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SLO2, a mitochondrial pentatricopeptide repeat protein affecting several RNA editing sites, is required for energy metabolism

Qiang Zhu[1,†], Jasper Dugardeyn[1,†,‡], Chunyi Zhang[1,§], Mizuki Takenaka[2], Kristina Kühn[3], Christian Craddock[4], Jan Smalle[1,¶], Michael Karampelias[1,**], Jurgen Denecke[5], Janny Peters[6], Tom Gerats[6], Axel Brennicke[2], Peter Eastmond[4], Etienne H. Meyer[3] and Dominique Van Der Straeten[1,*]

[1]*Laboratory of Functional Plant Biology, Department of Physiology, Ghent University, K L Ledeganckstraat 35, B-9000 Ghent, Belgium,*
[2]*Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany,*
[3]*Institut de Biologie Moléculaire des Plantes (IBMP) du CNRS, 12 Rue du Général Zimmer, 67084 Strasbourg Cedex, France,*
[4]*Department of Plant Sciences, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK,*
[5]*Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, Clarendon Way, Leeds LS2 9JT, UK, and*
[6]*Section of Plant Genetics, Institute for Wetland and Water Research, Faculty of Science, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands*

SUMMARY

Pentatricopeptide repeat (PPR) proteins belong to a family of approximately 450 members in Arabidopsis, of which few have been characterized. We identified loss of function alleles of *SLO2*, defective in a PPR protein belonging to the E+ subclass of the P-L-S subfamily. *slo2* mutants are characterized by retarded leaf emergence, restricted root growth, and late flowering. This phenotype is enhanced in the absence of sucrose, suggesting a defect in energy metabolism. The *slo2* growth retardation phenotypes are largely suppressed by supplying sugars or increasing light dosage or the concentration of CO_2. The SLO2 protein is localized in mitochondria. We identified four RNA editing defects and reduced editing at three sites in *slo2* mutants. The resulting amino acid changes occur in four mitochondrial proteins belonging to complex I of the electron transport chain. Both the abundance and activity of complex I are highly reduced in the *slo2* mutants, as well as the abundance of complexes III and IV. Moreover, ATP, NAD+, and sugar contents were much lower in the mutants. In contrast, the abundance of alternative oxidase was significantly enhanced. We propose that SLO2 is required for carbon energy balance in Arabidopsis by maintaining the abundance and/or activity of complexes I, III, and IV of the mitochondrial electron transport chain.

Keywords: *Arabidopsis thaliana*, growth retardation, mitochondria, mitochondrial electron transport chain, pentatricopeptide repeat, RNA editing.

INTRODUCTION

Pentatricopeptide repeat (PPR) proteins, which contain tandem arrays of a degenerate 35-amino-acid repeat, are uniquely amplified in plants (Lurin *et al.*, 2004; Andrés *et al.*, 2007; Schmitz-Linneweber and Small, 2008). In Arabidopsis, this family is composed of 450 members, and can be divided into two subfamilies based on the structure of the repeated

motif, called P and PLS subfamilies. Members of the P subfamily contain the canonical P motif common to all eukaryotes, while members of the PLS subfamily contain the P motif, as well as two P motif-derived variants, the short (S) and the long (L) motifs. Based on the presence of conserved domains in the C-terminal region, the PLS subfamily can be

further divided into the PLS, E, E+, and DYW subgroups (Lurin *et al.*, 2004; Andrés *et al.*, 2007). In plants, PPR proteins are predominantly localized in plastids (19%) or mitochondria (54%) (Lurin *et al.*, 2004). So far only one nuclear PPR protein and one dual-targeted protein have been identified (Ding *et al.*, 2006; Hammani *et al.*, 2011b). Given the number and slight variation of sequence repeats, PPR proteins were proposed to function as gene-specific regulators of plant RNA metabolism (Lurin *et al.*, 2004). In plant cell organelles, PPR proteins mainly play roles in RNA stability, cleavage, splicing, and editing; while also being involved in translational initiation and regulation (Andrés *et al.*, 2007; Schmitz-Linneweber and Small, 2008). Some PPR proteins appear to be essential for plant growth and development, as supported by the embryo lethality or severe growth defects associated with loss-of-function mutants (de Longevialle *et al.*, 2007; Liu *et al.*, 2010; Sung *et al.*, 2010). Many PPR proteins function in RNA editing (Schmitz-Linneweber and Small, 2008). The biochemical effect of RNA editing in plants is most often a site-specific C-to-U modification by cytosine deamination (Shikanai, 2006). To date, all but one of the PPR proteins involved in plastid or mitochondrial RNA editing belong to the E and DYW subgroups of the PLS subfamily; the exception is PPR596, which is in the P class (Doniwa *et al.*, 2010; Takenaka, 2010). In the plastid transcriptome, there are 34 editing sites (Tsudzuki *et al.*, 2001), while in mitochondria this number exceeds 500 (Giegé and Brennicke, 1999). In plastids, members of the E and the DYW class are implicated in RNA editing (Schmitz-Linneweber and Small, 2008; Yu *et al.*, 2009). In Arabidopsis and rice mitochondria, 14 RNA editing factors have been reported so far (Kim *et al.*, 2009; Zehrmann *et al.*, 2009; Sung *et al.*, 2010; Takenaka, 2010; Takenaka *et al.*, 2010; Tang *et al.*, 2010; Verbitskiy *et al.*, 2010; Hammani *et al.*, 2011a; Yuan and Liu, 2012).

Energy metabolism in plant cells encompasses mitochondria, plastids, and peroxisomes. Mitochondria are the main energy factories of the cell, performing oxidative phosphorylation driven by the electron transport chain (ETC). The electron transport chain of the cytochrome (classical) pathway is composed of four large complexes: complex I, complex II, complex III, and complex IV. Complex I, a NADH dehydrogenase, is the first protein complex in the electron transport chain, and catalyzes NADH oxidation while ubiquinone (UQ) is reduced; complex II is the only enzyme that participates in both the citric acid cycle and the electron transport chain; complex III is also known as cytochrome *c* reductase, and oxidizes ubiquinol while reducing cytochrome *c*. Complex IV is a cytochrome *c* oxidase, the terminal oxidase of the classical ETC. Complex I is composed of at least 49 subunits in Arabidopsis (Klodmann *et al.*, 2010), the majority of which are encoded by nuclear genes. Dysfunction in complex I results in various phenotypes, such as increased respira-

tion and decreased photosynthetic efficiency, thereby causing growth and developmental defects (Gutierres *et al.*, 1997; Brangeon *et al.*, 2000; Sabar *et al.*, 2000; Pineau *et al.*, 2005; Garmier *et al.*, 2008; Keren *et al.*, 2009; Meyer *et al.*, 2009; Liu *et al.*, 2010; Sung *et al.*, 2010); or changes in stress resistance (Sugioka *et al.*, 1988; Sabar *et al.*, 2000; Dutilleul *et al.*, 2003; Meyer *et al.*, 2009; Yuan and Liu, 2012). With the exception of apocytochrome B, which is encoded by the mitochondrial genome, complex III subunits are all encoded by the nuclear genome (Unseld *et al.*, 1997). Mutational analysis demonstrated that the ubiquinol-cytochrome *c* oxidoreductase activity of complex III is important for normal plant growth and stress response (des Francs-Small *et al.*, 2012). Complex IV is the terminal complex of the respiratory chain, composed of around 12–13 subunits, while complex V is the ATP synthase complex, which comprises around 15 distinct subunits (Dudkina *et al.*, 2006). Besides the classical cytochrome pathway, plants also possess alternative NAD(P)H dehydrogenases in the first part of the respiratory chain and an alternative oxidase (AOX) in the latter (Millar *et al.*, 2011). *AOX* can be induced by environmental stress or factors that inhibit the respiratory chain (Millar *et al.*, 2001).

In this study, we characterize a PPR protein, named SLOW GROWTH 2 (SLO2). In *slo2* mutants, seven editing changes were found which lead to four amino acid changes in subunits of complex I of the mitochondrial ETC, leading to a reduction of abundance and activity of complex I. Moreover, the abundance of complex III and complex IV were also reduced. We further demonstrate that *SLO2* plays a role in carbon and energy metabolism. To our knowledge, this is the only example of a single gene mutation leading to defects in three mitochondrial complexes.

RESULTS

Isolation of the *slo2-1* mutant

We previously demonstrated that the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) enhances leaf emergence on low-nutrient medium (LNM) (Smalle and Van Der Straeten, 1997). In search for ethylene mutants based on this observation, we identified the *slo2-1* mutant as one of the candidates by screening of a collection of 5000 T-DNA insertion lines (Feldmann, 1991) (Figure 1a,b). However, the subsequent characterization of *slo2-1* revealed only partial changes in ethylene sensitivity or biosynthesis. In addition to the delayed leaf emergence of *slo2-1*, a delay at various stages throughout the life cycle was observed compared with the wild type (Table S1). Due to this general developmental delay the mutant was named *slow growth 2-1* (*slo2-1*). The *slo2-1* mutation segregates according to Mendelian law of genetics, and is recessive – thus being a loss-of-function mutation. The *slo2-1* mutant was crossed (four
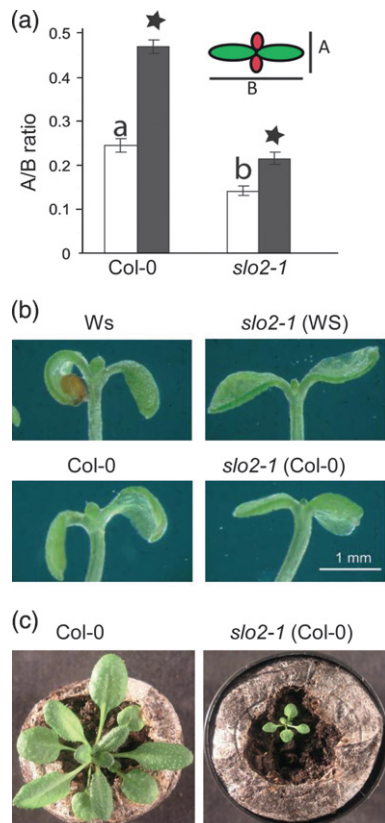
Figure 1. The *slo2-1* mutant shows reduced emergence of the first leaf pair.

(a) Degree of leaf emergence (DLE; A/B) in 2-week-old seedlings grown on low-nutrient medium (LNM) in the presence (black) or absence (white) of 50 μM 1-aminocyclopropane-1-carboxylic acid. Stars reflect a significant difference between treated and control plants ($P < 0.01$). Error bars represent standard error ($n = 50$).

(b) The *slo2-1* mutant displays a delayed leaf emergence. Close-up of leaf emergence of vertically grown 2-week-old Ws (wild type), *slo2-1* in Ws background, Col-0 (wild type) and *slo2-1* in Col-0 background. Seedlings were grown on LNM.

(c) Rosette phenotype of 3-week-old plants grown in soil.

times) into the Col-0 background, and phenotypic characteristics were preserved in both early and later development (Figure 1).

Map-based cloning of the *SLO2* gene

To uncover the molecular nature of the *slo2-1* mutation, we cloned the *SLO2* gene using a map-based cloning approach, since the mutation was not linked with the kanamycin resistance gene in the T-DNA. Details of the procedure are provided as Supporting Information. The SLO2 gene encodes a putative PPR protein with unknown function (*At2g13600*), The *slo2-1* mutant contains an in-frame deletion of 21-bp. We identified a second mutant allele, designated *slo2-2,* in the SALK SIGNAL collection, *SALK_521900*, containing a T-DNA insertion at 878 bp downstream of the start codon (Figure 2b). *slo2-2* shows an evident similarity to the *slo2-1* mutant phenotype albeit less pronounced (Fig-

ure 2c). A third allele, *slo2-3* (Tilling T94087), harbors an ethyl methanesulfonate (EMS)-induced point mutation (C to T) at position 247 downstream of ATG, causing a stop codon at amino acid 83 (Figure 2b). This mutation resulted in the strongest *slo2* phenotype (Figure 2c,d).

SLO2 encodes a member of the pentatricopeptide repeat protein family

Basic Local Alignment Search Tool (BLAST) analysis identified *SLO2* as a member of the PPR family, belonging to the P-L-S subfamily. It consists of seven PPR-like S, five PPR-like L, and four canonical P motifs with an E and E+ C-terminal extension (Lurin *et al.*, 2004) (Figure 2b). Unlike most plant PPR proteins, targeting prediction programs suggest inconclusive results on the subcellular localization of SLO2. A BLAST search of the full nucleotide sequence of *SLO2* against the complete Arabidopsis genome database did not result in closely similar genes. Given the low similarity of SLO2 with other PPR proteins and the evident phenotype exhibited by the *slo2* alleles, we conclude that *SLO2* encodes a unique PPR protein in Arabidopsis and therefore probably carries unique functions.

Ectopic expression of *SLO2* complements the growth defects of *slo2* mutants

To further confirm that the phenotypes of *slo2* mutants were caused by a mutation in the *At2g13600* locus, we performed complementation experiments for *slo2-1* and *slo2-2* using the full-length cDNA fused to GFP driven by its native promoter (*PSLO2:SLO2-GFP*) and the CaMV-35S promoter (*P35S:SLO2-GFP*), respectively. Both complementation lines showed reversal of the delay in leaf emergence and suppressed the reduced growth phenotypes in root and shoot (Figure 3a–d). The data above confirm that mutation of the *SLO2* gene is responsible for the mutant phenotype.

SLO2 protein is localized in mitochondria

To unequivocally determine the subcellular localization of SLO2, *P35S:SLO2-GFP* seedlings were stained with the mitochondrion-specific marker MitoTracker Orange, and analysed with a confocal microscope. The results show that the fusion protein co-localizes with the mitochondrial marker (Figure 4a), suggesting a mitochondrial localization for SLO2.

To corroborate these results, we crossed the mitochondrial marker line (ATPase-mCherry) with *P35S:SLO2-GFP* transgenic plants, and surveyed the F_1 progeny using confocal microscopy. The data confirmed that SLO2 resides in plant mitochondria (Figure 4b).

slo2 mutants are hypersensitive to sucrose

While lower levels of metabolizable sugars can stimulate seedling growth, high sugar concentrations have inhibitory effects (Rolland *et al.*, 2002). On a medium without
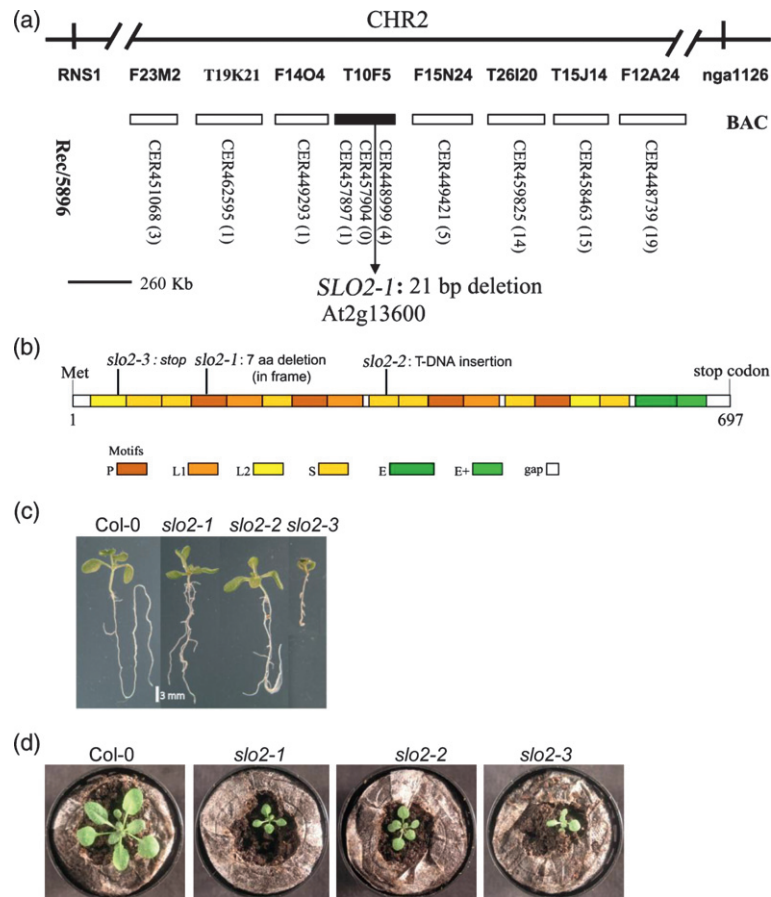
Figure 2. Map-based cloning of the *SLO2* gene.
(a) Scheme of the map-based cloning of the *SLO2* gene, detailed in supplementary data. The *slo2-1* mutation was mapped between InDel (Insertion-Deletion) markers CER457897 and CER448999 on BAC clone T10F5 on chromosome 2 (http://www.arabidopsis.org/Cereon/). The number of recombinants found in 5896 DNA samples is shown. Between BAC F23M2 and F12A24 are 2606499 nucleotides. The scale bar reflects the total length of the depicted part of chromosome 2 but not that of individual BAC clones.
(b) Schematic representation of SLO2 protein motif structure and position of different *slo2* mutations. Using the HMMER package, Lurin *et al.* (2004) presented PPR_2_5619962 as 17-L2-S-S-P-L1-S-P-L1-1-S-S-P-L1-6-S-P-L2-S-4-E-E+-28. Intervening figures give the number of amino acids in gaps between the detected motifs. The amino acid positions of the PPR and PPR-like domains in SLO2 are as follows, PPR:116th-150th, 217th-251st, 350th-384th, 457th-491st; S: 54th-84th, 85th-115th, 186th-216th, 288th-318th, 319th-349th, 426th-456th, 528th-558th; L1: 151st-185th, 252nd-286th, 385th-419th; L2: 18th-53rd, 492nd-527th; E:563rd-638th; E+: 639th-669th. The *slo2-1* mutant contains a 21-bp deletion in the first predicted PPR domain (127th-133rd amino acids). The *slo2-2* allele carries a T-DNA insertion in the fourth S domain 878 bp downstream of the start codon of SLO2-1 gene. The TILLING line *slo2-3* introduces a stop codon 247 bp downstream of the start codon. The mutation sites are indicated.
(c) Phenotypes of vertically grown 2-week-old wild-type seedlings and three alleles of *slo2*.
(d) Phenotypes of 3-week-old wild-type and mutant plants grown on soil.

sucrose, *slo2* mutants showed a severe growth arrest (Figure 5a). However, the growth stimulation induced by 1% sucrose was remarkably higher in *slo2* alleles than in the wild type (Figure 5a,b). Moreover, early post-embryonic growth was more strongly inhibited by 7% sucrose in *slo2* mutants (Figure 5a,b). To exclude an osmotic effect of sucrose, the response of *slo2* to mannitol and sorbitol, two non-metabolizable sugars, was tested. No difference in early post-germination development was observed. In addition, germination of *slo2* was delayed on sucrose in a dose-dependent manner, this effect being significantly stronger than in Col-0 (Figure 5c). Glucose

and fructose also play a positive role in the post-germination growth of *slo2*, albeit to a lesser extent than sucrose (Figure S1).

We also tested the changes in *SLO2* gene expression in response to sucrose in seedlings grown in the light and in darkness. As evidenced by *in situ* hybridization, the expression is enhanced by 1% sucrose in both conditions (Figure 5d). These results were corroborated by GUS histochemical analysis (Figure 5e).

To obtain additional molecular evidence for the hypersensitivity of *slo2* to sucrose, we analyzed the expression levels of two sugar-repressed genes, ribulose-1,5-bisphos-
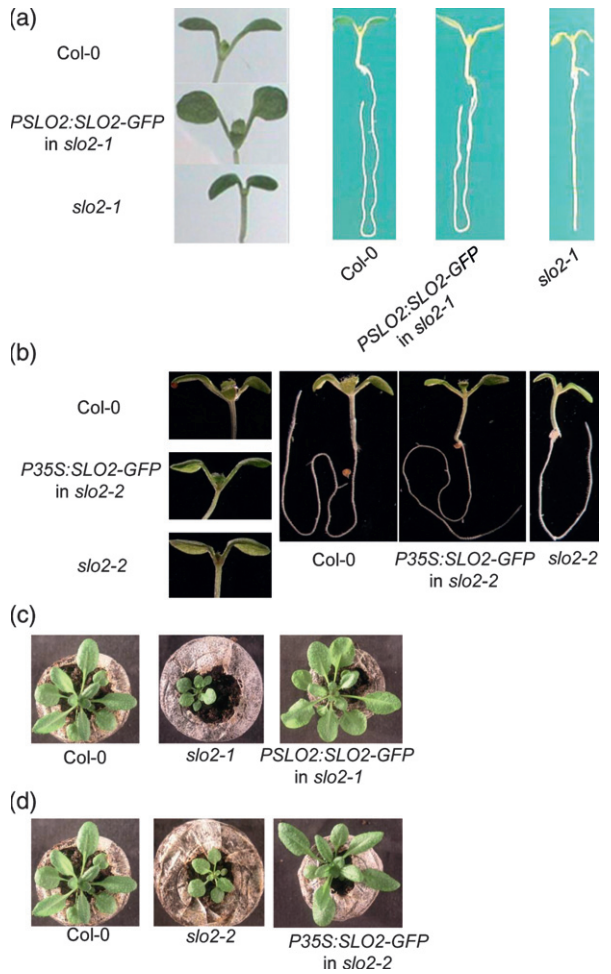
Figure 3. Complementation of *slo2* alleles with the wild-type *SLO2* gene.
(a) Complementation of the *slo2-1* seedling phenotype using the *SLO2* native promoter. Phenotype of Col-0, *slo2-1* complemented with *PSLO2:SLO2-GFP* and *slo2-1*. Left: close-up of the shoot. Right: 7-day-old seedlings.
(b) Complementation of the *slo2-2* seedling phenotype using the CaMV35S promoter.
(c) Phenotype of Col-0, *slo2-2* complemented with *P35S:SLO2-GFP* and *slo2-2*. Left: close-up of the shoot. Right: 7-day old seedlings.
(d) Four-week-old plants of wild-type, *slo2-1*, and *slo2-1/PSLO2:SLO2-GFP* complemented lines.
(e) Four-week-old plants of wild-type, *slo2-2*, and *slo2-2/P35S:SLO2-GFP* complemented lines.

phate carboxylase small subunit (*RBCS*) (Cheng *et al*., 1992; Sheen, 1994) and plastocyanin (*PC*) (Zhou *et al*., 1998), and one sugar-induced gene, chalcone synthase (*CHS*) (Nemeth *et al*., 1998) by quantitative RT-PCR. In the presence of 1% sucrose, the messenger levels of *RBCS* and *PC* in *slo2-1* were similar to that in Col-0; however, on medium containing 7% of sucrose, both genes were significantly more strongly down-regulated in the mutant than in Col-0 (Figure 5f). In addition, induction of transcription of the HXK-independent sugar-induced *CHS* gene (Sheen *et al*., 1999) was completely abolished in *slo2* (Figure 5f). The results indicate that *SLO2* has strong effects on sugar signaling.
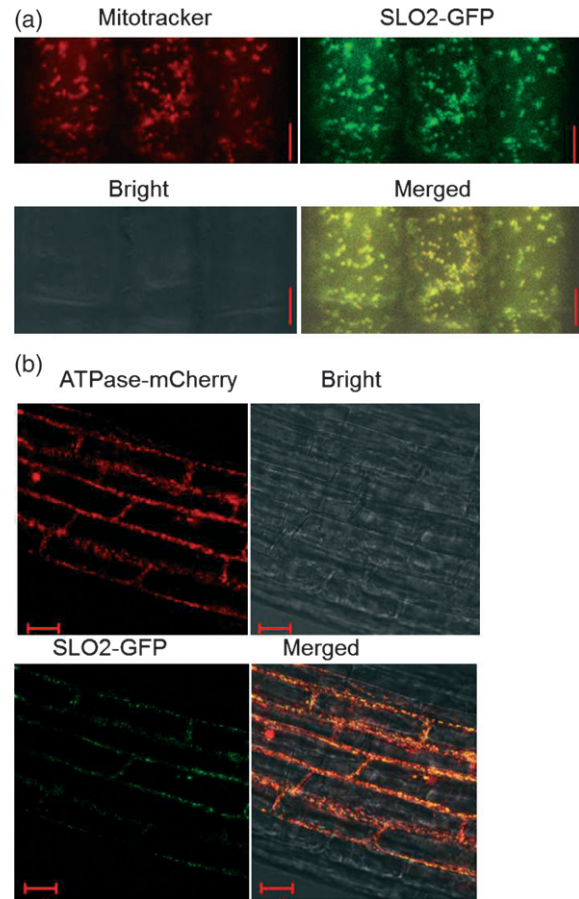

Figure 4. SLO2 protein is localized in mitochondria.
(a) The root of transgenic plants carrying the *P35S:SLO2-GFP* construct were stained with MitoTracker Orange, and observed under a confocal microscope. Red: MitoTracker Orange; green: GFP; bright and merged field.
(b) The transgenic line carrying *P35S:SLO2-GFP* was crossed with a mito-chondrial marker line carrying ATPase-mCherry. The root of F_1 individuals was observed using a confocal microscope. Red: mCherry; green: GFP; bright and merged field.

The *slo2* phenotype is dependent on the carbon status

Since the *slo2* mutant phenotype is affected by external sugars, we hypothesized that the slow growth of *slo2* may be directly linked to a disturbance of carbon/energy metabolism. We used three methods to test this point. First, we tested the effect of CO_2 fertilization on the growth of *slo2* mutants. Rubisco (RBC), which is a dual-activity enzyme, is the main entrance for carbon into organic matter. The RBC carboxylation reaction occurs under high CO_2 and low O_2 conditions, while its oxygenation needs a high O_2 concentration (Holland, 2006). The *slo2* mutant phenotype was largely recovered under high CO_2 conditions (3000 p.p.m.), reflected by the plant size (Figure 6a). Furthermore, CO_2 fertilization partially complemented the developmental delay in *slo2*. Under control conditions (450 p.p.m. CO_2), *slo2-1* shows a developmental delay of 36 days compared to
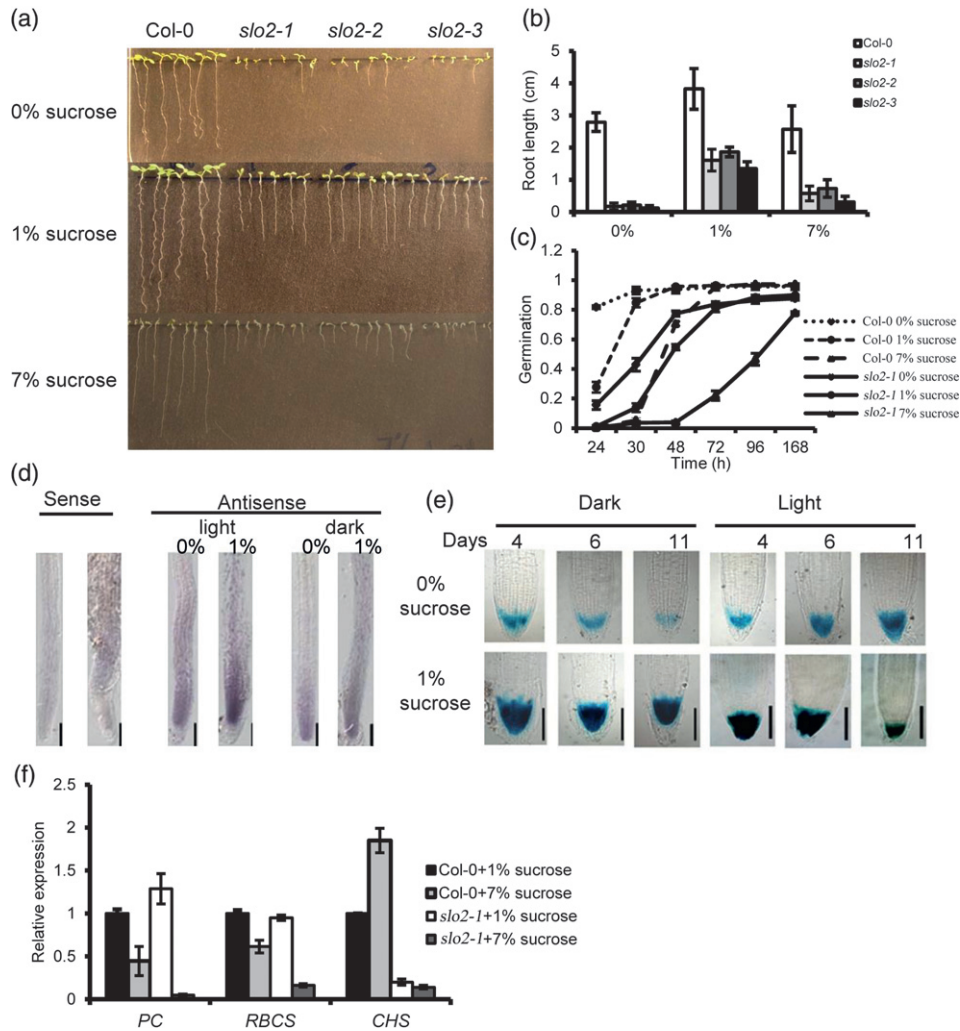
Figure 5. *slo2* mutants are hypersensitive to sucrose.

(a) Effect of sucrose on *slo2* mutants. Seeds of the wild type and three *slo2* alleles were sown on ½ MS medium containing different concentrations of sucrose: top 0%, middle 1%, bottom 7% sucrose. Pictures were taken after 7 days.

(b) Statistic analysis of root length from (a).

(c) Delay in seed germination caused by sucrose is enhanced in *slo2-1* mutant. Data are averages ± SEM (*n* = 3). Dotted lines represent the wild type; full lines represent *slo2-1*. Symbols: diamonds correspond to 0% sucrose, dots to 1% sucrose and triangles to 7% sucrose.

(d) Whole mount *in situ* hybridization for *SLO2* transcripts in roots. Six-day old individuals were hybridized with sense and antisense probes. Bar = 100 μm.

(e) *SLO2* promoter-driven expression of the GUS reporter gene in roots. Bar = 100 μm.

(f) Effect of the *slo2* mutation on sugar-related gene expression. Expression of plastocyanin (*PC*), ribulose-1,5-bisphosphate carboxylase small subunit (*RBCS*) and chalcone synthase (*CHS*) in 6-day-old etiolated wild-type (Col-0) and *slo2-1* seedlings relative to the expression in the wild type in the presence of 1% sucrose, obtained by quantitative PCR. Seedlings were grown on ½ MS medium containing 1 or 7% sucrose. To compensate for germination delay, *slo2-1* on 1% sucrose and wild type on 7% sucrose were sown 1 day earlier; *slo2-1* on 7% sucrose was sown 2 days earlier than the wild type on 1% sucrose.

Col-0 (Figure 6b, Table S1). Both genotypes take advantage of CO_2 fertilization (1700 p.p.m.), but the gap between them was reduced to 10 days only (Figure 6b). The same trend was noticed on the number of rosette leaves at initiation of flowering, proving that the restoration of flowering time is due to faster development (Figure 6c).

Secondly, we tested whether addition of an external carbon source could compensate for the reduced carbon fixation or higher loss of CO_2 in *slo2* mutants. In the presence of sucrose, *slo2* alleles grew normally and could complete their life cycle (Figure 6d, e). In the absence of sucrose, at least 80% of the Col-0 plants and two complemented lines could complete their life cycle, while in the case of *slo2* mutants, this ratio is reduced to 0–12% depending on the allele (Figure 6e). These data support the fact that SLO2 is essential for completion of the life cycle in the absence of sucrose. A similar beneficial effect of sucrose (1%) addition was seen on leaf emergence and root growth of mutant plants (six-fold increase in root length for *slo2*; 2.5-fold for Col-0).
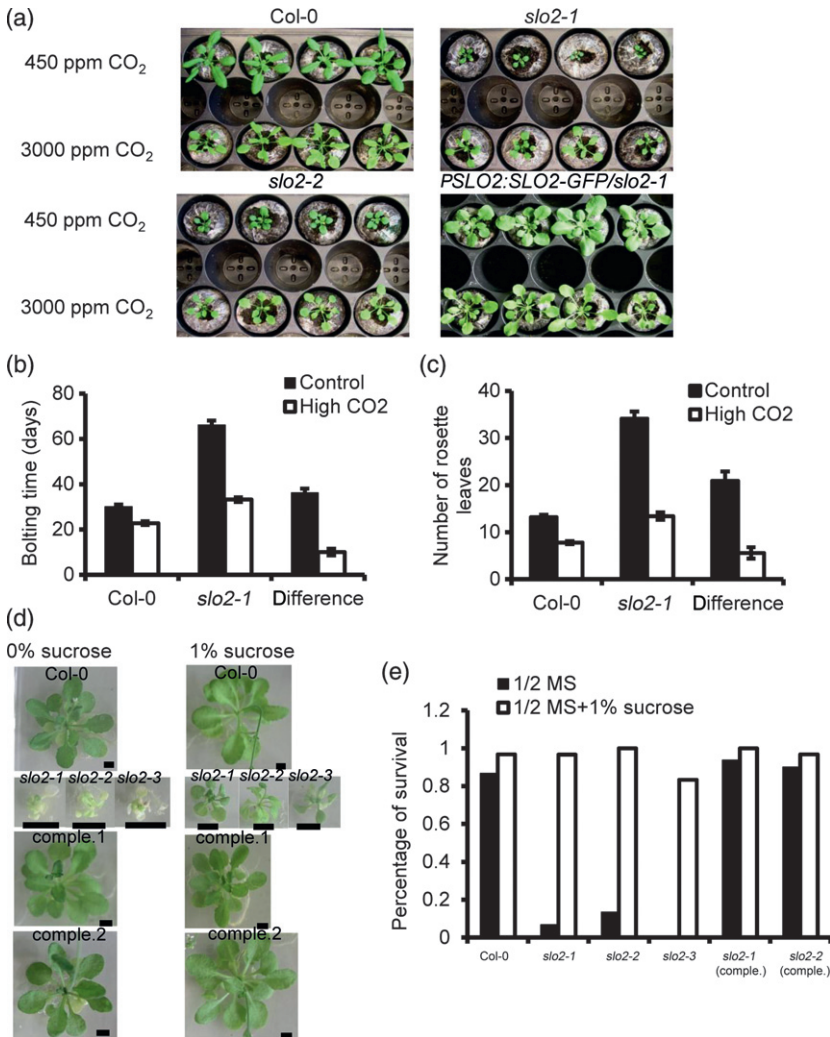
Figure 6. *SLO2* is involved in carbon and energy metabolism.

(a) The effect of elevated CO_2 on *slo2* rosette. Seven-day old seedlings were transferred into soil, and grown in atmosphere conditions or with 3000 p.p.m. CO_2. Pictures were taken 1 month later.

(b) Elevated CO_2 stimulates flowering in *slo2*. Figure shows the bolting time of wild-type and *slo2-1* under normal and elevated CO_2 concentrations. Elevated CO_2 concentrations (white bars, 1700 p.p.m. CO_2) have a stronger stimulatory effect on the mean bolting time in *slo2-1* than in the wild type compared with control conditions (black bars, 450 p.p.m. CO_2). Stars reflect a significant difference ($P < 0.01$) between Col-0 and *slo2-1* in the given condition. 'Difference' reflects the difference between the bolting time of the wild type and *slo2-1* in the respective conditions. Squares reflect a significant difference ($P < 0.01$) between the two conditions. Error bars represent standard error ($n = 15$).

(c) Elevated CO_2 stimulates flowering in *slo2*. Figure shows the number of rosette leaves upon bolting in the wild type and *slo2-1*. Conditions and calculations are identical to (a).

(d, e) *slo2* mutants cannot complete their life cycle in the absence of an external carbon source. Plants were grown on ½ MS + 1% sucrose (white bars) or in the absence of sucrose (black bars) until flowering. The percentage of plants that could complete their life cycle is shown. (e) Phenotype of Col-0, 3 *slo2* mutants and two complemented lines on 0 and 1% sucrose. Scale bar corresponds to 1 cm.

A third way of investigating the effect of alteration of the carbon status on the mutant was by growing the plants under different light intensities and photoperiods. Plants were grown under five different light conditions and their bolting time was assessed (Table S2). An increase in light dose (long days, high light) accelerates the growth and development of *slo2* mutants, the strongest positive effect being under continuous light. Overall, we conclude that in *slo2* mutants, addition of an external carbon source (CO_2 or sucrose) or an increased light dose largely compensates for growth retardation.
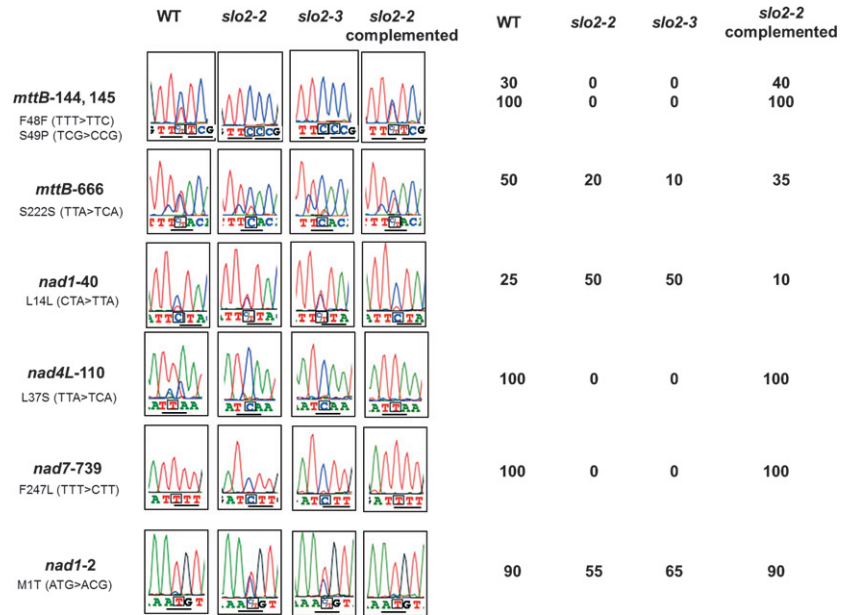
RNA editing changes in the *slo2* mutants

Since *SLO2* encodes an E-type PPR protein, we tested the possibility that SLO2 might be involved in RNA editing. A multiplexed SNaPshot approach, monitoring 315 mitochondrial editing sites was used (Takenaka and Brennicke, 2009). The screen identified several editing sites affected in mitochondria of *slo2* mutants. One of these, the C to U editing at position *nad4L*-110, was not detectable in

mutant alleles (Figure 7). This editing event leads to a serine to leucine amino acid change (S37L) in the predicted NAD4L subunit protein of the NADH dehydrogenase (complex I). The editing deficiency of the mutant allele is restored in complemented *slo2-2* (containing *P35S:SLO2-GFP*; Figure 7).

All other editing defects were likewise observed similarly in both examined *slo2* alleles, such as the two adjacent Cs in *mttB* (membrane targeting and translocation or *orfx*) at positions *mttB*-144 and *mttB*-145. The editing event at nucleotide 144 does not change the predicted amino acid (phenylalanine). As often observed for silent editing events, this site is edited to only about 50% in the steady-state mRNA population in Col-0 plants. In both mutant alleles, editing at this site is not detectable. At nucleotide 145, editing changes the predicted amino acid residue from proline to serine (P49S) in the entire population of steady-state transcripts. Like the preceding nucleotide, editing at this nucleotide is also lost in plants homozygous for either of the two mutant *slo2* alleles (Figure 7). A third editing change

Figure 7. SLO2 is involved in multiple mitochondrial RNA editing events.

RNA editing changes were detected using a multiplexed SNaPshot approach; the editing sites of the respective genes and the amino acid changes are listed. Sequencing chromatograms are displayed. The codons containing the editing sites are underlined, and the editing sites are marked with a square. The RNA editing efficiencies are shown on the right.

	WT	slo2-2	slo2-3	slo2-2 complemented	WT	slo2-2	slo2-3	slo2-2 complemented
mttB-144, 145 F48F (TTT>TTC) S49P (TCG>CCG)					30 100	0 0	0 0	40 100
mttB-666 S222S (TTA>TCA)					50	20	10	35
nad1-40 L14L (CTA>TTA)					25	50	50	10
nad4L-110 L37S (TTA>TCA)					100	0	0	100
nad7-739 F247L (TTT>CTT)					100	0	0	100
nad1-2 M1T (ATG>ACG)					90	55	65	90

occurs in *nad7* at site 739, the editing at this site leading to an amino acid change from leucine to phenylalanine. NAD7 is essential for accumulation of complex I (Pineau *et al.*, 2005). The restoration at these sites in the complemented line confirms that SLO2 plays an essential role in these editing events.

We also noticed some changes in editing efficiency in several mRNAs, most of which lower the level of steady-state editing in *slo2* mutants such as in the *mttB* RNA at site 666 (50 and 35% in wild-type and complemented line versus 20 and 10% in the *slo2-2* and *slo2-3* mutants, respectively) and *nad1* at site 2 (90% in the wild-type and complemented line versus about 60% in the mutants) (Figure 7). In one instance, an increase in the level of editing was observed, namely in *nad1* at site 40 (25 and 15% in wild-type and complemented line versus more than 50% in the mutants) (Figure 7). Among those sites with altered editing efficiency, some are silent, such as *mttB666* and *nad1-40*, while *nad1-2* is non-silent, with an amino acid substitution from threonine to methionine. Our results clearly show that the SLO2 protein is a mitochondrial RNA editing factor involved in several editing events.

Complex I, complex III, and complex IV are reduced in *slo2* mutants

The proteins NAD1, NAD7, and NAD4L are components of the mitochondrial complex I, which may play a role in its assembly. A previous report showed that the absence of NAD7 directly leads to the lack of complex I in the CMSII mutant (Pineau *et al.*, 2005). As we find RNA editing changes in these genes, which lead to amino acid changes, we speculate that RNA editing defects in those sites may lead to mitochondrial dysfunction in *slo2* mutants. To test this

hypothesis, we isolated mitochondrial membrane complexes from rosette leaves of Col-0, three *slo2* alleles, and the *slo2-1* complemented line and subjected these to blue native PAGE analysis. The result demonstrates that the abundance of complex I is highly reduced in all three *slo2* mutants compared with Col-0, and that this reduction is restored in the complemented line (Figure 8a). In-gel activity staining of NADH dehydrogenase further indicated a strong reduction in complex I activity (Figure 8b). To investigate whether other complexes of the electron transport chain are affected in the mutants, we performed western blot using antibodies against subunits of complexes III (cytochrome *c1*), IV (COX II), and V (α-ATPase). The results clearly showed that the levels of complex III and complex IV were much reduced in the mutants, while being partially restored in the complemented line (Figure 8c). Moreover, western blot results from SDS-PAGE gel were consistent with the immunodetection results from blue native gel electrophoresis (Figure 8e). Overall, our data indicate that SLO2 is necessary to sustain a proper level of complexes I, III, and IV in mitochondria.

slo2 mutants contain less ATP/ADP, NAD$^+$ and sugars

In plants, deficiency of complexes I and III leads to diminished proton translocation and a lower phosphorylation efficiency. The major energy source in living cells is ATP; lack of ATP obviously has negative effects on plant growth and development. Based on the stunted growth of the mutants and the observed deficiency in mitochondrial electron transfer, we speculated that *slo2* mutants contain reduced energy pools. To test this hypothesis, we measured the ATP and ADP levels in Col-0, *slo2* mutants and in the complemented *slo2-2* line. A significant reduction in ATP, ADP and the ATP/ADP ratio is seen in *slo2* mutants, while in
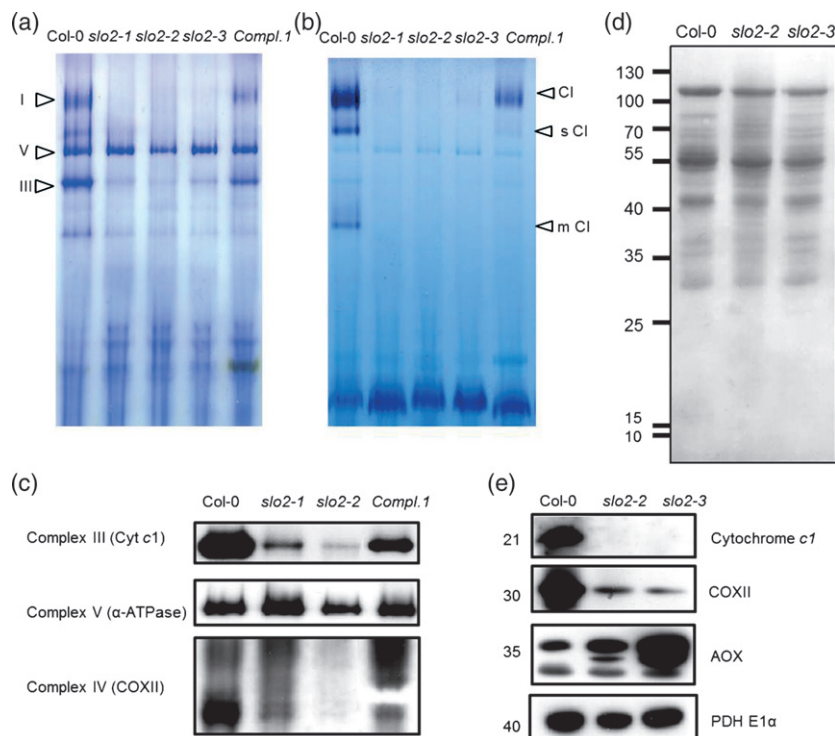
Figure 8. Complex I, complex III, and complex IV are affected in the *slo2* mutants.
(a) Coomassie-stained blue native gel of mitochondrial membrane complexes from Col-0, three *slo2* mutants and the *slo2-1* complemented line: I, complex I; V, complex V; III, complex III.
(b) Gel stained for NADH dehydrogenase activity prepared as in (a): CI, complex I; s CI, smaller version of complex I; m CI, matrix arm of complex I.
(c) Western blot analysis of complex III, complex IV, and complex V.
(d) The SDS-PAGE analysis of mitochondrial fractions from Col-0, *slo2-2*, and *slo2-3*.
(e) Western blot analysis of cytochrome c_1, COXII, and AOX. PDH E1α is used as a loading control. The molecular weight is indicated on the left.

the complemented line the levels were reverted to that in Col-0 (Figure 9). This suggests that *SLO2* is necessary to maintain the normal energy pool.

The pyridine nucleotides NAD+ and NADH act as primary redox carriers in metabolism, and the balance of NADH/NAD+ is critical for central redox control and to prevent the generation of reactive oxygen species (ROS) (Shen *et al.*, 2006). In *slo2* mutants, a significantly higher NADH content and concomitantly decreased NAD+ content were observed compared with that in Col-0 and the complemented *slo2-2* line (Figure 9). Consequently, the NADH/NAD+ ratios of the mutants are much higher than that in Col-0. Thus, *SLO2* is necessary for maintaining steady-state cellular NADH/NAD+ ratios.

During seed germination and seedling establishment, the storage reserves will be utilized. For example, triacylglycerides (TAG) will break down into sugars (such as sucrose, glucose, and fructose), which act as the main carbon and energy resources. We noticed that in 3-day-old *slo2* mutant seedlings the levels of sucrose, glucose, and fructose are much reduced compared with Col-0, while these changes were reversed in the complemented line (Figure 9). Our data suggest that *slo2* mutants contain fewer fuel sources for germination and seedling establishment.

DISCUSSION

In this work we have characterized the PPR protein SLO2 which plays a role in several mitochondrial RNA editing events, affecting complexes I, III, and IV, and as a result, the

cellular energy and carbon status. Physiological and molecular analysis of three *slo2* alleles indicates that *SLO2* is important for normal plant growth and sugar response. Given that the *slo2-1* allele carries a deletion of seven amino acids in the first P domain, the latter may play an important role through stabilizing the SLO2 protein or by specifically interacting with part of the nucleotide recognition sequence in either target RNAs or an essential protein interactor.

SLO2 is involved in multiple RNA editing events in mitochondria

Despite the lack of a recognizable mitochondrial targeting domain, the mature SLO2 protein is located in mitochondria and plays a specific role in mitochondrial RNA editing. To date, 14 RNA editing factors have been identified. Seven factors are involved in RNA editing of single sites (Takenaka, 2010; Takenaka *et al.*, 2010; Verbitskiy *et al.*, 2011; Yuan and Liu, 2012), five have two to four changes in editing in Arabidopsis (Zehrmann *et al.*, 2009; Bentolila *et al.*, 2010; Sung *et al.*, 2010; Verbitskiy *et al.*, 2010; Hammani *et al.*, 2011a), while in rice OGR1 controls seven specific editing sites on five distinct mitochondrial transcripts (Kim *et al.*, 2009). Our analysis demonstrates that SLO2 is involved in seven specific editing events on four distinct mitochondrial transcripts. As far as we know, this is the highest number of RNA editing changes ever identified in a single mutant in Arabidopsis. Interestingly, most of the identified mitochondrial editing factor (MEF) mutants do not exhibit obvious phenotypes under normal conditions (Zehrmann *et al.*, 2009;

Figure 9. Metabolic changes in *slo2* mutants. Five-day-old seedlings grown on ½ MS medium containing 1% sucrose were used for ATP, ADP, NAD$^+$, and NADH analysis. Three-day-old seedlings grown on ½ MS medium were used for sugar content analysis. Values are the mean ± SE of measurements on four repeats of 100 seedlings each.
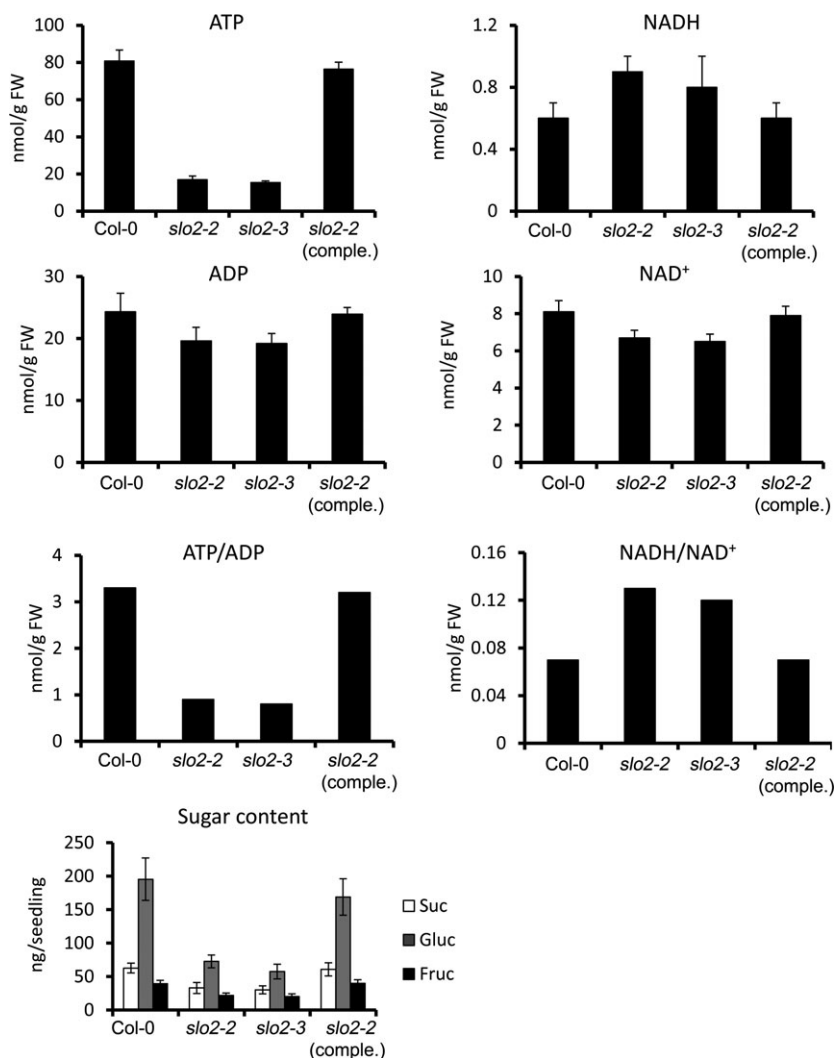
Takenaka *et al.*, 2010). In Arabidopsis, until now only three MEF mutants have been reported to show growth retardation: *slo1*, *otp 87*, and *slg 1* (Sung *et al.*, 2010; Hammani *et al.*, 2011a; Yuan and Liu, 2012). *slo2* mutants show growth retardation comparable to these three mutants, but have more editing defects. The editing changes in *slo2* result in amino acid changes in NAD4L (S37L), NAD1 (T1M), NAD7 (L247F) and MTTB (P49S), which are all, with the exception of MTTB, important components of the NADH dehydrogenase complex I in the mitochondrial membrane. The amino acid changes resulting from RNA editing defects have a strong impact on the abundance and function of complex I (Figure 8). Furthermore, our results clearly support a reduction of complex III and complex IV in *slo2* mutants (Figure 8), concomitant with a higher accumulation of AOX (Figure 8e). Hence, we propose that these RNA editing defects result in the dysfunction of mitochondrial electron transfer chain complexes, contributing to the observed *slo2* phenotype.

SLO2 plays a unique role in the mitochondrial electron transfer chain

As stated above, defects in *slo2* probably result in the dysfunction of mitochondrial complexes I, III, and IV, affecting mitochondrial electron transport. The question remains how a mutation in the *SLO2* gene can affect the other complexes of the mitochondrial ETC. We propose several possibilities. First, the possibility that more RNA editing defects may exist in *slo2* mutants cannot be completely ruled out. In mitochondria, the number of RNA editing sites exceeds 500 (Giegé and Brennicke, 1999), while our multiplexed SNaPshot analysis monitored 315 mitochondrial editing sites. It is therefore reasonable to assume that a mutation in *slo2* may affect RNA editing of components of complexes III and IV, thereby leading to the dysfunction of these complexes. However, combining the results from SNaPshot analysis and cDNA sequencing (for sites that are not covered by SNaPshot analysis), we find no differences in editing of

transcripts of mitochondrial genome encoded subunits of complexes III and IV between the wild type and *slo2* mutants. This indicates that the observed decreases in complexes III and IV are not related to altered RNA editing (Table S3). Second, impaired metabolism caused by a defect in one mitochondrial ETC complex may alter the levels of other complexes. Several reports in non-plant species support this possibility. For instance, in mammalian species, the majority of complex I is associated with complexes III and IV (Schägger and Pfeiffer, 2000). In *Caenorhabditis elegans*, mitochondrial complex I mutations lead to deficiency of cytochrome *c* oxidase (complex IV) (Grad and Lemire, 2004), while in yeast, the levels of COX subunits COXI, -II and -III were also reduced in mutants that affect the assembly of complex IV (Glerum *et al.*, 1995; Shoubridge, 2001). However, this scenario is not supported by reports on Arabidopsis mutants with reduced or absent complex I (i.e. *ndufs4*, *bir6*, *rug3*) in which other respiratory chain complexes were unaffected (Meyer *et al.*, 2009; Koprivova *et al.*, 2010; Kühn *et al.*, 2011). Similarly, *rpoTmp* mutants with primary defects in complexes I and IV showed no reduction in complex III (Kühn *et al.*, 2009), and *wtf9* mutants with primary defects in complexes III and IV were unaffected in complex I (des Francs-Small *et al.*, 2012). Although complexes III and IV are much reduced in *slo2*, the remaining proteins may be sufficient to sustain plant life under normal conditions. Meanwhile, it also opens the question of how these major changes in the mitochondrial ETC complexes are compensated for in order to allow normal growth. Third, we identified three RNA editing changes in *mttB* (membrane targeting and translocation, a mitochondrial analog of the plastidial *tatC* protein), one of which results in an amino acid change in the MTTB protein (van der Merwe and Dubery, 2007). In bacteria, MTTB is an essential integral membrane protein which functions in membrane targeting and secretion of cofactor-containing proteins, such as iron–sulfur clusters (Weiner *et al.*, 1998). In higher plants, MTTB exhibits high similarity to its ortholog in bacteria, and may have a similar function in the mitochondrial membrane of plants (Sünkel *et al.*, 1994; Giegé and Brennicke, 2001). So it is plausible that the mutations in the MTTB protein which result from RNA editing defects in *slo2* mutants may inhibit the mitochondrial import of functional proteins for the formation of mitochondrial ETC complexes, such as nuclear encoded iron–sulfur containing proteins or co-factor-containing redox proteins, possibly resulting in the reduced function of those complexes. Although the functional mechanism still needs to be investigated, our current study shows that *slo2* is an example in plants of a single gene mutation leading to comprehensive changes in three mitochondrial ETC complexes, yet remaining viable. To the best of our knowledge, no other PPR proteins have been characterized with such multiple effects on the mitochondrial ETC.

Loss of SLO2 protein function leads to defects in plant growth and development

The status of mitochondrial electron transport and CO_2 fixation rate are two vital factors determining plant growth (Stitt, 1986; Griffin *et al.*, 2001). In plant mitochondria, the alternative respiration pathway is activated when the classical mitochondrial ETC is impaired (Juszczuk and Rychter, 2003), but this process produces significantly less ATP. Our results showed that the levels of ATP and ADP are much reduced, while the ATP/ADP ratio is lower in *slo2* mutants compared with that in Col-0 (Figure 9), suggesting that the plants have an impaired energy status. Clearly, energy shortage is probably the major cause of the growth defects in *slo2*. Carbon supply is another vital factor for plant growth. Our results showed that SLO2 is essential for survival of Arabidopsis in the absence of an external carbon source (Figure 6d,e). External carbon sources, such as CO_2, sugars or high light, partially alleviate *slo2* defects, indicating disruption of the carbon balance in mutants. A lower endogenous energy level and net carbon fixation obviously negatively affect growth and development.

The NADH/NAD$^+$ ratio is vital for the central redox control and to prevent the accumulation of ROS (Shen *et al.*, 2006). Thus, the imbalance of NADH/NAD$^+$ in *slo2* mutants may lead to higher ROS accumulation. Reactive oxygen species play important roles in normal plant growth and development (Foreman *et al.*, 2003), and were proposed to have a pivotal role in environmental sensing and hormonal signaling (Swanson and Gilroy, 2010). Therefore, the observed severe growth defects of *slo2*, may also result from an enhanced level of H_2O_2.

In conclusion, we propose that SLO2 (directly or indirectly) modulates carbon and energy balance, thereby playing an essential role in a plant's life cycle under certain growth conditions. Future research will reveal the precise nature of the genes influenced by SLO2 as well as its interaction partners. This will help to further elucidate the roles of SLO2 in plant development. The observed defects probably resulting from an impaired mitochondrial ETC could be instrumental in unraveling the inter-organellar network of mitochondria, plastids, and peroxisomes.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Plants were grown as described previously (Smalle and Van Der Straeten, 1997). Unless stated otherwise, seeds were stratified for 2 days at 4°C. Growth conditions were a 16 h light/8 h dark photoperiod, white fluorescent light (75 μmol m^{-2} sec^{-1}), and a growth temperature of 21°C. Plants on soil were grown in a growth chamber at 22°C. High-light conditions were at a light intensity of 650 μmol m^{-2} sec^{-1}, low light conditions were at a light intensity of 60 μmol m^{-2} sec^{-1}. Long-day conditions were 16 h light/8 h darkness; short-day conditions were 8 h light/16 h darkness. In order to

avoid seed batch effects, seeds harvested from plants grown simultaneously were used for analysis.

A collection of 5000 T-DNA insertion lines (Feldmann, 1991) and the Wassilewskija (Ws) wild type of *Arabidopsis thaliana* (L.) Heynh. were obtained from the Nottingham Arabidopsis Stock Center and used for screening. SALK_021900 (*slo2-2*) was verified by PCR amplification using primers: forward 5′-TCTTTGATTGC GAAATCGCCT-3′ and reverse: 5′-CGCATGCAGAAAGAACACCAA-3′. The primer specific to left border region of the construct which was used to generate the T-DNA insertion lines is 5′-GCGTGGACCGCTT GCTGCAACT-3′. The Tilling line T94087 (*slo2-3*) which contains a T to C mutation leading to a stop codon at position 247 was verified by sequencing.

Map-based cloning of *SLO2*

The mapping population was generated by crossing *slo2* (Ws) to Col-0 or L*er*. The position of *slo2* on the Arabidopsis genetic map was established by determining linkage between the *slo2* phenotype and simple sequence length polymorphism (SSLP) markers in F_2 (Bell and Ecker, 1994). The segregation of polymorphic markers and mutant phenotype was analyzed using MAPMAKER 2.0 for Macintosh (Lander *et al.*, 1987) using the Kosambi mapping function. Fine-mapping was performed with InDel (insertion/deletion) markers released by TAIR-Cereon. The InDel primer sequences and the PCR fragment sizes (Col/L*er*/Ws, in bp) are listed in Table S4.

The forward primers were labeled with γ-^{33}p-ATP and the PCR products were separated and visualized on 6% acrylamide sequencing gel after exposure to phosphorimager cassettes on a Storm 820 phosphorimager (GE Healthcare, http://www.gehealthcare.com).

Constructs and transgenic *A. thaliana* plants

All constructs used for generating transgenic plants were made using the Gateway system (Invitrogen, http://www.invitrogen.com/). The PCR amplified fragments were first cloned into pDONR221 via BP reactions and then confirmed by sequencing, followed by LR reactions to obtain plant expression vectors. For the complementation experiment in *slo2-1* background, a 3.92 kb genomic fragment, including 1.67 kb of 5′ upstream and 0.17 kb of 3′ downstream regions, was amplified by PCR using the primers 5′-TACGCTTCCAACACAACACG-3′ and 5′-TCGTACAGCAACCGAAGA TG-3′, then cloned into destination vector *p*HGW (Karimi *et al.*, 2002). For the *PSLO2:GUS* transgenic line, the 1.67 kb promoter region was transcriptionally fused to *eGFP-GUS* using the pKGWFS7 vector using primers 5′-TACGCTTCCAACACAACACG-3′ and 5′-CCATTATCGTCGTTTGCAGA-3′ (Karimi *et al.*, 2005). For the *35S:SLO2-GFP* construct, full-length *SLO2* cDNA was amplified using the primers 5′-ACCATGGCAACAAAATCATTTC-3′ and 5′-ACATGGCGTTGTCCCAAAG-3′, the verified fragment was used for C-terminal fusion with GFP under control of the 35S promoter in vector pK7WGF2. All the binary vectors were introduced into *Agrobacterium tumefaciens* strain C58C1 (pMP90) or GV3101 by electroporation. The *slo2-1* and *slo2-2* alleles as well as wild-type plants were transformed using the floral-dip method (Clough and Bent, 1998).

The full-length cDNA was isolated using 5′ and 3′ rapid amplification of cDNA ends according to the manufacturer's instructions (SMART RACE cDNA Amplification Kit; Clontech, http://www.clontech.com/). The primers were located at 62891–62910 and 63689–63708 in the bacterial artificial chromosome (BAC) clone T10F5, with a 778-bp overlap.

Quantitative RT-PCR analysis

Total RNA was extracted using TRIzoL® reagent (GIBCO/BRL, http://www.invitrogen.com/site/us/en/home/brands/Gibco.html) and 5 μg

RNA was further purified and concentrated using a DNA-free RNA kit (Zymo Research). Complementary DNA was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, http://www.fermentas.com/). Quantitative RT-PCR was performed using a Cybergreen fluorescence-based assay kit (Platinum SBYR Green qPCR kit, cat. no. 11733-046l; Invitrogen). The PCR reactions were performed on a Rotor Gene 6 (Corbett, http://www.corbettlifescience.com/) or MyiQ® Two-Color Real-Time PCR Detection System (Bio-Rad, http://www.bio-rad.com/). At least two runs (technical repeats) were done for each data set and two to three biological repeats were performed. Data represent mean values and standard errors (SE). The primers are listed in Table S4.

Subcellular localization

For the analysis of the subcellular localization of the SLO2 protein, transgenic Arabidopsis containing *P35S:SLO2-GFP* was stained with 250 nM MitoTracker Orange for 1 h. The root was visualized by confocal microscopy. To confirm the mitochondrial localization, transgenic plants containing *P35S:SLO2-GFP* construct were crossed with a mitochondrial marker line expressing ATPase-mCherry (kindly provided by D. Logan, University of Saskatchewan, Saskatoon, SK, Canada). The F_1 progeny were observed under a confocal microscope. Green fluorescent protein fluorescence was detected with excitation at 488 nm and emission at 525 nm; for the Mito-Tracker stain, fluorescence was detected with excitation at 543 nm and emission at 615 nm; for mCherry, fluorescence was detected with excitation at 568 nm and emission at 580–700 nm.

RNA editing analysis

Total RNA was extracted from 4-week-old Arabidopsis rosette leaves using RNeasy Mini Kit (Qiagen, http://www.qiagen.com/), and was further purified and concentrated using a DNA-free RNA kit (Zymo Research, http://www.zymoresearch.com/) according to the manufacturers' protocols. Specific cDNAs were generated as described previously (Takenaka and Brennicke, 2007). The SNaPshot assay and RNA editing sites analysis were performed according to the established protocol (Takenaka and Brennicke, 2009). The editing defects were confirmed by sequencing specific RT-PCR products.

Blue native-PAGE and complex I activity assay

Eight-week-old plants grown in greenhouse conditions were used for isolation of mitochondria; Blue native PAGE of solubilized mitochondrial membranes, complex I activity assay, and western blots were performed as described previously (Meyer *et al.*, 2009).

NAD$^+$, NADH, ATP, ADP and sugar measurements

The trichloroacetic acid–ether extraction method was used to extract ATP and ADP from 5-day-old seedlings (Jelitto *et al.*, 1992), with measurements as described previously (Stitt *et al.*, 1989). Three-day old seedlings grown on half-strength MS medium were used for sugar measurement. Sugar measurements were as described previously (Pritchard *et al.*, 2002). Five-day-old seedlings grown on half MS salts plus 1% sucrose were used for pyridine nucleotide extraction and the concentration of NAD$^+$ and NADH determined as described by Shen *et al.* (2006).

ACCESSION NUMBER

Sequence data from this article can be found in the GenBank/EMBL data libraries or the Arabidopsis Genome Initiative database under accession number *SLO2* (At2g13600).

ACKNOWLEDGEMENTS

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Map-based cloning of the *SLO2* gene.

Figure S1. Effect of sugars on *slo2* mutants.

Table S1. Life cycle timing in *slo2-1* and wild type (Ws).

Table S2. Flowering time of *slo2-1* and Col-0 in different light conditions.

Table S3. List of the RNA editing sites detected by SNaPshot analysis or sequencing.

Table S4. Map-based cloning of the *SLO2* gene.

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