Pleiotropic Modulation of Carbon and Nitrogen Metabolism in Arabidopsis Plants Overexpressing the NAD kinase2 Gene¹[W]

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Nicotinamide nucleotides (NAD and NADP) are important cofactors in many metabolic processes in living organisms. In this study, we analyzed transgenic Arabidopsis (Arabidopsis thaliana) plants that overexpress NAD kinase2 (NADK2), an enzyme that catalyzes the synthesis of NADP from NAD in chloroplasts, to investigate the impacts of altering NADP level on plant metabolism. Metabolite profiling revealed that NADP(H) concentrations were proportional to NADK activity in NADK2 overexpressors and in the nadk2 mutant. Several metabolites associated with the Calvin cycle were also higher in the overexpressors, accompanied by an increase in overall Rubisco activity. Furthermore, enhanced NADP(H) production due to NADK2 overexpression increased nitrogen assimilation. Glutamine and glutamate concentrations, as well as some other amino acids, were higher in the overexpressors. These results indicate that overexpression of NADK2 either directly or indirectly stimulates carbon and nitrogen assimilation in Arabidopsis under restricted conditions. Importantly, since neither up-regulation nor down-regulation of NADK2 activity affected the sum amount of NAD and NADP or the redox state, the absolute level of NADP and/or the NADP/NAD ratio likely plays a key role in regulating plant metabolism.

Carbon (C) and nitrogen (N) assimilation are closely linked fundamental processes for plant growth. The emerging details of C and N assimilation suggest that a regulatory system coordinates uptake and distribution of these nutrients in response to both metabolic and environmental cues (Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001). Because the amounts of assimilated C and N largely influence plant growth and crop yields, there have been many attempts to engineer C and N assimilation by overexpression of a single enzyme. However, in only a few cases have significant improvements in C and N assimilation been achieved (Miyagawa et al., 2001; Sinclair et al., 2004), possibly because synchronous activation of a series of metabolic pathways might be necessary to influence assimilation. One successful example of metabolic engineering is in transgenic Arabidopsis (Arabidopsis thaliana) lines expressing the maize DoF1 (for DNA-binding with one finger 1) transcription factor, which have increased N contents and improved growth rates under N starvation conditions (Yanagisawa et al., 2004). An increase in the biosynthesis of cofactors could also be used to modify C and N assimilation, because high levels of a cofactor may stimulate multiple enzymatic reactions, resulting in synchronous metabolic pathway activation. However, the effectiveness of such a cofactor strategy, to our knowledge, has not been reported.

NAD and NADP are pyridine nucleotides that are essential for electron transport and that serve as cofactors in numerous metabolic processes in many organisms. We recently showed that modification of the NADP/NAD ratio impacted cell growth, implying that NAD and NADP play different roles in energy transduction (Takahashi et al., 2006b; Hashida et al., 2009). Although our previous data, obtained with an Arabidopsis mutant, suggested that the NADP/NAD

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ratio might affect metabolic processes as well (Takahashi et al., 2006b), this possibility remains to be evaluated.

NAD kinase (NADK; EC 2.7.1.23) catalyzes the de novo biosynthesis of NADP from NAD and ATP. Genes encoding NADK have been cloned from Homo sapiens (Lerner et al., 2001), Escherichia coli (Kawai et al., 2001a), Saccharomyces cerevisiae (Kawai et al., 2001b; Outten and Culotta, 2003), and other species (Kawai et al., 2000; Raffaelli et al., 2004; Sakuraba et al., 2005). Characterization of NADK isoforms indicates that the enzyme is involved in biotic and abiotic stress responses. For instance, cold stress induces calmodulin (CaM)-dependent NADK activity, but not CaM-independent NADK activity, in green bean (Phaseolus vulgaris) plants (Ruiz et al., 2002). Decreases in NADK activity in response to salinity and drought were observed in tomato (Solanum lycocephalum) and wheat (Triticum aestivum), respectively (Zagdanska, 1990; Delumeau et al., 2000). Furthermore, cultured cells of tobacco (Nicotiana tabacum) treated with elicitors, including cellulase, harpin, and incompatible bacteria, elevated CaM-dependent NADK activity and NADPH concentrations (Harding et al., 1997). Since NADPH is a substrate of NADPH oxidase, which regulates reactive oxygen species (ROS) production in defense signaling, these observations integrate NADK into the plant defense response system.

In Arabidopsis, three genes encoding NADK have been identified (NADK1, NADK2, and NADK3; Berrin et al., 2005; Turner et al., 2005). NADK2 is localized in chloroplasts, whereas NADK1 and NADK3 are in the cytosol (Chai et al., 2005, 2006). Chloroplastic NADK2 transcripts are abundant in leaves and are not affected by abiotic stresses. The genes encoding the cytosolic NADKs, NADK1 and NADK3, are ubiquitously expressed and are induced by abiotic stresses such as oxidative stress (Berrin et al., 2005; Chai et al., 2006). Based on these observations, the physiological role of NADK2 is likely different from that of NADK1 and NADK3. Moreover, the activity of NADK2 appears to be responsible for a major part of NADP production in photosynthetic cells, because a knockout mutant of NADK2 (nadk2) produced far less NADK activity and NADP(H) (Chai et al., 2005; Takahashi et al., 2006b). NADP produced by NADK2 is reduced to NADPH in the photosynthetically electron transport chain in chloroplasts, and then NADPH is transported from chloroplasts to the cytosol and other organelles via the malate/oxaloacetate shuttle (Heinke et al., 1991; Scheibe, 2004). The reducing energy of NADPH is used for biological processes in chloroplasts, including chlorophyll synthesis and CO2 fixation in the Calvin cycle, and for various processes in the cytosol and other organelles. Thus, NADK2 likely plays a central role in the metabolism of photosynthetic cells.

The nadk2 mutant produces pale green rosette leaves and does not grow as well as wild-type Arabidopsis. This mutant also has lower leaf chlorophyll contents, reduced expression of the NADPH:protochlorophyllide oxidoreductase genes (Chai et al., 2005), and high accumulation of zeaxanthin under low-light conditions, probably through inhibition of zeaxanthin epoxidase activity (Takahashi et al., 2006b). Consequently, the nadk2 mutant has decreased photosynthetic activity, presumably due to both decreased chlorophyll contents and aberrant energy dissipation in the xanthophyll cycle (Takahashi et al., 2006b), suggesting a tight linkage between NADK2 activity and CO2 fixation. On the other hand, nadk1, nadk2, and nadk3 mutants all showed an increased sensitivity to ROS stress (Berrin et al., 2005; Chai et al., 2005, 2006), suggesting that the NADKs play a conserved role in defense response. Hence, unlike other NADKs, NADK2 might contribute to both sustainable growth and defense responses in Arabidopsis. These observations prompted us to evaluate the effects of overexpression of the chloroplastic NADK2 on the metabolic alteration in Arabidopsis plants.

In this work, we generated and characterized NADK2-overexpressing transgenic Arabidopsis plants to increase intracellular NADP(H) concentrations and then investigated the effects of this metabolic disturbance. We found that the concentrations of several Calvin cycle metabolites and some amino acids increased in these transgenic plants. Since the total amounts of NAD and NADP as well as the redox state were similar in the wild-type Arabidopsis, the nadk2 mutant, and the NADK2 overexpressors, either NADP and/or the NADP/NAD ratio could be the key factor regulating plant metabolism. Moreover, our results also indicated that elevation of NADP concentrations leads to coordinated activation of both C fixation and N assimilation in planta. Although observed metabolic enhancement would be pleiotropic, we propose here that genetic manipulation of cofactors is a viable strategy for engineering plant metabolism.

RESULTS

Generation of Transgenic Arabidopsis Plants Overexpressing NADK2

We initially generated NADK2-overexpressing transgenic Arabidopsis plants using full-length NADK2 cDNA (2,958 bp) under the control of the cauliflower mosaic virus 35S promoter to investigate the effect of NADK2-mediated NADP production on C and N assimilation. Two transgenic lines with significant expression of the transgene (NADK2-OX1 and -OX2; Fig. 1, A–C) were used for further analysis. The nadk2 mutant showed a severe morphological phenotype, with smaller rosette leaves and shorter and fewer siliques (Fig. 1A; Table I); however, there was almost no change in the soil-grown NADK2 overexpressor plants. Amplification of transgene-derived and endogenous NADK2 mRNA was much higher in leaves of both NADK2-OX1 and -OX2 than in wild-type plants, and there was no detectable PCR product from the T-DNA insertion nadk2 mutant (Fig. 1B; Chai et al.,}
NADK activity in the nadk2 mutant was half the level of the wild type but 2.1- to 2.8-fold higher in the NADK2 overexpressors (Fig. 1C). Bolting and anthesis were delayed in the nadk2 mutant, but the timing of floral development was normal in the NADK2 overexpressors (Table I). Chlorophyll content was lower in rosette leaves of the nadk2 mutant, consistent with a previous observation (Chai et al., 2005), but tended to be higher in the NADK2 overexpressors (Fig. 1D).

Overexpression of NADK2 Increases the NADP(H)/NAD(H) Ratio But Has No Effect on Redox State

To measure the effect of NADK2 overexpression on pyridine nucleotide pools, NAD(P)(H) and their precursors, nicotinate mononucleotide (NaMN) and nicotinate adenine dinucleotide (NaAD), were quantified in leaf tissues by capillary electrophoresis mass spectrometry (CE-MS; Fig. 2A). NaMN concentrations were about the same in all plants analyzed, whereas NaAD was lower in both the nadk2 mutant and the NADK2 overexpressors. NAD and NADH concentrations were higher in the nadk2 mutant, but NADP and NADPH were significantly lower. This observation supports our previous suggestion that NADP synthesis in leaf tissue largely depends on NADK2 activity (Takahashi et al., 2006b). In the NADK2 overexpressors, the NAD content was unchanged, NADH concentrations were lower, and NADP and NADPH concentrations were higher, resulting in a 1.5- to 1.7-fold higher NADP(H)/NAD(H) ratio than in the wild type. The ratios of NADP(H)/NAD(H) in the wild type and nadk2 were 0.41 and 0.11, respectively (Fig. 2B). However, the sum of oxidative and reduced forms of NAD and NADP, and the (NADH+NADPH)/(NAD+NADP) ratio, which indicates the redox state, were not affected by modulation of NADK2 activity (Fig. 2C). These results indicate that modulation of

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>nadk2</th>
<th>NADK2-OX1</th>
<th>NADK2-OX2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf length (mm)</td>
<td>8.9 ± 1.4</td>
<td>6.5 ± 1.2</td>
<td>9.3 ± 0.7</td>
<td>9.6 ± 1.0</td>
</tr>
<tr>
<td>Leaf width (mm)</td>
<td>7.8 ± 1.3</td>
<td>6.5 ± 1.2</td>
<td>7.9 ± 0.8</td>
<td>8.1 ± 0.9</td>
</tr>
<tr>
<td>Leaf fresh weight (mg)</td>
<td>10.6 ± 2.0</td>
<td>5.5 ± 1.3</td>
<td>10.9 ± 1.5</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>Specific leaf fresh weight (mg cm⁻²)</td>
<td>14.1 ± 1.1</td>
<td>11.1 ± 0.4</td>
<td>13.6 ± 1.2</td>
<td>13.9 ± 0.7</td>
</tr>
<tr>
<td>Start of bolting (d)</td>
<td>28.9 ± 1.3</td>
<td>31.3 ± 2.2</td>
<td>28.8 ± 1.8</td>
<td>28.3 ± 1.1</td>
</tr>
<tr>
<td>Start of anthesis (d)</td>
<td>32.5 ± 2.0</td>
<td>36.0 ± 2.6</td>
<td>33.5 ± 2.3</td>
<td>32.0 ± 1.6</td>
</tr>
<tr>
<td>Number of siliques per plant</td>
<td>32.2 ± 4.7</td>
<td>19.6 ± 6.0</td>
<td>35.6 ± 6.0</td>
<td>35.8 ± 5.8</td>
</tr>
<tr>
<td>Silique length (mm)</td>
<td>10.3 ± 1.4</td>
<td>9.3 ± 1.3</td>
<td>10.2 ± 1.6</td>
<td>10.3 ± 1.5</td>
</tr>
</tbody>
</table>
NADK2 activity fundamentally influences only the NAD/NADP ratio, although the reason that NaAD was similarly reduced in both the nadk2 mutant and NADK2 overexpressors is unknown.

NADK2 Overexpression Affects Rubisco Activity and Calvin Cycle Intermediates

The effective quantum yield of electron transport through PSII was lower in the nadk2 mutant than in the wild type (Takahashi et al., 2006b) but was the same as in the wild type in NADK2 overexpressors (data not shown). However, CO2 uptake under constant light conditions indicated a tendency to higher rates in the NADK2 overexpressors (data not shown). In contrast, Rubisco activities in the NADK2 overexpressors were 1.1- or 1.2-fold higher than in the wild type. The activities of the other Calvin cycle enzymes, including phosphoglycerate kinase (PGK; EC 2.7.2.3), NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH; EC 1.2.1.13), aldolase (EC 4.1.2.13), stromal fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11), and phosphoribulokinase (PRK; EC 2.7.1.19), were not affected.

Rubisco catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP) and produces 3-phosphoglycerate (3PGA), which is reduced to triosephosphates, which are then converted to intermediates in glycolysis, the TCA cycle, and other pathways. Therefore, we measured the concentrations of intermediates of these central metabolic pathways (Fig. 3). The nadk2 mutant accumulated about half the wild-type concentrations of most intermediates, except for pyruvate and citrate. However, Glc-6-P, Rib-5-P, ribulose-5-phosphate, RuBP, pyruvate, and 2-oxoglutarate were all higher in the NADK2 overexpressors. The exceptions were Fru-1,6-bisphosphate, dihydroxyacetone phosphate, 3PGA, and phosphoenolpyruvate, which were unchanged. Despite an increase in total Rubisco activity, RuBP levels were much higher in the NADK2 overexpressors than in the wild type. On the other hand, no significant difference was observed in the metabolite contents of the other pathways, including the shikimate, glutathione, and polyamine pathways (data not shown).

Increased Accumulation of Glu Family Amino Acids by NADK2 Overexpression

In plants, amino acids are synthesized from intermediates of the central metabolic pathway. Therefore, there is a tight relationship between amino acid biosynthesis and Calvin cycle activity (Stitt and Schulze, 1994; Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001). Amino acids can be categorized into the Asp, Glu, Ser, pyruvate, and His families and the aromatic amino acids. Amino acids were measured in the nadk2 mutant and in the NADK2 overexpressors, and the largest differences were found in the Glu family amino acids, especially Gln and Glu (Table III). Glu, and to a larger extent Gln, concentrations were higher in both NADK2 overexpression lines in proportion to NADK activity. Because Gln and Glu are good molecular markers for N utilization efficiency (Foyer et al., 2003), this result indicates that NADK2 overexpression also affects N assimilation. Total amino acid contents were also higher in the NADK2 overexpressors and lower in the nadk2 mutant, possibly

![Figure 2. Pyridine nucleotide pools in leaves of the wild type (WT), the nadk2 mutant, and NADK2 overexpressors (OX1 and OX2). A, Quantification of the nucleotide contents. B, NADP/H/NAD(H) ratio. C, (NADPH+NADH)/(NADP+NAD) ratios. Analyses were performed on leaves harvested from 23-d-old plants grown in soil, and nucleotide levels were quantified by CE-MS. Each value represents the mean ± se of three independent extracts. Significant differences from the wild type are shown (* P < 0.05, ** P < 0.01; t-test). FW, Fresh weight; UD, under the detection limit.](https://www.plantphysiol.org/doi/fig/10.1104/pp.109.147225)
reflecting the overall influence of N in the metabolic system. Furthermore, NADK2 activity levels affected the levels of other amino acid families. Thr, Cys, Gly, and Ser concentrations were higher in the NADK2 overexpressors. Ala concentrations were higher in the nadk2 mutant.

**NADK2 Overexpression Increases Light-Dependent Accumulation of Gln and Glu**

To further characterize amino acid biosynthesis in the NADK2 overexpressors, we examined time-dependent changes in leaf concentrations of Gln and Glu with

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
<th>Wild Type</th>
<th>nadk2</th>
<th>NADK2-OX1</th>
<th>NADK2-OX2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubisco</td>
<td>µmol min⁻¹ g⁻¹ fresh weight</td>
<td>9.5 ± 0.2</td>
<td>9.0 ± 0.1**</td>
<td>10.5 ± 0.3**</td>
<td>11.2 ± 0.3*</td>
</tr>
<tr>
<td>PGK</td>
<td></td>
<td>14.4 ± 0.4</td>
<td>11.6 ± 0.4**</td>
<td>15.7 ± 0.3***</td>
<td>17.5 ± 0.4*</td>
</tr>
<tr>
<td>NADP-GAPDH</td>
<td></td>
<td>13.7 ± 2.5</td>
<td>13.6 ± 2.7</td>
<td>13.1 ± 1.0</td>
<td>11.2 ± 0.1</td>
</tr>
<tr>
<td>Aldolase</td>
<td></td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Stromal FBPase</td>
<td></td>
<td>2.5 ± 0.4</td>
<td>2.3 ± 0.8</td>
<td>2.1 ± 0.5</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>PRK</td>
<td></td>
<td>14.9 ± 2.1</td>
<td>16.6 ± 2.2</td>
<td>15.3 ± 2.0</td>
<td>12.8 ± 1.1</td>
</tr>
</tbody>
</table>

**Table II. Activity of Calvin cycle enzymes in rosette leaves of the wild type, the nadk2 mutant, and NADK2 overexpression (NADK2-OX1 and -OX2) lines**

Activities were determined in 4-week-old leaves. Data presented are means ± se of measurements from five independent plants per genotype. Statistical significances relative to the wild type by t test are as follows: * P < 0.01, ** P < 0.05, *** P < 0.1.

**Figure 3.** Glycolysis, TCA cycle, and Calvin cycle intermediates in leaves of the wild type (WT), the nadk2 mutant, and NADK2 overexpression (OX1 and OX2) lines. Analyses were performed with rosette leaves harvested from 23-d-old plants grown in soil. Each value represents the mean ± se of three independent experiments (nmol g⁻¹ fresh weight). Significant differences from the wild type are shown (* P < 0.05, ** P < 0.01; t test). DHAP, Dihydroxyacetone phosphate; FBP, Fru-1,6-bisphosphate; GA3P, glyceraldehyde-3-phosphate; G6P, Glc-6-P; 2OG, 2-oxoglutarate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Ru5P, ribulose-5-phosphate; R5P, Rib-5-phosphate.
Effects of NADK2 Overexpression on Gene Expression Associated with the GS/GOGAT Pathway

In plants, N assimilation proceeds through the Gln synthetase (GS)/Glu synthase (GOGAT) pathway (Lam et al., 1996). In order to determine whether the increased amino acid biosynthesis of the NADK2 overexpressors was caused, at least in part, by affecting the expression levels of GS/GOGAT pathway genes, the expression levels of several related genes were analyzed by real-time PCR. Levels of transcripts from the Glu dehydrogenases (GDH1 and GDH2; EC 1.4.1.13) were also compared. The expression of NADH-dependent GOGAT (GLT1; EC 1.4.1.13), increased amino acid biosynthesis of the NADK2 overexpressors was caused, at least in part, by affecting the expression levels of GS/GOGAT pathway genes, the expression levels of several related genes were analyzed by real-time PCR. Levels of transcripts from the Glu dehydrogenases (GDH1 and GDH2; EC 1.4.1.13) were also compared. The expression of NADH-dependent GOGAT (GLT1; EC 1.4.1.13),

Increased amino acid biosynthesis of the NADK2 overexpressors was caused, at least in part, by affecting the expression levels of GS/GOGAT pathway genes, the expression levels of several related genes were analyzed by real-time PCR. Levels of transcripts from the Glu dehydrogenases (GDH1 and GDH2; EC 1.4.1.13) were also compared. The expression of NADH-dependent GOGAT (GLT1; EC 1.4.1.13),

Table III. Amino acid accumulation in rosette leaves of the wild type, the nadk2 mutant, and NADK2 overexpression (NADK2-OX1 and -OX2) lines

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Wild Type</th>
<th>nadk2</th>
<th>NADK2-OX1</th>
<th>NADK2-OX2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol g⁻¹ fresh weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu family</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>4,519.7 ± 581.6</td>
<td>443.4 ± 75.5**</td>
<td>5,890.5 ± 382.0**</td>
<td>6,375.7 ± 1,258.9</td>
</tr>
<tr>
<td>Gln</td>
<td>4,236.9 ± 537.0</td>
<td>1,071.5 ± 55.6**</td>
<td>6,490.2 ± 546.7*</td>
<td>6,555.0 ± 813.8***</td>
</tr>
<tr>
<td>Glu</td>
<td>2,786.6 ± 265.3</td>
<td>2,460.3 ± 519.2</td>
<td>3,917.8 ± 190.5**</td>
<td>4,388.1 ± 142.8**</td>
</tr>
<tr>
<td>Pro</td>
<td>984.1 ± 99.7</td>
<td>137.6 ± 9.1*</td>
<td>2,070.1 ± 641.2</td>
<td>1,075.5 ± 251.4</td>
</tr>
<tr>
<td>Ser family</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>4.8 ± 0.6</td>
<td>13.1 ± 1.1***</td>
<td>7.6 ± 0.2**</td>
<td>9.1 ± 1.9</td>
</tr>
<tr>
<td>Gly</td>
<td>259.6 ± 72.1</td>
<td>113.2 ± 14.1***</td>
<td>485.1 ± 56.7**</td>
<td>282.9 ± 80.4</td>
</tr>
<tr>
<td>Ser</td>
<td>3,961.0 ± 272.0</td>
<td>2,971.5 ± 172.4**</td>
<td>5,069.3 ± 490.9**</td>
<td>4,300.2 ± 211.1</td>
</tr>
<tr>
<td>Pyruvate family</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>271.5 ± 54.7</td>
<td>822.2 ± 129.8**</td>
<td>368.5 ± 49.9</td>
<td>385.6 ± 29.2**</td>
</tr>
<tr>
<td>Leu</td>
<td>75.2 ± 14.7</td>
<td>69.3 ± 5.9</td>
<td>66.9 ± 6.7</td>
<td>58.0 ± 2.5</td>
</tr>
<tr>
<td>Val</td>
<td>97.1 ± 12.1</td>
<td>116.7 ± 12.6</td>
<td>114.1 ± 12.6</td>
<td>102.3 ± 5.5</td>
</tr>
<tr>
<td>Aromatic amino acids</td>
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<tr>
<td>Phe</td>
<td>36.3 ± 6.0</td>
<td>36.3 ± 3.9</td>
<td>45.7 ± 2.1</td>
<td>35.2 ± 2.5</td>
</tr>
<tr>
<td>Trp</td>
<td>4.6 ± 1.2</td>
<td>8.6 ± 0.7***</td>
<td>4.8 ± 0.9</td>
<td>6.3 ± 1.0</td>
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<tr>
<td>Tyr</td>
<td>15.1 ± 1.8</td>
<td>12.6 ± 0.5</td>
<td>16.2 ± 1.6</td>
<td>12.7 ± 0.8</td>
</tr>
<tr>
<td>His family</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>116.7 ± 9.4</td>
<td>78.8 ± 9.7***</td>
<td>130.5 ± 12.4</td>
<td>108.6 ± 17.7</td>
</tr>
<tr>
<td>Total</td>
<td>21,027.8 ± 1,867.6</td>
<td>12,425.6 ± 1,014.1**</td>
<td>29,502.4 ± 482.6**</td>
<td>28,124.8 ± 1,927.1***</td>
</tr>
</tbody>
</table>

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GDH1, and GDH2 consistently mirrored the amino acid contents of the wild type, the nadk2 mutant, and the NADK2 overexpressor lines (Fig. 5A). Expression levels decreased among the isoenzymes of cytosolic GS (GLN1; EC 6.3.1.2), GLN1;1 (high-affinity form) and GLN1;3 (low-affinity form), in the nadk2 mutant, whereas no significant difference was observed in overexpressors. The expression levels of GLN1;5; chloroplastic GS (GS2; EC 6.3.1.2), and ferredoxin-dependent GOGAT (GLU1 and GLU2; EC 1.4.7.1) differed some from the wild type in both the nadk2 mutant and overexpressors, but not with any readily discernible pattern. Furthermore, we investigated GS and GDH activities in leaves (Fig. 5B). However, both GS and GDH activities were similar in the wild type, the nadk2 mutant, and NADK2 overexpressors.

NADK2 Overexpression Affects Both Gene Expression Associated with Nitrate Reduction and Nitrate Accumulation

Nitrate absorbed from soils is reduced to ammonium in sequential reactions catalyzed by nitrate reductase (NR; EC 1.7.1.1) and nitrite reductase (EC 1.7.7.1). This nitrate reduction process is essential to N assimilation. For example, overexpression of nitrite reductase affects N assimilation in tobacco plants (Takahashi et al., 2001). Although the overexpression of NR scarcely influences N assimilation (Ferrario-Méry et al., 1998), it has recently been reported that overexpression of the constitutively active form of NR leads to high N assimilation, implying a critical role for posttranslational regulation of NR in N assimilation (Lea et al., 2006). To investigate the possibility that NADK2 overexpression increases N assimilation via nitrate reduction, we examined the expression of genes related to nitrate reduction and the contents of inorganic N in leaves. The transcript levels of NIA1 and NIA2, which encode the two isoforms of the NR, were 4- and 2.5-fold higher in the nadk2 mutant, respectively, but decreased in the NADK2 overexpressors (Fig. 6A). Nitrite reductase gene (NIR) expression was also higher in the nadk2 mutant but lower in the NADK2 overexpressors. This result ruled out the possibility that up-regulation of NIA and NIR caused the increase in N assimilation by the NADK2 overexpressors but suggested that the expression levels of NIA and NIR are responsive to the modified metabolic balances in nadk2 and the NADK2 overexpressors. NO3⁻ induces
NIA and NIR expression (Pouteau et al., 1989; Lin et al., 1994; Scheible et al., 1997). In fact, NO$_3^-$ concentrations were approximately 10-fold higher in the nadk2 mutant (Fig. 6B). Although NO$_3^-$ was also 2- to 3-fold higher in the NADK2 overexpressors, concentrations of Gln, a known repressor of NIA (Vincentz et al., 1993), were also higher in these lines. NH$_4^+$ was about the same in the wild type and NADK2 overexpressors but slightly lower in the nadk2 mutant. However, NR activity of the unphosphorylated form, total NR activity, and the NR activation state were unchanged in the nadk2 mutant and in the NADK2 overexpressors (Fig. 6C).

**Requirement for Abundant N in NADK2 Overexpression-Mediated Activation of N Assimilation**

Transgenic Arabidopsis lines that are able to assimilate N at higher rates than the wild type grew better under low-N conditions (Yanagisawa et al., 2004). Therefore, we examined the growth of Arabidopsis plants with different NADK activities under different N conditions. Arabidopsis plants were grown on plates containing 1, 10, 30, or 60 mM KNO$_3$ (Fig. 7A). Both NADK2 overexpressors grew better than the wild type with 30 mM KNO$_3$. The improved growth of the NADK2-OX1 line was observed even under the 10 and 60 mM KNO$_3$ conditions (Fig. 7B). The growth of NADK2 overexpressors with 60 mM KNO$_3$ was not as good as with half the KNO$_3$, implying that 60 mM KNO$_3$ in the medium exceeds the optimal concentration for the NADK2 overexpressors. The growth of the nadk2 mutant was inferior to that of the wild type under any N conditions, and increasing the amount of supplied N did not increase growth. Since the growth of the nadk2 mutant, the wild type, and NADK2 overexpressors was comparable under low-N conditions, NADK2-dependent stimulation of N assimilation would likely only be evident under high-N conditions. The results of the Gln content analysis supported these observations in that Gln was higher in the NADK2 overexpressors than in the wild type grown in 30 or 60 mM KNO$_3$ but was comparable in each of the lines grown in 1 mM KNO$_3$ (Fig. 7C).

**DISCUSSION**

In this report, Arabidopsis plants with enhanced NADK activity were used to investigate the effects of NADP overproduction on plant metabolism, and specifically on C and N assimilation. Although both NAD and NADP are redox regulators of living cells, these compounds likely play overlapping but different roles in other biological processes. NAD is also a substrate for mono- and poly-ADP-ribosylation reactions, which are posttranslational protein modifications. Further analysis of such NAD-degrading reactions in mammals revealed that they are involved in other cellular processes, including energy metabolism and cell death.
Figure 7. Growth and Gln concentrations in the wild type (WT), the nadk2 mutant, and NADK2 overexpression (OX1 and OX2) lines grown on a low-N medium. A, Plants grown on a modified half-strength MS agar medium containing 1, 10, 30, or 60 mM KNO$_3$ as the sole N source. Bars = 1 cm. B and C, Comparison of growth and Gln concentrations in rosette leaves in plants grown on modified MS medium containing different concentrations of KNO$_3$. Plants were grown on MS medium for 5 d after germination and transferred to MS medium containing 1, 10, 30, or 60 mM KNO$_3$. Plants grown for a further 7 d were compared for growth and Gln contents. Black squares, the wild type; white squares, the nadk2 mutant; black triangles, the NADK2-OX1 line; black diamonds, the NADK2-OX2 line. Values represent means ± SD of at least 25 plants (B) or six independent experiments (C). Significant differences from the wild type are shown (* $P < 0.05$, ** $P < 0.01$; t test). FW, Fresh weight.

(Hassa et al., 2006; Alvarez-Gonzalez, 2007; Hassa and Hottiger, 2008). It is noteworthy that alteration of ADP-ribosylation activity directly affects intracellular NAD concentrations independently of redox reactions. On the other hand, NADK activity regulates the NAD/P/NAD ratio and is induced by environmental stresses in higher plants (Zagdańska, 1990; Delumeau et al., 2000; Ruiz et al., 2002). In cyanobacteria, illumination-dependent alterations in the NADP(H)/NAD(H) ratio induce dissociation of the PRK/CP12/GAPDH complex to regulate the activities of PRK and GAPDH (Tamoï et al., 2005). Based on these findings, changes in NAD(P)(H) concentrations would be expected to affect cellular processes in plants independently of the redox state. In this work, we show that transgenic Arabidopsis plants overexpressing NADK2 have elevated levels of NADP(H) in their leaves. However, the total contents and the redox state remained almost unchanged in both the nadk2 mutant and the NADK2 overexpressors. These plants can thus be used for studying the regulation mediated by NADP(H). We found several NADK2 overexpression phenotypes, including modified C and N metabolism, which are primarily due to the NADP content itself or to the modified NADP/NAD ratio, rather than to variations in redox states. Because the manipulation of NADK2 activity induced pleiotropic effects, NADK2 appears to be critical for proper metabolic regulation in Arabidopsis.

Modification of NADK2 expression also induced several morphological phenotypes in Arabidopsis. Under the growth conditions used, the nadk2 mutant produced small and pale green leaves with decreased chlorophyll content (Chai et al., 2005). Conversely, the chlorophyll content of NADK2 overexpression plants was higher than in the wild type, although overexpression of NADK2 exerted only a limited impact on leaf size when plants were grown in soil. NADK2 deficiency in the nadk2 mutant decreased photosynthetic electron transport in leaves, probably through severe inhibition of zeaxanthin epoxidation (Takahashi et al., 2006b), but NADK2 overexpression did not obviously affect photosynthetic electron transport (data not shown). These observations suggest that the effects of elevated NADP content are not perfectly opposite to the effects generated by its reduction, possibly because some effects might be induced only when the NADP content or the NADP/NAD ratio reaches some threshold. This explanation might be applicable to other phenomena that are inconsistent in the nadk2 mutant and the NADK2 overexpressors.

To identify pleiotropic effects caused by modifications of NADP(H) contents, transcriptomes of wild-type Arabidopsis, the nadk2 mutant, and the NADK2 overexpressor were compared by DNA microarray analysis (Fig. 8; Supplemental Tables S1 and S2). Broad effects on gene expression in the nadk2 mutant were revealed. In this mutant, the expression of genes involved in metabolic regulation, stress responses, cellular homeostasis, and morphogenesis was strongly modified. This finding is in accord with results obtained in previous reports, because the mutant shows a severe morphological phenotype and high sensitivity against ROS stress (Chai et al., 2005; Takahashi et al., 2006b). This suggests that decreased levels of NADP(H), which acts as an essential factor in a variety of processes, induces disorders at least partly through modulations of gene expression. On the other hand, limited and comparatively moderate effects on gene expression were found in the NADK2 overexpressor.
While the expression levels of 2,878 genes were significantly modified in the nadk2 mutant, only 47 genes were affected in the NADK2 overexpressor (P < 0.01). Importantly, we found that intermediates of the Calvin cycle such as Rib-5-P and RuBP were higher in the NADK2 overexpressors, although NADK2 overexpression had only a limited effect on the expression level of related genes and photosynthetic electron transport. Therefore, some enzyme activity in the Calvin cycle must be different in the NADK2 overexpressors in vitro. Previously, it has been shown that expression of a cyanobacterial dual-function enzyme, FBPase/sedoheptulose-1,7-bisphosphatase, in chloroplasts of transgenic tobacco plants significantly increases Rubisco activity but not the activities of PRK, NADP-GAPDH, and aldolase (Miyagawa et al., 2001). This observation might indicate that there is some mechanism that coordinates Rubisco activity and the activity of Calvin cycle enzymes. One possible mechanism to explain this observation is that the increased NADP(H) content may improve conjugation of NADP(H) to NADP(H)-dependent enzymes or complexes, including the PRK/CP12/GAPDH complex. Indeed, some NAD(P)(H)-dependent enzymes are activated by increased NAD(P)(H) contents (White et al., 1983; Scagliarini et al., 1990; Stewart and Copeland, 1998; Graciet et al., 2002). Therefore, even if there was no change in the activities of PRK and NADP-GAPDH when activity was measured in vitro in the presence of identical concentrations of NAD(P)(H), there is still a possibility that the activity of these enzymes was different in the cells of the wild-type and NADK2 transgenic Arabidopsis plants. This hypothesis is comparable to the case of Rubisco activation, and it has been proposed that increases in RuBP play a role in the activation of Rubisco in vivo. We demonstrate here that significant increases in initial and total Rubisco activities and RuBP contents themselves were

Figure B. Effects of up- and down-regulation of the NADK2 gene on the gene expression profile in leaves. The transcript levels of genes associated with metabolic activities in the nadk2 mutant (A) or the NADK2-OX2 line (B) were compared with those in the wild type. The transcript levels of genes associated with cellular responses in the nadk2 mutant (C) and the NADK2-OX2 line (D) were also compared with those in the wild type. Genes that exhibited increased expression levels are shown in blue, while those with decreased expression are shown in red. The color scale applies to all panels. CHO, Carbohydrate; OPP, oxidative pentose phosphate.
induced in the NADK2 overexpressors. However, it is currently unknown whether these changes were induced by activation of the Calvin cycle or triggered the activation of the Calvin cycle. Therefore, further analysis (e.g. of possible modifications of enzyme activity by thiol-mediated regulation) would be necessary to elucidate the relationship between overexpression and modified activity of the Calvin cycle. Discovery of a regulatory facet associated with NADP(H) would then provide a new target for genetic engineering of the Calvin cycle.

Intermediates of the Calvin cycle are utilized in several biosynthetic pathways, including the GS/GOGAT pathway for Glu family amino acid synthesis via 2-oxoglutarate as a C skeleton (Hodges, 2002; Suzuki and Knaff, 2005; Forde and Lea, 2007; Tabuchi et al., 2007). Since 2-oxoglutarate levels were altered proportionally with NADP(H) contents, we hypothesized that Gln and Glu synthesis were also affected by NADK2 overexpression. Indeed, levels of Glu family amino acids were higher in the NADK2 overexpressors. There was no evident activation or repression of gene expression associated with the GS/GOGAT cycle in the nadk2 mutant or the NADK2 overexpressors, although there was a slight modulation of GLT1 and GDH expression proportional to NADK activity. Furthermore, no significant change was observed in GS or GDH activity of the nadk2 mutant or the NADK2 overexpressors, suggesting that other factors may be involved in producing higher Glu family amino acid concentrations in the NADK2 overexpressors. On the other hand, the overexpression of NADK2 significantly affected expression of genes encoding enzymes involved in nitrate reduction. However, NIA and NIR expression was high in the nadk2 mutant but low in the NADK2 overexpressors. This modified gene expression thus likely reflects the altered metabolic balance rather than activation of N assimilation. Despite the reduced levels of NIA and NIR transcripts, no significant difference among the wild type, the nadk2 mutant, and NADK2 overexpressors was found in terms of NR activity and NR activation state. However, nitrate reduction itself appears to be more active in the NADK2 overexpressors, judging from the Gln content in plants that were grown on medium containing nitrate as the sole N source. Thus, NADK2 overexpression appears to stimulate N assimilation not so much through changes in the phosphorylation state of the NR protein but rather through other factors, such as the supply of C skeletons derived from the Calvin cycle, at least in part.

Neither knockout nor overexpression of NADK2 affected Gln contents under low-N (1 mM KNO₃) conditions, whereas overexpression of NADK2 induced increases in the Gln content under high-N (30 or 60 mM KNO₃) conditions (Fig. 7C). Therefore, we speculate that NADP levels maintained by NADK1 and NADK3 in the cytosol may be sufficient for N assimilation at low levels under low-N conditions, while NADK2 activity in chloroplasts may dictate N assimilation rates under high-N conditions. This hypothesis may be supported by the observation that the growth of the nadk2 mutant is comparable to that of the wild type and the NADK2 overexpressors under low-N conditions but is not as good under high-N conditions (Fig. 7, A and B). Unexpectedly, we observed that the growth of NADK2 overexpressors, but not the wild type, decreased when the concentration of KNO₃ in the medium was 60 mM rather than 30 mM. Although the mechanism underlying this phenomenon is currently unknown, it may be that NADK2 is involved in controlling growth in response to exogenous N concentrations in the environment.

In summary, the overexpression of a single gene, NADK2, caused pleiotropic effects on numerous metabolic processes in Arabidopsis leaves. Although the observed effects on metabolism presumably included direct or indirect consequences that are not distinguishable at this stage, our results suggest that modulation of NADP levels would be integrated into the network that controls plant metabolism. Furthermore, our results also highlight the possibility that enhancement of cofactor synthesis is a workable strategy for improving C and N assimilation in planta, because the overexpression of NADK2 improved growth and increased the concentration of Gln, a storage material. More effective improvement of C and N assimilation might be achieved by identification and targeted modifications of key processes that are critical for C and N metabolism.

MATERIALS AND METHODS

Generation of Transgenic Plants

To generate Arabidopsis (Arabidopsis thaliana) plants overexpressing NADK2 under the control of the cauliflower mosaic virus 35S promoter, a cDNA clone containing the complete Arabidopsis NADK2 open reading frame was amplified by PCR using primers with an additional sequence for the attB site. The PCR product obtained was cloned into pDONR201 (Invitrogen) and then transferred into pH2GW7 (Karimi et al., 2002) using the Gateway recombination system. The resultant plasmid (pH2GW7-NADK2) was introduced into wild-type Arabidopsis (ecotype Columbia) by Agrobacterium tumefaciens-mediated transformation utilizing the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on 0.8% (w/v) agar plates containing Murashige and Skoog (MS) medium and 40 μg mL⁻¹ hygromycin. Homozygous plants harboring the transgene were used for further analysis.

Plant Materials and Growth Conditions

Plants were grown on MS agar plates or in soil at 23°C under continuous light conditions (60 μmol m⁻² s⁻¹). For analysis of metabolite contents and gene expression, leaves of 23-d-old soil-grown plants were frozen in liquid N and stored at −80°C. For evaluation of growth under different N conditions, seeds were germinated on modified half-strength MS medium. Five-day-old seedlings were transferred onto plates containing different concentrations of KNO₃ and grown for 7 d. For the analyses shown in Figure 4, the plants were grown with photoperiods of 16 h at 60 μmol m⁻² s⁻¹ and 25-d-old plants were used.

Reverse Transcription (RT)-PCR and Real-Time RT-PCR Expression Analysis

Total RNA was extracted from 23-d-old plants with an RNasey Plant Mini Prep Kit (Qiagen), and 1 μg of total RNA was reverse transcribed with oligo (dT) primer using Ready-To-Go You-Prime First-Strand Beads (GE Health-
incubated for 30 min at 30°C. Glutamine dehydrochloride were added. Nitrite concentration was measured at 540 nm, and NR activity was expressed as nmol nitrite h⁻¹ g⁻¹ fresh weight. The NR activation state was estimated by calculating the ratio of the activity in the presence of 6 mM MgCl₂ (activity of the unphosphorylated form) to the activity in the presence of 2 mM EDTA (total activity) and expressed as a percentage. For measurement of GS and GDH, a crude leaf extract was prepared, and GDH activity was measured by quantifying produced Gln, according to Ishiyama et al. (2004). GDH activity was determined by quantifying Gln according to Masclaux et al. (2000) with minor modifications. Protein concentration in the crude extract was measured with a protein assay kit (Bio-Rad Laboratories).

### Chlorophyll Estimation
Chlorophyll was extracted from leaves as described previously (Oka et al., 2004). Frozen leaves were homogenized with 80% acetone, and homogenates were centrifuged at 1,500 rpm for 5 min. Supernatants were measured at 645 nm per 1 g fresh weight. Chlorophyll content was calculated according to MacKinnon (1941) and expressed as mg chlorophyll g⁻¹ fresh weight.

### Enzyme Assays
Preparation of crude tissue lysates and NAD kinase assays were performed as described previously (Takahashi et al., 2006a). Briefly, frozen leaves were ground in liquid N and further homogenized in a buffer containing 50 mM tripotassium phosphate and 100 mM sodium ascorbate (pH 10.0). After centrifugation at 15,000 rpm for 5 min, the supernatant was measured at 465 and 663 nm. The chlorophyll content was calculated according to the Arabidopsis genome (The Arabidopsis Information Resource 7 version).

### NADK2 and Carbon and Nitrogen Metabolism
The following materials are available in the online version of this article.

**Supplemental Table S1.** Selected metabolism-related genes that differentially expressed between nakh2 and wild-type plant.

**Supplemental Table S2.** Selected metabolism-related genes that differentially expressed between NADK2-OX and wild-type plant.

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