Plant mitochondria possess alternative respiratory pathways mediated by the type II NAD(P)H dehydrogenases and alternative oxidases. Here, E3 SUMO ligase was shown to regulate alternative respiratory pathways and to participate in the maintenance of carbon and nitrogen balance in Arabidopsis. The transcript abundance of the type II NAD(P)H dehydrogenases NDA2 and NDB2 and alternative oxidases AOX1a and AOX1d genes was low in siz1-2 mutants compared to that in wild-type. The addition of nitrate or ammonium resulted in a decrease or an increase in the expression of the same gene families, respectively, in both wild-type and siz1-2 mutants. The amount of free sugar (glucose, fructose and sucrose) was lower in siz1-2 mutants than that in wild-type. These results indicate that low nitrate reductase activity due to the ASIZ1 mutation is correlated with an overall decrease in alternative respiration and with a low carbohydrate content to maintain the carbon to nitrogen ratio in siz1-2 mutants. [BMB Reports 2012; 45(6): 342-347]

INTRODUCTION

Large quantities of nitrogen are required to biosynthesize amino acids and secondary metabolites in plants. Plants absorb either nitrate or ammonium as nitrogen sources. Ammonium is assimilated directly in the roots, whereas nitrate is transported to the leaves for further reduction and assimilation (1).

In Arabidopsis, nitrate is reduced to nitrite by the nitrate reductases NIA1 and NIA2 using cytosolic NADH. This is followed by reduction to ammonium using three NADPH equivalents in plastids. Finally, ammonium is incorporated into the amino acid glutamate by glutamine synthetase (1).

Similar to other plant nitrate reductases, the Arabidopsis nitrate reductase is a member of the sulfite oxidase family, which includes both sulfite-oxidizing enzymes. Nitrate reductase contains three functional domains: a MoCo cofactor-binding domain, a heme-binding domain, which is similar to the one contained in the cytochrome b; super family, and a FAD-binding domain (2).

The primary features of the electron transfer chain found in plant mitochondria are similar to those of the electron transfer chain in mitochondria isolated from other eukaryotes. The electron transfer chain contains four integral multiprotein complexes (3). Complex I (NADH ubiquinone oxidoreductase) is an NADH dehydrogenase that oxidizes the NADH generated in the mitochondrial matrix via the tricarboxylic acid (TCA) cycle and transfers the resulting electrons to ubiquinone. Complex II (succinate:ubiquinone oxidoreductase) catalyzes the oxidation of succinate to fumarate during the TCA cycle and transfers the resulting electrons to ubiquinone. Complex III (ubiquinone-cytochrome c oxidoreductase) oxidizes the ubiquinone reduced by complexes I and II and transfers the resulting electrons to cytochrome c. Reduced cytochrome c is oxidized by complex IV (cytochrome c oxidase), the terminal electron transfer complex in the series. Three sites of energy conservation occur at complexes I, III, and IV during electron transfer. At these sites, protons are translocated across the inner membrane to generate the proton motive force that drives ATP synthesis.

However, plant mitochondria also contain an alternative respiratory pathway that bypasses one or more of the multiprotein oxidative phosphorylation complexes. Type II NAD(P)H dehydrogenases and alternative oxidases do not pump protons, and the proton flow through uncoupling proteins is not coupled to ATP synthesis (4). Respiratory bypass proteins are implicated in several physiological processes, including thermogenesis (5), the prevention of reactive oxygen species formation (6, 7), and the dissipation of excess redox equivalents (8).

In Arabidopsis, the respiratory bypass proteins are encoded by small gene families such as the type II NAD(P)H dehydrogenase genes NDA1-2, NDB1-4, and NDC1; alternative oxidase genes AOX1a-d and AOX2; and uncoupling protein genes UCP1-2 and UCP4 (9-12).

SIZ1 is a SP-RING finger protein that has a DNA-binding
SAP domain and a zinc finger Miz domain. Arabidopsis SIZ1 (AtSIZ1) is a key regulator of the signaling responses for nutrient deficiencies and environmental stress (13-20). AtSIZ1 is also involved in nitrogen assimilation by modulating nitrate reductase activity (21).

The Arabidopsis siz1 mutant displays a dwarf phenotype (15), early flowering (19) and abnormal seed development (21), high salicylic acid content, and enhanced resistance to bacterial pathogens (16). Additionally, nitrate reductase activity is much lower in siz1-2 plants than that in wild-type plants, resulting in high nitrate and low nitrogen content in siz1-2 mutants (21). Mutant phenotypes recover to wild-type phenotypes after adding exogenous ammonium but not after adding nitrate, and sumoylation of nitrate reductases by AtSIZ1 increases their activity (21), providing evidence for the involvement of AtSIZ1 in the positive regulation of plant growth through nitrate reduction.

Nitrate induces downregulation of the expression of an external type II NAD(P)H dehydrogenase and several alternative oxidases, which leads to lower respiratory reoxidation of matrix NADH (22). These results suggest that alternative respiration-related genes encoding type II NAD(P)H dehydrogenase and several alternative oxidases must be downregulated in the siz1 mutant and that AtSIZ1 can act as a regulator of an alternative respiratory pathway.

Post-germination seedling development requires efficient utilization of both endogenous storage reserves and resources from the environment. Nitrogen deficiency limits growth, respiration, and utilization of carbohydrates more severely than that of photosynthesis in barley, pea, lemmna, and tobacco (23, 24).

Interestingly, a recent study reported that the alternative respiratory pathway must be tightly connected with the balance in carbon and nitrogen metabolism (25). Starch content and the carbon to nitrogen ratio are higher in aox1a mutants compared to wild-type plants, indicating that a lack of alternative oxidase is linked to the difference in the carbon and nitrogen balance at low temperature. This result suggests that alternative oxidase is necessary for balanced metabolism.

To determine the regulatory mechanism responsible for lower nitrate reductase activity due to loss of E3 SUMO ligase AtSIZ1 and how this may affect respiratory bypass pathways, the transcript abundance of the type II NAD(P)H dehydrogenases and AOX genes was determined using real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. The quantity of free sugars was also monitored to evaluate whether low nitrogen content in siz1-2 plants results in decreased carbohydrate levels. The results demonstrated that nitrate reductase activity exhibited opposing effects on respiratory bypass. Furthermore, reduced levels of nitrate reductase activity in siz1-2 plants resulted in low free sugar content.

**RESULTS**

Plants possess respiratory bypass pathways, which are mediated by type II NADH dehydrogenase and alternative oxidases located in the mitochondria; inner membrane. These pathways are not found in other organisms. Interestingly, reorganization of the respiratory bypass pathway in response to nitrogen sources was recently reported (22).

Nitrate reductase activity decreases in siz1-2 plants (20). Based on this result, the transcript levels of two type II NADH dehydrogenases, NDA2 and NDB2, and two alternative oxidases, AOX1a and AOX1d, were evaluated by quantitative real-time RT-PCR using gene-specific primers (Table 1) in siz1-2 plants. The results demonstrated that the transcript levels of these genes decreased in siz1-2 mutants compared to those in wild-type plants (Table 2). Transcript levels were also examined in both wild-type and siz1-2 plants after nitrate or ammonium treatment. The results revealed that the transcript levels in both wild-type and siz1-2 plants decreased after nitrate treatment, whereas the transcript levels increased following ammonium treatment (Table 3).

Reduced expression of the genes encoding respiratory bypass pathway proteins in siz1-2 mutants can be explained by the following mechanisms. Seedlings grown on nitrate media downregulate the expression of an external type II NAD(P)H dehydrogenase and several alternative oxidases, which can lead to lower respiratory reoxidation of matrix NADH produced during 2-oxoglutarate synthesis (22). This lower respiratory chain activity causes the export of more reductant (NADH) to the cytosol via shuttles such as the malate/oxaloacetate shuttle (26). In contrast, alternative pathways of the respiratory chain are upregulated when nitrogen is supplied as ammonium (22).

These data suggest that nitrate concentrations may be higher in the cytosol of siz1-2 mutants than those in wild-type plants, leading to activation of the nitrate reduction pathway for reducing or scavenging nitrate. This activation may trigger the transport of reductants produced in the mitochondria to the cyto-

**Table 1.** Primer sequences used for real time RT-PCR reactions

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDA2</td>
<td>5'-gtcttgtagtacaaaaactcagcgc-3'</td>
<td>5'-acacaacagcagctgtagaggttac-3'</td>
<td>297</td>
</tr>
<tr>
<td>NDB2</td>
<td>5'-tggatccctccagcagc-3'</td>
<td>5'-gtcttgtagtacaaaaactcagcgc-3'</td>
<td>297</td>
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<td>5'-acacaacagcagctgtagaggttac-3'</td>
<td>303</td>
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<tr>
<td>AOX1d</td>
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<td>5'-gtcttgtagtacaaaaactcagcgc-3'</td>
<td>299</td>
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<td>5'-gtcttgtagtacaaaaactcagcgc-3'</td>
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