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Arabidopsis SIZ1 positively regulates alternative respiratory bypass pathways

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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 *AtSIZ1* 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).

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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_5 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, lemna, 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 cy-

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

Gene name	Forward primer	Reverse primer	PCR product (bp)
NDA2	5′-gttcatgatcaaaaacctcacacg-3′	5′-acacaaacgacgtcgtagagattc-3′	297
NDB2	5′-gagaaatttcagtgtcttcg-3′	5′-ggtagtaaaggagtgaaag-3′	297
AOX1a	5′-gtttcgtctcacgaggctttatca-3′	5′-cctgaaacagttccacttccattc	303
AOX1d	5′-atgtcctacagatcgatttaccgc-3′	5′-ggtttgtatgaatcccatggctga-3′	299
Tubulin	5′-gtgagcgaacagttcacagcgatg-3′	5′-ttattgctcctcctgcacttccac-3′	210

tosol for the reduction of cytosolic nitrate, resulting in down-regulation of respiratory bypass gene expression in *siz1-2* mutants. Overall, the patterns of ammonium- and nitrate-mediated changes in the alternative respiratory pathways are consistent with a role for these pathways in the maintenance of whole cell redox homeostasis during nitrogen assimilation.

The carbohydrate to nitrogen ratio plays a central and interactive role regulating post-germination growth. Carbon metabolites are critical for nitrate reduction, because carbon skeletons are required for ammonia fixation, and the energy from reduced carbon is needed to reduce nitrate. This close integration of nitrogen and carbon metabolism is essential for normal plant growth and development (27). Based on previous data showing that nitrate reductase activity and nitrogen content are low in *siz1-2* plants compared to those in wild-type plants, the carbohydrate amounts in *siz1-2* plants were assessed. The levels of free sugars such as glucose, fructose, and sucrose decreased in the leaves and roots of *siz1-2* plants (Table 4). Additionally, the amounts decreased in both the leaves and roots of wild-type and mutant plants following KNO_3 treatment, whereas the amounts increased in both parts of wild-type and *siz1-2* plants following $(NH_4)_2SO_4$ treatment. Moreover, the pattern of increase or decrease in the free sugar quantities was similar in wild-type and *siz1-2* plants, indicating that the carbon to nitrogen ratio is well maintained in *siz1-2* plants.

Table 2. Relative transcript levels of genes involved in alternative respiration in the leaves and roots of wild-type or *siz1-2* plants

	Leaf		Root	
	Wild type	*siz1-2*	Wild type	*siz1-2*
NDA2	1.00 ± 0.58	0.81 ± 0.38	1.00 ± 0.24	0.23 ± 0.16
NDB2	1.00 ± 0.28	0.72 ± 0.08	1.00 ± 0.17	0.22 ± 0.04
AOX1a	1.00 ± 0.16	0.83 ± 0.14	1.00 ± 0.11	0.22 ± 0.04
AOX1d	1.00 ± 0.15	0.54 ± 0.05	1.00 ± 0.23	0.18 ± 0.03

Numerical values represent the mean of three independent experiments and are expressed as mean ± standard deviation. The transcript levels of the genes in the mutant were normalized against the transcript levels of the genes in wild type plants. Thus, the normalized values in mutant plants indicate the relative transcript levels compared to those in wild-type plants. Values are expressed as 1.00 ± 0.00.

DISCUSSION

This study identified E3 SUMO ligase as a regulator of the alternative respiratory pathway through activation of nitrate reductase via its ligase activity.

Nitrate is a potent signaling molecule. Based on studies of a nitrate reductase double mutant, nitrate alters the transcript levels of 595 genes. For the genes investigated, nitrate signaling is wholly (*AOX1d*, *NDA2*) or partially (*AOX1a*) mediated by the nitrate ion itself (27). It was previously reported that the nitrate amount is much greater in *siz1-2* mutants than that in wild-type plants (21), suggesting that *siz1-2* mutants may re-

Table 3. Relative transcript levels of genes involved in alternative respiration in the leaves and roots of wild-type or *siz1-2* plants supplied with specific nitrogen sources

	Leaf					
	Wild type			*siz1-2*		
	K_2SO_4	KNO_3	$(NH_4)_2SO_4$	K_2SO_4	KNO_3	$(NH_4)_2SO_4$
NDA2	1.00 ± 0.52	0.31 ± 0.13	1.70 ± 0.48	1.00 ± 0.39	0.25 ± 0.10	1.16 ± 0.13
NDB2	1.00 ± 0.28	0.87 ± 0.08	1.37 ± 0.23	1.00 ± 0.16	0.84 ± 0.03	2.17 ± 0.58
AOX1a	1.00 ± 0.11	0.50 ± 0.12	1.58 ± 0.34	1.00 ± 0.22	0.85 ± 0.18	2.16 ± 0.54
AOX1d	1.00 ± 0.19	0.07 ± 0.01	1.13 ± 0.15	1.00 ± 0.10	0.11 ± 0.03	1.17 ± 0.36
	Root					
	Wild type			*siz1-2*		
	K_2SO_4	KNO_3	$(NH_4)_2SO_4$	K_2SO_4	KNO_3	$(NH_4)_2SO_4$
NDA2	1.00 ± 0.26	0.53 ± 0.12	1.20 ± 0.33	1.00 ± 0.17	0.12 ± 0.05	1.18 ± 0.16
NDB2	1.00 ± 0.21	0.88 ± 0.06	1.17 ± 0.16	1.00 ± 0.10	0.08 ± 0.03	1.10 ± 0.20
AOX1a	1.00 ± 0.18	0.86 ± 0.11	1.26 ± 0.24	1.00 ± 0.38	0.13 ± 0.03	1.38 ± 0.33
AOX1d	1.00 ± 0.08	0.66 ± 0.05	2.73 ± 0.50	1.00 ± 0.17	0.04 ± 0.01	2.20 ± 0.55

Numerical values represent the mean of three independent experiments, and values are expressed as mean ± standard deviation. The transcript levels of the genes were normalized against the transcript levels of the genes in wild-type and *siz1-2* mutant plants treated with K_2SO_4. Values are expressed as 1.00 ± 0.00.

Table 4. Levels of glucose, fructose, and sucrose in the leaves and roots of wild-type and *siz1-2* plants

	Leaf					
	Wild type			*siz1-2*		
	K_2SO_4	KNO_3	$(NH_4)_2 SO_4$	K_2SO_4	KNO_3	$(NH_4)_2 SO_4$
Fructose	2.86 ± 0.04	2.54 ± 0.07	3.24 ± 0.29	1.69 ± 0.11	1.42 ± 0.03	2.75 ± 0.25
Glucose	2.44 ± 0.08	2.20 ± 0.07	2.97 ± 0.13	1.18 ± 0.12	0.95 ± 0.15	2.47 ± 0.08
Sucrose	1.36 ± 0.05	1.23 ± 0.10	1.95 ± 0.09	0.80 ± 0.12	0.68 ± 0.15	1.17 ± 0.12
	Root					
	Wild type			*siz1-2*		
	K_2SO_4	KNO_3	$(NH_4)_2 SO_4$	K_2SO_4	KNO_3	$(NH_4)_2 SO_4$
Fructose	1.38 ± 0.06	1.16 ± 0.05	1.57 ± 0.04	0.88 ± 0.13	0.69 ± 0.07	1.36 ± 0.08
Glucose	1.24 ± 0.08	1.11 ± 0.08	1.58 ± 0.04	0.59 ± 0.12	0.50 ± 0.08	1.19 ± 0.11
Sucrose	0.88 ± 0.03	0.63 ± 0.08	0.84 ± 0.37	0.41 ± 0.08	0.37 ± 0.07	0.62 ± 0.14

Numerical values represent the mean of three independent experiments, and the values are expressed as mean ± standard deviation. The units of all numerical values are micromole of free sugar per gram of leaves or roots.

quire more reductants to reduce nitrate. Nitrate and ammonium reportedly cause changes in the expression of type II NAD(P)H dehydrogenases and alternative oxidases. For example, expression of alternative respiratory-related genes such as *AOX1a*, *AOX1d*, *AOX2*, *NDB2*, and *NDB4* is induced by ammonium but downregulated by nitrate (22). These ammonium- and nitrate-induced transcriptional changes occur in both shoot and root tissues. In addition, downregulation of a matrix-facing type II NAD(P)H dehydrogenase and several alternative oxidases following nitrate treatment results in low respiratory reoxidation of matrix NADH produced during 2-oxoglutarate synthesis. This allows the export of more reductant (NADH) to the cytosol via the malate/oxaloacetate shuttle (26). In contrast, the alternative pathways of the respiratory chain are upregulated under conditions of ammonium treatment. In other words, cytosolic NADH accumulates in the absence of nitrate reduction, resulting in activation of the ammonium-induced external type II NAD(P)H dehydrogenases and alternative oxidases. Thus, the increased capacities of alternative respiratory pathways in ammonium-grown seedlings may also result in more respiratory oxidation of matrix NADH and less export of redox equivalents to the cytosol. Results showing that more reductant must be oxidized by the respiratory chain under ammonium nutrition have also been reported for barley, wheat, maize, and pea roots (28-30).

In this study, the transcript levels of the genes encoding two type II NAD(P)H dehydrogenases and two alternative oxidases were downregulated in *siz1-2* plants (Table 1), suggesting that nitrate may accumulate in the cytosol of *siz1-2* plants. Additionally, the transcript levels of all genes increased or decreased in both wild-type and *siz1-2* plants following treatment with nitrate or ammonium, respectively (Table 3). Interestingly, the transcript levels in *siz1-2* mutants were still lower following ammonium treatment than those in wild-type plants. This implies that although the *siz1-2* mutant phenotype appears to have recovered to wild-type levels after ammonium treatment, the *siz1-2* mutant developmental systems have not completely recovered to wild-type levels (21). All of these results, including the data from this study, support the hypothesis that respiratory chain activity is altered by the nitrogen source, and that the reductant export from the mitochondria to the cytosol increases to reduce cytosolic nitrate (28, 31). Thus, it is speculated that sumoylation of nitrate reductase NIA1 and NIA2 by E3 SUMO ligase AtSIZ1 is a regulatory mechanism for the alternative respiratory pathway. This mechanism serves to reduce cytosolic nitrate and maintain reductant homeostasis in *Arabidopsis*.

Nitrogen is essential for normal seedling development, and nitrogen levels are continually evaluated or reorganized in response to the carbohydrate amount during development, indicating that nitrogen and carbohydrates interact. Interestingly, an alternative oxidase is suggested to consume excess sugar, and, thus, exert control over the carbon and nitrogen balance in cell culture (32) and in spinach leaves (33). This implies that the alternative pathway is tightly correlated with carbon and nitrogen balance. Notably, the alternative pathway-related genes including *AOX1a* were downregulated in *siz1-2* mutants. Additionally, a recent study reported that *siz1-2* mutants contain much lower nitrogen content than that of wild-type plants. The amount of free sugar was measured in *siz1-2* mutants to investigate whether AtSIZ1 contributes to maintenance of the carbon and nitrogen balance. The results showed that glucose, fructose, and sucrose contents decreased in *siz1-2* mutants,

and that the levels were down or upregulated by nitrate or ammonium treatment, respectively, in both wild-type and *siz1-2* mutant plants (Table 4). However, the carbohydrate levels in *siz1-2* mutants did not completely recover following ammonium treatment, as indicated by the phenotype reported previously (21). This result indicates that AtSIZ1 contributes to the maintenance of carbon and nitrogen levels by stimulating the activities of nitrate reductases NIA1 and NIA2.

The data presented here demonstrate that E3 SUMO ligase AtSIZ1 plays a key role modulating the alternative respiratory pathway by stimulating nitrate reductase activity. Sumoylation is also an important post-translational modification mechanism used to control the carbohydrate amount relative to the nitrogen content.

Finally, based on the above data, we tentatively suggest that the activities of alternative respiratory pathway proteins are directly regulated by E3 SUMO ligase AtSIZ1, and that the stabilities of these proteins are additionally controlled by E3 SUMO ligase activity. Therefore, further investigation of direct interactions between alternative respiratory pathway proteins and AtSIZ1 and analyses of AtSIZ1-mediated modulation of their activities will provide an answer as to whether or not AtSIZ1 directly participates in alternative respiration. It is also to be expected that an examination of mitochondrial shape and size and an evaluation of alternative respiration-related protein levels will provide clues regarding the regulatory roles of AtSIZ1 in alternative respiration.

MATERIALS AND METHODS

Plant material and culture conditions

The *A. thaliana* Columbia-0 (Col0) ecotype and *siz1-2* T-DNA insertion mutant plants were used in this study. Plants were grown in fully automated growth chambers under 16 h illumination either on 0.75% agar media [MS salts (Sigma-Aldrich, St. Louis MO, USA), 0.5 g/l Mes, and 10 g/l sucrose] or on soil (3:1 mix of Metro-Mix 200 to vermiculite; Scotts, Marysville, OH, USA). Plants were maintained at 22°C during the light period and at 20°C during the dark period. Wild type and mutant seeds were germinated and grown on MS agar media to investigate the effects of different nitrogen sources. After 4 days, the seedlings were transferred to soil, and a 5 mM solution of each K_2SO_4, KNO_3, and $(NH_4)_2SO_4$ was added at 10 day intervals. Samples were collected from the shoots or roots and frozen in liquid nitrogen until used for RT-PCR and carbohydrate measurement.

Quantitative RT-PCR analysis

The expression levels of the genes encoding alternative respiratory pathway-related proteins, alternative NAD(P)H dehydrogenase 2, alternative NAD(P)H dehydrogenase B2, alternative oxidase 1a, alternative oxidase 1d, and alternative oxidase 2 were examined by real-time RT-PCR in wild-type and *siz1-2* plants. Total RNA was isolated from shoots or roots of wild-type plants and the *siz1-2* mutant using TRIzol (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Two micrograms of total RNA were reverse-transcribed with 200 units of SuperScript TM (Invitrogen, San Diego, CA, USA). The resulting cDNA: RNA hybrids were treated with 10 units of DNase I (Roche, Waterville, OH, USA) for 30 min at 37°C, purified on a Qiaquick PCR column (Qiagen, Germantown, MD, USA), and used as templates for real-time PCR. Real-time PCR was conducted using the MyiQ RT-PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ SYBR Supermix (Bio-Rad). Amplification was monitored in real-time with the 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers for tubulin were added as an internal control together with gene-specific primers. All of the real-time PCR reactions were repeated three times using three independent RNA samples.

Carbohydrate measurement

Soluble carbohydrates were extracted with 0.6 M perchloric acid as described previously (34). The amounts of the free glucose, fructose, and sucrose were measured with a Glucose Assay kit, a Fructose Assay kit, and a Sucrose Assay kit (Sigma-Aldrich), respectively, according to the manufacturer's instructions.

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