Pleiotropic Modulation of Carbon and Nitrogen Metabolism in Arabidopsis Plants Overexpressing the *NAD kinase2* Gene[1][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 lycopersicum*) and wheat (*Triticum aestivum*), respectively (Zagdańska, 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 photosynthetic electron transport chain in chloroplasts, and then NADPH is transported from chloroplasts to the cytosol and other organelles via the malate/oxaloacetate shuttle (Heineke et al., 1991; Scheibe, 2004). The reducing energy of NADPH is used for biological processes in chloroplasts, including chlorophyll synthesis and CO_2 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 CO_2 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.,

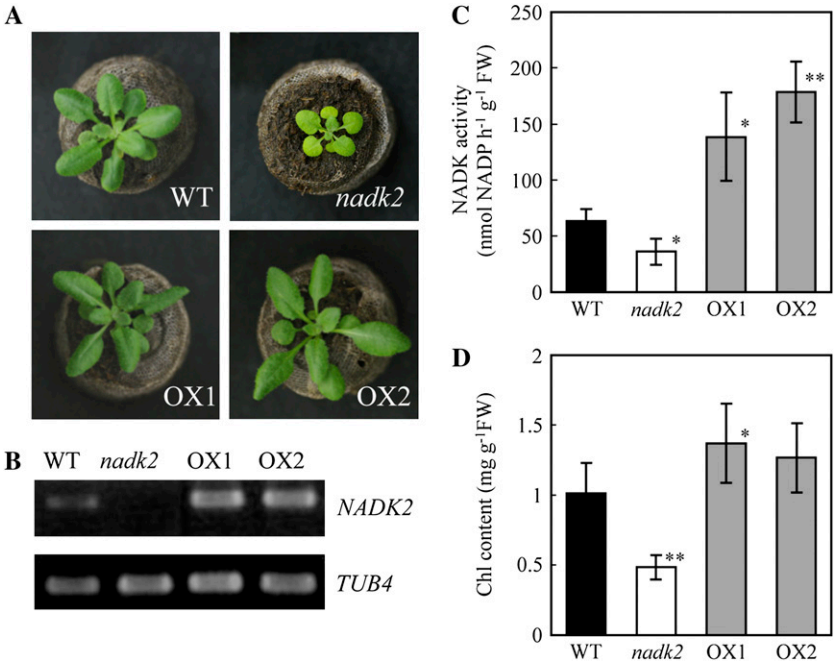

Figure 1. Phenotype of Arabidopsis wild type (WT), the *nadk2* mutant, and transgenic lines overexpressing *NADK2* (OX1 and OX2) grown for 23 d under continuous light. A, Plants grown in soil. B, Levels of the *NADK2* transcript in rosette leaves. RT-PCR was performed with 1 μg of total RNA. C, NADK activity in rosette leaves. D, Chlorophyll (Chl) contents of rosette leaves. Values are means ± SD calculated from three independent experiments. Significant differences from the wild type are evaluated by *t* test and shown by asterisks (* $P < 0.05$ or ** $P < 0.01$). FW, Fresh weight.

2005). 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 I. *Developmental characteristics of Arabidopsis wild type, the nadk2 mutant, and NADK2 overexpression (NADK2-OX1 and -OX2) lines under continuous light conditions*

Size and weight of leaves were measured using the fifth rosette leaf of 23-d-old plants. All other parameters were measured on 90-d-old plants. The data are means ± SD of at least 30 plants.

Parameter	Wild Type	*nadk2*	NADK2-OX1	NADK2-OX2
Leaf length (mm)	8.9 ± 1.4	6.5 ± 1.2	9.3 ± 0.7	9.6 ± 1.0
Leaf width (mm)	7.8 ± 1.3	6.5 ± 1.2	7.9 ± 0.8	8.1 ± 0.9
Leaf fresh weight (mg)	10.6 ± 2.0	5.5 ± 1.3	10.9 ± 1.5	12.0 ± 0.8
Specific leaf fresh weight (mg cm^{-2})	14.1 ± 1.1	11.1 ± 0.4	13.6 ± 1.2	13.9 ± 0.7
Start of bolting (d)	28.9 ± 1.3	31.3 ± 2.2	28.8 ± 1.8	28.3 ± 1.1
Start of anthesis (d)	32.5 ± 2.0	36.0 ± 2.6	33.5 ± 2.3	32.0 ± 1.6
Number of siliques per plant	32.2 ± 4.7	19.6 ± 6.0	35.6 ± 6.0	35.8 ± 5.8
Silique length (mm)	10.3 ± 1.4	9.3 ± 1.3	10.2 ± 1.6	10.3 ± 1.5

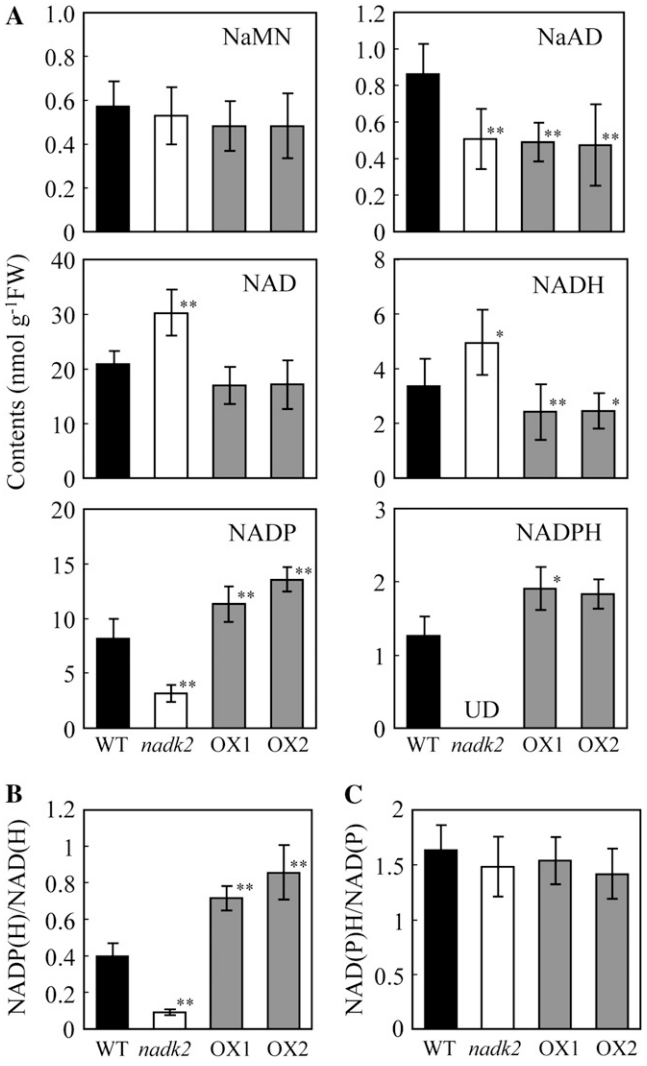

Figure 2. Pyridine nucleotide pools in leaves of the wild type (WT), the *nadk2* mutant, and *NADK2* overexpressors (OX1 and OX2). A, Quantification of the nucleotide contents. B, NADP(H)/NAD(H) ratio. C, (NADPH+NADH)/(NADP+NAD) ratios. Analyses were performed on leaves harvested from 23-d-old plants 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.

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, CO_2 uptake under constant light conditions indicated a tendency to higher rates in the *NADK2* overexpressors (data not shown). The activities of enzymes in the Calvin cycle were thus measured. Both the initial and total activities of Rubisco (EC 4.1.1.39) in the *nadk2* mutant were approximately 1.1- and 1.2-fold lower than in the wild type (Table II). 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 phospho*enol*pyruvate, 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

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: * $P < 0.01$, ** $P < 0.05$, *** $P < 0.1$.

Enzyme	Activity			
	Wild Type	*nadk2*	NADK2-OX1	NADK2-OX2
	μmol min^{-1} *g*$^{-1}$ *fresh weight*			
Rubisco				
Initial	9.5 ± 0.2	9.0 ± 0.1***	10.5 ± 0.3**	11.2 ± 0.3*
Total	14.4 ± 0.4	11.6 ± 0.4*	15.7 ± 0.3***	17.5 ± 0.4*
PGK	47.6 ± 4.8	52.4 ± 4.5	51.0 ± 4.2	46.4 ± 2.1
NADP-GAPDH	13.7 ± 2.5	13.6 ± 2.7	13.1 ± 1.0	11.2 ± 0.1
Aldolase	2.0 ± 0.1	1.7 ± 0.4	2.0 ± 0.2	2.0 ± 0.2
Stromal FBPase	2.5 ± 0.4	2.3 ± 0.8	2.1 ± 0.5	2.1 ± 0.5
PRK	14.9 ± 2.1	16.6 ± 2.2	15.3 ± 2.0	12.8 ± 1.1

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

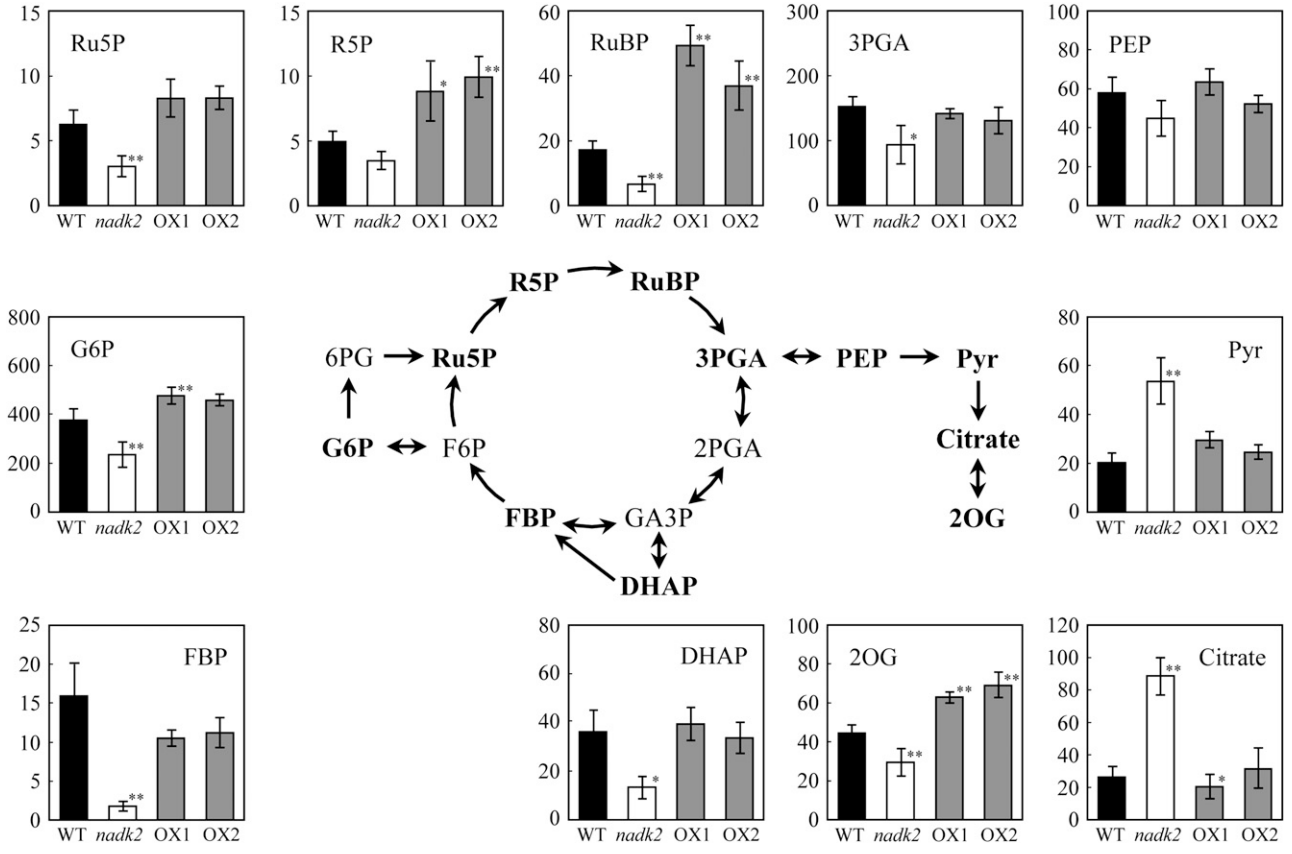

Figure 3. Glycolysis, TCA cycle, and Calvin cycle intermediates in leaves of the wild type (WT), the *nadk2* mutant, and *NADK2* overexpression (OX1 and OX2) lines. Analyses were performed with rosette leaves harvested from 23-d-old plants grown in soil. Each value represents the mean ± SE of three independent 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, phospho*enol*pyruvate; Pyr, pyruvate; Ru5P, ribulose-5-phosphate; R5P, Rib-5-phosphate.

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

Values indicate means obtained from three independent experiments. SE values are in all cases less than 35% of mean values. Statistical significances relative to the wild type by *t* test are as follows: * $P < 0.01$, ** $P < 0.05$, *** $P < 0.1$

Metabolite	Concentration			
	Wild Type	*nadk2*	NADK2-OX1	NADK2-OX2
	nmol g^{-1} fresh weight			
Asp family				
Asn	1,618.4 ± 54.7	671.7 ± 54.9**	2,074.2 ± 234.0	1,582.9 ± 350.5
Asp	1,238.4 ± 180.1	2,944.0 ± 477.6***	1,628.5 ± 168.9	1,719.6 ± 170.8
Lys	106.5 ± 13.9	50.2 ± 5.2**	90.9 ± 6.4	81.0 ± 23.4
Met	14.9 ± 3.0	7.4 ± 0.2***	19.0 ± 1.3	16.6 ± 0.4
Thr	635.5 ± 43.1	374.6 ± 22.5**	922.6 ± 53.6**	951.1 ± 104.6***
Glu family				
Arg	4,519.7 ± 581.6	443.4 ± 75.5**	5,890.5 ± 382.0**	6,375.7 ± 1,258.9
Gln	4,236.9 ± 537.0	1,071.5 ± 55.6**	6,490.2 ± 546.7*	6,555.0 ± 813.8***
Glu	2,786.6 ± 265.3	2,460.3 ± 519.2	3,917.8 ± 190.5**	4,388.1 ± 142.8**
Pro	984.1 ± 99.7	137.6 ± 9.1*	2,070.1 ± 641.2	1,075.5 ± 251.4
Ser family				
Cys	4.8 ± 0.6	13.1 ± 1.1**	7.6 ± 0.2**	9.1 ± 1.9
Gly	259.6 ± 72.1	113.2 ± 14.1***	485.1 ± 56.7**	282.9 ± 80.4
Ser	3,961.0 ± 272.0	2,971.5 ± 172.4**	5,069.3 ± 40.9**	4,300.2 ± 211.1
Pyruvate family				
Ala	271.5 ± 54.7	822.2 ± 129.8**	368.5 ± 49.9	385.6 ± 29.2**
Leu	75.2 ± 14.7	69.3 ± 5.9	66.9 ± 6.7	58.0 ± 2.5
Val	97.1 ± 12.1	116.7 ± 12.6	114.1 ± 12.6	102.3 ± 5.5
Aromatic amino acids				
Phe	36.3 ± 6.0	36.3 ± 3.9	45.7 ± 2.1	35.2 ± 2.5
Trp	4.6 ± 1.2	8.6 ± 0.7***	4.8 ± 0.9	6.3 ± 1.0
Tyr	15.1 ± 1.8	12.6 ± 0.5	16.2 ± 1.6	12.7 ± 0.8
His family				
His	116.7 ± 9.4	78.8 ± 9.7***	130.5 ± 12.4	108.6 ± 17.7
Total	21,027.8 ± 1,867.6	12,425.6 ± 1,014.1**	29,502.4 ± 482.6**	28,124.8 ± 1,927.1***

exposure to light irradiation (60 μmol m^{-2} s^{-1}; Fig. 4). Because N assimilation is a light energy-dependent process, an expanded capacity for N assimilation should lead to more rapid production of larger amounts of Gln and Glu in the overexpressor line with light irradiation. Initial Gln concentrations were higher in NADK2-OX2 and by 30 min were already increasing. Wild-type Gln concentrations began to increase starting at the 1-h time point. Initial Gln concentrations were lower in the *nadk2* mutant than in the wild type. Concentrations of Gln peaked in all three plant lines at 1 h, although the *nadk2* mutant produced very little.

Glu concentrations were somewhat higher in NADK2-OX2 than in wild-type Arabidopsis at the 1-h time point. The rates of Glu synthesis were 551 nmol g^{-1} fresh weight h^{-1} in the wild type and 1,992 nmol g^{-1} fresh weight h^{-1} in NADK2-OX2. The Glu concentration in the *nadk2* mutant was only slightly increased at the 30-min time point.

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 1.4.1.3) were also compared. The expression of NADH-dependent GOGAT (*GLT1*; EC 1.4.1.13),

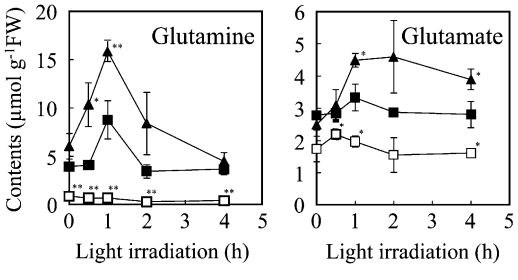

Figure 4. Gln and Glu synthesis stimulated by light irradiation. Rosette leaves of 25-d-old plants were harvested at each time point indicated after light irradiation (60 μmol m^{-2} s^{-1}), and Gln and Glu contents of the leaves were quantified. Black squares, the wild type; white squares, the *nadk2* mutant; black triangles, the *NADK2*-OX2 line. Each value represents the mean ± SE of three independent experiments. Significant differences from the wild type are shown (* $P < 0.05$, ** $P < 0.01$; *t* test). FW, Fresh weight.

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. NO_3^- induces

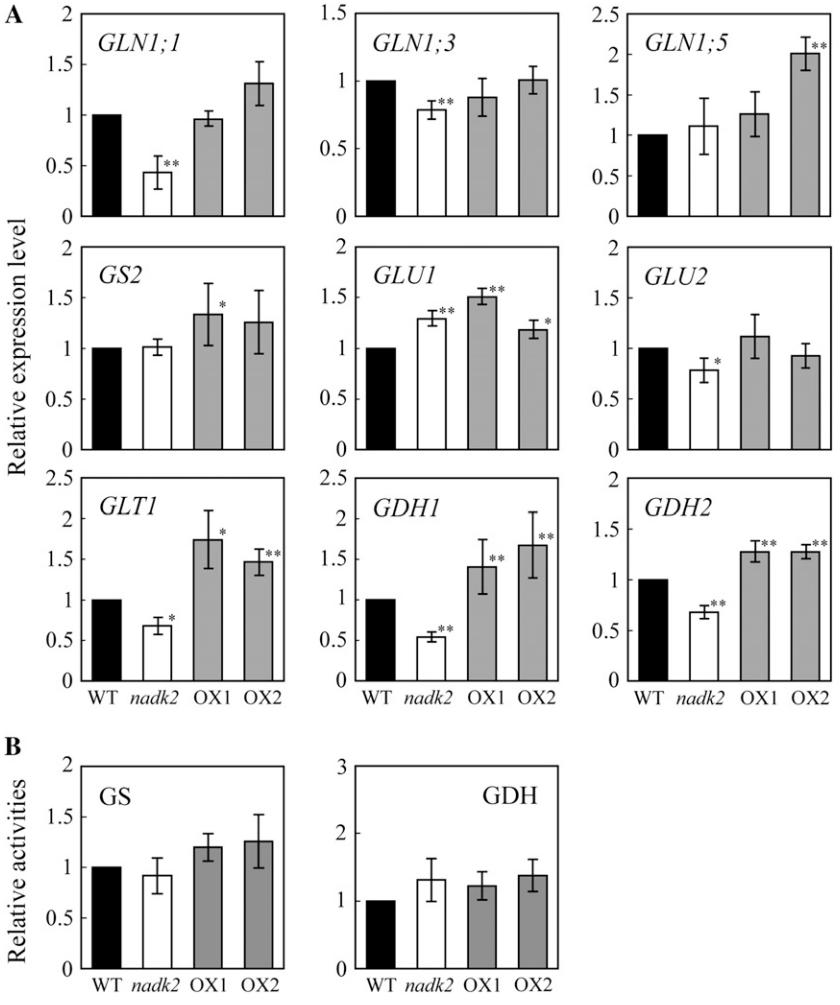

Figure 5. Effects of *NADK2* overexpression on GS/GOGAT pathway-related gene expression. A, Levels of *GLN1;1*, *GLN1;3*, *GLN1;5*, *GS2*, *GLU1*, *GLT1*, *GDH1*, and *GDH2* transcripts in rosette leaves of 23-d-old wild-type plants (WT), the *nadk2* mutant, and *NADK2* overexpressors (OX1 and OX2) were compared. Values were obtained from real-time PCR and expressed as relative to the wild type. B, GS and GDH activities in leaves are expressed relative to the wild type. GS and GDH activities in the wild type are 692.9 nmol Gln min^{-1} g^{-1} fresh weight and 36.3 nmol Glu min^{-1} g^{-1} fresh weight, respectively. Each value represents the mean ± SD calculated from three replicates. Significant differences from the wild type are shown (* $P < 0.05$, ** $P < 0.01$; *t* test).

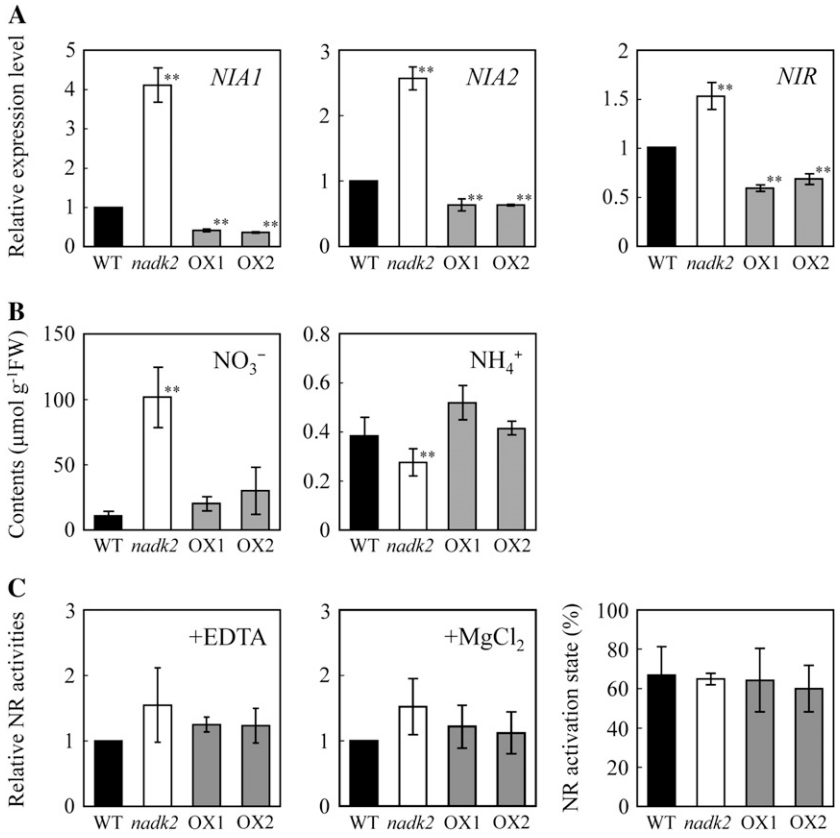

Figure 6. Effects of *NADK2* overexpression on nitrate reduction. Transcript and metabolite levels associated with nitrate reduction in rosette leaves of 23-d-old wild-type plants (WT), the *nadk2* mutant, and *NADK2* overexpression (OX1 and OX2) lines were analyzed. A, Transcript levels of nitrate reductase (*NIA1* and *NIA2*) and nitrite reductase (*NIR*) genes estimated by real-time PCR are expressed relative to the wild type. Each value represents the mean ± SD calculated from three replicates. B, Accumulation of NO_3^- and NH_4^+ in leaves determined by ion chromatography. C, NR activity and the ratio in the activation state. Values are expressed relative to the wild type in the presence of EDTA (668.0 nmol nitrite h^{-1} g^{-1} fresh weight [FW]) and in the presence of $MgCl_2$ (454.8 nmol nitrite h^{-1} g^{-1} fresh weight), respectively. Each value represents the mean ± SE of three independent experiments. Significant differences from the wild type are shown ** $P < 0.01$; *t* test).

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

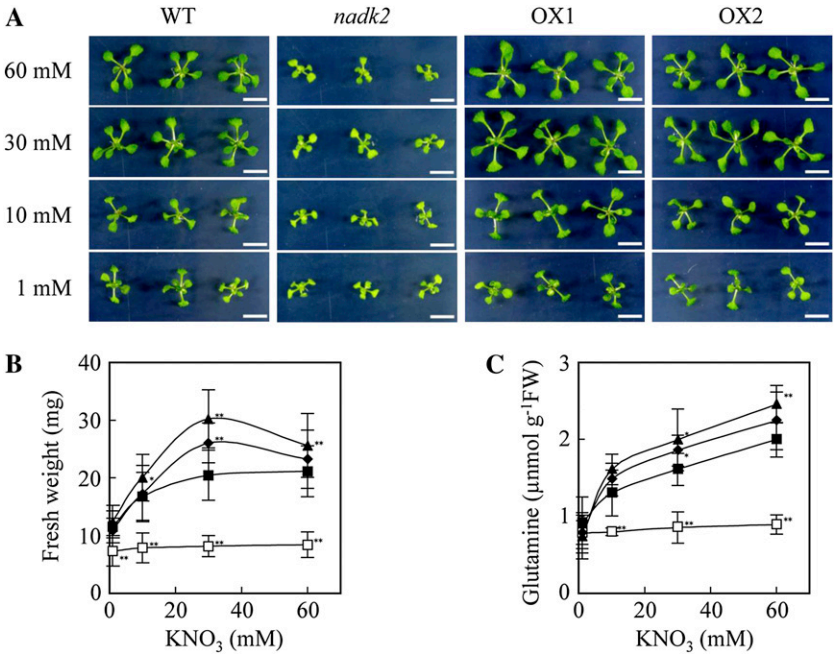

Figure 7. Growth and Gln concentrations in the wild type (WT), the *nadk2* mutant, and *NADK2* overexpression (OX1 and OX2) lines grown on a low-N medium. A, Plants grown on a modified half-strength MS agar medium containing 1, 10, 30, or 60 mM KNO_3 as the sole N source. Bars = 1 cm. B and C, Comparison 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/NADP 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 (Tamoi 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* overexpressor 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.

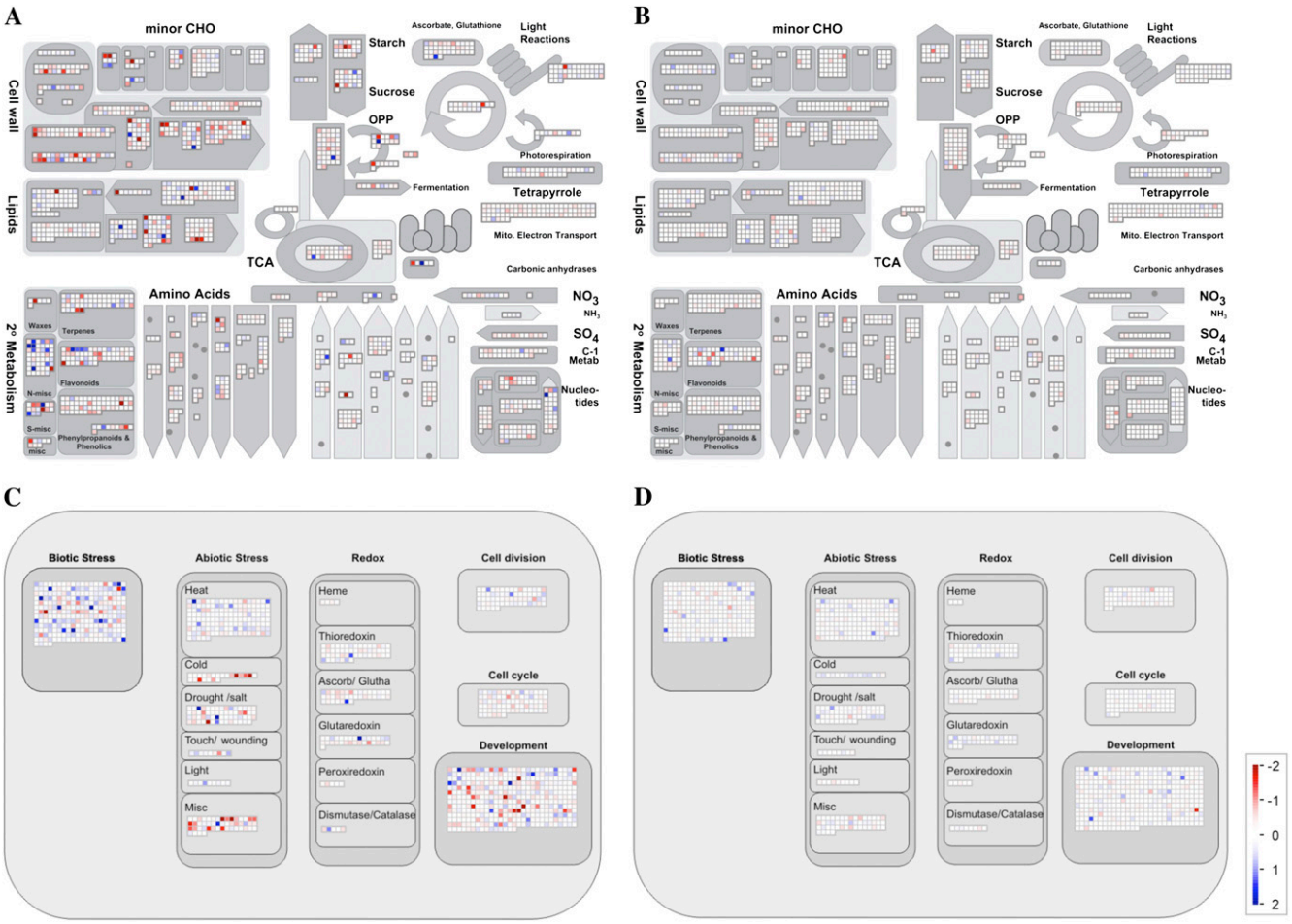

Figure 8. Effects of up- and down-regulation of the *NADK2* gene on the gene expression profile in leaves. The transcript levels of genes associated with metabolic activities in the *nadk2* mutant (A) or the NADK2-OX2 line (B) were compared with those in the wild type. The transcript levels of genes 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.

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 coordinately regulates 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

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 *NADK*2 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 KNO_3) conditions, whereas overexpression of *NADK2* induced increases in the Gln content under high-N (30 or 60 mM KNO_3) 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 KNO_3 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-*AtNADK2*) 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 $\mu g\ mL^{-1}$ 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 $\mu mol\ m^{-2}\ s^{-1}$). 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 KNO_3 and grown for 7 d. For the analyses shown in Figure 4, the plants were grown with photoperiods of 16 h at 60 $\mu mol\ m^{-2}\ s^{-1}$ 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 RNeasy 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-

care). First-strand cDNA was amplified by PCR with specific primer sets for *NADK2* and for the constitutively expressed *β-tubulin4* (*TUB4*) gene as a control. The following primers were used: *NADK2* (forward, 5′-GGCTTCTCTGCAGCCCCTATTGCTGTGCC-3′; reverse, 5′-GACTCGTTTGAGGTCTTGCCTGAAGTCCT-3′) and *TUB4* (forward, 5′-AGAGGTTGACGAGCAGATGA-3′; reverse, 5′-CCTCTTCTTCCTCCTCGTAC-3′). For real-time RT-PCR, total RNA was treated with RNase-free DNase I (Qiagen) to eliminate genomic DNA contamination according to the manufacturer's instructions. First-strand cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with the QuantiTect SYBR Green PCR Kit (Qiagen) and an ABI Prism 7500 sequence detection system (Applied Biosystems). *Actin8* was used as an internal control in each experiment. The following gene-specific primers were used: *GLN1;1* (forward, 5′-TGTCATGTGCGATGCGTACA-3′; reverse, 5′-CCGCAGCGTGTCTTTTGTTA-3′), *GLN1;3* (forward, 5′-CACATTGCTGCTTACGGTGAA-3′; reverse, 5′-TGTGTTGATGTCTGCGGTTTC-3′), *GLN1;5* (forward, 5′-GGTCGTGACATTGTCGATGCT-3′; reverse, 5′-CACCATTGGCACCACCAATA-3′), *GS2* (forward, 5′-TGGTGAAGTTATGCCTGGACAGT-3′; reverse, 5′-CATGATCACCTGCATCAATTCCT-3′), *GLU1* (forward, 5′-ATGTCCTCCGTGACCTGCTT-3′; reverse, 5′-AACGGCCGGTTCTACTTTCC-3′), *GLU2* (forward, 5′-GCATTTAGCCAACCGTCCTATT-3′; reverse, 5′-CAGGGATAGGGTTACGGTCACT-3′), *GLT1* (forward, 5′-GCTATCTCAGAAGGACGTCAAGCT-3′; reverse, 5′-AGCTTGGCGTCTTCGTCATC-3′), *GDH1* (forward, 5′-GGGAAGATTGTTGCCGTGAGT-3′; reverse, 5′-TGAGCAAGGCCGGGATATC-3′), *GDH2* (forward, 5′-CGAAGCTGATCCACGAGAAAG-3′; reverse, 5′-AGGGTTCCTGATTGCACCAGTA-3′), *NIA1* (forward, 5′-TTTGGTCAACCCACGTGAGAA-3′; reverse, 5′-CTTACGAACGTCGTGCGAGAT-3′), *NIA2* (forward, 5′-GGCGTGGTTCGTTCTTACAAA-3′; reverse, 5′-TTGGTTCTGGTGCGCCTTA-3′), and *NIR* (forward, 5′-TCGCAGTTTATCACCGCTAATTC-3′; reverse, 5′-CCACCACACACACATTCCACTT-3′).

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 and 663 nm. The chlorophyll content was calculated according to Mackinney (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 resulting supernatant was used as crude extract. NADK activity was determined in 1 mL of reaction mixture containing 34 mM Tricine-KOH (pH 8.0), 5 mM NAD, 5 mM ATP, 7 mM $MgCl_2$, 6 mM nicotinamide, and 400 to 500 μg of total protein from the crude extract incubated for 30 min at 30°C. NADK activity was expressed as nmol NADP h^{-1} g^{-1} fresh weight. For measurement of Rubisco activity, frozen leaf discs were ground with a chilled mortar and pestle and further homogenized in an extraction buffer containing 100 mM HEPES-KOH (pH 8.0), 1 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Initial and total activities were measured with a spectrophotometer at 340 nm as described by Sharkey et al. (1991). Stromal FBPase was extracted in 50 mM HEPES-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF and assayed as described by Hurry et al. (1995). To assay the activity of the other Calvin cycle enzymes, frozen tissues were homogenized in an extraction buffer containing 50 mM HEPES-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. Aldolase, PGK, and PRK activities were measured according to the methods described by Haake et al. (1998), Leegood and Walker (1980), and Latzko et al. (1970), respectively. NADP-GAPDH activity was measured in a reaction mixture containing 100 mM Tricine-KOH (pH 8.0), 30 mM $MgCl_2$, 20 mM KCl, 1 mM EDTA, 5 mM DTT, 0.5 mM NADPH, 5 mM ATP, 6 mM phosphoglyceric acid, and 10 units of PGK. Crude extracts were prepared to measure NR activity by the method of Ferrario-Méry et al. (1998). NR activity was assayed in the reaction mixture containing 50 mM HEPES-KOH (pH 7.5), 0.1 mM NADH, 5 mM KNO_3, and either 6 mM $MgCl_2$ or 2 mM EDTA. After incubation at room temperature for 15 min, an equal volume of 1% (w/v) sulfanilamide in 3 N HCl and 0.02% (w/v) *N*-naphthylethylenediamime 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 $MgCl_2$ (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 GS activity was measured by quantifying produced Gln, according to Ishiyama et al. (2004). GDH activity was determined by quantifying Glu production 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).

Metabolite Analysis

Samples were ground to fine powder in liquid N and further homogenized with ice-cold 50% (v/v) methanol containing PIPES and Met sulfone as internal standards. Homogenates were then centrifuged at 15,000 rpm for 5 min, the supernatant was filtered through a Millipore 5-kD cutoff filter (Amicon), and the filtrates were used for analysis. Separation and quantification of organic acids, nucleotides, and amino acids were performed by CE-MS (Agilent Technologies), as described previously (Takahashi et al., 2006a, 2006b). For the determination of organic acids and nucleotides, a polyethylene glycol-coated capillary (DB-WAX; J&W Scientific) with 20 mM ammonium acetate (pH 6.8) as a run buffer was used. Amino acids were separated in an uncoated fused-silica capillary using 1 M formic acid (pH 1.9) as a run buffer. Accuracy was verified with known concentrations of reference standard compounds. Nitrate and ammonia contents were measured with an ICS-3000 ion chromatography system (Dionex). Accuracy was established using cation mixed standard solution II and anion mixed standard IV (Kanto Chemical).

Agilent Oligomicroarray Analysis

Triplicate independent biological replicates of microarray experiments were performed on an Agilent Technologies 44K Arabidopsis 3 Oligo Microarray. Total RNA was extracted by the TRIzol method. Total RNA was further purified using the RNeasy Plant Mini Kit (Qiagen). The purified RNA was quantified by 260/280/320-nm UV light absorption measurements with a spectrophotometer (Ultrospec 3000; Pharmacia Biotech) and by an Agilent 2100 bioanalyzer (Agilent Technologies). The purified RNA was used for the preparation of Cy5- and Cy3-labeled cDNA probes. All experiments and data analysis were performed according to the manufacturer's manual and instructions. Feature-extraction and image-analysis software (version 7.5; Agilent Technologies) was used to locate and delineate every spot in the array and to integrate each spot's intensity, using filtering and normalization by the Lowess method. Microarray data were annotated according to the Arabidopsis genome (The Arabidopsis Information Resource 7 version). Genes were classified into functional categories and were visualized using MapMan, mapping version 2.2.0 (http://gabi.rzpd.de/projects/MapMan/; Thimm et al., 2004).

Reference Reagents

Analytical-grade dihydroxyacetone phosphate, Fru-1,6-bisphosphate, ribulose-5-phosphate, Rib-5-P, RuBP, Glc-6-P, 3PGA, phospho*enol*pyruvate, pyruvate, citrate, 2-oxoglutarate, and amino acids were used as reference reagents. Standard solutions were prepared by adjusting these compounds to 50 μM.

Supplemental Data

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

Supplemental Table S1. Selected metabolism-related genes that differentially expressed between *nadk2* 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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