**ABA overly-sensitive 5 (ABO5), encoding a pentatricopeptide repeat protein required for cis-splicing of mitochondrial nad2 intron 3, is involved in the abscisic acid response in Arabidopsis**

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**SUMMARY**

To study the molecular mechanism of abscisic acid (ABA) regulation of root development, we screened the root growth of Arabidopsis mutants for sensitivity to ABA. ABA overly-sensitive 5 (ABO5/At1g51965) was identified, and was determined to encode a pentatricopeptide repeat protein required for cis-splicing of mitochondrial nad2 intron 3 (nad2 is one subunit in complex I). Under constant light conditions (24-h light/0-h dark photoperiod), abo5 mutants exhibited various phenotypes and expressed lower transcripts of stress-inducible genes, such as RD29A, COR47 and ABF2, and photosynthesis-related genes proton gradient regulation 5 (PGR5) and PGR5-like photosynthetic phenotype (PGRL1), but higher levels of nuclear-encoded genes alternative oxidase 1a (AOX1a) and oxidative signal-inducible 1 (OXI1). Prolonged ABA treatment increased the expression of the cox2 gene in complex IV and nad genes in complex I to a higher level than no ABA treatment in the wild type, but only to a moderate level in abo5, probably because abo5 already expressed high levels of mitochondrial-encoded cox2 and nad genes under no ABA treatment. More H₂O₂ accumulated in the root tips of abo5 than in the wild type, and H₂O₂ accumulation was further enhanced by ABA treatment. However, these growth phenotypes and gene-expression defects were attenuated by growing abo5 plants under short-day conditions (12-h light/12-h dark photoperiod). Our results indicate that ABO5 is important in the plant response to ABA.

**Keywords:** mitochondria, PPR protein, ABA signaling, oxidative stress.

**INTRODUCTION**

Plants have evolved a series of mechanisms to limit stress and damage caused by unfavorable environmental conditions. Abscisic acid (ABA), a hormone produced when plants are stressed by drought, salt and cold, is an important signal molecule. ABA helps plants cope with these unfavorable stresses, and also plays essential roles in seed development and seedling growth. Genetic screening using seed germination sensitivity to ABA has identified several key mediators in the ABA signaling pathway, including (ABA insensitive 1) ABI1, ABI2, ABI3, ABI4 and ABI5 (Finkelstein and Lynch, 2000; Finkelstein et al., 1998). ABI1 and ABI2, which are phosphatase type-2C proteins with negative regulation roles in ABA signaling, physically interact with and inhibit downstream target proteins of ABA signals, such as serine/threonine protein kinase OPEN STOMATA1 (OST1), when ABA content is limited. The increased levels of ABA under abiotic stress cause the ABA receptors PRY1/PYLs to interact with these PP2C proteins and relieve the inhibition on their downstream targeted protein kinases (Fuji et al., 2009; Ma et al., 2009; Nishimura et al., 2009; Park et al., 2009; Santiago et al., 2009). ABI5 is a basic leucine zipper transcription factor that can be phosphorylated and activated...
by SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3 to regulate the expression of stress-responsive genes (Nakashima et al., 2009). ABI3 encodes a transcriptional factor that displays high homology with maize viviparous 1 (Giraudat et al., 1992). ABI4 is a member of the ERF/AP2 transcription factor family (Finkelstein et al., 1998). ABA can stimulate the accumulation of H$_2$O$_2$, which acts as a signal molecule to induce stomatal closure for controlling water consumption by activating calcium channels (Murata et al., 2001; Pei et al., 1997). ABI1 and ABI2 are sensitive to H$_2$O$_2$, which is partially mediated and transduced by a general oxyradical scavenger glutathione peroxidase, ATGPX3 (Miao et al., 2006).

The chloroplast and mitochondrion are the two main cellular organelles with independent genomes. Under unfavorable conditions, both chloroplasts and mitochondria cause the accumulation of reactive oxygen species (ROS), which increases oxidative stress and also act as signals to help the plant respond/adapt to the unfavorable environment. Pentatricopeptide repeat (PPR) proteins form a large protein family, are mostly targeted to mitochondria or chloroplasts in plants, and play diverse roles in RNA metabolism (Lurin et al., 2004; O’Toole et al., 2008; Schmitz-Linneweber and Small, 2008). For example, PPR4 from Zea mays is associated with a chloroplast-encoded pre-mRNA, and mediates its splicing (Schmitz-Linneweber et al., 2006). OTP51 is necessary for splicing intron 2 in the putative chloroplast open reading frame 3 (ycf3) mRNA and other introns (de Longevialle et al., 2008). PPR5 binds to the chloroplast trnG-UCC precursor group-II intron and stabilizes it in maize (Beick et al., 2008). PPR10 also stabilizes chloroplast mRNA in Arabidopsis (Pfalz et al., 2009). AtECB2 (Yu et al., 2009), LPA 66 (Cai et al., 2009), RARE1 (Robbins et al., 2009), CHLORORESPERATORY REDUCTION 22 (CCR22), CRR28 (Okuda et al., 2009) and CRR4 edit certain RNAs in the chloroplast (Kotera et al., 2005). Both OGR1 (opaque and growth retardation 1) and MITOCHONDRIAL RNA EDITING FACTOR 1 (MEF1) are essential for RNA editing in mitochondria of rice (OGR1) or Arabidopsis (MEF1) (Kim et al., 2009; Zehrmann et al., 2009). PPR40 mediates ubiqinol-cytochrome c oxidoreductase activity in complex III, and its impairment leads to the accumulation of ROS and enhanced sensitivity to stress (Zsigmond et al., 2008). PPR-B regulates the translation of orf138 mRNA in radish mitochondria to control cytoplasmic male sterility (Uyttewaal et al., 2008). Most PPR proteins are essential for plant organelle biogenesis, and the disruption of some PPRs leads to embryo lethality (Cushing et al., 2005; Lurin et al., 2004).

Mitochondria consist of four major complexes localized on the membranes. Complex I is responsible for transporting electrons from the NADH pool to ubiquinone in the electron transport system. There are 30–40 NADH dehydrogenase subunits encoded by both mitochondrial and nuclear genes. Plant mitochondria have specific alternative respiratory pathways, including the non-proton-pumping NAD(P)H dehydrogenases, which bypass complex I for diminishing ROS production, and an alternative oxidase (AOX), which directly accepts electrons from the ubiquinone pool for reducing ROS production. These alternative pathways increase the tolerance of plant mitochondria to respiratory defects, and loss of complex I has diverse effects on plant cells. For example, mutation in frostbite 1 (fro1), a nuclear gene encoding NADH dehydrogenase co-enzyme in the mitochondrial electron transfer chain, causes reduced transcription of cold-inducible genes, a high accumulation of ROS and decreased cold acclimation (Lee et al., 2002). In contrast, loss of complex-I function in a cytoplasmic male-sterile mutant (CMSII) did not cause a large increase in ROS accumulation, but instead caused a marked increase in antioxidant activities and the re-establishment of a new redox homeostasis in tobacco cells (Dutilleul et al., 2003b). Non-chromosomal stripe (NCS) mutants truncated in the nad4 gene of complex I show increased expression of AOX. PPR protein OTP43 is necessary for splicing intron 1 in the nad1, a central anchor component of mitochondrial complex I in Arabidopsis thaliana, and mutation in OTP43 increases levels of AOX (de Longevialle et al., 2007). Mutations affecting different subunits of complex I result in various growth phenotypes (de Longevialle et al., 2007; Dutilleul et al., 2003b; Lee et al., 2002; Nakagawa and Sakurai, 2006), indicating the diverse effects of complex I.

Organellar proteins are encoded by both organellar and nuclear genes. Communication between organelles and the nucleus is essential for plant responses to stressful environmental conditions, and for adjusting the requirements for plant development (Pogson et al., 2008). Retrograde signals from organelles that regulate the expression of nuclear genes represent an important feedback control for plant responses to environmental and developmental limitations. Several signaling pathways involved in chloroplast-to-nucleus retrograde signaling have been described previously (Surpin et al., 2002; Woodson and Chory, 2008). The existence of multiple mitochondrial retrograde signaling pathways that are capable of initiating specific gene expression in the nucleus is also suggested by plant responses to the dysfunction of specific mitochondrial proteins (Rhoads and Subbaiah, 2007). One target of mitochondrial retrograde signaling is the upregulation of AOX expression, which helps to ameliorate the ROS stress initiated by mitochondrial dysfunction (Umbach et al., 2005). Cross-talk occurs between mitochondria and chloroplasts, and retrograde signals from one organelle that directly or indirectly influence another organelle require further study (Dutilleul et al., 2003b; Jiao et al., 2005; Matsuo and Obokata, 2006).

Using a root-bending assay we have identified mutants in which root growth is sensitive to ABA, and have isolated several mutants, including those with a mutation in DNA
polymerase ε and several elongator subunits (Yin et al., 2009; Zhou et al., 2009). Here, we report on a new PPR protein, ABA overly-sensitive 5 (ABO5), that is located in mitochondria and participates in the cis-splicing of intron 3 of nad2 in mitochondrial complex I. Abo5 showed various growth phenotypes and changed the expression of both nuclear and mitochondrial genes in a photoperiod-dependent manner. Our results suggest that ABO5 is an important mediator in plant responses to ABA.

RESULTS

The abo5 mutant shows growth retardation and is sensitive to ABA

The abo5 mutant was identified during a genetic screen for mutants exhibiting root-growth sensitivity to 30 μM ABA in a root-bending assay performed on a T-DNA insertion mutant pool. ABA inhibits the early seed germination process, including testa and endosperm rupture, post-germination growth (PGG), and the growth of root and shoot. Here, we define PGG as the percentage of seeds that produce seedlings with green cotyledons during early seedling growth. Genetic analysis indicated that the abo5 mutant was caused by a single recessive mutation. Because PGG is more sensitive to ABA than seedling growth (i.e. growth subsequent to PGG), we first compared PGG on MS medium supplemented by a single recessive mutation. Because PGG is more sensitive to ABA overly-sensitive 5 (ABO5), that is located in mitochondrial complex I.

PGG, we first compared PGG on MS medium supplemented with 0.1 μM ABA under a photoperiod of 24-h light/0-h dark (24/0). As shown in Figure 1a,b, PGG without ABA was lower in abo5 than in the wild type. On MS medium containing 0.1 μM ABA, the relative PGG (PGG with ABA expressed as a percentage of PGG without ABA) was much lower for abo5 than for the wild type during 128 h (Figure 1c). Because sugar could increase ABA sensitivity during seed germination, we measured the effects of sugar on PGG of abo5 and the wild type (Figure 1d,e). Without sugar, PGG differed only slightly between abo5 and the wild type. After 72 h on medium containing 3% sucrose, only 12.5% of abo5 exhibited PGG compared with 97.3% of the wild type. After 96 h on MS medium containing 3% sucrose, PGG had increased to 72.2% in abo5. After 72 h on MS medium containing 3% glucose, the germination percentage was 48.6% for abo5 and 97.2% for the wild type. After 96 h on MS medium containing 6% glucose, neither abo5 nor the wild type exhibited any PGG. Next, we transferred 5-day-old seedlings to MS containing different concentrations of ABA and measured the root growth after 6 days. As shown in Figure 1f,g, the root growth of abo5 was more inhibited by ABA than the wild-type root growth. The contents of ABA, sucrose, and total soluble sugars did not differ between abo5 and the wild type (Figure 1i–k). Similarly, the sucrose content of the CMSII mutant is not different from the wild type (Dutilleul et al., 2003a). These results indicate that abo5 is more sensitive than the wild type to both ABA and sugar in terms of PGG, but is especially sensitive to ABA in terms of root growth.

Genetic analysis of abo5 with abi1-1, abi2-1, abi3-1, abi4-1 and abi5 mutants

To clarify the role of ABO5 in the ABA response, we analyzed the double mutants of abo5 combined with each of five classic ABA-insensitive mutants: abi1-1, abi2-1, abi3-1, abi4-1 and abi5. abi1-1 and abi2-1 are two dominative negative mutants (Leung et al., 1997; Meyer et al., 1994), whereas abi3-1 (Giraudat et al., 1992), abi4-1 (Finkelstein et al., 1998) and abi5 (Finkelstein and Lynch, 2000) are loss-of-function mutants. Seed germination of all five mutants is insensitive to ABA. The seed germination of the abo5 abi1-1, abo5 abi2-1, abo5 abi3-1, abo5 abi4-1 and abo5 abi5 double mutants had the same ABA-insensitive phenotype as the abi1 single mutants (Figure 1h, right lane). When the response of root growth to ABA was measured, abo5 abi1-1 and abo5 abi2-1 were similar to abi1-1 and abi2-1 in that they were insensitive to ABA, but abo5 abi3-1, abo5 abi4-1 and abo5 abi5 differed from abi3-1, abi4-1 and abi5 in that they were as sensitive to ABA as abo5 (Figure 1h, left lane). Given that root growth of abi3-1, abi4-1, and abi5 is insensitive to ABA, we conclude that the sensitivity of root growth to ABA of the abo5 abi3-1, abo5 abi4-1 and abo5 abi5 mutants is mainly caused by the abo5 mutation. These results suggest that ABO5 is part of the general response to ABA.

ABO5 encodes a PPR protein localized in the mitochondrion

Because the abo5 mutant was obtained from a T-DNA insertion library, we used TAIL-PCR to clone the targeted gene. The T-DNA was inserted in the second exon of At1g51965 (Figure 2a). Insertion of T-DNA caused premature transcription termination, and the product of ABO5 mRNA was shorter in the abo5 mutant than in the wild type (Figure 3a). We acquired three T-DNA insert SALK lines, and each T-DNA was inserted in the promoter region of At1g51965. RT-PCR using the total RNA isolated from each T-DNA and abo5 indicated that the transcripts were equally detected in the wild type and three SALK lines, but not in the abo5 mutant (Figure 2b). SALK lines with homozygous T-DNA did not exhibit any growth phenotype or ABA-sensitive phenotypes (data not shown). These results indicate that At1g51965 is not disrupted by T-DNA insertions in three SALK lines. To further confirm that abo5 is caused by At1g51965, we crossed abo5 with the Landsberg accession, and performed map-based cloning using the segregated abo5 mutants identified from F2 seedlings, based on the root sensitivity to ABA. As shown in Figure 2c, abo5 was limited to the same T-DNA locus region.

ABO5 cDNA was obtained by RT-PCR from total RNAs isolated from young seedlings. ABO5 encodes a putative P-subfamily PPR protein with 650 amino acids. ABO5 contains one signal peptide for mitochondria at the N terminal, two PPR repeats in the middle and four PPR repeats at the C terminal (Figure 2d). Because the full-length ABO5
fused with GFP failed to emit any fluorescence, we constructed a fused protein containing the ABO5 N-terminal signal peptide fused in frame with GFP, and transiently expressed it in leaf protoplast cells. The fused protein was co-localized with mitochondrial-specific staining marker (Figure 2e), suggesting that ABO5 is localized in mitochondrion.

In the complex-I mutant NADH dehydrogenase (ubiquinone) fragment S subunit 4 (ndufs4) and the complex-III mutant ppr40, a similar delay in seed germination was observed, indicating that the components in complex I and III are critical for seed germination (de Longevialle et al., 2007; Meyer et al., 2009). Seed germination was even more sensitive to ABA in ndufs4 than in abo5 (Figure 2f), suggesting that both ABO5 and NDUFS4 are critical components in plant response to ABA.

We overexpressed the cDNA of At1g51965/ABO5 in abo5 under the control of a cauliflower mosaic virus 35S promoter, and isolated five independent transgenic lines: all of them complemented the retarded-growth phenotypes and the ABA-sensitive phenotypes. Figure 2g,h show one transgenic line (line 3) of the T3 generation that

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**Figure 1.** The phenotypes of abo5 mutants.

(a) The wild type and abo5 were germinated on and grown on MS medium without or with 0.1 µM ABA, and with a photoperiod of 24/0 (24 h of light and 0 h of dark). Seedlings were photographed after 10 days.

(b) Post-germination growth (PGG) of the wild type and abo5 on MS medium. PGG indicates the percentage of total seeds (>40 per plate) that germinated and produced green cotyledons. Values are means ± SEs (n = 3).

(c) PGG of the wild type and abo5 as affected by ABA. Relative PGG is PGG with ABA expressed as a percentage of PGG without ABA. Values are means ± SE. (d) and (e) Relative PGG of the wild type and abo5 on MS medium containing different concentrations of glucose (d) and sucrose (e). Data are means ± SEs (n = 3).

(f) Root growth of the wild type and abo5 on MS medium containing 0 or 50 µM ABA. (g) Root growth of the wild type and abo5 as affected by ABA. Relative root growth is root length in the presence of ABA expressed as a percentage of root length in the absence of ABA. Values are means ± SE (n = 35).

(h) Genetic analysis of abo5 with abi1-1, abi2-1, abi3-1, abi4-1 and abi5. Four-day-old seedlings were transferred to MS medium (upper lane, left) or MS medium containing 30 µM ABA for 5 days (lower lane, left), or different seeds were directly sown on MS medium (upper lane, right) or MS medium containing 1 µM ABA (lower lane, right) for 8 days before seedlings were photographed.

(i) Comparison of soluble sugar contents in the wild type (WT) and abo5 under 24/0 and 12/12 photoperiods.

(j) Comparison of sucrose contents in the wild type (WT) and abo5 under 24/0 and 12/12 photoperiods.

(k) ABA contents in the wild type and abo5 under a 24/0 photoperiod.
complemented the abo5 mutant in both ABA hypersensitivity and growth.

Because PGG and root growth of abo5 are sensitive to ABA, we analyzed the transcripts of ABO5 under ABA treatment. Northern blot indicated that the expression of ABO5 was not influenced by ABA (Figure 3a). We constructed a promoter-GUS binary vector and transformed it into the wild type. GUS staining was determined using transgenic seedlings expressing pABO5-GUS. As shown in Figure 3b–f, GUS activity was greater in flowers, siliques, root tips and young leaves than in mature leaves, stems and elongated roots. Quantitative RT-PCR indicated that ABO5 was differentially expressed in various parts, which was consistent with GUS staining (Figure 3g). These results suggest that ABO5 is universally expressed in Arabidopsis.

**abo5 impairs nad2 pre-RNA splicing and increases the transcripts of mitochondrial genes**

Out of more than 40 proteins in complex I of mitochondria, nine are encoded by genes (nad1, nad2, nad3, nad4, nad4L, nad5, nad6, nad7 and nad9) in the mitochondrial genome (Heazlewood et al., 2003). Among them, the exons of nad1, nad2 and nad5 are scattered on different sites of the genome, and require both cis- and trans-splicing for mRNA maturation. A previous study suggested that the PPR protein
OTP43 is required for the trans-splicing of the *nad1* intron 1 (de Longevialle et al., 2007). Because PPR proteins are mainly involved in organelle RNA metabolism, we first examined the transcripts of mitochondrial genes *nad1*, *nad2* and *nad5*. For each gene, we selected two fragments that cover two different exons separated by other genes, and we used these fragments as probes. As shown in Figure 4a, the splicing of *nad2* was impaired in the *abo5* mutant, but a very low level of correctly spliced *nad2* transcripts could still be detected. *nad2* encodes NADH dehydrogenase subunit 2 in complex I. qRT-PCR was performed to identify the intron(s) in which the splicing was affected. The qRT-PRC conditions used here (which were suitable for amplifying a 300–500-bp fragment) could not amplify the fragment containing the large introns in *nad2* (Figure 4bi). We found that cis-splicing of *nad2* intron 3 was defective in the *abo5* mutant, and the splicing of other introns was not affected by *abo5* mutation (Figure 4bii). Instead, of the transcripts covering introns 1 and 2, two were elevated to higher levels in *abo5* than in the wild type, whereas the transcripts covering intron 4 were unchanged (Figure 4bii). To determine whether the fragment covering exons 3 and 4 was amplified because of stabilization of *nad2* precursor transcripts, we compared the intron-3 transcripts in *abo5* with the wild type by using primers inside intron 3. qRT-PCR indicated that there were twice as many intron-3 transcripts in *abo5* than in the wild type (Figure 4biii). We also compared two other complex-I cis-splicing genes in *abo5* and the wild type, *nad4* and *nad7* (Figure 1a), and complex IV cis-splicing gene *cox2* (Figure 4c), but we did not find a splicing defect. However, all of the genes tested exhibited higher transcriptional levels in *abo5* than in the wild type, including *nad6*, which does not contain any intron (Figure 4d). Complementing the *abo5* mutant with a wild-type *ABO5* cDNA fully restored the splicing of *nad2* (Figure 4e). These results indicate that the *abo5* mutation impairs the cis-splicing of the *nad2* intron 3, and increases the transcripts of all mitochondrial genes tested, but determining whether *ABO5* has other biological roles besides the splicing of the *nad2* intron 3 will require further study.

The transcripts of *nad* genes are highly induced by ABA in the wild type, but are only moderately induced in the *abo5* mutant during prolonged ABA treatment

We analyzed the transcript changes of *cox2* and *nad* genes under ABA treatment for 5 h by northern blot, but did not detect a clear difference between ABA-treated and untreated samples (Figure 4a,c,d). We then monitored the expression of *cox2* and *nad* genes after prolonged ABA treatment. The 7-day-old seedlings of *abo5* and the wild type grown on MS medium were transferred to MS medium containing 10 μM ABA or no ABA: after 7 days, the expression of *cox2* and *nad* genes was examined by qRT-PCR (Figure 5a). Consistent with the results in Figure 4a,c,d, the *abo5* mutation led to an enhanced expression of all *nad* genes and *cox2* relative to the wild type, but *nad* and *cox2* transcripts in *abo5* were only slightly increased by ABA treatment. In contrast, *nad* and *cox2* transcripts were significantly increased by ABA treatment in the wild-type seedlings. The transcripts induced by ABA in the wild type were comparable with those in the *abo5* mutant (Figure 5a). These results suggest that the transcripts of *cox2* and *nad* genes are induced by ABA only with prolonged treatment, and that the *abo5* mutation reduces the response of *cox2* and *nad* genes to such prolonged ABA treatment.

The *abo5* mutant increases the transcripts of *AOX1a*

Complex-I dysfunction usually increases alternative pathways as an adaptive response to mitochondrial stress, as shown in several previous studies (de Longevialle et al., 2007; Dutilleul et al., 2003b; Karpova et al., 2002). AOX is a terminal oxidase that can be induced by various stresses. AOX in Arabidopsis includes two subfamilies with five members, including AOX1a, AOX1b, AOX1c, AOX1d and AOX2 (Strodtkotter et al., 2009). AOX1a is a marker for mitochondrial retrograde response. Previous study indicates that the expression of *AOX1a* is directly regulated by ABI4 (Giraud et al., 2009). We used a northern blot to determine whether the *abo5* mutant alters the transcripts of *AOX1a* (Figure 5b). Under 24 h of light, more transcripts...
were detected in the abo5 mutant than in the wild type. ABA treatment increased AOX1a expression in both abo5 and the wild type. Because the basic level of AOX1a is higher in abo5 than in the wild type, it is likely that ABA did not induce more transcripts of AOX1a in abo5 than in the wild type. qRT-PCR confirmed that the relative increase of AOX1a caused by ABA was similar in abo5 and in the wild type (Figure 9g), suggesting that the high expression of AOX1a in abo5 might not be directly affected by ABI4 transcription because ABI4 expression in abo5 was not changed by ABA treatment (data not shown). The OXI1 gene, which encodes a serine/threonine kinase, is induced by both biotic and abiotic stress, and is an important component in oxidative burst-mediated signaling (Rentel et al., 2004). We compared the expression of OXI1 at different times after ABA treatment (Figure 5c). Before ABA treatment, OXI1 expression was very low in abo5 and almost undetectable in the wild type. ABA treatment for 24 h increased the expression of OXI1 more in abo5 than in the wild type, suggesting that abo5 might suffer from oxidative stress.

Figure 4. Expression levels of mitochondria complex-I subunit genes in abo5 and the wild type. (a) Expression and splicing pattern of complex-I subunit genes that require trans- and/or cis-splicing with or without ABA treatment. Probes were designed to detect each independent pre-mature transcript. rRNAs stained with ethidium bromide were used as loading controls (lower lane). Two-week-old seedlings were treated with 50 μM ABA for 5 h. Expression and splicing patterns were not affected by 5 h of ABA treatment. Note that the mature mRNA (1.497 kb) band (indicated by an arrow) of nad2 was greatly reduced in abo5. (b) i, Diagram of nad2 transcripts. The pre-transcript of nad2 requires both cis- and trans-splicing, as indicated. ii, Quantitative RT-PCR for ABO5 potential target position. Primers were designed as shown in the upper diagram for nad2 to amplify the fragment covering each intron. The relative expression of each fragment amplified by qRT-PCR was compared. Fragments 1, 3 and 4 require cis-splicing, whereas fragment 2 requires trans-splicing. Only the mature mRNAs could be amplified; the longer intron of pre-mRNA could not be amplified under the experimental conditions. iii, Measurement of intron 3 by qRT-PCR. The primers for intron 3 amplification are indicated with arrows in b–i. Values are means ± SEs (n = 3). (c) Expression of complex IV cox2, which requires cis-splicing. Samples were treated with or without 50 μM ABA for 6 h. rRNAs stained with ethidium bromide served as loading controls. (d) Expression pattern of complex I subunit nad6, which does not require splicing. Samples were treated with or without 50 μM ABA for 5 h. rRNAs stained with ethidium bromide served as loading controls. (e) Defect in nad2 splicing was recovered in complementary line 3 (Figure 2g). rRNAs stained with ethidium bromide served as loading controls.
abo5 mutants accumulate increased levels of proline and exhibit increased expression of P5CR and other nuclear genes that encode mitochondrial proteins

To adapt to various environmental stresses, plants accumulate the compatible osmolyte proline. In the biosynthesis of proline in the cytosol, the enzymes pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) catalyze the last two successive reductions from glutamate. Northern blot analysis indicated that P5CS was induced to the same level in abo5 and in the wild type by ABA treatment (Figure 5c). P5CR was not induced by ABA, but its expression was higher in abo5 than in the wild type. GDH1 (glutamate dehydrogenase 1) is localized in mitochondria and catalyzes the reversible amination of 2-oxoglutarate to glutamate in vitro. The expression of GDH1 was also higher in abo5 than in the wild type, and was not induced by ABA. Proline is oxidated by proline dehydrogenase (AtProDH) to produce pyrroline-5-carboxylate (P5C), which is subsequently oxidated by P5C dehydrogenase (P5CDH). The expression of AtProDH was higher in abo5 than in the wild type, and was not induced by ABA. Proline content in both abo5 and the wild type was induced by ABA treatment, and abo5 accumulated more proline than the wild type, regardless of the ABA concentration in the medium (Figure 5d). Similarly, various metabolites including proline are accumulated to a higher level in the complex-I mutant ndufs4 than in the wild type (Meyer et al., 2009).

abo5 mutants accumulate increased levels of H2O2 in root tips

The growth of abo5 primary roots is sensitive to ABA, and ABO5 is involved in the splicing of intron 3 in the complex-I nad2 gene, suggesting that, like the fro1 mutant, the abo5 mutant might accumulate more ROS than the wild type (Lee et al., 2002). We measured the H2O2 level in root tips by...
PROTEIN 1 (AREB1) is an ABA-induced ABRE-binding bZIP (ABF2)/ABSCISIC ACID-RESPONSIVE ELEMENT BINDING protein.

In the root tip region, abo5 accumulated more H₂O₂ under a 24/0 photoperiod (Figure 6a,c) than under a 12/12 photoperiod (Figure 6b,c), and abo5 accumulated much more H₂O₂ than the wild type after ABA treatment under a 24/0 photoperiod (Figure 6a,c), but not under a 12/12 photoperiod (Figure 6b,c). We also measured H₂O₂ content in roots by another quantitative method and obtained similar results (Figure 6d). These results suggest that abo5 accumulates more H₂O₂ than the wild type in the root tips under a 24/0 photoperiod.

abo5 mutants produced fewer transcripts of some ABA-inducible genes

Previous study with fro1 indicates that impairment of complex I increases ROS accumulation while suppressing cold-inducible genes (Lee et al., 2002). We compared the transcripts of several ABA-inducible marker genes between abo5 and the wild type under a 24/0 photoperiod. COR47 and RD29A are induced by various abiotic stresses such as cold, ABA and drought. We found that ABA induced more transcripts of both COR47 and RD29A at 3 h in the wild type than in abo5, and that the response to ABA was delayed in abo5, i.e. the level attained at 3 h in the wild type was attained at 5 h in abo5 (Figure 7a,b). ABRE-binding bZIP proteins (ABF2)/ABSCISIC ACID-RESPONSIVE ELEMENT BINDING PROTEIN 1 (AREB1) is an ABA-induced ABRE-binding bZIP transcriptional factor that regulates the expression of genes in the downstream of the ABA signaling pathway (Choi et al., 2000; Fujita et al., 2005; Kim et al., 2004). The expression of ABF2 was less in abo5 than in the wild type (Figure 7c). ABF2-regulated RD29B is strongly induced by ABA, and its expression was also less in abo5 than in the wild type (Figure 7d). Other stress-inducible genes such as RD22 (Yamaguchi-Shinozaki and Shinozaki, 1993), RAB18 (Lang and Palva, 1992), DREB2A (Liu et al., 1998) and MYB2 (Abe et al., 2003) were all less induced by ABA in abo5 than in the wild type (Figure 7e–h). These results suggest that abo5 mutants were impaired in ABA-regulated gene expression.

abo5 phenotypes are attenuated by reducing the length of light exposure

In the mutant CMSII, plant growth was greatly inhibited by strong light, suggesting that the defect in complex I impairs photosynthetic carbon assimilation under higher irradiance (Praught et al., 2006). We found that the retarded growth and ABA-sensitive phenotype of abo5 could be attenuated (i.e. growth could be increased) when the photoperiod was changed from a 24/0 to a 12/12 photoperiod. Wild-type seedlings grew more slowly under a 12/12 photoperiod than under a 24/0 photoperiod (Figure 8a). In contrast, abo5 seedlings grew better under a 12/12 photoperiod than under a 24/0 photoperiod, and the growth rates of abo5 and the wild type were almost the same under a 12/12 photoperiod. Wild-type growth could be increased) when the photoperiod was changed from a 24/0 to a 12/12 photoperiod. Wild-type seedlings grew better under a 12/12 photoperiod than under a 24/0 photoperiod, and the growth rates of abo5 and the wild type were almost the same under a 12/12 photoperiod on MS medium (Figure 8a). A 12/12 photoperiod also reduced the difference in growth (PGG and relative root growth) between abo5 and the wild type on a medium containing ABA (Figure 8b,c). We also measured proline...
contents under a 12/12 photoperiod, and found no obvious difference between the wild type and abo5 (Figure 8d).

**Gene expression patterns change under a 12/12 photoperiod**

To clarify the impact of photoperiod on gene expression, we used qRT-PCR to analyze mitochondrial gene expression of nad1, nad4, nad5, nad6 and nad7 under different photoperiods. Consistent with the northern blot results, the transcript levels of the five genes were greater in abo5 than in the wild type under a 24/0 photoperiod. Under a 12/12 photoperiod, however, transcriptional levels of these genes were similar for abo5 and the wild type (Figure 9a–e) because gene expression levels were much greater under a 12/12
than under a 24/0 photoperiod for the wild type, but remained unchanged for abo5 (Figure 9a–e). For the nad2 gene, we measured the transcripts of different exons covering intron 1, 2, 3 or 4 (Figure 9f). The transcripts covering introns 1 and 2 were higher in abo5 than in the wild type under a 24/0 photoperiod, but were similar under a 12/12 photoperiod. Because abo5 impaired the intron-3 splicing, transcripts covering intron 3 were not detected in abo5 but were detected in the wild type. Because of the impairment of intron-3 splicing, the transcripts covering intron 4 were unaffected by photoperiod in abo5. Transcripts covering introns 1, 2, 3 and 4 were higher under a 12/12 photoperiod than under a 24/0 photoperiod in the wild type, which is consistent with the expression patterns of other nad genes in complex I.

We also compared the transcripts of AOX1a and OXI1 in seedlings treated or not treated with 50 μM ABA for 24 h under a 12/12 or 24/0 photoperiod; we found that their expression levels were lower under a 12/12 than under a 24/0 photoperiod in both the wild type and the mutant (Figure 9g,h), and that ABA treatment increased the expression of AOX1a and OXI1 in abo5 and the wild type under both photoperiods. However, expression differences between abo5 and the wild type under ABA treatment occurred under the 24/0 photoperiod but not under the 12/12 photoperiod. There was no clear difference in OXI1 expression without ABA treatment (Figure 9h).

Expression of nuclear genes in photosynthesis is changed under constant light conditions in the abo5 mutant

Chloroplasts and mitochondria engage in close crosstalk during photosynthetic respiration. Previous studies indicate that impairment of mitochondrion function will reduce the photosynthetic performance in chloroplasts (Noctor et al., 2007). Because abo5 exhibited strong retarded-growth phenotypes under constant light (a 24/0 photoperiod) but not under short days (a 12/12 photoperiod), we suspected that the abo5 mutation might inhibit photosynthesis by influencing the expression of photosynthetic genes. We compared the expression of two nuclear photosynthetic genes in abo5 and the wild type. Proton gradient regulation 5 (PGR5) encodes a thylakoid membrane protein important in the ferredoxin-dependent cyclic electron flow...
genes, and AtnMat2 is responsible for the splicing of intron 2 and mitochondrial intron 1, AtnMat1a is responsible for the splicing of mitochondrial intron 3 in mitochondria. nad2 encodes NADH dehydrogenase subunit 2, one of nine mitochondrial gene-encoded proteins in complex I. The huge PPR protein family is considered to have expanded before the evolutionary divergence of monocots and dicots (O’Toole et al., 2008). In Arabidopsis and rice, the sizes of the PPR families are similar (450 members in Arabidopsis and 477 in rice) (O’Toole et al., 2008), suggesting that these proteins might have conserved biological roles in the monocot and dicot plants. As of now, however, the biological roles of only a small number of PPR proteins have been characterized.

In plants, because of the presence of alternative NAD(P)H dehydrogenases, the electron transport chain can bypass complex I when complex I is not efficiently working or is under stress. In Arabidopsis, six nuclear mutants involved in complex I have been described, including abo5 (this study), fro1/ndufs4 (an 18-kDs subunit of complex I) (Lee et al., 2002; Meyer et al., 2009), a T-DNA insertion mutant in At1g47260 (encoding a carbonic anhydrase) (Perales et al., 2005), opt43 (de Longevialle et al., 2007), css1 (changed sensitivity to cellulose synthesis inhibitors 1, At1g30010, encoding a maturase) (Nakagawa and Sakurai, 2006) and AtnMat2/At5g46920 (Keren et al., 2009). The PPR protein OPT43 is responsible for the trans-splicing of nad1 intron 1, AtnMat1a is responsible for the splicing of mitochondrial nda4, and probably also other mitochondrial genes, and AtnMat2 is responsible for the splicing of cox2, nad1 intron 2 and nad7 intron 2 (de Longevialle et al., 2007; Keren et al., 2009; Nakagawa and Sakurai, 2006). The plant size of the At1g47260 T-DNA mutant is normal, i.e. similar to that of the wild type. The css1 mutant grows slower that the wild type, but finally reaches the same size as the wild-type plant. abo5, fro1/ndufs4, opt43 and AtnMat2 mutants are smaller, and have delayed germination and retarded growth. The inhibition of abo5 germination by high sugar concentration was also observed in the fro1/ndufs4, and css1 mutants (Lee et al., 2002; Meyer et al., 2009; Nakagawa and Sakurai, 2006). Previous studies indicate that sugar has a strong connection with ABA regarding seed germination.

Screening for sugar-insensitive (sis) or glucose-insensitive (gin) mutants identified several ABA-insensitive or biosynthesis-deficient mutants, including abaa2, abad and abai5 (Arenas-Huertero et al., 2000; Laby et al., 2000). Indeed, besides being sensitive to high sugar concentration, the germination of both abo5 and ndufs4 was sensitive to ABA treatment. Furthermore, the seed germination sensitivity to ABA in abo5 could be reversed by each of five classic abi mutations, including two dominant negative mutations in ABI1 and ABI2, and the mutations in ABI3, ABI4 and ABI5. Previous study in analyzing the ABA-sensitive mutant era1 combined with abi1-1 or abi2-1 mutants indicates that the seed germination of abi1/1era1 and abi2/1era1 double mutants is sensitive to ABA (Brady et al., 2003). These results indicate that ABO5 and ERA1 are at different genetic positions with respect to their response to ABA. In the ppr40 mutant that impairs the function of complex III, seed germination is also sensitive to ABA. These results suggest that the energy-requirement processes including both complex I and complex III play important roles in regulation of seed germination by ABA (Meyer et al., 2009; Zsigmond et al., 2008).

It is interesting that abo5 was isolated during a genetic screen for mutants in which the roots were sensitive to ABA. ABI1 and ABI2 are two negative regulators in the ABA signaling pathway that have various roles in regulating guard cell movement, plant growth and development, whereas the functions of ABI3, ABI4 and ABI5 have mainly been studied relative to seed germination, but also relative to early seedling development (Bossi et al., 2009; Brady et al., 2003; Lopez-Molina et al., 2001). These results suggest that seed germination and seedling growth respond differently to ABA, and that ABO5 and other ABA-related genes may play different roles in these biological processes.

The abo5 mutation increased the expression of nad genes in complex I and of the cox2 gene in complex IV relative to the wild type. However, in the CMSII mutant (lacking a functional mitochondrial complex I) and in the css1 mutant (that impairs the splicing of nad4), the expression of cox2 and/or nad genes was not enhanced (Lelandais et al., 1998; Nakagawa and Sakurai, 2006). These results suggest that unlike the CMSII mutant, which lacks only nad7, the abo5 mutation might not only impair the splicing of nad2 intron 3 but might also play other unidentified biological roles, most probably in mitochondrion.

Although the nad genes in complex I and the cox2 gene in complex IV of the mitochondrial genome were expressed at higher levels in abo5 than in the wild type under 24 h of light, the expression of these genes was not increased by a short treatment with ABA. Interestingly, prolonged ABA treatment was able to induce the expression of cox2 and nad genes to higher levels in the wild type, but only to moderate levels in abo5, probably because cox2 and nad genes were already highly expressed as a result of the abo5 mutation.
These results suggest that the ABA-sensitive phenotype of abo5 might result from the direct impact of ABA on gene expression in the mitochondrion. However, ABO5 expression was not influenced by ABA treatment (5 h in Figure 3a; 7 days, data not shown). The results suggest that ABA affects the expression of cox2 and nad genes in abo5 probably through post-transcription regulation. It is possible that impairment of complex I and/or other biological functions in mitochondria would also impose severe stresses on other cellular components such as chloroplasts, peroxisomes, the cytosol and the nucleus. We found that the expression of some stress-inducible nuclear genes was less induced in abo5 than in the wild type by ABA treatment in a 24/0 photoperiod. Previous study indicates that the cold-stress induction of several cold-induced genes is reduced by the fro1 mutation relative to the wild type (Lee et al., 2002). Because lesions of both FRO1/NDUFS4 and ABO5 lead to the accumulation of ROS, it is possible that ROS act as signals to change gene expression levels (Lee et al., 2002; Meyer et al., 2009). Because both AB11 and AB12 proteins are sensitive to ROS, the accumulated ROS would inhibit the activities of the negative regulators AB11 and AB12 (Meinhard and Grill, 2001; Meinhard et al., 2002). We observed a higher accumulation of ROS in the root tips of abo5 than in the root tips of the wild type, especially in ABA treatment under constant-light conditions. It is therefore possible that the root growth of the ABA-sensitive phenotype might partially result from an increase in ROS accumulation in abo5 relative to the wild type. Consistently, the disruption of two ROS production genes, NADPH oxidase catalytic subunits AtrbohD and AtrbohF in Arabidopsis, impairs the ABA inhibition of seed germination and root growth (Kwak et al., 2003).

Disruption of electron flow in the respiratory chain usually leads to cellular redox imbalance, with a resulting overproduction of ROS. AOX is considered to be important for minimizing ROS generation. Induction of AOX is also closely linked to ROS overproduction in mitochondria. In abo5, more AOX1α was expressed under a 24/0 than under a 12/12 photoperiod, which is consistent with enhanced redox stress caused by a defect in complex I. Previous studies indicate that AOX is necessary for dissipating redox equivalents from chloroplasts (Noctor et al., 2007). Induction of AOX1α in abo5 could help prevent the photoinhibition caused by the overproduction of redox equivalents in chloroplasts. Mutants with a defect in complex I, like opt43, CMSII and maize non-chromosomal stripe (NCS) mutants, have high rates of AOX transcription and translation (de Longevialle et al., 2007; Dutilleul et al., 2003b; Karpova et al., 2002; Vidal et al., 2007). Interestingly, a recent study suggests that the expression of AOX1α is directly regulated by ABI4 (Giraud et al., 2009). However, unlike the CMSII mutant, which does not accumulate more H₂O₂ than the wild type, the abo5 mutant, like fro1/ndufs4, accumulated slightly more H₂O₂ than the wild type (Lee et al., 2002; Meyer et al., 2009). Consistent with a slightly higher accumulation of H₂O₂ in abo5, OXI1, which is another important oxidative burst-mediated gene, was also expressed at a slightly higher level in abo5 than in the wild type under a 24/0 photoperiod. OXI1 is an important mediator for activating two important mitogen-activated protein kinases, MPK3 and MPK6, for ROS-mediated root hair growth (Rentel et al., 2004). The expression of OXI1 is induced by various stresses that stimulate the production of H₂O₂ (Rentel et al., 2004).

Our study suggests that the abo5 mutation, probably partially through impairing complex I as a result of a splicing defect in nad2 intron 3, leads to various stresses at both molecular and growth phenotypic levels, and that the severity of these stresses largely depends on light. A reduced light period alleviates all these different phenotypes, including retarded plant growth and expression of various genes. Complex I in the mitochondrion is the major entry point for the electron transport chain, which burns off the excess reducing NAD(P)H that is produced by photorespiration (from glycine oxidation or by respiration carbon flow), or that is exported directly from chloroplasts during photosynthesis (Noctor et al., 2007). This defect of complex I would alleviate the efficiency of NADH oxidation, which would lead to the accumulated reduced equivalents, which in turn would reduce the photosynthetic activity by regulating the expression of nuclear genes through the retrograde signal pathway. The reduced expression of two important genes, PGR5 and PGR1A, in photosynthesis supports this hypothesis. Previous studies with the Nicotiana sylvestris CMSII mutant suggest that plants with a loss of function of complex I caused by a mutation of nad7 are not lethal, but decrease the ratio of photosynthesis to respiration with a lower glycine oxidation in a light strength-dependent manner (Noctor et al., 2007; Priault et al., 2006; Sabar et al., 2000). Because mitochondria are closely related to chloroplasts, in that both organelles can convert glycine to serine and provide ATP for cytosol sucrose synthesis during photosynthesis, and as required for cellular redox balance, it is reasonable that the mutants defective in complex I or other mitochondrial mutants will greatly affect photosynthetic activities, just as CMSII and the abo5 mutant do (Noctor et al., 2007, 2004; Pogson et al., 2008; Priault et al., 2006; Sabar et al., 2000). However, a biochemical analysis is needed to determine the degree to which the abo5 mutation changes complex I activity.

**EXPERIMENTAL PROCEDURES**

**Plant growth conditions and mutant isolation**

*Arabidopsis thaliana* (Columbia accession) seedlings were grown in forest soil and vermiculite (1:1) under long-day conditions (16-h light/8-h dark photoperiod) at 22°C in a glasshouse. Seedlings in plates were grown on MS medium (M5519; Sigma-Aldrich, http://www.sigmaaldrich.com) containing 3% (w/v) sucrose and 0.8% 761
(w/v) agar with a 24/0 or a 12/12 photoperiod in a plant growth chamber at 22°C.

A T-DNA insertion Arabidopsis pool was screened for root bending as described previously (Yin et al., 2009). Seedlings grown on MS medium containing 1% agar for 4 days were transferred onto the medium containing 1% agar and 30 μM ABA. abo5 was selected as an ABA-sensitive mutant.

Germination and root growth assay

Seeds (>40) were placed on MS medium containing different concentrations of ABA, sucrose and glucose, with three plates for each treatment. Samples were kept at 4°C for 2 days and then moved to a plant growth chamber with different photoperiods (24/0 or 12/12). Seedlings with green cotyledons were counted after 2 days, and data were collected every 12 or 24 h. The relative level of PGG (PGG in the absence of ABA) was calculated. This experiment was independently repeated three times.

Five-day-old seedlings grown on MS medium were transferred onto plates containing different concentrations of ABA under a 240 or a 12/12 photoperiod. Root growth was measured after 7 days. Relative root growth (root length in the presence of ABA expressed as a percentage of PGG in the absence of ABA) was calculated. This experiment was independently repeated three times.

TAIL-PCR for abo5 cloning

The T-DNA flank sequence in abo5 was determined by thermal asymmetric interlaced PCR with pSK1015-specific primers on the left border and a random primer. Primers were as follows: AtLB1, AT-AGCCGAGTGATTTGTGC; AtLB2, TAAT AACGTCGGA CCTAC-TAC; AtLB3, TTTGAGTATCACTGTTGC; random primer DEG1, WGCNAGNAGWANAAAG (W = A or T; N = A, C, G or T). The reaction program for each round was previously described by Qin et al. (2003).

Map-based cloning of abo5

abo5 mutants were crossed with the Landsberg accession, and 1192 abo5 mutants were picked out from the F2 population for their root growth sensitivity on MS medium containing 30 μM ABA. We used simple sequence length polymorphism markers to narrow the mutated site between F5D21 (forward, 5'-ACCCTGCGCACCTCTCCTAACT-3') and F6D8 (forward, 5'-GTTAGAAGCTGAAAGCCAAAACA-3'); reverse, 5'-AGGATTAATGAGAAAGCTTGGGAC-3') and further mapped using markers T14L22 (forward, 5'-CCAACAAGCTCCTATGATA-CACCA-3'); reverse, 5'-CCCCATCATCGATCAGGATG-3') and F19K6 (forward, 5'-GTGGAGATGCAGTGCCCTAACAAGTGT-3'); reverse, 5'-CCGTCTTTGCGGATATACCTTGGATG-3') and F5F19 (forward, 5'-CCAATCACGAAACTGAGTCCGACG-3'); reverse, 5'-GATTGG-GCCAAGCCCATAACAC-3'). The abo5 mutation was located at the top of BAC F5F19.

RNA gel blot analyses

Seedlings grown on MS medium under a 24/0 or a 12/12 photoperiod were transferred into double-distilled water containing 0 or 50 μM ABA. ABA under a 24/0 or a 24/0 photoperiod were ground in liquid nitrogen, and free proline contents were determined according to Chen et al. (2006). Soluble sugar and sucrose contents were determined as previously described (Zhang et al., 2008), and ABA content was determined as described by Chen et al. (2006). These experiments were independently repeated three times.

nad2 splicing analysis

nad2 premature transcript splicing was analyzed by qRT-PCR. Total RNA was isolated, and its reverse transcription was amplified by qRT-PCR. If introns between the two exons were too long to be amplified, detected PCR products stand for mature transcripts that have been spliced already. Primers used for each fragment were as follows: fragment 1, forward, 5'-CTCCTCCTCCTCACTACCTTCT-3'; reverse, 5'-AGGGATCCCGGATTGAAAGT-3'; fragment 2, forward, 5'-CGCTTGGCAGATACCTTGGATG-3'; reverse, 5'-GATTGG-GCCAAGCCCATAACAC-3'. The abo5 mutation was located at the top of BAC F5F19.

H2O2 assays

For the DCFH-DA staining assay, 1-week-old seedlings were amplified with forward primer 5'-Pst1 (CTCATTGACATTTTGGTGA-3') and reverse primer 5'-EcoR1 (TCTTGGGAGAAGGAGAC-3'). PCR products were digested and constructed into the pCAMBIA1391 vector. abo5:GUS was transformed into Arabidopsis by floral dip. Histochemical staining was performed as described previously by Zhou et al. (2009). Twenty-six T2 transgenic lines were subjected to the GUS staining assay.

GUS staining assay

Total RNA was extracted with Trizol reagent and digested with DNase I. A 6-μg quantity of total RNA was transcribed by M-MLV reverse transcriptase (Promega, http://www.promega.com). qRT-PCR was performed as previously described by Zhou et al. (2009). ACTIN4 was used as the internal control to normalize the samples. Each experiment was independently repeated three times, and the primers used for qRT-PCR are listed in Table S2. The primers used to amplify AOX1a, OX11, cox2 and nads were the same as those used to amplify fragments for the northern blot.

Determination of proline, sugar and ABA content

Two-week-old seedlings grown on medium containing 0 or 0.1 μM ABA under a 12/12 or a 24/0 photoperiod were ground in liquid nitrogen, and free proline contents were determined according to Chen et al. (2006). Soluble sugar and sucrose contents were determined as previously described (Zhang et al., 2008), and ABA content was determined as described by Chen et al. (2006). These experiments were independently repeated three times.
performed as follows: one cycle at 95°C for 5 min followed by 40 cycles of 95°C for 20 s, 57°C for 20 s and 72°C for 20 s. This experiment was independently repeated three times.

**Subcellular localization**

A 488-bp fragment at the N terminal of ABO5 was amplified and fused with GFP in frame in front of the 35S promoter. Primers used for amplifying the ABO5 N-terminal fragment were: 5′-AT-GAACTCTCCCGCGCC-3′; 5′-CACCATGAAATGAGAATCGAC-3′. Plasmids were extracted and introduced into protoplasts prepared from Arabidopsis leaves following the protocol previously described by Jin et al. (2001). Mitochondria were stained by Mito-tracker (Invitrogen) for co-localization. GFP fluorescence was detected with a confocal microscope, 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.

**Isolation of double mutants of abo5 with each of five classic abi mutants**

abo5 was crossed with abi1-1, abi2-1, abi3-1, abi4-1 and abi5. The F2 plants were used to identify double mutants. Primers used for confirming the mutation in abi1-1, abi2-1, abi3-1, abi4-1 and abi5 were as follows: for ABI1, forward, 5′-GATAATCTCGCGGGAGATT-3′, reverse, 5′-CCATTCCACAGTACCCTT-3′; for ABI2, forward, 5′-CATCATTGCCTATGGAGG-3′, reverse, 5′-CCGGAGCATGAGCCACAG-3′; for ABI3, forward, 5′-CGGTTTCTCTCGGAAAAGTCTTTCTG-3′, reverse, 5′-TTGCCCTCTAGCTCGCCGAAGT-3′; for ABI4, forward, 5′-ATGGGACCTTATGGTCGAC-3′, reverse, 5′-AGTTACCCAGAATCATGGAC-3′; for ABI5, forward, 5′-AGCTGACCGAAGGAAAAGTCTGCTT-3′, reverse, 5′-CTGACACCACTGCTTGTACCTATG-3′. PCR products of abi1-1, abi2-1, abi3-1, abi4-1 and abi5 were digested by Ncol, Ncol, SalI, NlaIV and MseI, respectively. abo5 was checked with the same ABO5-F and ABO5-R primers used for qRT-PCR (see Tables S1 and S2).

**Accession number**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_148578 (At1g51965).

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**SUPPORTING INFORMATION**

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

**Table S1.** Primers used to amplify fragments in the northern blot.

**Table S2.** Primers used for quantitative RT-PCR.

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**REFERENCES**


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