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RESEARCH PAPER

Isolation of *Arabidopsis ahg11,* a weak ABA hypersensitive mutant defective in *nad4* RNA editing

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Abstract

The phytohormone abscisic acid (ABA) plays pivotal roles in the regulation of developmental and environmental responses in plants. Identification of cytoplasmic ABA receptors enabled the elucidation of the main ABA signalling pathway, connecting ABA perception to either nuclear events or the action of several transporters. However, the physiological functions of ABA in cellular processes largely remain unknown. To obtain greater insight into the ABA response, genetic screening was performed to isolate ABA-related mutants of *Arabidopsis* and several novel ABA-hypersensitive mutants were isolated. One of those mutants—*ahg11*—was characterized further. Map-based cloning showed that *AHG11* encodes a PPR type protein, which has potential roles in RNA editing. An AHG11-GFP fusion protein indicated that AHG11 mainly localized to the mitochondria. Consistent with this observation, the *nad4* transcript, which normally undergoes RNA editing, lacks a single RNA editing event conferring a conversion of an amino acid residue in *ahg11* mutants. The geminating *ahg11* seeds have higher levels of reactive-oxygen-species-responsive genes. Presumably, partial impairment of mitochondrial function caused by an amino acid conversion in one of the complex I components induces redox imbalance which, in turn, confers an abnormal response to the plant hormone.

Key words: *Arabidopsis*, abscisic acid, mitochondria, pentatricopeptide repeat protein, RNA editing.

Introduction

The phytohormone abscisic acid (ABA), beside playing important roles in developmental processes such as seed maturation, germination control, and lateral root initiation, is also critically involved in various abiotic stress responses in plants, such as drought, salinity, and low temperature (Finkelstein *et al*., 2002; De Smet *et al*., 2006). Because of the importance of ABA in plants, its biosynthesis, mechanism of action, and its degradation have been vigorously investigated. Most enzymes involved in the biosynthesis and catabolism of ABA have been identified and the main pathways in both processes have been established (Nambara and Marion-Poll, 2005). Many putative signalling factors involved in the ABA response have been identified, but the signal transduction pathway itself remained unclear for many years (Hirayama and Shinozaki, 2007). A recent breakthrough in ABA signalling—the identification of a member of ABA receptors—greatly enhanced our understanding of the mechanisms by which ABA stimuli are translated into transcriptional responses or ion movements (Fujii *et al*., 2009; Ma *et al*., 2009; Park *et al*., 2009; Umezawa *et al*., 2009; Vlad

Abbreviations:ABA, abscisic acid; GFP, green fluorescent protein; PCR, polymerase chain reaction; PP2C, protein phosphatase 2C; PPR, pentatricopeptide repeat protein; qRT-PCR, quantitative reverse transcription-PCR; ROS, reactive oxygen species; SnRK2, SNF1-related protein kinase 2.

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et al., 2009). According to the recent studies, ABA recognition by PYR/PYL/RCAR-type receptors enhances the physical interaction between ABA receptors and 2C type protein phosphatases such as ABI1, ABI2, AHG3, HAB1, or HAB2 and inhibits PP2C activity, which, in turn, activates ABA-activated subclass III SnRK2s such as SnRK2.2/SRK2D, SnRK2.3/SRK2I, and SnRK2.6/SRK2E/OST1 in *Arabidopsis*. Activated SnRK2s then phosphorylate transcription factors such as ABF/AREB and ion-transporters such as SLAC1 (Furihata *et al*., 2006; Geiger *et al*., 2009).

Although the main ABA signalling pathway has been established, there is a lot more to be learned about ABA action. For example, the identified targets of SnRK2s or PP2Cs do not fully account for the diverse cellular responses to ABA. Identification of the ABA transporters strongly indicates the presence of an ABA regulatory system at the tissue or whole-body level (Kang *et al*., 2010; Kuromori *et al*., 2010), but we have almost no insight into such a system. In addition, the mechanisms that regulate germination, a process in which ABA is deeply involved, remain to be established. Therefore, further studies are required to obtain a more holistic understanding of the ABA action.

To obtain insights into the ABA action, a genetic screening was conducted to isolate *Arabidopsis* mutants with an abnormal response to ABA. ABA hypersensitive mutants have previously been reported: *ABA hypersensitive germination* (*ahg*)*1, ahg2, ahg3* (Nishimura *et al*., 2004, 2005, 2007; Yoshida *et al*., 2006). In this study, the *aba2-1* mutant that produces less ABA was used to skew our screen toward the identification of mutants that disrupt ABA signalling rather than those that affect ABA production (González-Guzmán *et al*., 2002). Consequently, three novel mutants were obtained that showed an enhanced ABA hypersensitivity at germination and they were named *ahg11, ahg12,* and *ahg16*. These mutants exhibit weaker but more unique phenotypes when compared with the pre-existing ABA-hypersensitive mutants. The *AHG11* gene was analysed further and it was found that it encodes a pentatricopeptide repeat protein (PPR). Genes encoding PPR proteins make up one of the largest gene family in plants. The PPR proteins are classified into two subfamilies—P and PLS—on the basis of the context of the motifs. Accumulated evidence supports the idea that PPR is involved in plastid and mitochondrial RNA processing, including splicing, RNA editing, and translational regulation (Saha *et al*., 2007; Schmitz-Linneweber and Small, 2008). The class of PPR proteins that can edit RNA belong to the PLS subfamily, which consist of a tandem array of PPR motifs and three additional C-terminal motifs, namely, E, E+, and DYW. The E+ motif is required for the conversion of C to U rather than for the recognition of the *cis*-elements for editing sites (Okuda *et al*., 2007). Being an E+ type PLS, AHG11 could be responsible for an RNA editing(s). An RNA editing site was successfully identified in mitochondrial *nad4* transcripts as the target of AHG11. The *ahg11* seeds accumulate more mRNAs for oxidative stress-responsive genes. These results suggest that mitochondrial function interacts with and regulates the action of stress-related hormones in plants.

Materials and methods

Plant materials, growth conditions, and mapping of loci

Arabidopsis thaliana (L.) Heynh. ecotypes Columbia (Col) and Landsberg *erecta* (L*er*) were used. Plant growth conditions were as described previously (Nishimura *et al*., 2004). Ethyl methanesulfonate (EMS)-mutagenized M_2 seeds were obtained as follows: *aba2-1* seeds were treated with 0.3% EMS for 16 h at room temperature (*c*. 2000 seeds per parental group), washed extensively with water, and sown on soil. M_2 generation seeds were separately harvested and formed independent pools. For germination assays, approximately 50 seeds were sown on plates containing 1× Murashige and Skoog salt mix, 2% sucrose, and various concentrations of plant hormones, NaCl, or mannitol. Seeds were imbibed at 4 °C for 4 d. Germination (emergence of radicles) and post-germination growth (green and expanded cotyledons) were scored at the indicated time points. The extraction and quantitative analyses of ABA were performed as described previously (Nishimura *et al*., 2005). For mapping loci, the *ahg11, 12,* or *16* mutant was crossed with L*er*, and F_2 progeny were obtained. ABA-hypersensitive individuals were selected on a medium containing 0.2 μM ABA and grown on a normal medium. The isolation of genomic DNA and PCR conditions for PCR-based DNA markers are described elsewhere (Hirayama *et al*., 1999).

Complementation analysis of ahg11

The genomic *AHG11* DNA fragment was obtained by PCR using high-fidelity DNA polymerase (KOD-Plus; Toyobo, Osaka, Japan) with primers, F-*Bam*Hpro2 and R-ter*Xba*I (see Supplementary Table S1 at *JXB* online), and subcloned into pBluescript SK(–). After the confirmation of nucleotide sequence, this *AHG11* clone was re-introduced into the binary vector pBI101. *Agrobacterium tumefaciens* strain GV3101 was transformed with the resultant plasmid and used for the infection of *Arabidopsis* plants by the flower-dipping method (Clough and Bent, 1998). Transgenic lines were screened by kanamycin tolerance in the next generation.

Northern blotting

Total RNA isolated from wild-type or *ahg11* seeds which were imbibed at 4 °C for 4 d and incubated for 48 h were separated on 1.2% agarose gel, transferred to a nylon membrane, and hybridized with a DIG labelled *nad4* DNA probe. DNA labelling and detection were performed with a DIG High Prime DNA labelling and Detection Starter Kit II (Roche Diagnostics Japan, Tokyo) following the manufacturer's instructions.

Analysis of GFP-fusion proteins

DNA segments containing the AHG11 N-terminal portion (18505110nt–18505358nt of the chromosome II sequence) or the AHG11 open reading frame (18505110nt–18506903nt) were amplified using F-*Bam*HINdeI3/R*Xba*2 primer set or F-*Bam*HINdeI3/AHG11-N-*Xba* primer set, respectively (see Supplementary Table S1 at *JXB* online), and fused to the SGFP gene downstream of the CaMV 35S promoter in pTH-2 (Chiu *et al*., 1996). The resultant plasmid was introduced into *Arabidopsis* mesophyll protoplasts obtained according to a previously described method (Yoo *et al*., 2007). To stain mitochondria, protoplasts were incubated with 100 nM Mitotracker Red CMXRos (Life Technologies Corporation, Tokyo, Japan) for 15 min and washed with the WI buffer thrice. Fluorescence was observed under a confocal microscope (FV1000-D, Olympus, Tokyo, Japan).

Analysis of editing status of organelle mRNA

Total RNA was isolated from *ahg11,* wild type, and *ahg11* harbouring pBI101-*AHG11,* using the TRIzol extraction reagent (Invitrogen Japan KK, Tokyo, Japan). cDNA was synthesized from 1 μg of total RNA using a ReverTra Ace Kit (Toyobo, Osaka, Japan). After DNase treatment, first strand cDNA was synthesized from total RNA (~1 μg) with random primers. Using cDNA as a template, a segment containing the edited RNA site was amplified by PCR with gene-specific primers (see Supplementary Table S1 at *JXB* online) and its sequence was determined.

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qRT-PCR

Total RNA was isolated from *ahg11* and wild-type seeds which were imbibed at 4 °C for 4 d and incubated in the growth chamber for 48 h. After DNase treatment, first strand cDNA was synthesized from total RNA (~1 µg) using the oligo dT(15) primer. Real-time PCR was performed on a LightCycler (Roche Diagnostics, Mannheim, Germany) in a total volume of 20 µl containing 10 µl of SYBR Green Real-Time PCR Master Mix -Plus- (Takara Bio Inc., Otsu, Japan), 8 pmol of each primer, and 1/40 of the cDNA mixture. The amplification program consisted of 50 cycles of 95 °C for 10 s and 60 °C for 1 min. The primers used are listed in Supplementary Table S1 at *JXB* online. *At4g02080* was used to normalize the expression levels (Dekkers *et al*., 2012).

Results

Isolation of ABA hypersensitive mutants from an aba2-1 *background*

Accumulation of ABA in seeds causes ABA hypersensitivity at germination. It was previously shown that the seeds of *ahg2-1*, an ABA hypersensitive mutant, have higher endogenous ABA levels (Nishimura *et al*., 2005). It is postulated that higher levels of endogenous ABA cause an enhanced ABA response. In order to isolate mutants that have defects downstream of ABA synthesis rather than in ABA synthesis or catabolism, factors that affect endogenous ABA levels need to be eliminated or reduced. To this end, the *aba2-1* mutant was used, which has reduced endogenous ABA levels due to a defect in the short-chain dehydrogenase enzyme and hence in ABA biosynthesis (Léon-Kloosterziel *et al*., 1996; González-Guzmán *et al*., 2002). The *aba2-1* seeds were mutagenized with EMS, and M_2 progeny were obtained. M_2 seeds were sown on agar medium supplemented with the ABA analogue PBI-51 or ABA and germination was induced (Nishimura *et al*., 2004). After several days, ungerminated or no-green individuals were collected and tested for their ABA hypersensitivity during germination in the next generation. This screening isolated five ABA hypersensitive candidates, which were named as *ABA hypersensitive germination* (*ahg11*, *12, 13, 15,* and *16*). These mutants exhibited similar degrees of ABA hypersensitivity in the presence of the *aba2-1* mutation (Fig. 1).

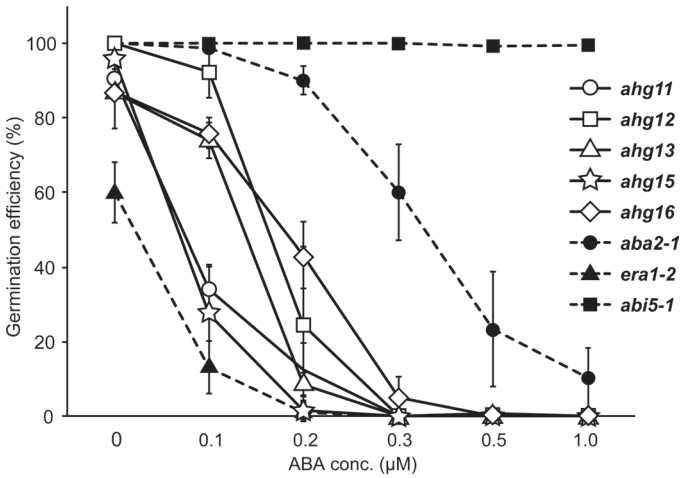

Fig. 1. ABA sensitivity of new ABA-hypersensitive mutants at germination. Various mutant seeds were sown on the medium containing various concentrations of ABA. Germination efficiency is determined by counting seedlings (n >40) with green cotyledons every day for a week. The data shows the germination efficiency at day 7—a mean of four independent experiments. Error bars indicate standard deviation.

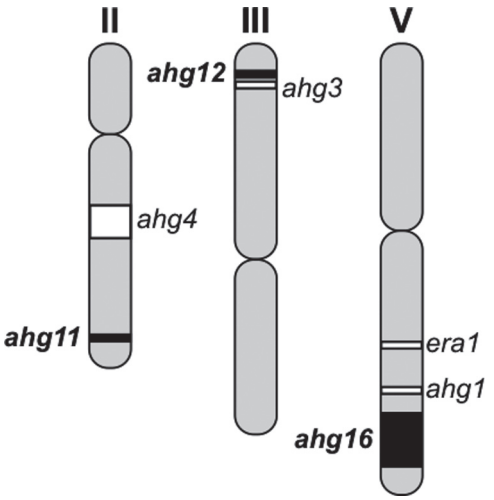

Fig. 2. Chromosomal positions of *ahg11, ahg12,* and *ahg16.* Schematic representation of the position of *ahg11, ahg12,* and *ahg16* loci (black boxes). Neighbouring known ABA hypersensitive loci are shown as white boxes.

Mapping of ahg11, 12, *and* 16 *loci*

After segregation of the *aba2-1* mutation and a backcross to the wild type, three mutants, namely, *ahg11*, *ahg12*, and *ahg16* retained strong phenotypes, enough to conduct mapping procedures. These test crosses revealed that *ahg11* and *ahg16* are recessive while *ahg12* is dominant. For genetic mapping, these mutants were crossed with L*er* wild-type plants and F_2 progeny were obtained. PCR-marker-based rough mapping with approximately 100 F_2 lines with ABA hypersensitivity indicated that *ahg11*, *ahg12*, and *ahg16* are located at the bottom of chromosome 2, the top of the chromosome 3, and the bottom of chromosome 5, respectively (Fig. 2). Previously, several other ABA-hypersensitive mutants were reported (Nishimura *et al*., 2004, 2005, 2007; Yoshida *et al*., 2006). The locus of *AHG3* is close to that of *ahg12*, but sequencing of the *AHG3* gene in the *ahg12* mutant confirmed that these mutants are not allelic. *AHG1* and *AHG4* were mapped to the lower arm of chromosome 5 and the middle part of chromosome 2, respectively, but these regions were unlinked to *ahg11* and *ahg16* loci. Therefore, *ahg11, 12, 16* are new ABA-hypersensitive loci.

Characterization of ahg11

In this study, *ahg11* was chosen for further analysis because of the relative clarity of its phenotype. It was postulated that *ahg11* does not increase the endogenous ABA levels because this mutant was isolated in the *aba2-1* background that has reduced ABA biosynthetic activity. Indeed, the endogenous ABA levels in *ahg11* dry seeds were lower than the wild type and those in imbibed seeds were the same as wild type (Fig. 3), implying that *ahg11* affects ABA

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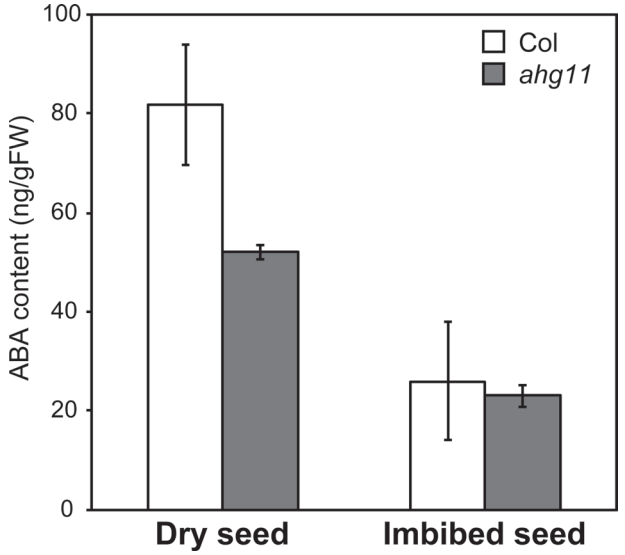

Fig. 3. Endogenous ABA content of *ahg11.* Endogenous ABA content of dry seeds and imbibed seeds was measured. The data shows the mean of three independent experiments. Error bars indicate standard deviation.

signalling or cellular responses to ABA rather than ABA biosynthesis or catabolism. Next, the responses of *ahg11* mutants to osmotic and salinity stresses were examined. As shown in Fig. 4, the germination levels of *ahg11* are slightly lower than those of the wild-type controls on an agar plate containing mannitol but are not as low as the strong ABA hypersensitive mutant *era1-2*. When subjected to salinity stress, *ahg11* apparently germinates less effectively than the wild type but again, not as little as *era1-2*. These data imply that *ahg11* affects not only ABA response but also abiotic stress response at germination. Sugar response is closely linked with ABA response (Rook *et al*., 2006; Rolland *et al*., 2006). Therefore, the sensitivity of *ahg11* to glucose and sucrose was examined and it was found that it is slightly enhanced at germination (see Supplementary Fig. S1 at *JXB* online). By contrast with the phenotypes observed at germination, the ABA hypersensitivity of *ahg11* is hardly detected in seedlings or adult plants (see Supplementary Fig. S2 at *JXB* online), suggesting that AHG11 function is required for germination or early seedling growth stages.

Responses to other plant hormones were also examined. Interestingly, the germination of *ahg11* was more strongly inhibited by jasmonic acid than wild type (see Supplementary Fig. S3 at *JXB* online). The responses to auxin, cytokinin, gibberellin, ethylene, and brassinosteroid were examined using elongation of the main root or hypocotyl. However, *ahg11* mutant seedlings did not exhibit any detectable abnormalities in their responses to these plant hormones (see Supplementary Fig. S4 at *JXB* online).

The results described above suggest that *ahg11* demonstrates an enhanced sensitivity to ABA during germination. Next, the *ahg11* responses to ABA were examined in adult plants. Root elongation, and the expression of ABA responsive genes, *RD29A* and *RAB18,* in *ahg11* mutants displayed no apparent differences when compared with those responses in wild-type plants (data not shown). These data are consistent with the idea that AHG11 function is required only in seeds or young seedlings.

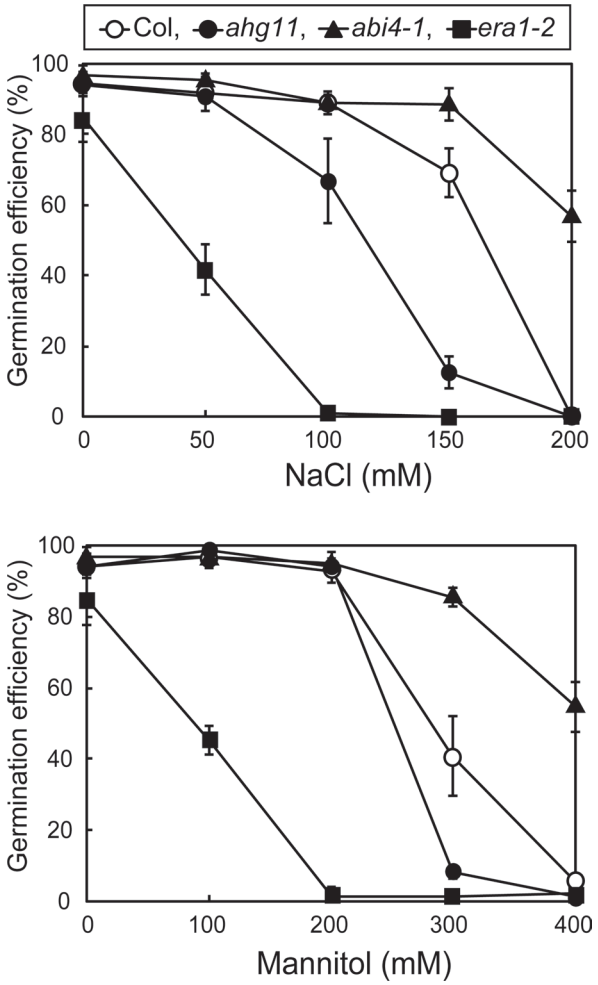

Fig. 4. Salt or hyper-osmotic stress sensitivity of *ahg11.* Germination efficiency was analysed in the presence of NaCl or mannitol. Wild-type (Col), *ahg11, abi4-1,* or *era1-2* seeds (>50) were sown on MS-sucrose plates containing various concentrations of NaCl or mannitol. Germinating seedlings with greening cotyledons were counted. The data shown are means of three independent experiments. Error bar indicates standard deviation.

Identification of AHG11 *locus*

Using PCR-based molecular markers, the predicted *AHG11* locus was fine-mapped by analysing the chromosomal DNA obtained from ABA-hypersensitive F_2 progeny from a test cross with the L*er* wild type. The putative *AHG11* region was narrowed to the region spanning about 80 kbp, where 17 genes are located according to The *Arabidopsis* Information Database (TAIR). The genomic regions corresponding to each of these genes were sequenced in *ahg11* backgrounds and two base substitutions were found in the gene *At2g44880*, which is predicted to encode a pentatricopeptide repeat protein (PPR). While the wild-type At2g44880 protein is predicted to have 12 PPR repeats, the upstream base substitution generates a nonsense mutation [Trp 338 (TGG) -> stop codon (TAG)] resulting in a truncated polypeptide. It has been shown that plants have numerous PPR genes (Saha *et al*., 2007; Schmitz-Linneweber and Small, 2008).

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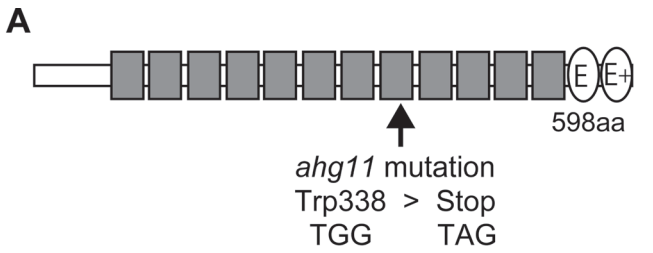
Arabidopsis has 450 PPR genes (Lurin *et al*., 2004). Among them, dozens of PPRs have been intensely investigated and their targets identified. PPRs are categorized into several groups or classes on the basis of the motif composition of the proteins (Lurin *et al*., 2004). AHG11 belongs to the E+ PLS family, most of which are involved in RNA editing, and has the highest similarity to CRR4, which edits the second cytosine residue of plastid *ndhD* transcripts (Kotera *et al*., 2005). The C-terminal E-motif is required for the editing function of CRR4 and another E+-class PPR, CRR21 (Okuda *et al*., 2007). Therefore, the nonsense *ahg11* mutation, which causes the lack of 4 C-terminal PPR repeats, E, and E+ motifs, is most likely responsible for the malfunction of the *AHG11* gene product (Fig. 5).

In order to confirm that *At2g44880* is *AHG11,* a complementation analysis was conducted. The genomic DNA containing *At2g44880* with a 500 bp 5' untranslated region and a 300 bp 3' untranslated region was introduced into the *ahg11* mutant. The resultant transgenic lines showed a normal ABA response at germination (see Supplementary Fig. S5A at *JXB* online). The transgenic lines also exhibited normal sensitivities to glucose and methyl jasmonic acid at germination (see Supplementary Fig. S5B, C at *JXB* online). Based on these data, it is concluded that *AHG11* is indeed *At2g44880.*

The expression level of *AHG11* is quite low according to public transcriptome databases. Database searches also revealed no previously submitted cDNAs for this gene. An attempt was made to detect transcripts by using RT-PCR and it was found that *AHG11* is expressed at quite a low level but ubiquitously (see Supplementary Fig. S6 at *JXB* online).



B

86-121	P	FLSNSMIKAYLETRQYPDSFALYRDLRKETCFAPDN
122-156	L	FTFTTLTKSCSLSMCVYQGLQLHSQIWRFGFCADM
157-187	S	YVSTGVVDMYAKFGKMGCARNAFDEMPHRSE
188-219	S	VSWTALISGYIRCGELDLASKLFDQMPHVKDV
220-250	S	VIYNAMMDGFVKSGDMTSARRLFDEMTHKTV
251-281	S	ITWTTMIHGYCNIKDIDAARKLFDAMPERNL
282-317	P	VSWNTMIGGYCQNKQPQEGIRLFQEMQATTSLDPDD
318-352	L	VTILSVLPAISDTGALSLGEWCHCFVQRKKLDKKV
353-383	S	KVCTAILDMYSKCGEIEKAKRIFDEMPEKQV
384-417	S	ASWNAMIHGYALNGNARAALDLFVTMMIEEKPDE
418-452	L2	ITMLAVITACNHGGLVEEGRKWFHVMREMGLNAKI
453-484	S	EHYGCMVDLLGRAGSLKEAEDLITNMPFEPNG

Fig. 5. Structure of AHG11.
(A) Schematic representation of AHG11 protein. Grey box indicates the PPR motif. The approximate position of the *ahg11* mutation site is shown.
(B) Alignment of PPR motifs of AHG11; 12 PPR motifs are shown with the position and type of PPR motif. The amino acid positions followed the annotation of At2g44880 in The *Arabidopsis* Information Resource (TAIR).

Subcellular localization of AHG11

The genome sequence of *AHG11/At2g44880* reveals multiple in-frame ATG codons. Since information on transcript sequences is lacking, it is not known which ATG codon is used as the start codon. When the most upstream ATG codon is utilized as the initiation codon, the polypeptide is predicted to localize mostly to the cytoplasm. On the other hand, when a downstream ATG codon is utilized, the gene product is predicted to function in plastids or the secretory pathway. AHG11 is closely related to CRR4, which is known to function in plastids. However, a recent proteomic study suggested that AHG11 localizes at the plasma membrane (Mitra *et al*., 2009). To determine the subcellular localization of AHG11, the N-terminal portion of AHG11 (83 aa from the most upstream ATG codon) was fused to a GFP gene to form the AHG11-GFP fusion gene. When a plasmid DNA harbouring this fusion gene was introduced to the *Arabidopsis* mesophyll protoplast, GFP fluorescence was observed in a granule-like structure in the cytoplasm (Fig. 6). Most of the GFP-fluorescence overlapped with a Mitotracker-Red signal, implying that AHG11 localizes to the mitochondria. However, the number of the AHG11-GFP signals was apparently less than the mitochondrial signal obtained with Mitotracker-Red. Presumably, AHG11 localizes to mitochondria only under some conditions. Similar, but weaker spatial fluorescence patterns were observed with a full-length AHG11-GFP fusion protein (data not shown). Hardly any GFP fluorescence was detected when the subcellular localization of AHG11-GFP fusion proteins that use the downstream ATG codon as the initiation codon was examined.

nad4 *transcript is the AHG11 target*

The results described above indicate that AHG11-fused GFP localizes to mitochondria, suggesting that AHG11 plays a role in mRNA processing in mitochondria, particularly in RNA editing. There are about 500 editing sites in mitochondrial mRNA (Giegé and Brennicke, 1999; Bentolila *et al*., 2008). In addition, the possibility cannot be excluded that AHG11 is involved in RNA editing in the plastid because AHG11 most resembles CRR4, which

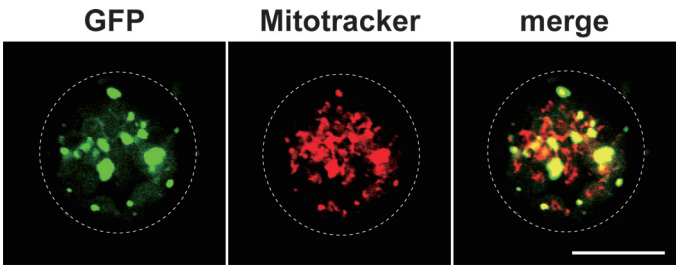


Fig. 6. Subcellular localization of an AHG11-GFP protein. AHG11-GFP fusion protein was expressed in the *Arabidopsis* mesophyll protoplast. Left, GFP fluorescent signal; middle, Mitotracker Red staining; right, merged picture. Bar indicates 20 μm. Dotted line outlines the cell shape.

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functions in the plastid. An examination of the status of each potential editing site one by one would be both time and labour-consuming. Recently, the RNA recognition code of editing PPR proteins was identified, namely the 'PPR code'. The PPR code enables *in silico* prediction of the candidate target site for PPR protein (Y Yagi *et al*., unpublished data). The prediction using the PPR code allowed us to narrow the candidate AHG11 editing sites to around 20 sites. cDNA fragments containing these candidate editing sites were synthesized using total RNA isolated from *ahg11* and wild-type seedlings and sequenced. Consequently, it was found that a C to U edit in the *nad4* transcript at nucleotide position 376 (*nad4*-376) was absent in the mutant while neighbouring sites *nad4*-362 and *nad4*-403 were edited correctly (Fig. 7A). More than 400 mitochondrial editing sites were examined and all of the known plastid editing sites, including *accD* and *PsbZ* (Yu *et al*., 2009), and no other differences in the RNA editing states between *ahg11* and wild-type RNA were detected. It should be noted, however, that not all RNA editing sites could be examined. In addition, some of the known RNA editing sites were missed and a few novel RNA editing candidates found in mitochondrial transcripts obtained from germinating wild-type seeds. Therefore, the possibility cannot be excluded that AHG11 is also responsible for other RNA editing sites of mitochondrial transcripts. Proper RNA editing at *nad4*-376 was observed in the transgenic *ahg11* mutants possessing the wild-type *AHG11* transgene, confirming that AHG11 is required for *nad4*-376 RNA editing (Fig. 7A). Since the other editing sites of *nad4* are edited as reported previously and any abnormally spliced mRNAs were not detected (Fig. 7B), the *ahg11* mutation presumably does not affect any other RNA processing steps of *nad4* transcripts. Although *ahg11* exhibits an ABA hypersensitive phenotype at the seed or young seedling stages, the defect of *nad4*-376 RNA editing was detected in the adult *ahg11* plant, implying that AHG11 functions at any stages consistent with the result of RT-PCR (see Supplementary Fig. S6 at *JXB* online). The C to U editing at *nad4*-376 converts Arg126 to Cys, where hydrophobic residues are found in other organisms (Fig. 7C). Presumably, editing *nad4*-376 is required for its proper function in complex I.

ahg11 *has an impaired redox balance*

Previous reports on the PPR proteins functioning in mitochondria demonstrated that dysfunction of these PPR proteins resulted in higher reactive oxygen species (ROS) accumulation or redox imbalance in the cell (Zsigmond *et al*., 2008). Presumably, an impaired respiration chain produces ROS. It is possible that the

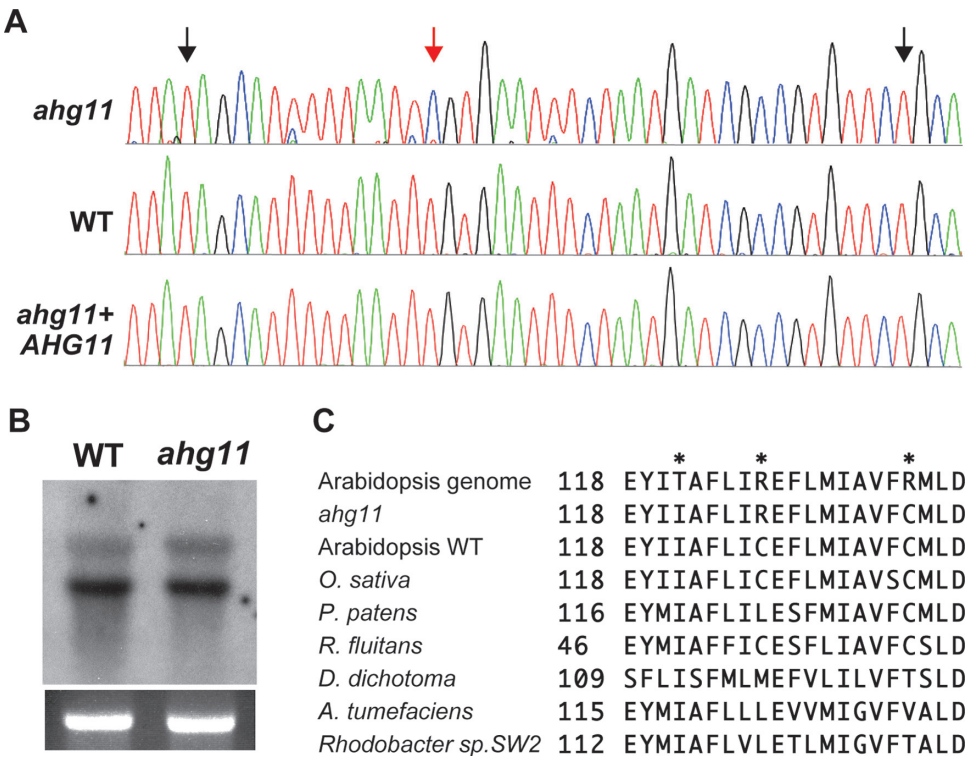

Fig. 7. Target RNA editing site of AHG11.
(A) Sequence chromatograms around the *nad4*-376 editing site of the *nad4* cDNA obtained from wild-type or *ahg11* plants. Red, green, blue or black peaks indicate T, A, C, or G, respectively. The vertical red arrow indicates *nad4*-376 and black arrows indicate *nad4*-362 and *nad4*-403 editing sites.
(B) Northern blotting of *nad4.* Total RNA was isolated from wild-type or *ahg11* seeds which were imbibed at 4 °C for 4 d and incubated for 48 h and used for the Northern blotting experiment. EtBr-stained rRNA was shown as loading control.
(C) Alignment of NAD4 amino acid sequence around the region that corresponds to the *nad4*-376 editing site. Asterisks indicate the amino acid residues corresponding to the RNA-editing sites in *Arabidopsis* (left to right, *nad4*-362, *nad4*-376, *nad4*-403). The number indicates the position of the first amino acid in the alignment.

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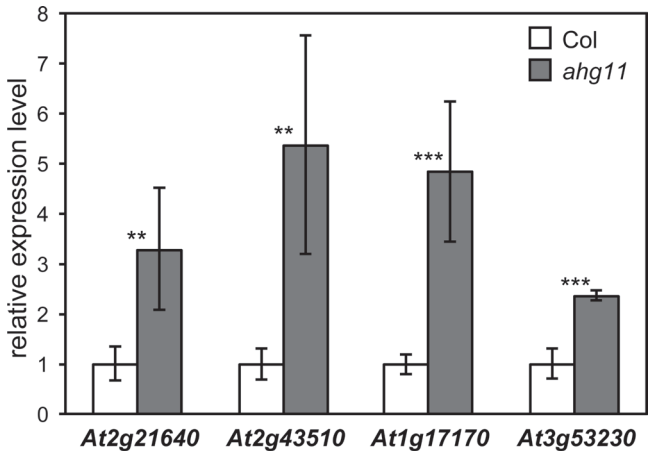

Fig. 8. Gene expression of ROS responsive genes in *ahg11*. Expression levels of ROS-inducible genes in *ahg11* seeds (black bars) and in wild-type seeds (white bars) quantified by qRT-PCR are shown. The expression level of each gene is normalized to that of wild-type seeds. Asterisks (**) and (***) indicate the *P*-values <0.05 and <0.01 by Student's *t* tests, respectively. The data shown are the means of three independent experiments. Error bars indicate standard deviation.

ahg11 mutation also confers such effect in germinating seeds. To see the cellular redox imbalance, the expression patterns of four reactive oxygen stress inducible genes were examined. *Gadjev et al.* (2006) reported various ROS inducible genes. Among the major ROS-inducible genes, *At2g21640* encoding an unknown protein, *At2g43510* encoding a trypsin inhibitor protein, *At1g17170* encoding glutathione *S*-transferase tau 24, and *At3g53230* encoding AtCDC48, were chosen because these genes express in seeds according to the public microarray database. To see the expression level in germinating seeds, total mRNA was prepared from seeds which were imbibed at 4 °C for 4 d and incubated at 22 °C for 48 h under the lights and used for qRT-PCR experiments. As shown in Fig. 8, mRNA levels of all these genes were higher in *ahg11* than the wild type. It was confirmed that the difference in those mRNA levels were not due to the small difference in germination timing between *ahg11* and the wild type (see Supplementary Fig. S7 at *JXB* online). These results suggest that the defect in *nad4*-376 editing caused by *ahg11* induces a redox imbalance in geminating seeds, as reported in other PPR defect mutants.

Discussion

Identification of new ABA receptors and the determination of the major ABA signalling pathway has resulted in a better understanding of ABA action and the cellular response to this phytohormone at the molecular level. However, to describe the diverse actions of ABA, it was neessaryto identify more components involved in the ABA-signalling pathway. A novel genetic screening was conducted to identify weak ABA hypersensitive mutants with an ABA biosynthetic mutant, *aba2-1,* and at least three novel *ABA hypersensitive germination* (*ahg11, 12,* and *ahg16*) loci were obtained.

ahg11 was analysed in detail in this study. It was demonstrated that *ahg11* showed a weak ABA hypersensitivity only in germination and post-germination growth, suggesting that the AHG11 function is particularly required only for seeds or young seedlings. Fine mapping of the *AHG11* gene revealed that *At2g44880* encodes an E+ PLS class PPR protein. AHG11 is related most closely to CRR4, which is involved in mRNA editing in the chloroplast. The fluorescence signal of AHG11-GFP fusion proteins, however, coincided with Mitotracker staining and its target RNA is mitochondrial *nad4* mRNA, suggesting that AHG11 functions in the mitochondria. However, not all mitochondrial Mitotracker signals overlapped with the AHG11-GFP signals. This observation could imply that only some mitochondria co-localize with AHG11-GFP. If this is indeed the case, AHG11 localizes to mitochondria only under specific conditions.

This idea is consistent with the stage-specific *ahg11* phenotype. The *ahg11* mutation is a nonsense mutation that truncates the PPR protein and lacks the C-terminal half that contains the E+ motif important for RNA editing. Therefore, *ahg11* is very likely a null mutation but its phenotype is restricted to germination and the young seedling stages. *nad4*-376 RNA editing is detected not only at the germination stage but also in the adult, and the defect in *nad4*-376 RNA editing in *ahg11* is seen in both adult plants as well as germinating seedlings. The RNA editing of *nad4*-376 converts the amino acid residue from Arg to Cys, which probably influences NAD4 protein function through a change in the electrostatic potential or the hydrophobicity of NAD4. Thus, activity of NAD4 (and its nursing components) in complex I can be required in mitochondria under certain specific cellular conditions or developmental stages. Germination requires vigorous mitochondrial metabolic function, especially in beta-oxidation of the seed-storage lipid. It is worth noting that impairment in beta-oxidation induces deeper seed dormancy and ABA and JA hypersensitivity at germination (Dave *et al*., 2011). Alternatively, defects in NAD4 activity can be compensated by other factors in adult plants. Similar observations have been reported previously. The *css1* mutation impairs a *nad4* transcript splicing factor At-nMat1a that confers pleiotropic effects such as sugar hypersensitivity and growth retardation at the young seedling stage but no significant effects at the adult stage (Nakagawa and Sakurai, 2006). MEF18, an *Arabidopsis* PPR, edits *nad4* mRNA at 1355 which is responsible for the change of a conserved amino acid residue. A T-DNA insertion mutant of *MEF18,* however, does not exhibit any visible phenotype (Takenaka *et al*., 2010). *slo1* and *mef11* mutants are also defective in RNA editing of the *nad4* transcript (Sung *et al*., 2010; Verbitskiy *et al*., 2010). Although *slo1* shows severe growth retardation and *mef11* shows slightly slow growth, these mutants have an additional defect in RNA editing in the *nad9* transcripts (*slo1*), or in *cox3* and *ccb203* (*mef11*). Therefore, the physiological effects of the loss of RNA editing in *nad4* are obscure in these mutants. Further analysis can potentially shed light on the physiological or developmental uses of RNA editing in NAD4 mRNA.

The connection between ABA-hypersensitive phenotypes and mitochondrial function has not received much attention until recently. The *abo5* mutant, which lacks another PPR protein responsible for *nad2* pre-mRNA splicing, exhibits an

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ABA-hypersensitive phenotype at germination and root elongation, and shows severe growth retardation (Liu *et al*., 2010). It is possible that redox imbalance caused by impaired mitochondrial function activates the ABA signalling in which ROS plays important roles (Zsigmond *et al*., 2008; Yuan and Liu, 2012). Consistent with this idea, *abo5* mutants accumulate higher levels of ROS (Liu *et al*., 2010). Our data presented here also suggest that *ahg11* has redox imbalance (Fig. 8). Alternatively, impaired mitochondrial function disrupts the metabolic balance that, in turn, affects the ABA response. Indeed, altered sugar sensitivity is observed in *abo5* and *css1* (Nakagawa and Sakurai, 2006; Liu *et al*., 2010). Since strong cross-talk between sugar and ABA responses have been demonstrated (Rook *et al*., 2006; Rolland *et al*., 2006), the ABA hypersensitive phenotype of *ahg11* could result from a metabolic imbalance. In actuality, *ahg11* showed slightly enhanced sensitivity to glucose at germination (see Supplementary Fig. S1 at *JXB* online). In addition, *fro1* mutants, which are defective in the 18 kDa Fe–S subunit of complex I, exhibit hypersensitivity to sugars, and osmotic and salinity stresses at germination (Lee *et al*., 2002). If this is a universal phenomenon, most of the mitochondria-related mutants would be expected to have an altered ABA response phenotype. Therefore, it would be interesting to examine the ABA sensitivity of known mitochondria-related mutants. It should be noted that another ABA hypersensitive mutant, *ahg2-1*, also displays a phenotype related to mitochondrial function (Nishimura *et al*., 2009).

Recent studies have revealed that mitochondria have important functions, besides their established roles in metabolism. In animal cells, mitochondria have a pivotal role in an apoptosis response to various extracellular stimuli (Kroemer *et al*., 1998; Wang, 2001; Wang and Youle, 2009). In many other organisms, mitochondrial function is highly involved in various cellular processes influenced by environmental conditions (Lee *et al*., 2002; Atkin and Macherel, 2009; Green *et al*., 2011; Gleason *et al*., 2011; Yang *et al*., 2011; Schwarzländer *et al*., 2012). ABA-related phenotypes of *ahg11, abo5,* and other mutants with impaired mitochondrial function suggest that mitochondria play an important role in stress response in plants as well. Further analysis of these mutants with biochemical and cell biological studies on the relationship between mitochondria and stress responses will shed new light on the strategies utilized by plants that have two different symbiotic organelles, mitochondria and plastids.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Sugar sensitivity of *ahg11*.

Supplementary Fig. S2. ABA and salt sensitivity of *ahg11* seedlings.

Supplementary Fig. S3. Jasmonic acid sensitivity of *ahg11*.

Supplementary Fig. S4. Response to various hormones of *ahg11* seedlings.

Supplementary Fig. S5. Complementation of *ahg11* by a wild-type *AHG11* gene.

Supplementary Fig. S6. Detection of *AHG11* transcripts by RT-PCR.

Supplementary Fig. S7. Expression levels of ROS inducible genes in wild-type seeds.

Supplementary Table S1. Primers used in this study.

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