeny of two independent \textit{adk1-1} transformants carrying the \textit{pKWG-ADK1} complementation construct (F3-A and F3-N, respectively; \(n = 90–107\)). E, Presentation time of \textit{Ler} and \textit{adk1-1} (\(n = 86–98\)). The thick lines correspond to the best-fit logarithmic functions associated with the observed data. The x-intercepts of these lines correspond to the predicted presentation time. Correlation coefficients are 0.93 for \textit{Ler} and 0.97 for \textit{adk1-1}. In A through D, asterisks indicate statistically significant differences (\(P < 0.05\)), and error bars represent SEs. The data shown here are derived from one representative experiment, which was repeated once in A, C, and D and twice in B and E, with similar results.}
\end{figure}
only a minor enhancement of their sensitivity to 5 nM IAA, and wild-type sensitivity to naphthylphthalamic acid (NPA), an inhibitor of auxin efflux activity during polar auxin transport (Muday and DeLong, 2001). These data suggest that the auxin response or NPA-sensitive components of its transport are not substan-

tially affected in the mutant. Consistent with this conclusion, the AGR1/EIR1/PIN2/WAV6 efflux facilitator, which contributes to basipetal auxin transport in roots, was properly expressed and distributed at the correct location within transporting cells of adk1-1 root tips (Supplemental Fig. S3, A and B).

**ADK1 Contributes to Gravity Signal Transduction in the Root Statocytes**

To investigate the possibility that adk1-1 might be affected in early phases of gravity signal transduction, we analyzed the root curvature response to short doses of gravistimulation and used a logarithmic best-fit model (Perbal et al., 2002) to evaluate gravitropic sensitivity. Results shown in Figure 2E indicate that the minimal gravistimulation time needed for induction of gravitropic curvature (presentation time) is 0.86 min for wild-type Landsberg erecta (Ler) roots (correlation coefficient of 0.93) and 8.94 min for adk1-1 roots (correlation coefficient of 0.97).

Because gravity signal transduction occurs in the columella cells of the root cap, we analyzed possible effects of adk1-1 on root cap morphology. While wild-type root caps typically contain three tiers of columella cells (Supplemental Fig. S4A), most adk1-1 root caps are disorganized, containing varying numbers of differently sized columella cells in each tier (Supplemental Fig. S4A). However, mutant columella cells still contain starch-filled plastids (Supplemental Fig. S4B, left), suggesting their differentiation is not altered. Interestingly, the morphological defects of adk1-1 root caps closely resemble those of scr-1 (Sabatini et al., 2003). However, scr-1 roots display wild-type kinetics of gravitropism (Supplemental Fig. S4C), as previously reported (Fukaki et al., 1998), and wild-type presentation time (Supplemental Fig. S4D). Therefore, the reduced graviresponse associated with adk1-1 is likely not a direct result of its cap morphological defect, although it remains possible that the mechanism that governs altered cap morphology in adk1-1 differs from that responsible for the same phenotype in scr-1 and affects root gravitropism.

**adk1-1 Affects PIN3 Expression and Relocalization to the Lower Membrane of Root Statocytes upon Gravistimulation**

Because gravistimulation promotes a redistribution of the PIN3 auxin efflux facilitator to the lower membrane of the root statocytes (Friml et al., 2002), we immunolocalized the PIN3 protein in vertically grown and gravistimulated adk1-1 and Ler roots. In vertically oriented Ler root tips, the PIN3 signal was often seen symmetrically distributed at the periphery of statocytes in tiers 2 and 3 of the cap without preferential bias toward specific sides (Fig. 4A). On the other hand, in vertically oriented adk1-1 root tips, PIN3 signals often showed uneven distribution, displaying asymmetrical localization to one or more sides of the statocytes.
Furthermore, while four columns of S2 and S3 columella cells consistently displayed PIN3 signals in mid-plane optical sections of Ler root tips (Fig. 4A), only three to five central statocytes detectably expressed PIN3 in adk1-1 roots (Fig. 4B).

After 30 min of gravistimulation, PIN3 was localized to the new bottom side of the columella cells in 10 out of the 17 Ler root tips observed (Fig. 4E) and to the new upper side in three other Ler roots, whereas the remaining four roots retained symmetrical PIN3 localization (data not shown; Table I). These data give a localization index (RI PIN3) of 41 for 30-min gravistimulated Ler root tips (see “Materials and Methods”; Table I). In contrast, analysis of PIN3 distribution in 30-min gravistimulated adk1-1 root tips revealed a slightly negative RI PIN3 for this mutant (Table I; Fig. 4F), suggesting a defect in PIN3 relocalization. Importantly, the effect of adk1-1 on PIN3 expression and localization in the root cap appears specific, as expression and distribution of two other root-tip-expressed efflux facilitators (Blilou et al., 2005) appear normal in this mutant (Supplemental Fig. S3, A and B, and C and D, respectively). Furthermore, abnormal root-tip morphology does not appear to be directly responsible for adk1-1’s inability to relocalize PIN3 in the statocytes upon gravistimulation because similar gravistimuli promote PIN3 relocalization in scr-1 root statocytes despite abnormal root cap morphology (Fig. 4, G and H; Table I). However, we cannot exclude the possibility that the mechanism that drives altered cap morphology in adk1-1 also affects expression and relocalization of PIN3 in mutant statocytes.

adk1-1 Affects Expression of the Auxin-Responsive DR5-GUS Reporter Construct in the Root Tip

Altered distribution of PIN3 in the statocytes of vertical and gravistimulated adk1-1 roots suggests

**Table I. Patterns of PIN3 accumulation in the columella cells of 30-min gravistimulated wild-type and mutant root tips**

<table>
<thead>
<tr>
<th></th>
<th>Bot</th>
<th>Top</th>
<th>No Bias</th>
<th>Total</th>
<th>RI PIN3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ler</td>
<td>10</td>
<td>3</td>
<td>4</td>
<td>17</td>
<td>41%</td>
</tr>
<tr>
<td>adk1-1</td>
<td>1</td>
<td>2</td>
<td>10</td>
<td>13</td>
<td>~8%</td>
</tr>
<tr>
<td>Ws</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>75%</td>
</tr>
<tr>
<td>scr-1</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>75%</td>
</tr>
</tbody>
</table>

*Localization of PIN3 toward the new bottom side of columella cells in gravity-stimulated root tips. †Localization of PIN3 toward the new top side of columella cells in gravity-stimulated root tips. ‡No bias of PIN3 localization toward top or bottom sides of the columella cells. ‡RI PIN3 of PIN3 = 100 × (no. of samples showing bottom localization of PIN3 – no. of samples showing top PIN3 localization)/total.*
potential alterations in lateral auxin transport in the root cap, with possible effects on auxin accumulation in the root tip. To investigate this possibility, we analyzed expression of an auxin-responsive DR5-GUS (Boonsirichai et al., 2003) reporter construct in wild-type and adk1-1 mutant roots. In wild-type root tips, strong GUS signal was detected in the central region of the root cap and in the vasculature (Fig. 4I). Upon gravistimulation, the GUS signal extended into the lower flank of the root cap, indicating gravistimulus-induced lateral auxin transport (Boonsirichai et al., 2003). When analyzed after 6 h of gravistimulation (a time sufficient to promote a visible curvature response that can be used to unambiguously define the “up” and “down” flanks of most root tips under a microscope), the GUS signal had extended to the lower flank of the root tip in 47 wild-type seedlings out of the 104 tested, whereas it extended to the opposite, upper flank of only 19 gravistimulated seedlings, leading to an expression index for DR5-GUS (EI_{DR5-GUS}) of 27 (see “Materials and Methods”; Table II).

When stained in the same vial as the wild type, vertically grown adk1-1 roots showed patchy GUS staining in the root cap (Fig. 4K), and staining was less intense in the mutant than in the wild type (Fig. 4, I and K). These data suggest that adk1-1 root tips accumulate less auxin in a smaller number of statocytes than wild type, likely explaining their cap morphological defects (Blilou et al., 2005). After gravistimulation, adk1-1 roots showed no evidence of preferential lateral GUS activation at the bottom flank of the cap (Fig. 4L; Table II), indicating a defect in gravity-induced auxin redistribution for this mutant.

It should be noted here that almost 50% of wild-type or mutant seedlings displayed asymmetrical DR5-GUS expression extending to one of the root-cap flanks even in the absence of gravistimulation (Table II). Such differential expression could reflect some random fluctuations in DR5-GUS expression in lateral root cap cells, represent a response to uncharacterized lateral stimuli, or be an experimental artifact during GUS staining. However, the preferential activation of DR5-GUS expression on the lower flank of 6-h gravistimulated wild-type roots is highly significant (P < 0.05, t test), strongly supporting a role for the gravity signal transduction pathway in promoting DR5-GUS expression in lateral cap cells at the lower flank of horizontal roots.

adk1-1 Does Not Affect Cytoplasmic Alkalinization of the Root Statocytes upon Gravistimulation

Gravistimulation also promotes a cytoplasmic alkalinization of wild-type root-cap statocytes, and this output is necessary for full graviresponse (Scott and Allen, 1999; Fasano et al., 2001). Therefore, we investigated whether adk1-1 also affects gravity-induced cytoplasmic alkalinization of root-cap statocytes. Results showed no significant differences in cytoplasmic pH between Ler and adk1-1 root statocytes, both before (t = 0 min; pH = 7.17 ± 0.6 and 7.05 ± 0.5, respectively) and during (t = 2.5 min; pH = 7.6 ± 0.7 and 7.4 ± 0.5, respectively) a graviresponse (P > 0.05, t test). Hence, adk1-1 does not affect this energy-consuming physiological output of gravity signal transduction, implying that the gravitropic defect associated with adk1-1 is not a simple consequence of altered energy balance within the statocytes. This result also suggests either that ADK1 functions between cytoplasmic alkalinization and PIN3 relocalization in a linear pathway, or that gravity-induced cytoplasmic alkalinization and PIN3 redistribution in root-cap statocytes are outputs of distinct gravity signal transduction pathways. Recent observations of a differential effect of adk1-1 on the transcriptional responses to gravistimulation of three fast gravity-respecting, root-tip-expressed genes support the existence of branched pathways in root gravity signal transduction (Yester et al., 2006).

Spermine Partially Rescues the Root Gravitropic Defect of adk1-1

As discussed above, the main substrate of ADK in plants, Ado, feedback inhibits the AdoMet cycle (Moffatt et al., 2000). Interestingly, at least two secondary products derived from this pathway were previously proposed to modulate polar auxin transport: polyamines and ethylene (Morgan and Gausman, 1966; Luschnig et al., 1998; Clay and Nelson, 2005). To examine a potential role for these compounds in the gravitropic defect of adk1-1, we analyzed root gravi tropism in wild-type and adk1-1 mutant seedlings in the presence of 1-aminoacyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, and spermine. Adding ACC to the medium at concentrations varying

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**Table II. Patterns of DR5-GUS expression in the root cap of 6-h gravistimulated 4- to 5-d-old wild-type Ler and adk1-1 mutant seedlings**

Cumulative data from three separate experiments, each showing similar trends.

<table>
<thead>
<tr>
<th></th>
<th>Bottoms</th>
<th>Top</th>
<th>Bias</th>
<th>No Bias</th>
<th>Total</th>
<th>EI_{DR5-GUS}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertical</td>
<td>Ler</td>
<td>adk1-1</td>
<td>Ler</td>
<td>adk1-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-h GS</td>
<td>47</td>
<td>42</td>
<td>19</td>
<td>45</td>
<td>104</td>
<td>69</td>
</tr>
</tbody>
</table>

*Seedlings were gravistimulated for 6 h.*

*Number of gravistimulated roots showing DR5-GUS expression in lateral cap cells at the lower sides of the root.*

*Number of gravistimulated roots showing DR5-GUS expression in lateral cap cells at the upper sides of the root.*

*Number of vertical roots showing asymmetrical activation of DR5-GUS expression in the lateral cap cells on one side of the root.*

*Number of vertical roots showing symmetrical DR5-GUS expression limited to the central columns of columella cells.*

*Total number of root tips analyzed.*

*EI_{DR5-GUS} = 100 × (no. of roots showing DR5-GUS expression in cells at the bottom flank of the cap − no. of roots showing DR5-GUS expression in cells at the top flank of the cap)/total number of roots analyzed.*
from 0.1 to 10 \( \mu M \) did not rescue the gravitropic and cap morphogenesis defects of \( \text{adk1-1} \) (Supplemental Fig. S5; data not shown). Spermine, on the other hand, appeared to rescue the gravitropic defect of \( \text{adk1-1} \) in a dose-dependent manner (Fig. 5A). However, increasing its concentration from 100 to 200 \( \mu M \) did not result in additional increases in \( \text{adk1-1} \) curvature after 11 h of gravistimulation (57° ± 2.2 at 200 \( \mu M \) versus 59° ± 2.9 at 100 \( \mu M \); \( P = 0.47 \), \( t \) test; \( n = 112–116 \)), indicating that the spermine dose-response curve of \( \text{adk1-1} \) reached saturation at 100 \( \mu M \). Spermine had no affect on the gravitropic response of wild-type \( \text{Ler} \) roots (Fig. 5A), implying that its ability to partially rescue the gravitropic defect of \( \text{adk1-1} \) roots is specific to plants with a lesion in ADK. To further investigate the possible role of spermine in the regulation of root gravitropism, we analyzed the effect of an inhibitor of polyamines synthesis, methyl glyoxal bis(guanylhydrazone) (MGBG; Williams-Ashman and Seidenfeld, 1986), on \( \text{adk1-1} \) and wild-type \( \text{Ler} \) roots. Figure 5B shows that MGBG at concentrations varying from 50 to 500 \( \mu M \) significantly inhibits wild-type root gravitropism but does not affect the response of \( \text{adk1-1} \).

Together, our data indicate that spermine is important for full graviresponse in roots and suggest that spermine deficiency in \( \text{adk1-1} \) is partially responsible for altered kinetics of root gravitropism. Consistent with this interpretation, \( \text{adk1-1} \) mutant seedlings were found to contain less spermine than wild type (Table III; \( P = 9.4 \times 10^{-5} \), \( t \) test), whereas putrescine and spermidine levels were not affected by the mutation.

### Table III. Levels of putrescine, spermidine, and spermine in 2-week-old wild-type \( \text{Ler} \) and \( \text{adk1-1} \) mutant seedlings

<table>
<thead>
<tr>
<th>Sample</th>
<th>Putrescine ( \text{nmol g}^{-1} \text{ fresh weight} )</th>
<th>Spermidine ( \text{nmol g}^{-1} \text{ fresh weight} )</th>
<th>Spermine ( \text{nmol g}^{-1} \text{ fresh weight} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Ler} )</td>
<td>45.5 ± 4.1</td>
<td>99.3 ± 3.9</td>
<td>26.3 ± 0.8</td>
</tr>
<tr>
<td>( \text{adk1-1} )</td>
<td>44.6 ± 1.5</td>
<td>99.0 ± 3.4</td>
<td>21.7 ± 0.5</td>
</tr>
</tbody>
</table>

*Statistically significant difference between \( \text{adk1-1} \) and wild type \(( P = 9.4 \times 10^{-5} \), \( t \) test).
morphogenesis (Schoor and Moffatt, 2004). The latter model is compatible with our observation of decreased auxin level, as defined by DR5::GUS expression in root-cap columella cells of adk1-1 relative to wild type. Work is under way to test these models.

While regulating Ado levels, ADK also converts ATP into AMP (Moffatt et al., 2000), sensitizing its activity to the energy balance of a cell. Hence, its positive contribution to gravitropic sensitivity may allow roots exposed to stressful conditions to deploy growth behaviors that are less dependent upon gravitropism, thus enabling better responses to other, more relevant directional parameters that guide them toward more favorable environments (Massa and Gilroy, 2003).

CONCLUSIONS AND PERSPECTIVES

In summary, our data indicate that adk1-1 alters root gravitropism by affecting both gravitropic sensitivity and curvature kinetics. The curvature kinetics defect is partly rescued by adding spermine to the medium, suggesting a role for this polyamine in the process. This conclusion is in line with recent observations suggesting a role for polyamines in the control of polar auxin transport in roots (Clay and Nelson, 2005). While adk1-1 shows almost wild-type root growth sensitivity to auxin and NPA (Fig. 3, C and D), and the AGR1/EIR1/PIN2/WAV6 auxin-efflux facilitator is normally expressed and distributed in mutant roots (Supplemental Fig. S3), it is possible that distribution and/or activity of auxin influx carriers are altered in lateral root cap or epidermal cells of adk1-1 roots due to spermine deficiency. Alternatively, adk1-1 could also affect the transport or activity of other gravitropic signals. For instance, cytokinins, which may provide such a signaling function in roots (Aloni et al., 2004), are likely to be affected by adk1-1, as they serve as substrates for ADK activity and are targeted by several AdoMet-dependent methyltransferases (Schoor and Moffatt, 2004).

On the other hand, gravitropic sensitivity and cap morphology defects of adk1-1 could not be rescued by addition of spermine or ACC to the medium, suggesting that different mechanisms might modulate this aspect of gravitropic regulation by ADK. Gravity-induced PIN3 redistribution to the lower membrane of root-cap statocytes was proposed to involve the cycling of vesicles between the plasma membrane and endosome (Friml et al., 2002; Boutte et al., 2006). In animal models, ADK-controlled extracellular Ado regulates multiple cellular processes, including neurotransmitter release (Buck, 2004). Thus, it is tempting to speculate that similar Ado effects on vesicular trafficking might be responsible for the phenotypes of adk1-1. Alternatively, reduced Ado salvage in adk1-1 may inhibit AdoMet regeneration, leading to reduced capping of newly synthesized mRNAs upon gravistimulation (Kimbrough et al., 2004) and inadequate homeostasis (through AdoMet-dependent transmethylation reactions) of plant regulatory molecules, such as auxin, cytokinins, and flavonoids, with demonstrated or potential roles in gravitropism and root tip

MATERIALS AND METHODS

Materials

Estland wild-type seed stocks were used for the proteomic screen (Guan et al., 2003). adk1-1 was isolated from a Ler population containing a gene-trap construct (Wilson, 1993) and provided to us by Dr. Sundus-Larsson (Uppsala University, Sweden). adk2-1 and pin3-4 were obtained from the SALK T-DNA insertion collection from the Arabidopsis Biological Resource Center (SALK_000565 and SALK_038609, respectively; Alonso et al., 2003). pgm-1 (TC7), DR5::GUS-transformed wild-type Columbia seeds, and scr-1 were previously described (Di Laurenzio et al., 1996; Ulmavov et al., 1997; Boonsirichai et al., 2003; Peer et al., 2004). Plant growth conditions were as described (Boonsirichai et al., 2003; Guan et al., 2003).

Two-Dimensional Electrophoresis of Root-Tip Proteins

Gravitropism was performed on vertically grown 4-d-old seedlings by gently rotating the plates 90° in a clockwise direction. Seedlings were allowed to respond to the stimulus for an additional 12 min. Mechanostimulation was also carried out by gently rotating the plates 90° clockwise for 15 to 30 s, then rotating them back counterclockwise to their original orientation and incubating them for another 12 min. At the end of this period, 3- to 5-mm root tips were dissected and stored in liquid nitrogen. To minimize the biological and technological variance, for each experiment, protein samples were extracted from approximately 600 seedlings from 10 different plates per extraction (50–60 mg of tissue). This protocol was repeated three times.

Root tips were ground in liquid nitrogen and subjected to a three-step extraction protocol (Molloy et al., 1998). Tris-soluble proteins were fractionated by two-dimensional electrophoresis (2-DE) as recommended by the supplier (Amersham Pharmacia) and silver stained as described (Blum et al., 1987). Silver-stained gels were scanned and digitized. Digitized 2-DE gel images were analyzed using image-analysis software (PDQuest; Bio-Rad) for spot-intensity comparison between stimulated and unstimulated samples. Protein spots present on a gel were detected using the Spot Detection Wizard in the software. Relative intensity of a protein spot was calculated by a Gaussian arithmetic model and normalized with the total intensity of the gel images from the three experimental repeats. Identical protein spots in both the control and experiment gel images were land-marked manually to generate a match-set image. Proteins included in specific 2-DE spots were identified by nano-liquid chromatography tandem mass spectrometry, as described in the legend to Supplemental Figure S1.

Western-Blot Analysis of ADK Protein Expression in Arabidopsis Root Tips

Total proteins were extracted from control and 30-min gravistimulated root tips as described previously (Kagaya et al., 2002), and approximately 30 μg protein from each extract was subjected to western-blot analysis using the procedures outlined by the manufacturer (Qiagen). Anti-ADK (Moffatt et al., 2000) and mouse monoclonal anti-histone H3 (Abcam) antibodies were used
as primary antibodies at dilutions of 1:7,500 and 1:3,000, respectively. Horse-radish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Pierce) were used as secondary antibodies at a dilution of 1:10,000.

**Phenotypic Characterization of Mutant Seedlings**

Phenotypic analyses were conducted on 4-d-old seedlings, unless otherwise stated. Analyses of etiolated growth and of gravitropism in light or in darkness were as described (Sedbrook et al., 1999; Boonsirichai et al., 2003). Analyses of gravitropic sensitivities were carried out using previously reported procedures (Blancalter et al., 1998; Perbal et al., 2002). Phytotormone-, NPA-, spermine-, and ACC-response assays and starch staining of the columella cells were performed as described previously (Guan et al., 2003; Clay and Nelson, 2005). Cellular organization of wild-type and mutant root tips was analyzed by fluorescent labeling of the cell walls, using 10 μg mL⁻¹ propidium iodide (Sigma) for 1 to 3 min.

Histochemical staining for GUS activity was described previously (Boonsirichai et al., 2003). To account for some wild-type root caps showing DR5-GUS expression in their upper lateral cap cells upon gravitumulation, we established EDR5-GUS, which is defined as 100 times the ratio of the number of root caps showing preferential DR5-GUS expression in the lower lateral cap cells minus the number of caps showing expression in the upper lateral cap cells, to the total number of caps analyzed.

Analyses of cytoplasmic pH in control and gravistimulated root-cap sections (S3 layer) were performed on at least 10 separate roots, as reported previously (Boonsirichai et al., 2003).

**Determination of ADK Activity**

ADK enzymatic activity was measured in protein extracts from rosette leaves of 3.5- to 4-week-old plants grown in soil, or from 5- to 6 mm-long, dissected root tips of vertically grown or 12-min gravistimulated, 5-d-old, GM-agar-grown seedlings (Boonsirichai et al., 2003), using previously published procedures (Moffatt et al., 2000). For each sample, ADK activity was determined in three reactions involving 0.2, 0.4, and 0.6 μL of the initial extract. Each determination was performed in triplicate. Each experiment was repeated. The data shown in this article derive from one representative experiment that included two biological replicates of each tested genotype. Activities are represented in units, which correspond to nanomoles of AMP produced per milligram of total protein in the extract per minute of reaction.

**Determination of Polyamine Levels in Seedlings**

To determine the levels of free putrescine, spermidine, and spermine, wild-type Ler and adk1-1 mutant seedlings were germinated and grown for 2 weeks on vertical GM media solidified with 0.7% agar (Sigma, type E). Growth conditions were: 16 h day, 8 h night; light from cool-white fluorescent tubes at 70 μE m⁻² s⁻¹; 22°C; and 80% relative humidity. Seedlings were harvested, frozen in liquid nitrogen, and ground. Ground tissue was resuspended in 1 mL of cold perchloric acid/500 mg of tissue and kept frozen in liquid nitrogen. The supernatant was diluted 1:1,000 and used as purified anti-PIN3 polyclonal antibody for Western blotting experiments. Anti-PIN2 and anti-PIN4 antibodies were described previously (Boonsirichai et al., 2000; Peer et al., 2004). Immunolocalization experiments were performed on 4- to 6-d-old seedlings, as described (Boonsirichai et al., 2003). To keep track of the seedlings' orientation within the gravity field, gravistimulated roots were cut diagonally along the vertical axis. Images were obtained using a Bio-Rad MRC1024 laser scanning confocal microscope with excitation/emission filters of 560/585 nm (Keck Biological Imaging Laboratory, University of Wisconsin-Madison).

To account for several gravistimulated wild-type root caps that displayed asymmetrical accumulation of PIN3 at the upper side of the columella cells, possibly as a consequence of stochastic effects unrelated to gravitumulation, we established EDR5, which is defined as 100 times the ratio of the number of root caps showing preferential PIN3 accumulation along the new physical bottom of the columella statocytes, minus the number of root caps showing PIN3 accumulation on the upper side of the statocytes, to the total number of root caps analyzed.

**Statistical Analysis**

Experiments described in this article were repeated once or twice, as outlined in the figures legends. All biometrical data were subjected to statistical analysis of significance, using the F- and t-test functions provided by Microsoft Excel version X.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Identification of graviresponsive ADK protein spot by mass spectrometry.

**Supplemental Figure S2.** Root-tip ADK protein abundance increases relative to a histone H3 loading control upon gravitumulation.

**Supplemental Figure S3.** adk1-1 shows wild-type response to exogenously applied IAA and NPA.

**Supplemental Figure S4.** Columella cell morphology defects in adk1-1.

**Supplemental Figure S5.** ACC does not rescue the gravitropic defect of adk1-1.

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LITERATURE CITED


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