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NADK2, an *Arabidopsis* chloroplastic NAD kinase, plays a vital role in both chlorophyll synthesis and chloroplast protection

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Abstract

As one of terminal electron acceptors in photosynthetic electron transport chain, NADP receives electron and H^+ to synthesize NADPH, an important reducing energy in chlorophyll synthesis and Calvin cycle. NAD kinase (NADK), the catalyzing enzyme for the *de novo* synthesis of NADP from substrates NAD and ATP, may play an important role in the synthesis of NADPH. NADK activity has been observed in different sub-cellular fractions of mitochondria, chloroplast, and cytoplasm. Recently, two distinct NADK isoforms (NADK1 and NADK2) have been identified in *Arabidopsis*. However, the physiological roles of NADKs remain unclear. In present study, we investigated the physiological role of *Arabidiposis* NADK2. Sub-cellular localization of the NADK2–GFP fusion protein indicated that the NADK2 protein was localized in the chloroplast. The *NADK2* knock out mutant (*nadk2*) showed obvious growth inhibition and smaller rosette leaves with a pale yellow color. Parallel to the reduced chlorophyll content, the expression levels of two POR genes, encoding key enzymes in chlorophyll synthesis, were down regulated in the *nadk2* plants. The *nadk2* plants also displayed hypersensitivity to environmental stresses provoking oxidative stress, such as UVB, drought, heat shock and salinity. These results suggest that NADK2 may be a chloroplast NAD kinase and play a vital role in chlorophyll synthesis and chloroplast protection against oxidative damage.

Introduction

NADP is essential for biosynthetic pathways, energy metabolism, and signal transduction in living organisms (Garavaglia *et al.*, 2003). NAD kinase (NADK, EC 2.7.1.23) catalyzes the NADP formation from substrates NAD and ATP through phosphorylation. This enzyme has been regarded as the sole catalyzer for the *de novo* biosynthesis of NADP (Kawai *et al.*, 2001a), suggesting a regulatory role in the NADP-dependent anabolic/biosynthetic pathways. NADKs have been found in all organisms investigated to date, suggesting an essential role in all organisms (McGuinness and Butler, 1985; Zielinski, 1998). They may be the key enzymes in the synthesis of NADPH by providing NADP. NADPH plays important roles in energy metabolism and reductive biosynthesis in all organisms. In chloroplast, NADPH, produced from NADP in photosynthetic electron transport chain, provides important reducing energy in chlorophyll (chl) synthesis and Calvin cycle.

In plants, NADK has been found in chloroplasts (Muto *et al.*, 1981; Jarrett *et al.*, 1982), mitochondria (Dieter and Marme, 1984) and cytoplasm (Simon *et al.*, 1982). Moreover, plants possess both calmodulin (CaM)-independent and CaM-dependent NADK isoforms (Roberts and

Harmon, 1992; Harding *et al*., 1997; Pou De Crescenzo *et al*., 2001). Indeed, NADK was the first CaM-regulated enzyme identified in plants, it has been used as a tool for studying CaMs in plants, because it is sensitive to a number of sequence changes in CaMs isolated from different organisms (Anderson *et al*., 1980). Developmental changes and environmental signals, which increase metabolic demand for NADP, have been proposed as physiological rationale for the regulation of NAD kinase activity. In chloroplasts, NADK catalyzes the conversion of NAD to NADP probably in a light-induced way (Jarrett *et al*., 1982), and is involved in the production of active oxygen species through altering NAD(H)/NADP(H) homeostasis (Harding *et al*., 1997). In addition, a CaM-dependent NADK is proposed to induce the foliar accumulation of proline in response to cold-shock by its modulator role in metabolic pathways (Ruiz *et al*., 2002).

However, all these above studies had no molecular data. The lack of information about the NAD kinase gene has hindered definitive understanding of NADP metabolism in organisms. By now, most studies on NADKs remain at biochemical level, the role of NADK itself remains unclear. Recently, cDNAs encoding NADKs were cloned from bacteria (Kawai *et al*., 2000, 2001a), yeast (Kawai *et al*., 2001b), and human (Lerner *et al*., 2001). In plants, the cloning and characterization of *NADKs*, *NADK1*, *NADK2* and *NADHK*, has been described only in *Arabidopsis* (Turner *et al*., 2004a, b). In these studies, recombinant proteins of *Arabidopsis* NADKs have been partially purified from *E. coli* and some enzyme properties have been preliminarily investigated. However, the physiological roles of plant NADKs are still unknown. In plants, chloroplast and mitochondria are major sources of ROS under normal and abnormal conditions. NADPH is required for antioxidant protection and for specific biosynthetic pathways. In yeast mitochondria, Pos5p, the first identified NADH kinase with NADK-like domain, has been revealed as an important antioxidant factor and key source of the cellular reductant NADPH. Subsequent work with deletion mutants and recombinant protein expression has shown that Pos5p plays important roles in providing the reducing energy for yeast to scavenge ROS in mitochondria by catalyzing the synthesis of NADPH from the substrates NADH and ATP *in vivo* (Caryn *et al*., 2003).

To obtain new insights into the regulation of the cellular redox system in plants, our works focus on the *Arabidopsis* NADK genes. Based on protein homology analysis and biochemical property comparison of NAD(H) kinases between yeast and higher plants, we speculated that NAD(H)K maybe play similar roles in higher plants. PSORT (http://www.psort.org) analysis predicts that the *Arabidopsis* NADK2 protein should be targeted to the chloroplast, suggesting its potential role in providing the reducing energy for plants to scavenge ROS in chloroplast. Consistent with this hypothesis, light increases the NADP: NAD ratio in the chloroplast stroma, where NADP serves as a terminal electron acceptor during photosynthesis, then becomes NADPH (Heber and Santarius, 1965). Otherwise, NADPH:Pchlide oxidoreductase (POR), a light-dependent enzyme that reduces photoactive Pchlide to chlorophyllide, plays a key role in chloroplast differentiation during greening. An *Arabidopsis porB porC* double mutant lacking light-dependent NADPH:protochlorophyllide oxidoreductases B and C is highly chlorophyll-deficient and developmentally arrested (Frick *et al*., 2003). POR uses NADPH as its coenzyme. These studies reveal that the NADK is a vital and possibly limiting step in the process of NADPH generation and many biosynthesis. The multiple functions of NADP(H) make it obvious that nicotinamide dinucleotide is a key molecule for cell viability, which implies that the cellular concentration of this compound must be precisely regulated.

To investigate the physiological roles of plant NADKs, in present study, we isolated a *NADK2* knock out mutant (*nadk2*) and cloned this gene in *Arabidopsis*. Our study on the *nadk2* of *Arabidopsis* suggests that NADK2, an *Arabidopsis* chloroplastic NAD kinase, is a key enzyme in both chlorophyll synthesis and chloroplast protection.

Materials and methods

Plant growth, genetic analysis

Arabidopsis seeds were planted onto sterile media containing 1 × MS (Murashige-Skoog's) medium and 2% (W/V) sucrose. After 3 days of cold treatment, the plants were grown under 16 h light, 22 °C; 8 h dark, 20 °C , and a relative humidity of 70%. To obtain etiolated wild-type and *nadk2*

seedlings, surface-sterilized seeds were sown on MS agar, which was supplemented with 2% (W/V) sucrose. The seeds were placed in the dark at 4 °C for 36 h, illuminated for 90 min with white light 22 °C and thereafter returned to darkness at room temperature for 5 days. Etiolated plants were illuminated with a single saturating light flash or continuous normal white light of luminescence tubes. T-DNA insertion line for the *NADK2* gene, Salk_122250, was obtained from the Salk Institute Genomic Analysis Laboratory collection (Alonso *et al.*, 2003). *nadk2* homozygous mutant plants were screened with PCR amplification, and the single insertion was further confirmed using Southern blot analysis using T-DNA as the probe. Genomic DNA was digested with three restriction enzymes: *Kpn*I, *Eco*RI and *Bam*HI, respectively. Southern blot analysis was performed as described earlier (Lin *et al.*, 2003). The cross was made to investigate the segregation of the mutant in F2: wild-type × *nadk2*.

Sub-cellular localization of NADK2–*GFP fusion protein*

A portion of *NADK2* cDNA encoding amino acids (1–287) was amplified by PCR using the primers (5′-GCTCTAGAATGT TCCTATGCTTTTGCCCTTGCC-3′) and (5′-CGCGGATCCCCTTCAATCCTTCTTGGGTT-ACCTGTCC-3′), which allowed cloning of the PCR product at *Xba*I–*Bam*HI sites of a modified pBI221 vector (Clontech, Palo Alto, CA, USA). This vector contains a soluble modified red-shifted GFP ('SmRS-GFP') at *Bam*HI–*Sac*I sites (Davis and Vierstra, 1998). Protoplasts transformation was performed as described earlier (Sheen, 2001). The autofluorescence (red) and the GFP images of chloroplasts were observed under fluorescence light microscopy (Zeiss LSM510)

Measurement of chlorophyll contents

Chlorophyll was extracted with acetone at 4 °C from 0.2 g 4-week-old seedlings. The extracts were subjected to spectrophotometric measurements at 603, 645 and 663 nm (Mochizuki *et al.*, 2001).

NADPH measurements

Pyridine nucleotides were extracted from 30 mg *Arabidopsis* leaves with 1 ml of 0.1 M NaOH. The extracts were boiled for 6 min, cooled on ice, and centrifuged at 12,000*g* for 6 min, soluble fraction was neutralized by adding 0.1 M HC1, followed by incubation on ice for 15 min (Gogorcena *et al.*, 1995; Hajirezaei *et al.*, 2002). The whole neutralized mixture was then used to quantify the nucleotides by the enzyme-cycling method (Matsumura and Miyachi, 1980).

Chlorophyll intermediates measurements

Thirty milligrams of frozen leaf samples were ground with a microtube pestle in 1 ml 9:1 acetone:0.1 M NH_4OH, and centrifuged at 3000 rpm for 15 min at 0 °C. The supernatant was extracted with hexane three times. Fluorescence measurements were performed on the recovered supernatants with an SPEX fluorometer (Metuchen, NJ) using Ex485:Em632 for Protoporphyrin IX, Ex440:Em633 for protochlorophyllide, Ex440:Em672 for chlorophyllide, and Ex420:Em595 for Mg-protoporphyrin (Santiago-Ong *et al.*, 2001; Masuda *et al.*, 2003). The result is represented by relative fluorescence μg^{-1} chl.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 3-week-old *Arabidopsis* leaves by using the TRIzol reagent. First strand cDNA was reverse-transcribed using M-MLV reverse transcriptase in the presence of 1 unit RNase inhibitor. To detect gene expression in the *nadk2* seedlings by RT-PCR, gene-specific primers were used as below:

PORB forward primer (5′-AACCGCTGC-GACTTCAAGCCCTA-3′) and reverse primer (5′-ACCCGCCAATGTATTCGTGTTCC-3′); PORC forward primer (5′-ACAGACA GTTA-CAGCCACGCCGC-3′) and reverse primer (5′-TTTTGCCCATTCAATCCTGACGCT-3′); CZSOD2 forward primer (5′-CTTCAAC TCTCCGTTCCTCTTTC-3′) and reverse primer (5′-CATTTATGTTTCCCAGGTCACCC-3′); tAPX forward primer (5′-TTTCCCCCG CCGTCACCTCTTCG-3′) and reverse primer (5′-CAGCATCGTGCCACCCCAATCTA-3′);

GPX1 forward primer (5′-AAAACCGTTCACGATTTCACCG-3′) and reverse primer (5′-CACCCAAGAATCCTCCTGCGTT-3′); Peroxiredoxin forward primer (5′-TGGACTTCA CTTTTGTCTGCCC-3′) and reverse primer (5′-GGTTTCATTGATTTCTCCCCTG-3′); NADK2 forward primer (5′-TGTCTAGAATGTTCCTATGCTTTTGCCCTTGCC-3′) and reverse primer (5′-GACCCGGGAGAGAGCCTTTTGATC AAGACGC-3′). The β-tubulin gene was used as an internal control to determine the cDNA quality and quantity using these primers: forward primer (5′-CGTGGATCACAGCAATACAGAGCC-3′) and reverse primer (5′-CCTCCTGCACT TCCACTTCGTCTTC-3′) (Chinnusamy *et al.*, 2003). All the trials were repeated three times and have the similar results.

Abiotic stress treatments

For UV treatment, plants were transferred to continuous normal lighting of white fluorescent light, supplemented with 0.4 W^{-2} UVB light from one fluorescent 15 W UVB bulb (Spectronics, USA) for indicated time. For leaf drying test, matured leaves were cut from seedlings, and were dried on tissue paper on bench under room temperature. The weight of the leaves was weighted at the designated time. Leaf water loss rate was expressed as percent of initial weight. For heat shock test, seedlings were pretreated at 38 °C for 30 min, followed by a severe heat shock at 44 °C for 3 h, and then returned to 22 °C for recovery 2 days. For salt stress test, plants were watered with 150 mM NaCl solution for 0 day or 7 days before being photographed. Five-week-old *nadk2* and wild-type plants were used for all above stress treatment experiments. Each treatment was repeated three times.

Results

NADK genes in Arabidopsis and sequence alignment

Although NADK activity has been reported in various plant species in recent years, to our knowledge, genetic evidence for their functions is very limited. A recent work identified *Arabidopsis* NADK1 and NADK2 as the NAD kinases by cDNA cloning and characterization (Turner *et al.*, 2004a, b). Based on the sequences of bacteria, yeast and human NADKs, we also cloned the putative NADK genes from the model plant *Arabidopsis*, *NADK1, NADK2* and *NADK3* , and they correspond to loci At3g21070, At1g21640 and At1g78590, respectively. As shown in Figure 1, *Arabidopsis* NADK1 and NADK2 contain a conservative kinase domain as those well-characterized NADKs from *E. coli*, yeast, and human, the NADK motifs, XXX-XXGGDG-XL and GDXXX-TPTGSTAY (where X represents a hydrophobic residue) are also largely conserved in both NADK1 and NADK2 (Kawai *et al.*, 2001a).

NADK2 is targeted to chloroplast

PSORT (http://www.psort.org) analysis predicts that NADK2 has a chloroplast transit peptide, suggesting that NADK2 might be targeted to the chloroplast. To determine sub-cellular localization of the NADK2, the sequence encoding the N-terminal region of the NADK2 protein (amino acids 1–287), which including the transit peptide, was fused in-frame with the GFP at the N-terminal region. Transient expression of this fusion protein was monitored by fluorescence in *Arabidopsis thaliana* protoplasts under confocal microscopy. GFP fluorescence spread throughout the cytoplasm (Figure 2A–C) and GFP is not in the chloroplasts but does seem to be somewhat localized. The 'cytoplasmic fluorescence' appears to be low. Perhaps this is due to the unevenness of the fluorescence. Whereas, chlorophyll autofluorescence was co-localized with GFP fluorescence after GFP was fused to NADK2 (Figure 2D–F). These results suggest that NADK2 is targeted into chloroplasts with its transit peptide.

Identification of an Arabidopsis *mutant with T-DNA insertion in the* NADK2 *gene*

To investigate the function of *Arabidopsis* NADK2, we obtained the knock out line from the SIGNAL project (signal.salk.edu/tabout.html). The genomic DNA cloning by T-DNA tagging and sequence analysis revealed a T-DNA insert in the third exon of the *NADK2* gene (Figure 3A). Southern blot analysis showed that one single band was hybridized with T-DNA probe in the *nadk2* mutant genomic DNAs following digestion with three different restriction enzymes

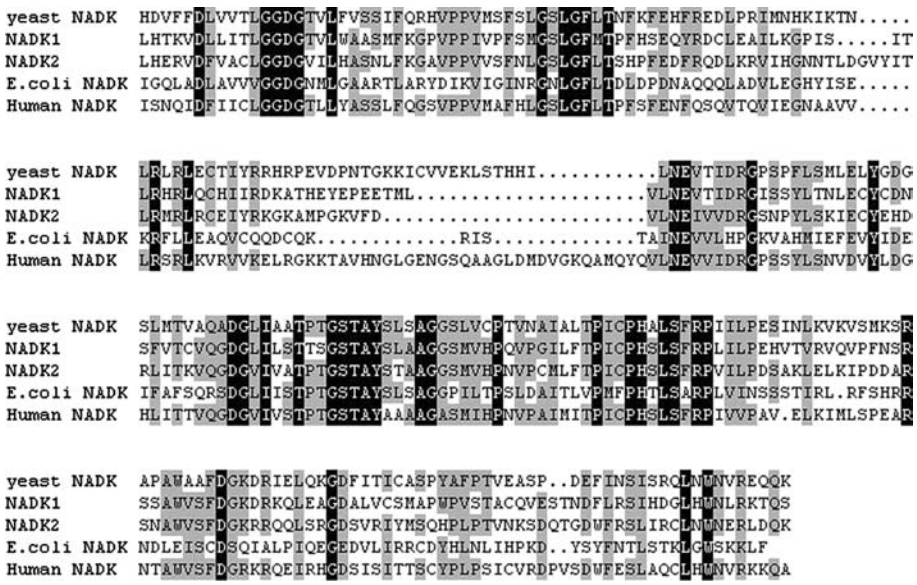

Figure 1. NADK2 belongs to the NAD kinase family. Sequence alignment of the NAD kinase domains of NADK2 and the related NAD kinases: *Arabidopsis* NADK1; yeast NADK (UTR1p; p21373); *E. coli* NADK (YfjB; NP_417105); human NADK (NP_075394). Black and gray boxes indicate identical or similar residues, respectively, conserved among at least 60% of the sequences.

(Figure 3B), suggesting only one T-DNA insert in the genome of the *nadk2* mutant. F2 segregation analysis of 116 individuals from the cross: *nadk2* × wild-type showed 86 with wild-type phenotype and 30 with the phenotype of the *nadk2* mutant, fitting an expected 3:1 segregation ratio of wild-type to mutant ($x^2 = 0.011$). In contrast to a considerate level of gene expression in the wild-type plants, no transcript was detected for the NADK2 gene expression in the *nadk2* mutant by RT-PCR (Figure 3C). All of these results indicated that the *nadk2* mutant selected in this study was a homozygous mutant at the *NADK2* locus.

The knock out of *NADK2* resulted in several alterations in the growth and development of the *Arabidopsis* plants (Figure 3D and E). Unlike wild-type seedlings, the *nadk2* mutant plants were characterized by pale-green leaves throughout their life cycle, reduced sizes of leaves and siliques, and reduced seed production. The *nadk2* plants were also much shorter than the control plants (Figure 3D and E).

The nadk2 *mutant decreased chlorophyll synthesis*

To uncover why the *nadk2* mutant leaves were yellowish, we observed the chloroplast structure under transmission electron microscope, but no obvious difference between the *nadk2* and wild-type plants was found under controlled conditions (data not shown). However, the analysis of chlorophyll content showed that the *nadk2* plants had significant lower levels of chlorophyll *a* and chlorophyll *b* as compared with wild-type plants and total chlorophyll in mutant was only 74% of wild-type (Figure 4). We searched further for defects in the chlorophyll biosynthetic pathway. It was found that the *nadk2* plants accumulated relative much more Pchlide and Mg-protoIX than wild-type (Figure 5). PORs catalyze Pchlide to Chlide using NADPH as reducing energy. The measurement of NADPH revealed that its concentration was reduced in the mutant and the total concentration of NADPH in mutant is 81% of wild-type (Figure 6A). Pchlide is the precursor of POR. The accumulation of higher concentration of Pchlide in the *nadk2* than that in wild-type suggests that the total PORs activities reduced in the *nadk2* plants (Figure 5). As an indicator of activities of PORs, the ratio of Chlide/Pchlide in *nadk2* mutant was only 47% of that in wild-type (Figure 6B). The amount of Pchlide reduction during the greening period depends on the amount of active POR. We determined pigment levels from etiolated seedlings

of wild-type and *nadk2* mutant before and after a saturating light flash. The transformation of Pchlide into chl *a* was reduced in the *nadk2* mutant (Table 1). This result shows that NADPH deficiency in *nadk2* mutant poses a major obstacle for pigments formation.

Relative quantitative RT-PCR analysis on mRNA expression of some chloroplast genes revealed that the expression levels of the two genes encoding critical enzymes in chlorophyll synthesis, *PORB* and *PORC*, were repressed in *nadk2* plants, while the expression levels of other chloroplast

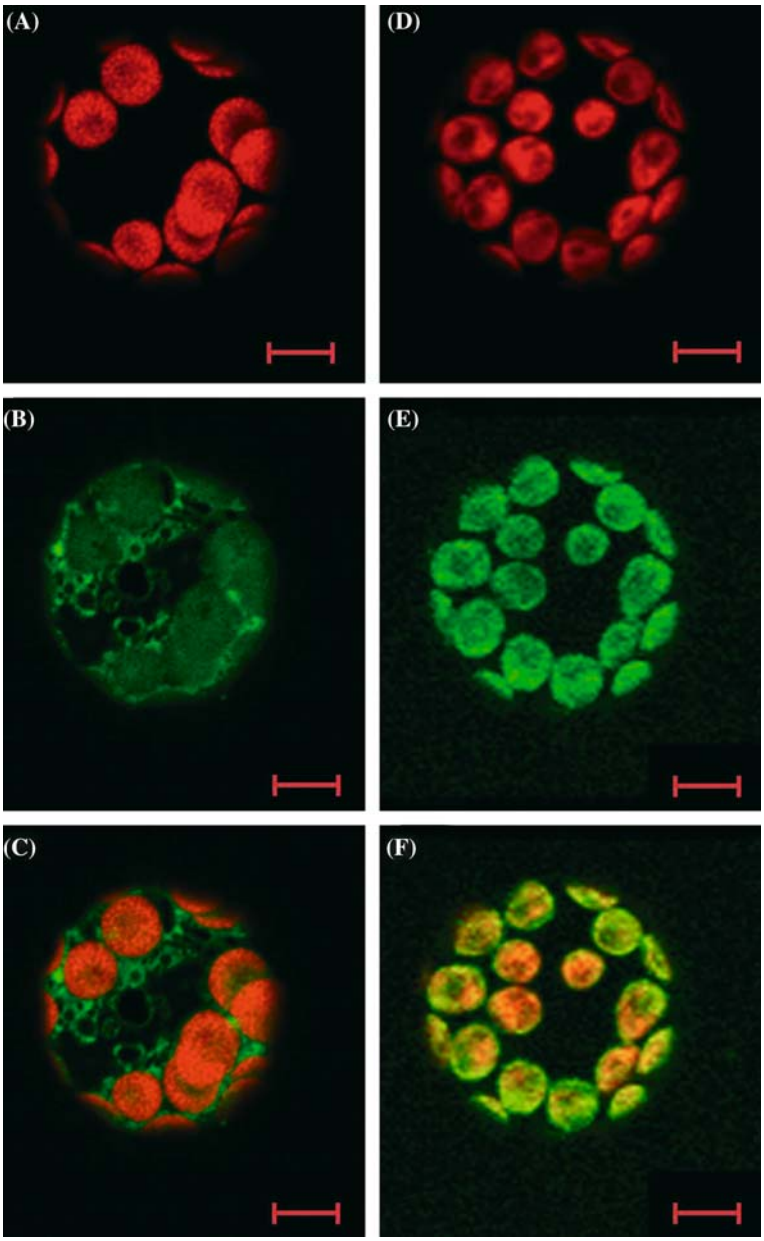

Figure 2. Sub-cellular localization of NADK2–GFP fusion protein. *Arabidopsis* cell protoplasts were transformed using a control vector 35S-GFP (A–C) and 35S-NADK2-GFP construct (D–F). Red autofluorescence (A and D) used as a chloroplast marker and green fluorescence signal (B and E) was detected by a laser confocal-scanning microscope. (C) is an overlap picture of (A) and (B). (F) is an overlap picture of (D) and (E). The bar indicates 5 μm.

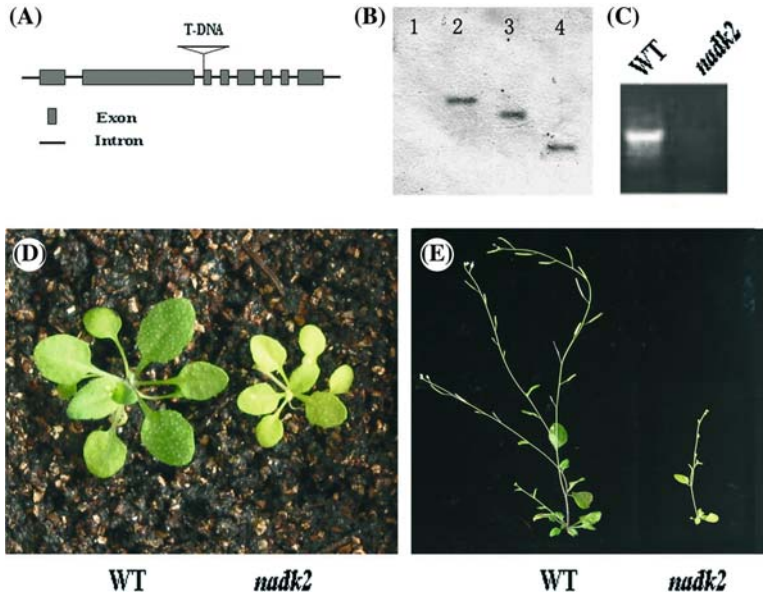

Figure 3. Identification of the *Arabidopsis nadk2* mutant. (A) Diagrammatic illustration of the *Arabidopsis NADK2* gene showing the site of the T-DNA insertion. (B) Southern blot analysis on the genomic DNAs of the wild-type control (1) and the *nadk2* mutant (2, 3 and 4) using the T-DNA as a probe. The genomic DNA was digested with restriction enzyme *Kpn*I (lane 1 and 2), *Eco*RI (lane 3), and *Bam*HI (lane 4), respectively. (C) RT-PCR analysis of gene expression of *NADK2* in the wild-type plants (WT) and the *nadk2* mutant (*nakd2*). (D) Four-week-old wild-type and *nadk2* seedlings are shown. (E) Eight-week-old wild-type and *nadk2* seedlings are shown.

genes, including *chloroplastic Cu/Zn superoxide dismutase* 2 (*CZSOD2*), *thylakoid-bound ascorbate peroxidase* (*tAPX*), *glutathione peroxidase* 1(*GPX1*) and *peroxiredoxin*, did not change much more (Figure 7), suggesting that the decreased chlorophyll content in the *nadk2* mutant may be attributable to the decreased expression of the *POR* genes.

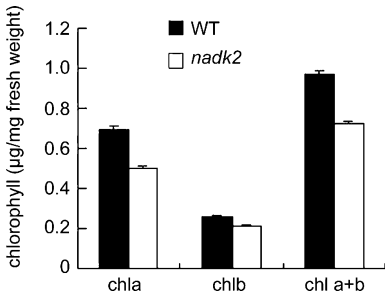

Figure 4. Comparison of chlorophyll levels between wild-type and *nadk2* mutants. Chlorophyll *a*/*b* (chl *a*/*b*) contents in *nadk2* leaves are approximately 74% (w/w) compared to wild-type.

The nadk2 *mutant is sensitive to environmental stresses*

It has documented that many environmental stresses can cause oxidative damages. The mechanism for scavenging reactive oxidative species (ROS) is very important for plants. NADPH is an essential factor for ROS removal. To explore the

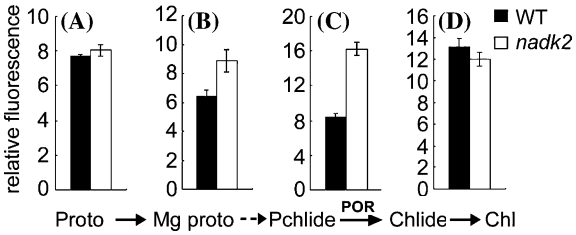

Figure 5. Levels of chlorophyll intermediates in the wild-type and *nadk2* seedlings. The relative levels of Protoporphyrin IX (ProtoIX), Mg-protoporphyrin IX (Mg-protoIX), Protochlorophyllide (Pchlide) and Chlorophyllide (Chlide) flux through the chlorophyll biosynthetic pathway were detected in the wild-type (WT) and *nadk2* seedlings. A schematic diagram of the chlorophyll synthetic pathway is shown and the graphic bar represents levels of ProtoIX (A), Mg-protoIX (B), Pchlide (C) and Chlide (D), respectively.

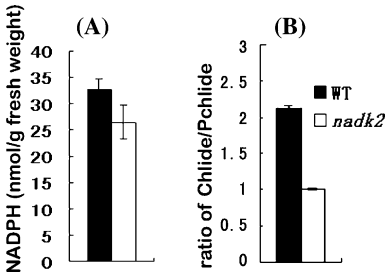

Figure 6. Measurements of NADPH levels and the ratios of Chide/Pchlide in the wild-type and *nadk2* seedlings. (A) Plants were grown under the conditions as described in Materials and methods. The total NADPH level of the *nadk2* mutant is lower than wild-type. (B) The ratios of Chlide/Pchlide represent the relative activity of NADPH:protochlorophyllide oxidoreductase (POR) in wild-type and *nadk2* seedlings.

physiological role of NADK2 related to chloroplast protection against oxidative damage, we tested the sensitivity of the *nadk2* mutant to different environmental stresses, including UVB, drought, heat shock and salinity. When the wild-type and *nadk2* mutant plants were exposed to UVB irradiation, *nadk2* mutant plants showed hypersensitivity to UVB (Figure 8). The time course of water loss rate showed that the detached leaves of *nadk2* plants dehydrated faster than those of wild-type plants (Figure 9). The *nadk2* plants lost about 58% of their initial weight 3 h post-dehydration treatment, while the wild-type plants only lost approximately 37% (Figure 9). When plants were transferred to 44 °C from 22 °C for 3 h and recovered for 2 days, the *nadk2* seedlings

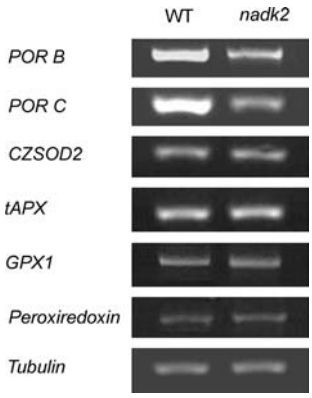

Figure 7. RT-PCR analysis on the transcript expression of chloroplast genes. The transcript levels of chloroplast genes *POR B*, *POR C*, *CZSOD2*, *tAPX*, *GPX1* and *Peroxiredoxin* are compared between the wild-type seedlings and the *nadk2* mutant. The tubulin gene was used as an internal control.

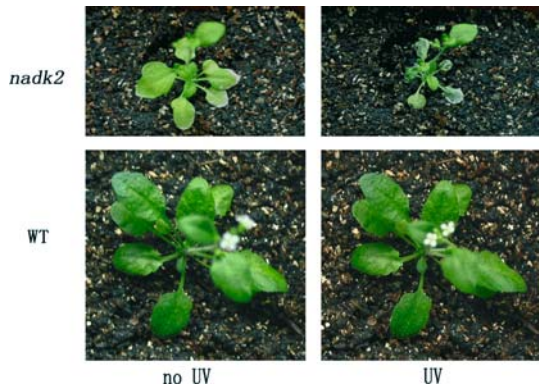

Figure 8. Effects of UVB irradiation on mature wild-type and *nadk2* mutant *Arabidopsis* plants. Five-week-old wild-type and *nadk2* plants were exposed to 0.40 W/m^2 UVB. The plants were photographed at 0 h (left, no UV), and 6 h (right, UV) post-treatment.

were strongly damaged, their shoot and leaves withered severely, whereas the wild-type seedlings only showed slightly delayed growth (Figure 10). The *nadk2* mutant was more strictly arrested than wild-type in 150 mM NaCl, which displayed sensitivity to salinity (Figure 11).

Discussion

NADPH, as an essential reducing energy, functions in both normal metabolic activities and plant defense mechanisms in response to various environmental stresses. It is logical to assume that the enzymes catalyzing the synthesis of NADPH play important roles. The only way to synthesize new NADPH is the reduction of NADP produced by NADK, or the phosphorylation of NADH by

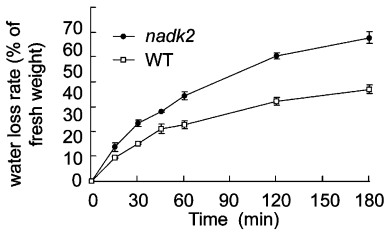

Figure 9. Water loss rates of detached leaves from wild-type and *nadk2* plants. Fully expanded leaves at similar positions were cut from wild-type and *nadk2* plants in the morning. The *nadk2* leaves showed drastic desiccation from 1 to 3 h post-drying treatment. Water loss rate is expressed as percent of initial weight.

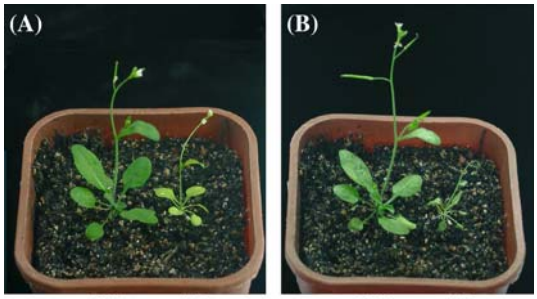

Figure 10. Heat shock effects on the *nadk2* mutant. Five-week-old wild-type and *nadk2* plants grown at 22 °C (A) were pretreated at 38 °C for 30 min, immediately subjected to a severe heat shock at 44 °C for 3 h, and then returned to 22 °C for recovery 2 days (B) before being photographed.

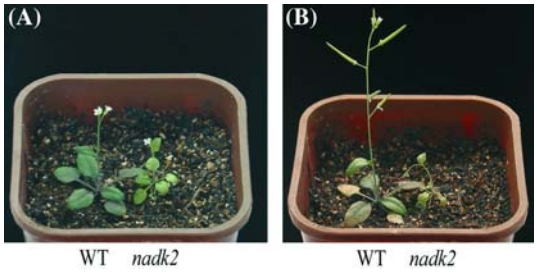

Figure 11. Effect of NaCl on the phenotype of *nadk2* mutant. Five-week-old wild-type and *nadk2* plants were watered with 150 mM NaCl solution, the photograph were taken at 0-day (A) and 7-day (B) post-treatment.

NADH kinase directly. The activation of this kind of kinases could be the first important step towards NADPH synthesis. In plants, sub-cellular compartmentation of NADPH differs significantly between the cytoplasm and chloroplast. In the chloroplast, light results in a reduction of NADP. In spinach and sugar beet, 40% of total cellular NADPH is in the chloroplasts, reaching 60–85% in the light (Hunt *et al*., 2004). NADK activities have been detected in mitochondrial fraction, chloroplastic fraction and the cytosolic fraction. The NADK with different sub-cellular locations may have different functions. Among the *Arabidopsis* NADKs, NADK2 has the predicted chloroplast transit peptide at the amino terminus, which is supported by the sub-cellular location of the NADK2–GFP fusion protein (Figure 2).

It is well known that NADP, as terminal electron acceptor of photosystem I (PSI), receives electron and H^+ to synthesize NADPH. Earlier biochemical data have suggested that an unknown NADK may be localized in the chloroplast to regulate a light-induced conversion of NAD to NADP, which is necessary for the NADPH production and photosynthesis (Jarrett *et al*., 1982). In this study, we first provide genetic evidence that the chloroplast NADK2 plays a vital role in both chlorophyll synthesis and chloroplast protection.

The abnormal leaf color produced in the *nadk2* mutant implies that NADK2 plays an essential role in chlorophyll synthesis. Examination of the chlorophyll content supported this hypothesis. Other evidence comes from the relative quantitative RT-PCR analysis. The gene expression of *PORB* and *PORC*, encoding the key enzymes in chlorophyll synthesis, are down regulated in the *nadk2* mutant (Figure 7). Coincidently, a recent work has demonstrated that PORB and PORC play a critical role in maintaining light-dependent chlorophyll biosynthesis in green plants, and are together essential for growth and development (Frick *et al*., 2003). As the coenzyme of PORs, NADPH was supplied at a lower level in the *nadk2*

Table 1. Light-induced Pchlide reduction and chlorophyll accumulation in cotyledons of the wild-type and the *nadk2* mutant.

Pigment	*nadk2*		Wild-type	
	Etiolated	Flash	Etiolated	Flash
Pchlide	41.0 ± 5.8	16.0 ± 4.9	37.6 ± 5.4	6.0 ± 3.8
	Light (5 h)	Light (48 h)	Light (5 h)	Light (48 h)
Chl *a*	6.0 ± 0.5	66.8 ± 2.3	11.0 ± 0.7	134.4 ± 2.8
Chl *b*	2.2 ± 0.4	30.2 ± 3.1	4.6 ± 0.4	65.3 ± 4.6

Etiolated plants were grown in complete darkness for 5 days. The amount of Pchlide is given in its relative fluorescence per 50 μg of fresh weight after a single saturating light flash. Chlorophyll accumulation is given in nanograms of pigment per microgram of fresh weight.

mutant (Figure 6A), PORs activities maybe decrease in the *nadk2* mutant. Our results indicated that the *nadk2* mutant accumulated much more Pchlide than the wild-type and the ratio of Chlide/Pchlide in wild-type was higher than that in the *nadk2* mutant (Figure 5). Because PORs catalyze synthesis of Chlide from Pchlide, our data suggest that the total PORs activities of *nadk2* be lower than those of the wild-type (Figure 6B and Table 1). Furthermore, we found that the intermediate product Mg-protoporphyrin IX also accumulated at a higher level than that in the wild-type plants (Figure 5). As a signaling molecule, Mg-protoporphyrin IX regulates the expression of many nuclear genes encoding chloroplastic proteins (Strand *et al*., 2003). Therefore, the accumulation of Mg-ProtoIX to an abnormal higher level is possible to trigger the repression of *PORs* gene expression in the *nadk2* plants. It needs further investigation to determine whether the suppressed synthesis of chlorophyll results from the reduced expression level of *POR* genes.

The inactivation of the chloroplast NADK2 resulted in the hypersensitivity of the *nadk2* mutant to abiotic stresses provoking oxidative stress, such as UVB, drought, heat shock and salinity (Figures 8, 9, 10 and 11), suggesting that NADK2 might be involved in chloroplast protection against oxidative stress. These abnormal phenotypes are probably related to a decreased level of NADPH in chloroplast. Since plant cells are exposed to ROS continuously, especially in extreme environments, ROS scavenging mechanisms in different organelles play key roles for cell survival. Chloroplast, a major source of ROS, has evolved many antioxidative enzymes such as catalase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione S-transferase, glutathione reductase, superoxide dismutase, ascorbate peroxidase and NADPH thioredoxin reductase. All these enzymes act synergistically in ROS scavenging in plants, and most of them utilize NADPH as a reducing energy directly or indirectly (Mittler, 2002). For example, an *Arabidopsis* mutant, in which the chloroplast NADPH thioredoxin reductase gene was knocked out, showed hypersensitivity to methyl viologen, drought and salt stress (Serrato *et al*., 2004). On the other hand, the loss of NADPH may cause significant increase in the production of reactive oxygen species. In particular, the increased levels of porphyrin precursors may also increase the rate of photosensitized production of singlet oxygen. The reduced level of $NADP^+$ would cause a backup of electron flow and consequently increased fluorescence of PSII would possibly also lead to greater photosensitization. Diminished rates of oxidation of ferredoxin by $NADP^+$ may lead to greater production of superoxide anion. Consistent with these observations, the *nadk2* mutant shows hypersensitivity to abiotic stresses provoking oxidative stress.

Since many antioxidative reductases employ NADPH as a reducing energy, what is the source of NADPH becomes a key question and challenge to understand the mechanisms of plant tolerance to oxidative stress. In the cytosol, NADPH comes primarily from the pentose phosphate pathway; many enzymes in this metabolic pathway play important protective roles under oxidative stresses in yeast and mammals (Pandol *et al*., 1995; Juhnke *et al*., 1996; Slekar *et al*., 1996). In yeast, Pos5p represents the first member of the NAD(H) kinase family that providing the synthesis of NADPH (Caryn *et al*., 2003). In plants, chloroplast is another major source of ROS. The results presented in this work suggest that in chloroplast NADK2 accelerates the formation of reducing energy by supplying the substrate for NADPH for ROS scavenging, as well as for chlorophyll synthesis and biosynthesis, consistent with the pleiotropic effect of NADPH.

In conclusion, NAD is phosphorylated to NADP by NADK2 in the chloroplast, the resultant NADP is converted to NADPH by PSI pathway. Then, NADPH, as a cofactor, is involved in chlorophyll synthesis, biosynthesis in normal conditions. Under abiotic stresses, NADPH-requiring reductases (e.g. chloroplast NADPH thioredoxin reductase) use NADPH to remove excess ROS for cell survival.

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References

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P. and Cheuk, R. 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana* Science 301: 653–657.

Anderson, J.M., Charbonneau, H., Jones, H.P., McCann, R.O. and Cormier, M.J. 1980. Characterization of the plant nicotinamide adenine dinucleotide kinase activator protein and its identification as calmodulin Biochemistry 19: 3113–3120.

Caryn, E., Outten, C.E. and Culotta, V.C. 2003. A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae* EMBO J. 9: 2015–2024.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X., Agarwal, M. and Zhu, J.K. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis* Genes Dev. 17: 1043–1054.

Davis, S.J. and Vierstra, R.D. 1998. Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants Plant Mol. Biol. 365: 21–528.

Dieter, P. and Marme, D. 1984. A Ca^{2+}, calmodulin-dependent NAD kinase from corn is located in the outer mitochondrial membrane J. Biol. Chem. 259: 184–189.

Frick, G., Su, Q., Apel, K. and Armstrong, G.A. 2003. An *Arabidopsis porB porC* double mutant lacking light-dependent NADPH:protochlorophyllide oxidoreductases B and C is highly chlorophyll-deficient and developmentally arrested Plant J. 35: 141–153.

Garavaglia, S., Galizzi, A. and Rizzi, M. 2003. Allosteric regulation of *Bacillus subtilis* NAD kinase by quinolinic acid J. Bacteriol. 185: 4844–4850.

Gogorcena, Y., Iturbe-Ormaetxe, I., Escuredo, P.R. and Becana, M. 1995. Antioxidant defenses against activated oxygen in pea nodules subjected to water stress Plant Physiol. 108: 753–759.

Hajirezaei, M.R., Peisker, M., Tschiersch, H., Palatnik, J.F., Valle, E.M., Carrillo, N. and Sonnewald, U. 2002. Small changes in the activity of chloroplastic $NADP^+$-dependent ferredoxin oxidoreductase lead to impaired plant growth and restrict photosynthetic activity of transgenic tobacco plants Plant J. 29: 281–293.

Harding, S.A., Oh, S.H. and Roberts, D.M. 1997. Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species EMBO J. 16: 1137–1144.

Heber, U.W. and Santarius, K.A. 1965. Compartmentation and reduction of pyridine nucleotides in relation to photosynthesis Biochim. Biophys. Acta 109: 390–408.

Hunt, L., Lerner, F. and Ziegler, M. 2004. NAD – new roles in signaling regulation in plants New Phytol. 163: 31–44.

Jarrett, H.W., Brown, C.J., Blazck, C.C. and Cormier, M.J. 1982. Evidence that calmodulin is in the chloroplast of peas and serves a regulatory role in photosynthesis J. Biol. Chem. 257: 13795–13804.

Juhnke, H., Krems, B., Kotter, P. and Entian, K.D. 1996. Mutants that show increased sensitivity to hydrogen peroxide reveal an important role for the pentose phosphate pathway in protection of yeast against oxidative stress Mol. Gen. Genet. 252: 456–464.

Kawai, S., Mori, S., Mukai, T., Suzuki, S., Hashimoto, W., Yamada, T. and Murata, K. 2000. Inorganic polyphosphate/ATP–NAD kinase of *Micrococcus favus* and *Mycobacterium tuberculosis* H37Rv Biochem. Biophys. Res. Commun. 276: 57–63.

Kawai, S., Mori, S., Mukai, T., Hashimoto, W. and Murata, K. 2001a. Molecular characterization of *Escherichia coli* NAD kinase Eur. J. Biochem. 268: 4359–4365.

Kawai, S., Suzuki, S., Mori, S. and Murata, K. 2001b. Molecular cloning and identification of UTR1 of a yeast *Saccharomyces cerevisiae* as a gene encoding an NAD kinase FEMS Microbiol. Lett. 200: 181–184.

Lerner, F., Niere, M., Ludwig, A. and Ziegler, M. 2001. Structural and functional characterization of human NAD kinase Biochem. Biophys. Res. Commun. 288: 69–74.

Lin, M., Behal, R. and Oliver, D.J. 2003. Disruption of plE2, the gene for the E2 subunit of the plastid pyruvate dehydrogenase complex, in *Arabidopsis* causes an early embryo lethal phenotype Plant Mol. Biol. 52: 865–872.

Masuda, T., Fusada, N., Oosawa, N., Takamatsu, K., Yamamoto, Y.Y., Ohto, M., Nakamura, K., Goto, K., Shibata, D., Shirano, Y., Hayashi, H., Kato, T., Tabata, S., Shimada, H., Ohta, H. and Takamiya K.-i., 2003. Functional analysis of isoforms of NADPH:protochlorophyllide oxidoreductase (POR), PORB and PORC, in *Arabidopsis thaliana* Plant Cell Physiol. 44: 963–974.

Matsumura, H and Miyachi, S. 1980. Cycling assay for nicotinamide adenine dinucleotides Methods Enzymol. 69: 465–470.

McGuinness, E.T. and Butler, J.R. 1985. NAD kinase: a review J. Biochem. 17: 1–11.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance Trends Plant Sci. 9: 405–410.

Mochizuki, N., Brusslan, J.A., Larkin, R., Nagatani, A. and Chory, J. 2001. *Arabidopsis genomes uncoupled 5* (*GUN5*) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction PNAS 98: 2053–2058.

Muto, S., Miyachi, S., Usuda, H., Edwards, G.E. and Bassham, J.A. 1981. Light induced conversion of nicotinamide adenine-dinucleotide to nicotinamide nicotinamide adenine-dinueleotide phosphate in higher-plant leaves Plant Physiol. 68: 324–328.

Pandol, P.P., Sonati, F., Rivi, R., Mason, P., Grosveld, F. and Luzzatto, L. 1995. Targeted disruption of the house-keeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress EMBO J. 14: 5209–5215.

Pou De Crescenzo, M.A., Gallais, S., Leon, A. and Laval-Martin, D.L. 2001. Tween-20 activates and solubilizes the mitochondrial membrane bound, calmodulin dependent NAD1 kinase of *Avena sativa* L J. Membr. Biol. 182: 135–146.

Roberts, D.M. and Harmon, A.C. 1992. Calcium-modulated proteins: targets of intracellular calcium signals in higher plants Annu. Rev. Plant. Physiol. Plant Mol. Biol. 43: 375–414.

Ruiz, J.M., Sánchez, E., García, P.C., López-Lefebre, L.R., Rivero, R.M. and Romero, L. 2002. Proline metabolism and NAD kinase activity in greenbean plants subjected to cold-shock Phytochemistry 59: 473–478.

Santiago-Ong, M., Green, R.M., Tingay, S., Brusslan, J.A. and Tobin, E.M. 2001. Shygrl1 is a mutant affected in multiple aspects of photomorphogenesis Plant Physiol. 126: 587–600.

Serrato, A.P., PérezRuiz, J.M., Spínola, M.C. and Cejudo, F.J. 2004. A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in *Arabidopsis thaliana* J. Biol. Chem. 279: 43821–43827.

Sheen, J. 2001. Signal transduction in maize and *Arabidopsis* mesophyll protoplasts Plant Physiol. 127: 1466–1475.

Simon, P., Dieter, P., Bonzon, M., Greppin, H. and Marme, D. 1982. Calmodulin dependent and independent NAD kinase activities from cytoplasmic fractions of spinach (*Spinacia oleracea* L.) Plant Cell Rep. 1: 119–122.

Slekar, K.H., Kosman, D.J. and Culotta, V.C. 1996. The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection J. Biol. Chem. 271: 28831–28836.

Strand, A., Asami, T. and Alonso, J. 2003. Chloroplast to nucleus communication triggered by accumulation of My-protorphyrinIX Nature 421: 79–83.

Turner, W.L., Waller, J.C. and Snedden, W.A. 2004a. Cloning and characterization of two NAD kinases from *Arabidopsis*. Identification of a calmodulin binding isoform Plant Physiol. 135: 1243–1245.

Turner, W.L., Waller, J.C. and Snedden, W.A. 2004b. Identification, molecular cloning and functional characterization of a novel NADH kinase from *Arabidopsis thaliana* Biochem. J. 385(Pt 1) 217–23.

Zielinski, R.E. 1998. Calmodulin and calmodulin-binding proteins in plants Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 697–725.