Identification of a Pentatricopeptide Repeat Protein Implicated in Splicing of Intron 1 of Mitochondrial nad7 Transcripts

Received for publication, May 24, 2010, and in revised form, August 3, 2010 Published, JBC Papers in Press, August 3, 2010, DOI 10.1074/jbc.M110.147603

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Splicing of plant organellar transcripts is facilitated by members of a large protein family, the pentatricopeptide repeat proteins. We have identified a pentatricopeptide repeat protein in a genetic screen for mutants resistant to inhibition of root growth by buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis and consequently named BIR6 (BSO-insensitive roots 6). BIR6 is involved in splicing of intron 1 of the mitochondrial nad7 transcript. Loss-of-function mutations in BIR6 result in a strongly reduced accumulation of fully processed nad7 transcript. This affects assembly of Complex I and results in moderate growth retardation. In agreement with disruption of Complex I function, the genes encoding alternative NADH oxidizing enzymes are induced in the mutant, and the mutant plants are less sensitive to mannitol and salt stress. Mutation in the BIR6 gene allowed normal root growth in presence of BSO and strongly attenuated depletion of glutathione content at these conditions. The same phenotype was observed with other mutants affected in function of Complex I, thus reinforcing the importance of Complex I function for cellular redox homeostasis.

Pentatricopeptide repeat (PPR) proteins are characterized by 2–27 tandem repeats of loosely conserved 35-amino acid motifs (1, 2). Arabidopsis and other angiosperms possess 450–600 genes encoding PPR proteins (2, 3). The PPR proteins are separated into two subfamilies and four subclasses depending on the motifs localized at their C termini. Although functions of several tens of these proteins have been identified, this is still only a small portion of the PPR gene family. The PPR proteins are usually involved in processing of mitochondrial or plastidic transcripts (reviewed in Ref. 4). Therefore, disruption of genes encoding PPR proteins often results in severe phenotypes, including seedling or embryo lethality (3, 5, 6). PPR proteins play roles in RNA editing (7, 8) as well as splicing (4, 9), transcription (6), and translation (10). Despite the large number of PPR protein genes, there seems to be very little, if any, redundancy in function.

Mitochondrial respiratory chain is composed of four complexes sequentially transferring electrons from NADH to molecular oxygen. Complex I functions as NADH-ubiquinone oxidoreductase. Although mutants of Complex II are embryolethal and no mutants of complexes III and IV have been identified (11, 12), several mutants of Complex I have been characterized and helped to demonstrate the plasticity of plant respiration (13–16). The best studied Complex I mutant, the Nicotiana sylvestris CMSII (for cytosomal male sterility), lacks NAD7 subunit of Complex I because of a deletion in mitochondrial DNA (16). The growth of CMSII mutant is retarded, and its photosynthetic capacity is reduced by 20–30% (17, 18). The defect of Complex I results in insensitivity of respiration to rotenone, in an increase in alternative oxidase, and in changes in redox properties leading to a higher tolerance to ozone and viruses (19, 20).

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) plays multiple roles in the plant life cycle, including detoxification of reactive oxygen species, xenobiotics, and heavy metals; storage and transport of reduced sulfur; regulation of plastid and nuclear gene expression; and resistance to pathogen infection (21–24). GSH is an essential component in the regulation of cellular redox homeostasis (22, 24). A mutation in GSH-synthesizing enzyme γ-glutamylcysteine synthetase, allowing synthesis of ~1–2% of wild type GSH levels, underlies the rml (root meristemless) phenotype in the Arabidopsis rml1 mutant (25). Thus, the reduced capacity to synthesize GSH results in a specific loss of root meristem activity and cessation of root growth (25). The rml phenotype can be mimicked by growing plants in the presence of buthionine sulfoximine (BSO) (25, 26), a specific nontoxic inhibitor of γ-glutamylcysteine synthetase (27).

Here we describe cloning of a mutant in a PPR protein isolated in a genetic screen to find mutants resistant to inhibition of root growth by BSO. We show that this protein is required...
for efficient splicing of nad7 transcripts encoding a subunit of Complex I of the mitochondrial respiratory chain.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Arabidopsis thaliana plants, ecotypes Columbia (Col-0) and Wassilewskija (Ws), were used as WT in this study. T-DNA insertion lines SALK_000310 and FLAG_426F01 (for bir6-2 and bir6-3, respectively) and SALK_010194C (ΔAt1g47260) were obtained from the Nottingham Arabidopsis Resource Centre, and homozygous plants were isolated by standard procedures as described before (28). The seeds of rml1 and css1 mutants were generous gifts from Dr. C. Cobbett and Dr. N. Nakagawa, respectively. For the genetic screen, Col-0 seeds were treated with pool of seedlings of 15–20 mg of fresh weight.

Microarray Analysis—The analysis of cysteine and GSH was performed exactly as described (29) with pools of seedlings of 15–20 mg of fresh weight.

Microarray Analysis—The RNA was extracted from 6-day-old seedlings grown in the presence of 1.25 mM BSO by phenol:chloroform:isoamylalcohol (25:24:1) extraction and LiCl precipitation, treated with DNase I, and repurified using the Qiagen RNeasy plant mini kit according to the manufacturer’s instructions. The labeling, hybridization, and detection using three biological replicates of both wild type Col-0 and bir6-2 plants and the Arabidopsis Gene Expression Microarray v.4 chip (Agilent) were performed by Cogenics service. Only genes flagged by the scanner software as “present” in all three replicates of either genotype were included in further analysis. The expression data were normalized according to the AtGenExpress recommendations using a global mean normalization excluding the top and bottom 2% of the data. Additional quality control was introduced to remove the genes with the most variable expression from further consideration. Standard deviations across the three replicates for each genotype were determined, and genes with a standard deviation greater than 15% of the signal intensity were eliminated from further analysis. Fold changes in expression levels were calculated from the means of the three biological replicates, and their statistical significance was tested by the Cyber-T test (30) on the log-transformed signal intensities. In addition, a false discovery rate control was performed according to Ref. 31, setting the threshold q value at 0.05.

PPR Protein Involved in Splicing of nad7 Transcript

was created as follows. Oligonucleotide primers BIR6B1 (aaaaagcagctccattcattcactcgtc) and BIR6B2 (agaaagctgggt-cagctggacacccaaag) were designed to amplify a genomic DNA fragment including a 1,100-bp 5’-promoter region (to the end of the adjacent gene) and to terminate just before translational stop site of BIR6 to form a C-terminal translational fusion with GFP. The BIR6_GFP::BIR6 fragment was amplified by PCR from Arabidopsis ecotype Col-0 genomic DNA using KOD plus DNA polymerase (Toyobo). The resultant fragment was cloned into pCR-BluntII-TOPO (Invitrogen) and fully sequenced. The fragment was introduced into pDONR207 donor vector and subsequently to pBin-GFP-C (a gift from J. Doonan, John Innes Centre) by Gateway® technology. For complementation of the mutant phenotype, the BIR6 was expressed under the control of its own promoter. The construct was prepared in the same way except that a primer BIR6C2 (agaaagctgggtaaatgagacataatac) was used instead of BIR6B2 to incorporate a stop codon between the BIR6 coding region and GFP. Arabidopsis transformation and selection was performed as described in Ref. 28. The increase in BIR6 transcript in the overexpressing lines was confirmed by quantitative RT-PCR. Two lines with the highest BIR6 mRNA levels were selected for further analysis.

Quantitative RT-PCR—To compare mRNA levels of alternative NADH oxidizing enzymes, total RNA was isolated by RNeasy plant mini kit (Qiagen) with on-column DNase I treatment as described by the manufacturer. First strand cDNA was synthesized using an oligo(dT) primer and SuperScript II reverse transcriptase, followed by a subsequent standard dissociation protocol to ensure that each amplicon was a single product. All of the quantifications were normalized to ubiquitin UBQ10. The RT-PCRs were performed in triplicate for each of the three independent samples.

Confocal Microscopy and Analysis—Arabidopsis plants were imaged on a Zeiss 510 meta laser scanning confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) using either ×10/0.45 air or ×63/1.2 water immersion objectives. The fluorescent z-stack of images of the plants was taken at 1024 × 1024 pixels (size, 1.16–0.04 µm2) with a 0.5–3-µm z step size. GFP was excited at 488 nm, emitted light was collected at 505–550 nm, and a transmitted light image was collected for reference. The ImageJ program was used to adjust image brightness, contrast, color merging, and maximum intensity projection of images from z sections.

Subcellular Localization of BIR6—The first 300 bp of the coding sequence of BIR6 were amplified according to the manufacturer’s instructions using the Expand High Fidelity PCR system (Roche Applied Science) and the primers 5’-ggggcaacagtgttgcacaaaaagcagcttcgaaggagataacagctgtacagatgacatcctg and 5’-ggggcaacctttggcataaagagcagcttcacctccccgatatcctcagatttccttgaagatattgat containing the attB sites for Gateway® cloning. The PCR product was cloned into the Gateway®
pDONR207 vector using the Gateway® BP Clonase™ enzyme mix (Invitrogen) and verified by sequencing. The entry clone and a Gateway® cloning cassette (pDest/pgem/CGFP; kindly provided by Prof. Whelan) (33) were used for LR recombination to clone the first 300 bp of BIR6 in frame with the coding region of the GFP. The mitochondrial targeting sequence of yeast Cox4 fused to mCherry in pBIN20 (34) was used as a mitochondrial control. The constructs were biolistically transformed into Arabidopsis cultured cells as previously described (35). In brief, the GFP and mCherry plasmids (5 μg each) were co-precipitated onto 1-μm gold particles and transformed using the biolistic PDS-1000/He system (Bio-Rad). Particles were bombarded onto 2 ml of Arabidopsis cultured cells resting on filter paper on osmoticum plates (2.17 g/liter Murashige and Skoog Modified Basal Salt Mixture, 30 g/liter sucrose, 0.5 mg/liter naphthalene acetic acid, 0.05 mg/liter kinetin, 36.44 g/liter mannitol). After bombardment, the cells were placed in the dark at 22 °C. Fluorescence images were obtained 24 h after transformation using an Olympus BX61 epifluorescence microscope with excitation wavelengths of 460/480 nm (GFP) and 535/555 nm (mCherry) and emission wavelengths of 495–540 nm (GFP) and 570–625 nm (mCherry) using CellR™ imaging software.

Analysis of Mitochondrial Transcripts—Arabidopsis seeds were germinated on soil and grown at 22 °C under an 8-h photoperiod for isolation of RNA. Leaf RNA was isolated using the plant RNeasy extraction kit (Qiagen) according to the manufacturer's instructions. The reverse transcription step was performed on 3 μg of DNase-treated RNA (Ambion DNase; for 1 h at 37 °C), using Superscript III (Invitrogen) and random hexamer primers as described before. To check whether we could detect the fully spliced NAD7 transcript in the bir6 mutants, RT-PCR was performed using primers designed to amplify between NAD7 exons 1 and 5. COX2 and actin 2–8 transcripts were used as controls for RNA quality (primer sequences in supplementary Table S2). To detect and quantify the splicing of mitochondrial mRNAs, primers were designed to amplify 100–200-bp regions spanning intron-exon regions (unspliced forms) and in exons (spliced forms) of each mitochondrial gene. The complete list of these primers is given in supplementary Table S3. Transcript levels of mitochondrial transcripts were determined using the primers listed in supplementary Table S4.

Quantitative RT-PCR was performed using LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) and a Roche LightCycler 480 real time PCR system with the following thermal cycling program: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. The data were analyzed using the LightCycler 480 software version 1.5 (Roche Applied Science).

Analysis of Complex I—Mitochondria were isolated from 3-week-old seedlings grown hydroponically in half-strength MS medium, and Blue native (BN)-polyacrylamide gel electrophoresis was performed as previously described (36). Proteins separated by BN-PAGE were transferred onto PVDF membrane (GE Healthcare) in cathode buffer for 15 h at 40 mA, using a Bio-Rad Mini transblot cell. The membrane was then destained with ethanol and re stained with Coomassie Blue to visualize the complexes and subsequently destained before any further treatment. Immunodetection was performed using either an anti-NAD9 antibody (37) at a 1/50,000 dilution or an
anti-NAD6 antibody at a 1/4,000 dilution, followed by a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1/10,000 dilution) and subsequently revealed using ECL reagents (GE Healthcare). The anti-NAD6 antibody was raised in rabbit by Auspep Pty. Ltd. (Parkville, Australia) using an 18-mer synthetic peptide from the plant-specific C-terminal hydrophilic part of NAD6 (KVKRQDVFRRNAIDFRRTIM-RRT), linked to the diphtheria toxoid.

RESULTS

Isolation of Mutant in PPR Protein—We performed a genetic screen to isolate mutants tolerant to inhibition of root growth by BSO (25, 26) (supplemental Fig. S1). Among the 10 bir mutants isolated, we chose the bir6 mutant for further analysis because, apart from maintaining normal root growth on BSO plates, the plants were significantly smaller than WT. High resolution mapping was used to identify the BIR6 gene as At3g48250. Sequencing the gene revealed a G-to-A mutation at position 746 after the ATG, resulting in a change of Trp-248 into a premature stop codon (Fig. 1). The gene encodes a member of a PPR protein family. BIR6 is a relatively small member of the family containing eight repeats of the pentatricopeptide motif (Fig. 1B).

To verify that the phenotype is caused by this mutation, we obtained two T-DNA insertion alleles of bir6. The T-DNA lines SALK_000310 (bir6-2; Col-0 background) and FLAG_426F01 (bir6-3; Wassilewskija background) contain T-DNA insertions in the BIR6 coding region (Fig. 1A). BSO does not inhibit root growth of bir6-2 (Fig. 1C) and bir6-3 (not shown) to the same extent as Col-0, similar to the original bir6 mutant (from now on denoted as bir6-1). To complement the bir6-1 mutant, we transformed it with a construct containing the BIR6 open reading frame under control of the native promoter. The roots of the complemented mutants were inhibited by BSO to the same extent as in WT plants (Fig. 1C).

Apart from the BSO-insensitive roots phenotype, the bir6-1 mutants, as well as both T-DNA mutants, were significantly smaller after 5 weeks with short days than corresponding WT plants (Fig. 1D). This phenotype was correlated with a lack of BIR6 transcript in leaves of the bir6-2 and bir6-3 plants (Fig. 1E). The premature stop codon in bir6-1 also resulted in a strong reduction of BIR6 mRNA levels, probably because of the degradation of the transcript through the nonsense mediated decay pathway (40). Because the phenotype of bir6-2 and bir6-1 was identical, and the original mutant was derived from EMS mutagenesis and could carry other mutations, we performed most other experiments with bir6-2.

BIR6 Is a Mitochondrial Protein—To find out the molecular function of BIR6, we analyzed Col-0 plants expressing BIR6 fused to GFP by confocal microscopy. The GFP signal (driven by the BIR6 promoter) was strong in all cells of shoots and roots of young seedlings, pointing to constitutive expression of the gene (supplemental Fig. S2, A–F). On a subcellular level, the GFP signal was localized to mitochondria of shoot and root cells (supplemental Fig. S2, G–L). To confirm the targeting of BIR6 to mitochondria, the BIR6::GFP construct was transfected into Arabidopsis cells together with a mitochondrial marker, COX4 fused to red fluorescent protein (Fig. 2). The overlay of the green and red fluorescence clearly shows that BIR6 is localized exclusively in the mitochondria.

To identify metabolic processes affected by the bir6 mutation, we performed a microarray analysis with bir6-2 and Col-0 seedlings grown for 6 days in the presence of BSO. However, despite the growth phenotype of the bir6-2 plants, only 32 genes were found to be differentially expressed (fold change

**FIGURE 2.** Subcellular localization of BIR6. Cultured Arabidopsis cells were biolistically transformed with expression constructs encoding C-terminal fusions of BIR6 with GFP and the mitochondrial marker COX4 with red fluorescent protein. Presented are fluorescence images 24 h after transformation. A, GFP fluorescence. B, red fluorescent protein (RFP) fluorescence. C, merge of both showing expression of BIR6 exclusively in mitochondria.

**FIGURE 3.** Expression analysis of alternative oxidase and NADH dehydrogenase. Total RNA was isolated from 7-day-old seedlings of Col-0 and bir6-2 grown on MS-phytagel with or without the addition of 1.25 mM BSO. The mRNA levels were determined by quantitative RT-PCR and standardized with ubiquitin transcripts. A, AOX1A. B, AOX1B. C, NDB3. D, NDB4. The data are presented as values relative to control Col-0 and means ± S.D. from at least three plants. Different letters mark data significantly different at p < 0.05.
threshold, 2-fold; q value threshold, 0.05). Of these, 16 genes were more highly expressed in bir6-2, and 16 were more highly expressed in the WT (supplemental Table S5). None of these genes, however, pointed to specific defects in the mutant that would explain the phenotype.

Iterative group analysis using the Aracyc classification of metabolic pathways revealed several pathways and enzyme activities up-regulated in bir6-2, such as indole-3-acetic acid synthesis, glyoxylate cycle, cyanate degradation, and most importantly alternative oxidase (supplemental Tables S6 and S8) as well as pathways down-regulated in bir6-2 (supplemental Tables S7 and S9). Among the up-regulated genes in the microarray analysis was an NADH oxidizing enzyme, NADH dehydrogenase (NDB3). Indeed, the transcript levels of two genes each from the families of alternative oxidases, AOX1A and AOX1B, and NADH dehydrogenases, NDB3 and NDB4, were higher in bir6-2 than in Col-0 as determined by quantitative PCR (Fig. 3). Three of the genes were induced by BSO in Col-0. Because genes corresponding to these two families are often induced in mutants in mitochondrial respiration, specifically Complex I (19, 36), we deduced that BIR6 might be functionally linked to mitochondrial respiratory Complex I.

**BIR6 Is Implicated in Splicing of nad7 Transcripts**—We analyzed the entire bir6-2 mitochondrial transcriptome for alterations in RNA abundance by RT-qPCR (supplemental Fig. S3). No obviously significant departures from wild type levels were observed. Such analyses, however, do not exclude alterations in RNA splicing because they do not differentiate between spliced and unspliced transcripts. Hence we also specifically examined splicing of the 23 introns in mitochondrial mRNAs from all three bir6 mutants (Fig. 4A). The results show a clear defect in splicing of nad7 intron 1 that disappears when the wild type BIR6 gene was reintroduced into bir6-1. nad7 is composed of five exons and four introns, which are cis-spliced (Fig. 4B). The splicing of the other three nad7 introns appears unaffected in bir6 mutants, and indeed none of the other mitochondrial splicing events are drastically altered in all three bir6 mutant alleles. The defect in nad7 splicing, although dramatic (decreased ~100-fold), is not total, as shown by the ability to amplify the fully processed transcript in bir6-1 and bir6-2 by RT-PCR (Fig. 4C). Nevertheless, the decrease in nad7 splicing would be expected to severely compromise assembly of mitochondrial Complex I, of which the nad7 gene product is a subunit.

To assay for Complex I, mitochondrial proteins were separated by BN-PAGE with subsequent in-gel NADH oxidase activity staining. Fig. 5A shows that the NADH oxidase activity at the position expected for Complex I was greatly reduced in bir6-1, as compared with mitochondria from Col-0 and complemented bir6-1 plants. A similar BN-PAGE gel was blotted, stained, and probed with NAD9 and NAD6 antibodies (Fig. 5, B–D), proving that the high molecular weight bands revealed by the NADH oxidase activity are Complex I.

**FIGURE 4. Expression analysis of mitochondrial transcripts.** A, quantitative RT-PCR of spliced mitochondrial transcripts. The histogram shows the relative proportions of spliced to unspliced forms of each transcript as log2 values of the ratio. Two biological repeats were performed for each of the three mutant genotypes as compared with their respective WT. B, structure of nad7 gene. The nad7 gene is composed of five exons and four introns that undergo cis-splicing. The size of exons and introns is not to scale. The primers used for the splicing tests are indicated on the figure, and their sequences are indicated in supplemental Table S3. C, detection by RT-PCR of fully processed nad7 transcript (i.e. with all four introns spliced out) in bir6-1 and bir6-2 mutants and the complemented line. The primers used for this experiment were e1F and e5R in B. Given the length of the RT-PCR product (953 bp) and the 35 amplification cycles employed, these results cannot be considered to be indicative of the relative quantities of spliced nad7 transcript in the different samples, unlike the results presented in A.
Stress Tolerance of bir6 Mutants

Mutants in the respiratory chain are often affected in resistance to oxidative stress (20, 41). Therefore, we compared the performance of the bir6-2 T-DNA insertion line with its corresponding wild type ecotype Col-0 under salt and osmotic stress, both of which result in the accumulation of reactive oxygen species leading to oxidative stress (Fig. 6). Salt treatment resulted in 52% reduction of growth (determined as seedling fresh weight) in Col-0, but bir6-2 was much less affected (reduction by 32%). A differential response to mannitol was less evident, although again bir6-2 was less affected than Col-0. Both treatments resulted in a reduction of total chlorophyll content in Col-0. bir6-2 had the same chlorophyll content in control conditions but was significantly less affected by the treatments. Remarkably, whereas osmotic stress led to a 40% decrease in chlorophyll in Col-0, it had no effect on chlorophyll in the mutant, despite the effect on fresh weight (Fig. 6).

Disruption of Complex I Affects Glutathione Levels in BSO-treated Plants

The bir6 mutant was isolated in a screen for resistance to BSO. Because BSO inhibits synthesis of GSH, we determined thiol content of bir6-2 seedlings. Although in Col-0 grown in the presence of the inhibitor GSH content is reduced to ~20% compared with control plants, the bir6-2 mutants grown on BSO retained significantly more GSH (Fig. 7A), correlating with strongly attenuated reduction of root growth by the inhibitor (Fig. 7B). The question arises whether the increased GSH and reduced sensitivity of root growth to BSO is caused specifically by the loss of BIR6 or by the consequently altered activity of Complex I. We therefore analyzed other mutants with defects in mitochondrial Complex I, css1 with disrupted intron maturation, and therefore impaired nad4 splicing (42) and a mutant in γ-carboxy anhydrase ΔAt1g47260 (15). Both mutants responded to BSO in the same way as bir6-2, i.e. the GSH content was significantly less reduced by BSO, and the primary roots were inhibited to a much lower extent than in Col-0 (Fig. 7). Thus, we can conclude that reduced activity of Complex I results in reduced sensitivity to BSO-induced depletion of GSH and consequently prevention from BSO-induced inhibition of primary root growth.

DISCUSSION

BIR6 Is Involved in Synthesis of the NAD7 Subunit of Complex I—The function of only a small fraction of the 450 Arabidopsis PPR proteins is known. They are usually involved in processing

FIGURE 5. Analysis of mitochondrial Complex I in bir6 mutant. Mitochondria were isolated from 3-week-old seedlings of Col-0, bir6-1 and complemented bir6-1 (bir6-1/BIR6) grown in liquid half-strength MS medium. Blue native-PAGE of mitochondrial membrane protein complexes (loading 70 μg) is shown. A, in-gel NADH oxidase activity staining. B, BN gel after transfer to PVDF membrane and staining with Coomassie Blue. C, Western blot of the membrane B probed with a NAD9 antibody. D, Western blot probed with a NAD6 antibody. The arrows indicate some of the mitochondrial membrane complexes or supercomplexes: I, supercomplex formed of Complex I + dimeric Complex III (1500 kDa); CI, Complex I (1000 kDa); V, ATP synthase complex (600 kDa); III, dimeric Complex III (500 kDa).

FIGURE 6. Resistance of bir6 mutant to abiotic stress. Col-0 and bir6-2 were grown for 2 weeks on horizontal MS-phytagel plates, transferred to fresh plates with MS-phytagel supplemented with 150 mM NaCl or 300 mM mannitol, and grown for a further 7 days. A, fresh weight of individual seedlings. B, total chlorophyll content. The data are presented as the means ± S.D. from at least eight (A) or from three pools of five (B) plants. Different letters mark data significantly different at p < 0.05.
PPR Protein Involved in Splicing of nad7 Transcript

The nad7 gene. Although the molecular defect in bir6 is similar, the consequences of the defect are much less severe in Arabidopsis. Indeed, in various crossing experiments with bir6-1 as a pollen donor, no alterations in viability of the progeny were observed. Again this is likely to be due to the fact that the CMSII mutant has lost nad7 and Complex I completely, whereas bir6 mutants retain some full-length nad7 transcripts (Fig. 4C) and detectable Complex I activity (Fig. 5). A recently characterized mutant in a PPR protein responsible for editing of nad7 transcripts also did not show any major growth or developmental alterations (47), although it is not known whether the amino acid alteration induced by the lack of editing affects the stability or function of the protein. NAD7 forms part of a peripheral 140-kDa subcomplex separate from the NADH oxidase activity but possibly involved in quinone reduction (48).

A Link between Complex I and Glutathione—The BIR6 PPR protein was isolated in a genetic screen to find mutants resistant to the inhibition of root growth by BSO (25, 26). BSO inhibits γ-glutamylcysteine synthetase, and prolonged exposure thus depletes GSH levels, specifically affecting root growth. We expected to find mutants in genes involved in GSH homeostasis, allowing them to maintain higher GSH content in the presence of the inhibitor and so overcome the root growth phenotype. Indeed, an independent genetic screen based on the same concept resulted in isolation of plastidic GSH transporters (26). Identification of the PPR protein responsible for nad7 splicing in our screen as well as the similar response of css1 and ΔAt1g47260 to BSO (Fig. 7) confirms a strong but probably only indirect effect of mitochondrial respiration on GSH homeostasis. The link between Complex I activity and GSH observed in bir6 is consistent with observations on Arabidopsis cell cultures treated with the Complex I inhibitor, rotenone (49). In these experiments GSH levels rose significantly after 48 h of rotenone treatment. Foliar GSH content was also higher in the N. sylvestris CMSII mutant than in the corresponding wild type in most measurements (20, 50). All three alleles of the bir6 mutant as well as the two other Complex I mutants analyzed maintained higher GSH levels during BSO treatment. In addition, the better tolerance of bir6 to osmotic and salt stress may also be due to a higher capacity to maintain GSH levels. However, the mechanism by which Complex I mutants maintain higher GSH levels on BSO remains to be elucidated.

In conclusion, we have identified a function of a previously uncharacterized mitochondrial PPR protein. The loss of At3g48250 results in inefficient splicing of intron 1 of the nad7 transcript and thus in disruption of function and assembly of Complex I. In addition we demonstrated a new phenotype of Complex I mutants, resistant to inhibition of root growth by BSO.

FIGURE 7. Comparison of bir6-2 phenotype with other mutants of mitochondrial Complex I. Col-0, bir6-2, css1, and ΔAt1g47260 were grown for 6 days on vertical MS-phytagel plates with or without 1.25 mM BSO. A, total GSH content. B, primary root length. The data are presented as the means ± S.D. from at least three pools of five plants (A) or 10 plants (B). Different letters mark significantly different data at \( p < 0.05 \).
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43. PPR Protein Involved in Splicing of nad7 Transcript

JOURNAL OF BIOLOGICAL CHEMISTRY 32199
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