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The Arabidopsis onset of leaf death5 Mutation of Quinolinate Synthase Affects Nicotinamide Adenine Dinucleotide Biosynthesis and Causes Early Ageing


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Leaf senescence in Arabidopsis thaliana is a strict, genetically controlled nutrient recovery program, which typically progresses in an age-dependent manner. Leaves of the Arabidopsis onset of leaf death5 (old5) mutant exhibit early developmental senescence. Here, we show that OLD5 encodes quinolinate synthase (QS), a key enzyme in the de novo synthesis of NAD. The Arabidopsis QS was previously shown to carry a Cys desulfurase domain that stimulates reconstitution of the oxygen-sensitive Fe-S cluster that is required for QS activity. The old5 lesion in this enzyme does not affect QS activity but it decreases its Cys desulfurase activity and thereby the long-term catalytic competence of the enzyme. The old5 mutation causes increased NAD steady state levels that coincide with increased activity of enzymes in the NAD salvage pathway. NAD plays a key role in cellular redox reactions, including those of the tricarboxylic acid cycle. Broad-range metabolite profiling of the old5 mutant revealed that it contains higher levels of tricarboxylic acid cycle intermediates and nitrogen-containing amino acids. The mutant displays a higher respiration rate concomitant with increased expression of oxidative stress markers. We postulate that the alteration in the oxidative state is integrated into the plant developmental program, causing early ageing of the mutant.

INTRODUCTION

In Arabidopsis thaliana, the onset of leaf senescence proceeds within a predictable time window (Jing et al., 2002). The leaf senescence program can be prematurely initiated when a plant is under attack or grows in unfavorable conditions (Gan and Amasino, 1997; Quirino et al., 2000; Schippers et al., 2007). Senescence contributes to relocation of valuable resources from old, dying tissue to young developing parts of the plant. For example, transfer of nutrients supports the filling of grains in order to afford the plant reproductive success. Recent expression profiling studies have identified thousands of transcripts involved in the onset and progression of developmental senescence, termed senescence-associated genes (SAGs) (Buchanan-Wollaston et al., 2003, 2005; Guo et al., 2004). Aside from the fundamental importance of understanding senescence, potential improvements for crop yield or the longevity of ornamental plants might be realized. In this light, a recently identified senescence-associated transcription factor in wheat (Triticum aestivum), which was shown to affect protein content by 30% (Uauy et al., 2006), underlines the impact of senescence research on future agriculture. Despite advances in the understanding of the process of senescence, little is known regarding the mechanism that determines its onset.

During leaf growth, age-related changes (ARCs) occur as a result of the differential regulation of developmental processes. The onset of developmental senescence occurs after certain ARCs have taken place in the leaf (Jing et al., 2005). Ageing occurs throughout leaf development, from leaf initiation to death; however, senescence only refers to the process that leads to the death of the leaf (Lim et al., 2003). By contrast, in ageing research on nonplant species, the terms senescence and ageing are interchangeable. Longevity in these other model organisms may not be regulated by a genetically controlled ageing program, since most organisms reproduce early and, therefore, the ageing process escapes from the force of natural selection (Rose, 1991; Kirkwood, 2002). Nevertheless, many genes have been identified that alter longevity in organisms ranging from yeast to worms, fruit flies, and mammals (Jing et al., 2003; Kirkwood, 2005). On the contrary, for plants, nutrient salvage during leaf senescence is a vital part of development and hence under evolutionary selection (Bleecker, 1998). Therefore, the longevity of plants may be fundamentally differently regulated than in other organisms. Although ageing of Arabidopsis is influenced by its reproductive strategy, there is only a weak correlation between the appearance of reproductive structures and the onset of leaf senescence. Male- or female-sterile mutant plants live 20 d...
longer (Nooden and Penney, 2001); conversely, the lifespan of individual leaves of these mutants is not affected. Thus, individual leaves are useful models for studying the regulation of the ageing program in plants (Jing et al., 2003; Lim et al., 2003; Schippers et al., 2007).

In general, ageing is controlled by programs involved in life maintenance, stress responses, and development (Schippers et al., 2007). In the mid-1950s, it was postulated that reactive oxygen species (ROS) are the main cause of animal ageing, beginning with cumulative damage that results in loss of viability (Harman, 1956). Interestingly, the Arabidopsis longevity mutants oresasr1, oresasr3, and oresasr9 (Woo et al., 2004) and gigantea (Kurepa et al., 1998) all show higher tolerance to oxidative stress. In nonplant species, ROS are mainly produced by mitochondria; however, during leaf senescence in plants, the main ROS source is the chloroplast (Quirino et al., 2000). That said, plant mitochondria produce considerable ROS during the hypersensitive response invoked by plant-pathogen interactions (Finkel and Holbrook, 2000) and under a variety of other cellular circumstances (Sweetlove et al., 2006, 2007). Increased tissue contents of ROS (Navabpour et al., 2003) and exogenously applied causal agents of oxidative stress such as UV-B light and ozone (Miller et al., 1999; John et al., 2001) lead to premature expression of SAGs. These combined data support the notion that ROS influence both the ageing process and the lifespan of Arabidopsis leaves.

The process of senescence correlates with a loss of antioxidant capacity and consequently with an increase in ROS (Zimmermann and Zentgraf, 2005). An important source of ROS production in animals and plants are the membrane-associated NAD(P)H oxidases that generate ROS via lipid oxidation (Finkel and Holbrook, 2000; Mittler, 2002). NAD(P)Hs are well-studied redox molecules that mediate hundreds of reactions and are the basis of almost every metabolic pathway in the cell (Noctor et al., 2006). Pyridine nucleotides are the main redox regulators, since they react slowly with oxygen species in an enzyme-dependent way. ROS production in the chloroplast has been clearly demonstrated to be under the influence of NADP/NADPH ratios in this compartment (Asada, 2006). Production of mitochondrial ROS by the electron transport chain is largely dependent on NAD(P) status as well (Duttileul et al., 2005; Shen et al., 2006; Sweetlove et al., 2006). The turnover of antioxidant pools such as those of glutathione and ascorbate are maintained by NAD(P)H (Noctor et al., 2006). The ascorbate-deficient mutant vtc1 shows premature senescence when grown under a long photoperiod or when leaves are placed in the dark (Barth et al., 2004). Moreover, a proteomics study of the early senescence mutant old1 revealed that most of the highly upregulated proteins in the mutant belong to the family of glutathione S-transferases (Hebeler et al., 2008). This suggests that glutathione S-transferases in the context of leaf senescence are needed to protect cells against ROS during the process. Taken together, these studies demonstrate that the redox status might be an important determinant for the onset of senescence.

The availability of senescence mutants has given us the opportunity to discover which processes play a role in plant ageing and determine the onset of leaf senescence (Jing et al., 2002, 2005). Here, we report that a mutation in the gene encoding quinolinate synthase (QS/OLDS) results in an early onset of developmental senescence in Arabidopsis. Despite the fact that the genetic lesion of this mutant is in the de novo pathway of NAD synthesis, the mutant exhibits increased content of pyridine nucleotides. Our data indicate that the increased NAD level is due to an increased activity of enzymes involved in NAD salvage. Further characterization of the mutant revealed changes in the levels of transcripts that are suggestive of oxidative stress, while the metabolic profile obtained for the mutant shows increased levels of metabolites of central metabolism. The results are discussed in the context of current theories of ageing and senescence that have been documented previously in the plant and mammalian literature.

RESULTS

Characterization of the old5 Mutant during Developmental Senescence

The old5 mutant was identified as a monogenic recessive trait during a screen of an ethyl methane sulphonate–treated population of Arabidopsis plants (Jing et al., 2002) and was shown to display an early onset of senescence and an enhanced senescence response after ethylene treatment (Jing et al., 2005). The mutant shows yellowing of the first leaf pair after 30 d (Figure 1A), whereas the wild type shows senescence symptoms after 40 d. The visual yellowing parallels a decline in chlorophyll content and photochemical efficiency (Figure 1B). Thus, the old5 mutant exhibits the physiological hallmarks of leaf senescence. The early senescence of the mutant results in a decrease in lifespan of ~2 weeks (Figure 1C).

To further characterize senescence in this mutant, the expression of two established senescence marker genes was followed by quantitative real-time PCR. First, we examined SAG13, whose expression is associated with oxidative stress and senescence (Weaver et al., 1998). SAG13 expression was detected from day 24 in both the mutant and the wild type. However, the expression in the mutant was ~10-fold higher at day 24, and this progressed to an ~10,000-fold higher expression at day 33 than in the wild type (Figure 2). Second, we examined the hallmark of age-induced senescence, SAG12 (Weaver et al., 1998). SAG12 was detected after 30 d only in old5 but not in the wild type. The SAG12 expression correlates with the first symptoms of yellowing at 30 d, implying, together with the expression of the SAG13 marker, that senescence in old5 mutants begins prematurely but still occurs in an age-dependent way.

OLD5 Encodes a QS

The old5 mutant was identified in the Landsberg erecta (Ler) accession and was crossed to Columbia (Col) to facilitate mapping. The mapping population segregated in a 1:3 ratio (mutant: wild type) (Figure 2). Second, we examined the hallmark of age-induced senescence, SAG12 (Weaver et al., 1998). SAG12 was detected after 30 d only in old5 but not in the wild type. The SAG12 expression correlates with the first symptoms of yellowing at 30 d, implying, together with the expression of the SAG13 marker, that senescence in old5 mutants begins prematurely but still occurs in an age-dependent way.

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In the sequence of At5g50210, a C to T change in the first exon of the old5 mutant was detected. This gene has previously been annotated as QS (Katoh et al., 2006), which is a component of the pathway of the de novo synthesis of NAD from Asp. A homozygous T-DNA knockout of QS is embryo-lethal (Katoh et al., 2006), demonstrating that the old5 mutation does not result in a complete loss of gene function.

The identity of At5g50210 as the QS/OLD5 gene was confirmed by a complementation test. A 5.1-kb Ler genomic fragment containing the coding sequence, a 2.1-kb 5’ promoter sequence according to the Arabidopsis Gene Regulatory Information Server database (Davuluri et al., 2003), and a 0.5-kb 3’ sequence was transformed into old5 mutant plants. Ten T1 lines were selected on kanamycin, and these all showed absence of the old5 phenotype, as expected since the mutation is recessive. By PCR, we showed that the lines contained both the kanamycin-resistance gene and the homozygous parental old5 point mutation (see Methods). The T2 segregated 3:1 (wild type:mutant), confirming the identity of the OLD5 mutant gene.

old5 Mutants Have an Amino Acid Change in the SufE/YgdK Domain

The QS/OLD5 protein consists of three domains: a chloroplast-targeting signal, a SufE/YgdK domain, and a QS domain (Figure 3A). The old5 mutation results in a Pro-to Ser amino acid substitution in the SufE/YgdK domain. This domain is highly homologous to the previously identified AtSufE/Cp SufE (Xu and Möller, 2006; Ye et al., 2006), the YgdK and SufE proteins of Escherichia coli (Loiseau et al., 2005), and a putative Arabidopsis gene, At1g67810, which was found by a BLAST search of the OLD5 SufE/YgdK domain (residues 84 to 214) against the Arabidopsis genome (Figure 3B). AtSufE/Cp SufE, SufE, and YgdK share functional homology as acceptors of sulfur atoms and stimulators of Cys desulfurase activity. SufE of E. coli (Loiseau et al., 2003) and Cp SufE of Arabidopsis (Ye et al., 2006) stimulate SufS Cys desulfurase activity. The Fe-S cluster, which is inserted by SufS into QS, is absolutely required for enzyme activity in E. coli (Ollagnier-de Choudens et al., 2005). In the mutated Arabidopsis old5 protein, Pro-101 is replaced by a Ser. The Pro-101 residue is present in all Arabidopsis SufE/YgdK-containing proteins, suggesting a conserved role. Utilizing the 3D-Jigsaw protein-modeling server (Bates et al., 2001), the SufE/YgdK domain of OLD5 protein could be modeled with high confidence on the resolved structure of YgdK (Liu et al., 2005). In the model, the mutation from Pro to Ser results in an altered start of the second α-helix (Figure 3C). This possible different folding of the protein might have an influence on its function.

OLD5 Interacts with a SufS-Like Protein

The YgdK/SufE proteins of E. coli interact with their SufS counterpart (Loiseau et al., 2003, 2005). By analogy, therefore, we expected the SufE/YgdK domain of OLD5 to interact with a SufS-like protein. Recent studies show that the Arabidopsis SufS-like protein Cp NifS interacts with SufE (Xu and Möller, 2006; Ye et al., 2006). Furthermore, it has been suggested that QS might work in...
an enzyme complex with aspartate oxidase (AO) (Sakuraba et al., 2005), since the product of AO is unstable (Yang et al., 2003).

By a database search, we found that the Arabidopsis genome contains two other putative plastidic SufS-like proteins, Cp NifS2 and Cp NifS3. In order to test whether OLD5 can interact with a Cys desulfurase, a yeast two-hybrid assay was performed. As a positive control, we tested the interaction between Cp NifS and SufE. The results of our screen are summarized in Table 1. On selection medium lacking either His or adenine (Ade), we found that the OLD5 protein interacts with Cp NifS and Cp NifS3. These interactions were found for the mutated old5 protein only on His selection medium but not on the more stringent Ade plates. Interestingly, an interaction with AO was also detected, suggesting that OLD5 works as part of an enzyme complex.

Taken together, these results suggest that OLD5 is part of a Cys desulfurase complex, and this was recently confirmed by Murthy et al. (2007).

The old5 Mutation Reduces Cys Desulfurase Activity

Since the homozygous T-DNA knockout line is embryo-lethal and the old5 allele we isolated is recessive and not lethal, we expected that the old5 mutation must either cause a reduction or a change of enzyme function. In order to assess this, we first performed complementation of an E. coli mutant deficient in QS (ΔNadA) (Murthy et al., 2007). Plasmids containing the full-length open reading frame for wild-type and mutant QS protein lacking the chloroplast localization signal were used (Murthy et al., 2007).

Expression of either the wild-type or mutant protein restored growth of the deletion strain on minimal medium lacking nicotinic acid. Subsequently, we wanted to understand if the old5 mutation affects enzyme activity. The QS domain is dependent on a Fe-S cluster, which is provided through its SufE/YgdK domain through stimulation of a Cys desulfurase (Loiseau et al., 2005; Murthy et al., 2007). Previously, the activity of both domains of OL5 was determined under anaerobic conditions (Murthy et al., 2007). Since the QS domain is not affected by the old5 mutation, we expected that the activity should be similar to that of the wild type. The ability of anaerobically isolated wild-type and old5 protein to catalyze the formation of quinolinic acid was assayed using an established protocol (Murthy et al., 2007). For this purpose, the substrate iminoaspartate, which is a required but highly unstable substrate of QS, was generated from l-Asp by AO. The formation of quinolinate by the mutated old5 protein is shown in Figure 4A and overlaps with that of the wild-type protein. Thus, the enzyme activity of the QS domain is not affected by the mutation. The function of the second domain of the OLD5 protein is to stimulate the Cys desulfurase activity of Cp NifS (Murthy et al., 2007). Testing this activity revealed that the old5 mutation results in an almost threefold lower stimulation than that observed in the wild type (Figure 4B). Taken together, the decreased activity of the SufE domain probably decreases overall activity of the protein, since continuous repairing/reconstituting of the Fe-S cluster is required for the maintenance of QS activity in planta (Murthy et al., 2007).

The old5 Mutation Results in Increased Steady State Pyridine Nucleotide Levels

The mutation in the SufE/YgdK domain might also decrease enzyme activity in planta. Given that a reduced QS activity may be anticipated to result in lower NAD steady state levels, we determined the pyridine nucleotide content in the first leaf pair of 21- and 27-d-old plants. At the 21-d time point, the old5 mutant leaves show no phenotypic difference in comparison with the wild type (Figure 1A), but at 27 d, some changes in gene expression between the wild type and the mutant are detectable (Figure 2). Measurement of pyridine nucleotide content revealed, indeed, that the mutant had significantly increased NAD, NADH, NADP, and NADPH levels after 27 d (Figure 5A). The values we report for NAD, NADP, and NADPH in the wild type are similar to those of previous studies (Chai et al., 2005; Wang and Pichersky, 2007). Interestingly, the NAD/NADH and NADP/NADPH ratios in the mutant do not differ from the wild-type value, suggesting a similar redox balance for the pyridine nucleotides. Complementation of the mutant with the wild-type gene restores the pyridine nucleotide levels (see Supplemental Figure 1 online), demonstrating that the observed changes are a direct result of the old5 mutation.
Figure 3. OLD5 Encodes QS.

(A) Schematic representation of the protein domain organization of OLD5. The first domain is a transit peptide that targets OLD5 to the chloroplast. The other two domains are a SufE/YgdK and a QS domain.

(B) The OLD5 SufE domain shows similarity to SufE proteins from E. coli (SufE and YgdK). This domain is also present in two other Arabidopsis genes, At4g26500, encoding SufE, and At1g67810, a putative pollen-specific SufE protein. The old5 mutation causes a change in a Pro residue that is conserved between all three Arabidopsis proteins (arrow).

(C) Modeling by 3D-Jigsaw server predicts an effect of the mutated Pro on the structure of the SufE/YgdK domain (arrow). The protein is modeled without the chloroplast targeting signal. The three overlapping structures are encoded as follows: yellow, E. coli YgdK structure (Liu et al., 2005); magenta, wild-type Arabidopsis OLD5; cyan, old5 mutant protein.
The maintenance of the NAD pool depends on the balance between de novo synthesis, active pyridine salvage, and degradation (Wang and Pichersky, 2007). The de novo synthesis and the pyridine salvage pathway result in the production of nicotinate mononucleotide, which by two enzymatic steps is converted to NAD (Figure 5B). The observed increase in NAD levels is surprising, since the in vitro Cys desulfurase activity is reduced. To understand the regulation of NAD biosynthesis in the mutant, we used expression profiling and enzyme activity measurements (see Methods). First, we determined the expression of genes encoding key enzymes in the pathway of de novo synthesis (Figure 5B). The first step in this pathway is the conversion of L-Asp into iminoaspartate mediated by AO. The transcript level of AO was threefold higher in the old5 mutant than in the wild type at all time points measured. The next step of de novo biosynthesis, the synthesis of the pyridine ring, is controlled by OLDS/QS. The expression of QS decreases with age in both the wild type and the mutant. However, during senescence, the expression of QS is upregulated in the mutant, reflecting a possible increased demand for NAD during the process. The final step, synthesis of nicotinate mononucleotide, requires the quinolinate phosphoribosyltransferase (QPRT), which for both the wild type and the mutant gradually increase in expression with age but do not differ from one another (Figure 5B). In addition, in vitro QPRT activity was tested on whole leaf extracts and no difference was found (Figure 6A). Given that the activity of this enzyme is similar for the wild type and the mutant, it is highly unlikely that the observed increase in pyridine nucleotides can be explained by an increase in de novo synthesis. Taken together, the results show that AO is upregulated at the expression level, while QS likely has a lower activity in vitro and QPRT activity is unchanged in leaf extracts.

Thus, the increased NAD pool suggests that enzymes in the salvage pathway might compensate for the mutation in the de novo pathway. Such a mechanism was previously observed for the enzymes of the salvage pathway of pyrimidine nucleotides following the antisense inhibition of an enzyme of the de novo pathway of pyrimidine biosynthesis (Geigenberger et al., 2005). The salvage of nicotinamide, released from NAD, starts with deamidation by nicotinamidases to nicotinate (Wang and Pichersky, 2007) and subsequent conversion by nicotinate phosphoribosyltransferase (NaPRT). In Arabidopsis, NIC1 is the best characterized nicotinamidase, which, when knocked out, almost completely abolishes the salvage of nicotinamide (Wang and Pichersky, 2007). Overexpression of NIC1 results in an increase in NAD levels; thus, the expression level of NIC1 correlates with NAD content (Wang and Pichersky, 2007). The expression of NIC1 is constitutively higher in the old5 mutant (Figure 5B). The product of NIC1, nicotinate, is converted by NaPRTases, and the Arabidopsis genome encodes two homologs. In old5, At2g23420 (encoding NaPRT2) is upregulated when compared with the wild type, while At4g36940, which encodes the other putative NaPRTase, is expressed at similar levels as the wild type (data not shown). The increase in expression of both NIC1 and At2g23420 suggests an increased activity of the salvage pathway in the old5 mutant. To get a direct measurement of NIC1 activity, we used leaf extracts of 21-, 27-, and 33-d-old plants (Figure 6B). The assay shows constitutively higher activities of NIC in old5, suggesting that the salvage pathway is more active. The final step of NAD biosynthesis occurs through the action of NAD synthetase (NADS), which is similarly expressed in the mutant and the wild type. However,
using leaf extracts to determine the activity, we show that NADS activity is 1.5- to 2-fold higher in the old5 mutant (Figure 6C). Thus, the increase in NAD levels is due to a more active salvage pathway in old5, which is supported by an increased activity in NADS. Taken together, these results demonstrate an increased activity of the salvage pathway in the old5 mutant; this activity causes an increase in the steady state levels of NAD. We show that the alternate biosynthetic routes for NAD production influence each other, either directly or indirectly, in order to maintain sufficient levels of NAD.

**old5 Is Characterized by Increased Tricarboxylic Acid Cycle Intermediates**

Since previous studies have shown that alterations in NAD/NADH levels can have dramatic consequences on cellular

![Figure 5. Pyridine Nucleotide Biosynthesis Is Increased during Leaf Development and Ageing of old5.](image-url)

(A) Pyridine nucleotide content in old5 and the wild type at days 21 and 27 (old5, gray bars; wild type, black bars). The values shown are means of six repeats ± SD (indicated by error bars). Asterisks indicate statistical significance versus the wild-type control (*P < 0.05, Student’s t test). The ratio of pyridine nucleotides is not affected by the old5 mutation. FW, fresh weight.

(B) Schematic representation of NAD biosynthetic pathways. Transcripts of AO, nicotinamidase (NIC1), and NaPRT2 accumulate in old5 mutants (old5, open circles; wild type, closed circles). The values shown are means of three repeats ± SD (indicated by error bars). Asterisks indicate statistical significance versus the wild-type control (*P < 0.05, Student’s t test).
metabolism (Dutilleul et al., 2005; Shen et al., 2006), we next performed a comprehensive metabolite profiling using an established gas chromatography–mass spectrometry protocol capable of quantifying the relative levels of >80 metabolites (Lisec et al., 2006). For this purpose, leaves were harvested, in the middle of the light period, from both the old5 mutant and the wild type at days 21 and 27 and metabolites were measured (see Supplemental Table 1 online). Interestingly, although sugar levels in the old5 mutant were similar to wild-type levels at 21 d, maltose and sucrose levels were significantly higher at 27 d (Figure 7A). Increased sugar levels have been observed previously before the onset of senescence (Diaz et al., 2005; Wingler et al., 2006), suggesting that the old5 lesion invokes an early change in metabolism reminiscent of senescence. Sugars are subsequently processed through glycolysis to form the pyruvate needed to support mitochondrial respiration through the tricarboxylic acid (TCA) cycle (Fernie et al., 2004). After 21 d, we found an increase in the TCA cycle intermediate succinate in the mutant in comparison with the wild type, whereas other intermediates of this cycle were invariant (Figure 7B). However, at this time point, amino acids derived from the TCA cycle intermediate α-ketoglutarate accumulate in old5 to higher levels than in the wild type. These amino acids include Glu, Gin, Pro, and the polyamine spermidine (Figure 7C). Importantly, Asp, which is the precursor for the de novo NAD synthesis, was also significantly upregulated in old5 after 21 d. From Asp, several other compounds are synthesized, of which Asn was observed to accumulate after 21 d in old5. Complementation of the mutant with the wild-type OLD5 gene restored the metabolites to wild-type levels (see Supplemental Table 2 online), demonstrating that the observed metabolite changes are a direct result of the old5 mutation.

At 27 d, all of the measured α-ketoglutarate–derived compounds, including 4-aminobutyric acid (GABA), Orn, putrescine, and β-Ala, were more abundant in old5. Furthermore, the branched chain amino acids Ile and Val, which derive from pyruvate (Binder et al., 2007), were significantly higher after 27 d of growth in the old5 mutant. Ile and Val have previously been documented to accumulate in mutants of the electron-transfer flavoprotein complex, which display accelerated dark-induced senescence (Ishizaki et al., 2005, 2006). Moreover, the Asp precursor oxaloacetate, as well as succinate and malate, are more abundant at 27 d in the mutant. These changes might be indicative of an increased mitochondrial metabolic rate in old5 mutants (Noctor et al., 2007). Previously, it was shown that redox manipulation by DTT treatment of Arabidopsis leaves increases the level of succinate, malate, and oxaloacetate (Kolbe et al., 2006), suggesting that the changes in NAD/NADH levels may be the direct cause of the increase found in TCA cycle intermediates for the old5 mutant.

Comparison of differences in the metabolite levels observed between 21 and 27 d may reveal differential metabolic regulation in the mutant with respect to the wild type. Although the levels of the TCA cycle intermediates are higher in old5 than in the wild type at both time points, the pattern of change (i.e., decrease) between the time points is the same for the wild type and mutants (Figure 7B). However, that is not true for pyridine nucleotide levels, which in the wild type decrease with time, while in old5 they remain stable (Figure 5A).

### Increased Respiration Rate and Antioxidant Accumulation in old5

Both increased amounts of organic acids and higher levels of pyridine nucleotide can be attributed to an increased rate of respiration (Priaulx et al., 2007). The metabolite profile suggests that the changed pyridine nucleotide levels in old5 lead to an altered respiration rate. To analyze that possibility, we determined the oxygen consumption of whole leaves in the dark. Figure 8A shows oxygen consumption at 21 and 27 d. At both time points, the old5 mutant consumes approximately twofold more oxygen than the wild type, demonstrating an altered respiration rate. An overreduction of the mitochondrial electron transport chain gives rise to increased ROS production, which can be alleviated by activation of ALTERNATIVE OXIDASE1 (AOX1) (Noctor et al., 2007). The transcript abundance of AOX1 is also activated directly by TCA cycle intermediates (Vanlerbergh et al., 1997; Gray et al., 2004). Figure 9 shows that AOX1 is constitutively more highly expressed in old5, further supporting the notion that the mutant has a higher respiration rate than the wild type. Higher respiratory rates are generally coupled to an increased production of ROS by complexes I and III of the mitochondrial electron transport chain (Apel and Hirt, 2004;
The first indication that *old5* suffers from ROS stress is SAG13 expression (Figure 2), which is associated with oxidative stress (Miller et al., 1999) and low antioxidant levels (Conklin and Barth, 2004) as well as with senescence. Moreover, the mutant showed a change in expression of two other well-established ROS marker genes: At2g43510 and At3g13610 (Figure 9). The defensin-like gene At2g43510, which has previously been shown to be ubiquitously induced by various ROS (Gadjev et al., 2006), was expressed at higher levels in *old5* leaves at all time points and was strongly upregulated after 30 d (Figure 9). The second marker, 2-oxoglutarate–dependent dioxygenase (At3g13610), which is specifically expressed during hydrogen peroxide–related stress (Gadjev et al., 2006), showed a distinct expression after 24 d in *old5* leaves that increased rapidly with age. ROS are continuously generated as a by-product of aerobic metabolism and need to be rapidly detoxified by either enzymatic or nonenzymatic pathways, since they are highly toxic (Apel and Hirt, 2004). Protection against oxidative stress is partially provided for by low molecular weight antioxidants such as glutathione and ascorbate. Both the total thiol pool (GSH + GSSG + X) as well as the GSH are significantly increased in the mutant in comparison with the wild type (Figures 8B and 8C). An increase in the reduced pool of GSH provides an efficient protection against the toxic effects of hydrogen peroxide (May and Leaver, 1993). Finally, ROS stress in *Arabidopsis* has previously been reported to affect nitrate assimilation catalyzed by glutamate dehydrogenase (GDH) (Skopelitis et al., 2006). The altered metabolite profile and increased expression of ROS marker genes coincide with an increased expression of GDH from day 27 in the *old5* mutant (Figure 9). Taken together, these results show that the *old5* mutation results in an increased respiration rate that is accompanied by an increase in oxidative stress, based on the expression of ROS-inducible genes and an increase in the pool of glutathione.

**DISCUSSION**

*old5* Reveals a Role for NAD in Plant Ageing

Plants differ fundamentally from animals in that they do not have a rigid body plan and that organs can easily be added or removed, making them highly adaptive to the environment. As a result of developmental and adaptive strategies that resist, avoid, and anticipate ageing, it has been suggested that plants do not age at all in any sense recognizable in animals (Thomas, 2002). However, the lifespan of individual leaves clearly resembles animal ageing (Gan, 2003; Jing et al., 2003; Lim et al., 2003; Noctor et al., 2007). The first indication that *old5* suffers from ROS stress is SAG13 expression (Figure 2), which is associated with oxidative stress (Miller et al., 1999) and low antioxidant levels (Conklin and Barth, 2004) as well as with senescence. Moreover, the mutant showed a change in expression of two other well-established ROS marker genes: At2g43510 and At3g13610 (Figure 9). The defensin-like gene At2g43510, which has previously been shown to be ubiquitously induced by various ROS (Gadjev et al., 2006), was expressed at higher levels in *old5* leaves at all time points and was strongly upregulated after 30 d (Figure 9). The second marker, 2-oxoglutarate–dependent dioxygenase (At3g13610), which is specifically expressed during hydrogen peroxide–related stress (Gadjev et al., 2006), showed a distinct expression after 24 d in *old5* that increased rapidly with age. ROS are continuously generated as a by-product of aerobic metabolism and need to be rapidly detoxified by either enzymatic or nonenzymatic pathways, since they are highly toxic (Apel and Hirt, 2004). Protection against oxidative stress is partially provided for by low molecular weight antioxidants such as glutathione and ascorbate. Both the total thiol pool (GSH + GSSG + X) as well as the GSH are significantly increased in the mutant in comparison with the wild type (Figures 8B and 8C). An increase in the reduced pool of GSH provides an efficient protection against the toxic effects of hydrogen peroxide (May and Leaver, 1993).
Schippers et al., 2007). That is especially visible in Arabidopsis, in which the onset of leaf senescence occurs in an age-dependent way (Gan and Amasino, 1997; Quirino et al., 2000; Jing et al., 2002). Moreover, the recent identification of genes that affect the onset of leaf senescence (Woo et al., 2001; Jing et al., 2005, 2007) reveals common mechanisms present in the regulation of ageing between plants and animals.

In this study, we show that the old5 mutant displays early age-induced leaf senescence, as indicated by the expression of SAG12. The cloning of old5 identified a mutation in the gene encoding the QS. QS/OLD5 is an essential enzyme in the de novo synthesis of the pyridine nucleotide NAD (Katoh et al., 2006). Pyridine nucleotides have well-characterized roles as redox carriers in processes such as oxidative phosphorylation, the TCA cycle, and as electron acceptors in photosynthesis (Hunt et al., 2004). Next to their role in metabolism, they play a part during several stress conditions, including oxidative stress (Moller, 2001; Hayashi et al., 2005), wound response (Sinclair et al., 2000), and ABA signaling and salt stress (Shen et al., 2006; Wang and Pichersky, 2007). Recently, it was found that the NAD-dependent histone deacetylase SIR2 influences the lifespan for several model organisms, including yeast, Caenorhabditis

Figure 8. Increased Respiration of old5 Coincides with Increased Glutathione Levels.

(A) The oxygen consumption in the dark was measured and quantified with a Clark-type electrode (old5, gray bars; wild type, black bars).
(B) The pool of reduced glutathione shows an approximately twofold increase in the mutant (gray bars) in comparison with the wild type (black bars) and the complemented line (white bars).
(C) Elevated total glutathione pool (GSH + GSSX + X; X = any thiol) is observed in old5 compared with the wild type. Measurement of total glutathione is performed by reducing all disulfides with DTT from leaf tissues of 21- and 27-d-old plants, and the analysis was performed using reverse-phase HPLC. Asterisks indicate statistical significance versus the wild-type control (\( P < 0.05 \), Student’s t test). FW, fresh weight.

Figure 9. Relative Expression of Marker Genes Indicative of Oxidative Stress.

The relative expression of several genes was determined by real-time PCR and calculated relative to the wild type. The AOX is constitutively higher expressed (old5, gray bars; wild type, black bars). Two oxidative stress marker genes were tested and found to be upregulated in the mutant (At2g23420 and At3g13610). Next to that, GDH is more abundant than in the wild type. The values shown are means of three repeats ± SD (indicated by error bars). Experiments were repeated and showed similar results. Asterisks indicate statistical significance versus the wild-type control in each case (\( P < 0.05 \), Student’s t test).
The old5 Mutation Results in a Metabolite Profile Characteristic of Early Ageing

Redox reactions are the fundamental metabolic processes through which cells convert and distribute the energy that is necessary for growth and maintenance (Noctor, 2006). Changes in the availability of pyridine nucleotide levels have previously been shown to have a dramatic effect on the metabolite profiles of plants (Dutilleul et al., 2005; Shen et al., 2006). In addition, increased NADH levels affect other reactions that produce NADH and disturb the cellular redox balance, resulting in oxidative stress (Moller, 2001; Shen et al., 2006). The increased NAD levels in the old mutant coincide with increases in the level of TCA cycle intermediates and Asp, which is a precursor for NAD biosynthesis. Moreover, nitrogen metabolism is affected, as evidenced by the fact that amino acids deriving from ketoglutarate are more abundant. These data are consistent with those of a previous study on the tobacco (Nicotiana tabacum) nad7 mutant, which also displayed higher NAD/NADH levels concomitant with increased levels of nitrogen-containing amino acids (Dutilleul et al., 2005).

Given that the onset of leaf senescence in old5 is age-induced, it was anticipated that metabolic profiling of the mutant could identify metabolites that are characteristic biomarkers for an ARC (Diaz et al., 2005; Jing et al., 2005). Indeed, several biomarkers specific for the onset of leaf senescence were found in old5. Notably, the accumulation of the branched chain amino acids Val and Ile was previously shown to occur before the onset of senescence (Masclaux et al., 2000; Diaz et al., 2005; Ishizaki et al., 2005). One of the most intriguing metabolites that show an age-related accumulation in both the wild type and old5 is GABA. Studies on Osl2, a mitochondrial GABA transaminase in rice (Oryza sativa) (Ansari et al., 2005), suggested a switch in metabolism of the chloroplast (Murthy et al., 2007). Therefore, the continuous repair of the Fe-S cluster in the Q domain by its SufE/Ydkd domain determines the activity of the protein. The analysis of the mutated old5 protein revealed that it weakly interacts with components of the Fe-S biogenesis complex, Cp NifS and Cp NifS3 in a yeast two-hybrid experiment. Moreover, the activity measurements reveal that the stimulation of Cys desulfurase activity is reduced in the old5 protein. Although the QS activity of the old5 protein in oxygen-free experimental conditions is not altered, the decreased Cys desulfurase activity likely affects the long-term catalytic competence of the enzyme in vivo.

Interestingly, we also observed an accumulation of polyamines in the old5 mutant with age. Spermidine has the same precursor as aminocyclopropane, an important source of ethylene that can induce senescence prematurely (Imai et al., 2004). However, the accumulation of the polyamines might not be an ARC per se but may rather reflect a protection mechanism against ROS generation and programmed cell death (Papadakis and Roubelakis-Angelakis, 2005). Moreover, polyamines such as cytokinins are well known for their senescence-retarding effect.

Pyridine Biosynthesis, Crosstalk, and Stress Programming in old5 Mutant

The QS is dependent on a 4Fe-4S cluster for its activity (Loiseau et al., 2005). In E. coli, three Fe-S assembly systems exists, of which one appears to be more efficient in maintaining Qs activity. The Arabidopsis OLDS protein consists of a Fe-S assembly domain fused with the Qs domain. This fusion is likely of physiological significance, since the OLDS protein is highly oxygen-sensitive and localized to the oxygen-rich environment of the chloroplast (Murthy et al., 2007). Therefore, the continuous repair of the Fe-S cluster in the Qs domain by its SufE/Ydkd domain determines the activity of the protein. The analysis of the mutated old5 protein revealed that it weakly interacts with components of the Fe-S biogenesis complex, Cp NifS and Cp NifS3 in a yeast two hybrid experiment. Moreover, the activity measurements reveal that the stimulation of Cys desulfurase activity is reduced in the old5 protein. Although the Qs activity of the old5 protein in oxygen-free experimental conditions is not altered, the decreased Cys desulfurase activity likely affects the long-term catalytic competence of the enzyme in vivo.

Despite the reduced activity of the mutated old5 protein, plants showed increased levels of NAD/NADH at 21 d. The maintenance of the NAD pool depends on both the de novo synthesis and pyridine salvage. Whereas de novo synthesis is absolutely required for plant survival (Katoh et al., 2006), the recycling pathway is mainly important during stress, when it recycles utilized NAD in an energy-efficient manner (Wang and Pichersky, 2007). In apparent response to the compromised QS, old5 plants exhibit increased expression of AO, the first enzyme in the de novo synthesis of NAD (Figure 5). Thus, these data suggest that the de novo synthesis is transcriptionally regulated at the first step, formation of iminoaspartate from Asp by AO. Iminoaspartate has to be generated in the presence of active QS, since it is a highly unstable intermediate (Murthy et al., 2007). To increase the rate of de novo synthesis, both an increase in substrate and active OLDS protein are needed. Interestingly, the reduced overall activity of QS in the mutant might mimic increased oxygen stress (Gardner and Fridovich, 1991). Next to that, AO expression was recently found to be highly upregulated during application of oxidative stress (Gadjev et al., 2006), suggesting that AO and QS might play a role in the detection or signaling of ROS.

As a result of the mutation in QS, the salvage pathway is upregulated in old5 mutants. Both NIC1 and a putative NaPR Tase show increased expression. Enhanced expression of the salvage pathway enzyme NIC1 has previously been shown to increase the NAD levels in Arabidopsis (Hunt et al., 2007; Wang...
and Pichersky, 2007). Increased NAD expression in the mutant correlates with the measured increased enzyme activity in leaf extracts, suggesting that its activity is regulated at the transcriptional level. By contrast, the increased NAD synthetase activity was associated with a reduced gene expression over time, suggesting that NAD synthetase activity is posttranscriptionally regulated. Despite the old5 mutation, pyridine nucleotide levels in the mutant were increased compared with the wild type. This might be due to the activation of a stress response program, since increased expression of the NAD salvage pathway is reminiscent of the need to replenish the NAD pool during stress conditions (Berglund et al., 1996; Hunt et al., 2007; Wang and Pichersky, 2007). One example of a NAD-consuming enzyme induced by oxidative stress is poly(ADP-ribose) polymerase (De Block et al., 2005), linking increased salvage metabolism to stress. Next to that, the salvage pathway was shown to be activated and necessary for response to osmotic stress in Arabidopsis (Wang and Pichersky, 2007). The compensation for reduced de novo synthesis of NAD by up-regulation of the salvage pathway of pyridine nucleotides is a mechanism that has also been observed for pyrimidine biosynthesis (Geigenberger et al., 2005).

Oxidative Stress and Early Ageing of old5

Longevity is clearly genetically controlled in many species, including yeast, C. elegans, mouse, and human (Jing et al., 2003). Almost 100 years ago it was postulated that there is an inverse relationship between metabolic rate and longevity (Rubner, 1908; Pearl, 1928). Today, it is clear that caloric restriction can greatly extend the maximum lifespan of many species (Sohal and Weintraub, 1996). The discovery of the first gene (PHA-4) absolutely required for lifespan extension by caloric restriction in C. elegans supports a role for ROS generated by mitochondrial oxidative energy metabolism in lifespan determination. This is especially evident by the fact that reduced PHA-4 expression does not suppress the long lifespan of animals with defective electron transport chains (Panowski et al., 2007). Moreover, generation of radical oxygen species by the mitochondria is dependent upon dietary intake (Lee et al., 2008). These results fit within the free radical theory of ageing, which proposes that ROS produced by respiration contribute to the ageing of all organisms (Harman, 1956).

In our study, we demonstrate that the mutant old5 has an increased respiration rate together with increased oxidative stress. The reducing equivalents that are generated from TCA cycle activity are used by the mitochondrial electron transport chain to power the synthesis of ATP (Fernie et al., 2004). The increased NADH levels in the mutant might directly influence the rate of mitochondrial electron transport (Hunt et al., 2004). This results in expression of the AOX, which bypasses phosphorylation and does not contribute to ATP production but alleviates overreduction of the enzymes in the transport chain (Moller, 2001). Transgenic tobacco cells that fail to induce AOX go rapidly into programmed cell death during oxidative stress (Vanlerbergh et al., 2002). It has to be noted that the induction of cell death in plants is highly dependent on genetic factors (Wagner et al., 2004) and may not merely be a direct consequence of ROS-induced damage. The increased glutathione levels and increased expression of hydrogen peroxide–induced stress markers in our mutant fit well with previous studies showing that during oxidative stress mitochondria produce increased amounts of hydrogen peroxide (Sweetlove et al., 2002; Tiwari et al., 2002; Gadjev et al., 2006). Developmental senescence of pea (Pisum sativum) leaves coincides with increases in the levels of superoxide and hydrogen peroxide (Pastori and del Rio, 1997). Furthermore, treatment of Arabidopsis leaves with the herbicide 3-amino triazole inhibits catalase activity and causes hydrogen peroxide stress and increased expression of SAG genes (Navabpour et al., 2003). Next to that, the senescence-specific transcription factor WRKY53 is induced by hydrogen peroxide (Miao et al., 2004; Miao and Zentgraf, 2007). In general, it is believed that oxidative stress results in damage to the cell, which loses its viability, resulting in early ageing, as denoted by the free radical theory of ageing (Harman, 1956). However, it is more likely that altered ROS levels are implemented in the developmental program of the leaf, causing an early onset of senescence (Wagner et al., 2004; Queval et al., 2007).

By utilizing Arabidopsis leaves as a model system for plants, we have exploited the opportunity to characterize the mechanisms and genetics of ageing. Our study supports a role for ROS signaling in leaf development and ageing. Furthermore, we demonstrate that NAD might have a conserved role in ageing across kingdoms. Since ageing is manifested in many ways, cloning and physiological characterization of other old mutants will allow a fuller elucidation of the subtle differences between the various mechanisms of ageing and senescence.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Ler was used throughout this study. The old5 mutant was obtained from an ethyl methanesulfonate–mutagenized collection (Jing et al., 2005). The T-DNA insertion mutant QS-1 (SALK_079205) was obtained from the Nottingham Arabidopsis Stock Centre. The old5 mutant was complemented by transforming a 5.1-kb Ler genomic fragment containing the wild-type OLDS gene to the mutant. The 5.1-kb DNA fragment was amplified by PCR using two oligonucleotides, 5'-GAATATCCCAATATAGTACACCACAATCAAATTAAATAC-3' and 5'-GTTGTTACTAAGTTGCCAAACATTATATGTCTACTATTATG-3', and cloned into the pGEM-T Easy vector (Promega). The insert was sequenced and transferred as a Nott–Nott fragment to the binary vector pgreen029 (Helliens et al., 2000). Plants were grown on either soil or half-strength Murashige and Skoog medium at 23°C and 65% RH with a daylength of 16 h. The light intensity was set at 120 μmol·m⁻²·s⁻¹. An organic-rich γ-ray–radiated soil was used (Hortimea Groep).

Pigment Determination and Measurement of Photochemical Efficiency

For extraction of chlorophyll and carotenoids, samples were incubated overnight with NN,N-dimethylformamide at 4°C in darkness. The chlorophyll content was quantified spectrophotometrically according to Wellburn (1994) at 647 and 664 nm. Chlorophyll fluorescence emission was measured from the upper surface of the first leaf at room temperature (23°C) with a pulse-amplitude modulation portable fluorometer (PAM-2000; H. Walz) according to Maxwell and Johnson (2000). Plants were
dark-adapted for 1 to 2 h before measurements to ensure complete relaxation of the thylakoid pH gradient. An attached, fully expanded rosette leaf was placed in the leaf clip, allowing air to circulate freely on both sides of the leaf. At the start of each experiment, the leaf was exposed to 2 min of far-red illumination (2 to 4 μmol photons m⁻² s⁻¹) for determination of Fo (minimum fluorescence in the dark-adapted state). Saturating pulses of white light (8000 μmol photons m⁻² s⁻¹) were applied to determine Fm or Fm' values. PSII efficiency was calculated as (Fm − Fo)/Fm.

RNA Isolation and Real-Time PCR
Total RNA was isolated using TRIZOL reagent (Sigma-Aldrich) according to the manufacturer’s protocol. Five hundred nanograms of total RNA was used as template for first-strand cDNA synthesis using 200 units of RevertAid H-minus Moloney murine leukemia virus reverse transcriptase (Fermentas) and an oligo(dT21) primer. Quantitative RT-PCR was performed with the Cycler real-time PCR system (Bio-Rad). Primer pairs for real-time PCR were designed with the open-source PCR primer design program PeriPrimer version 1.1.10 (Marshall, 2004) around an intron to obtain a PCR product of 100 to 300 bp. The primer sequences are available in Supplemental Table 3 online. The presence of a single PCR product was verified by melt-curve analysis and gel electrophoresis. Relative quantification of gene expression was performed using the iCycler iQ software and the gene expression macro of Bio-Rad. All reactions were performed in quadruplicate on separate plates. Expression levels were normalized using Ct values obtained for the housekeeping gene ACT2 (At3g18780). Briefly, real-time PCR amplification was performed with 50 μL of reaction solution containing 2 μL of 10-fold diluted cDNA, 0.5 μL of a 10 mM stock of each primer, 1 μL of 25 mM stock MgCl₂ (Fermentas), 5 μL of PCR buffer + Mg (Roche), 1 μL of a 1000× diluted SYBR Green stock (Sigma-Aldrich), 0.5 μL of 100× BSA (New England Biolabs), and 1 unit of Roche Taq polymerase. The amplification program was 94°C for 2 min, 40 cycles at 94°C for 10 s, and 72°C for 25 s, and amplification was followed by a melt curve analysis.

Bacterial Complementation, Enzyme Purification, and Activity Measurements
Bacterial strain Escherichia coli ΔNadA deletion mutant and plasmid pBAD/Myc-HisB containing the mature coding sequence of wild-type OLD5 were obtained from Murthy et al. (2007). The SuIE/Ygdk domain coding sequence of the wild type was replaced with that of the old5 mutant to obtain a pBAD vector that expresses the mutated protein. Cyst desulfurase activity was measured as described by Ye et al. (2006). QS enzymatic activity was assayed under anaerobic conditions at 27°C as described by Murthy et al. (2007). The AO protein was kindly provided by Sandrine-Ollagnier.

Determination of Metabolite Levels
Leaf samples were taken at the time points indicated, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis. Extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of the appropriate extraction buffer. The relative levels of metabolites were determined using an established gas chromatography coupled to a time-of-flight mass analyzer protocol as described by Lisec et al. (2006). Data are presented normalized as detailed by Roessler et al. (2001). In short, peaks were assigned and quantified, and all data were normalized to the mean response calculated for the wild-type control of each measured batch; to allow comparison between the samples, individual wild-type values were normalized in the same way. The procedure of extraction and assay of NADs was performed according to the method described by Gibon and Larher (1997). The determination of glutathione contents was performed as described by Kreft et al. (2003).

NAD Biosynthesis Enzyme Activity Assay
Enzyme extracts were prepared from frozen ground leaf tissue. All extraction procedures were performed at 4°C. Twenty-five milligrams of tissue was homogenized in 0.25 mL of an appropriate extraction buffer. The homogenate was centrifuged for 5 min at 2700g, and the supernatant was used in the assays. The extraction buffer for QPRT and NAD synthase consisted of 100 mM MOPS-NaOH, pH 7.4, 5 mM MgCl₂, and 0.1 mM EDTA. For nicotinamidase, the buffer was modified as follows: 200 mM MOPS-KOH, pH 7.5, 5 mM MgCl₂, 10 mM DTT, and 0.1 mM EDTA. Enzyme assays were performed at 30°C. Nicotinamidase activity was determined as described by Wang and Pichersky (2007). QPRT was assayed as described by Wang et al. (2006). NAD synthase was assayed as described by Wagner and Wagner (1985), with the exception that the NAD produced was determined enzymatically as described by Gibon and Larher (1997).

Yeast Two-Hybrid Experiments
The coding sequences of the selected genes were amplified from cDNA with gene-specific primers (see Supplemental Table 3 online) and cloned in-frame with the GAL4 binding domain of the pGBK7T vector and the GAL4 activation domain of the pGAD424 vector (Clontech) by restriction with Smal and Sall. For protein-protein interaction screening, PJ69-4A (James et al., 1996) was transformed according to the Clontech yeast protocols handbook (PR13103) and selected on synthetic dropout (SD) medium lacking Leu and Trp for transformants. Subsequent colonies were dissolved in SD medium and spotted on selection medium for interaction by testing growth on SD medium lacking either Ade or His with the addition of 2 mM 3-amino-1,2,4-triazole. Plates were incubated at 28°C for 2 to 5 nights.

Respiratory Measurements
Assays of oxygen consumption by whole leaves were performed using a Clark-type oxygen electrode. Leaves were placed on a filter containing CO₂ buffer, and subsequently the measuring room was filled after calibration with a 2% oxygen-containing gas before measurement. Measurements were performed in six independent samples, and fresh weight and chlorophyll content of the leaves were determined.

Accession Numbers
The Arabidopsis Genome Initiative locus numbers for the major genes discussed in this article are At1g08490 for Cp NifS1, At1g18490 for Cp NifS2, At1g55090 for NADS, At2g01350 for QPRT, At2g22570 for At NIC1, At2g23420 for NAPRT2, At2g29350 for SAG13, At2g43510 for defensin-like, At3g13610 for oxidoreductase, At4g26500 for At SufE, At4g36940 for NaPRT1, At5g07440 for SAG13, At5g14760 for AO, At5g26600 for Cp NifS3, At5g50210 for QS/OLD5, and At5g45890 for SAG12.

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

Supplemental Figure 1. Complementation of the old5 Mutation Reverses the Increased Pyridine Nucleotide Content.

Supplemental Table 1. Metabolite Levels in the First Leaf Pair of 21- and 27-d-Old Plants of old5 Relative to the Wild Type.
Supplemental Table 2. Metabolite Levels in the First Leaf Pair of 21-,
27-, and 33-d-Old Plants of Complemented old5 Relative to the Wild
Type.

Supplemental Table 3. Primers Used in This Study.

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