Arabidopsis UPF1 RNA Helicase for Nonsense-mediated mRNA Decay is Involved in Seed Size Control and is Essential for Growth

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UPF1 RNA helicase plays a central role in nonsense-mediated mRNA decay (NMD), which specifically recognizes aberrant mRNAs containing premature termination codons and targets them for degradation. Although NMD factors are highly conserved among eukaryotes, little is known about the role of NMD in plant growth and development. The lba1 mutant of Arabidopsis thaliana with a Gly^{531}→Glu missense mutation in AtUPF1 yielded seeds that were on average 22% longer in the long axis and 35% heavier than the wild-type Col seeds. Expression of the wild-type AtUPF1 in this mutant reduced the seeds to a size similar to those of Col seeds. However, the distance between seeds in siliques was greater in lba1 than in Col, suggesting that the lba1 mutation may affect ovule development. Self-pollinated lba1/atupf1-1 mutant — with a mutation in AtUPF1 maternally affects seed development and that AtUPF1 is essential for seedling growth.

Keywords: Arabidopsis thaliana — lba1/atupf1-1 mutant — Nonsense-mediated mRNA decay — Seed sac — Seed size — UPF1.

Introduction

The nonsense-mediated mRNA decay (NMD) pathway is an mRNA surveillance mechanism conserved among eukaryotes. It specifically recognizes mRNAs containing premature translational termination codons (PTCs) and targets them for degradation (reviewed in Baker and Parker 2004, Conti and Izaurralde 2005). In addition to PTC-containing mRNAs generated by mutation, various forms of natural transcripts containing PTCs are targets of NMD, and transcripts of as many as 4–10% of total genes in yeast (He et al. 2003). In human (Mendell et al. 2004) are estimated to be subjected to post-transcriptional regulation by NMD. Three major factors in NMD, namely, UPF1, UPF2 and UPF3, have been highly conserved during the evolution of eukaryotes. Of these, UPF1 RNA helicase is a key component of NMD, and it interacts with UPF2 and UPF3 to form a surveillance complex (reviewed in Culbertson and Leeds 2003, Conti and Izaurralde 2005). Although NMD is a highly conserved system among eukaryotes, various organisms evolved different mechanisms to recognize PTCs in mRNA and recruit NMD factors. In mammals, the majority of NMD occurs if a pioneer round of translation terminates >50 nucleotides upstream of the 3′-most exon–exon junction; however, in yeast, Drosophila and, most likely, Caenorhabditis elegans, NMD occurs independently of splicing. In yeast, the mRNA is targeted for degradation by NMD if specific cis-elements are present downstream of PTCs (reviewed in González et al. 2001, Culbertson and Leeds 2003, Baker and Parker 2004, Conti and Izaurralde 2005). In accord with the differences in the mechanisms for recognizing PTC-containing mRNAs, the target genes for NMD have not been conserved during evolution (Mendell et al. 2004, Rehwinkel et al. 2005). In several organisms, NMD factors are involved in functions other than eliminating PTC-containing mRNAs (Domeier et al. 2000, Nott et al. 2004), and UPF1 but not UPF2 or UPF3 is required for non-NMD post-transcriptional regulation (Kim et al. 2005).

The presence of an NMD-like pathway in plants has been suggested by studies showing reduced stability of mRNAs containing PTCs from several intronless genes (reviewed in Gutiérrez et al. 1999); however, intron splicing has been shown to be important for the rapid degradation of mutant PTC-containing waxy mRNAs in rice (Oryza sativa) (Isshiki et al. 2001). In contrast to the case in mammals, splicing of an intron upstream of PTC is important in NMD of mutant waxy mRNA, suggesting that NMD in plants may have unique features.
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An \textit{Arabidopsis} gene for UPF1

The \textit{lba1} mutant of \textit{Arabidopsis} (Mita et al. 1997) contains a missense mutation in a single-copy gene for UPF1 (\textit{AtUPF1}; Yoine et al. 2006). \textit{AtUPF1} shares amino acid identities of 56, 50 and 48\% with UPF1 RNA helicases from human, \textit{Drosophila} and yeast, respectively (Culbertson and Leeds 2003). The \textit{lba1} mutation in the 20th exon of \textit{AtUPF1} converts Gly851, which is an invariant residue in all 16 known eukaryotic UPF1s (Culbertson and Leeds 2003; \url{http://www.molbio.wisc.edu/culbertson/UPFsequences/UPF1.jpg}), to glutamate (Fig. 1; Yoine et al. 2006).

The sequence-indexed \textit{Arabidopsis} T-DNA insertion lines of the Salk Institute Genome Analysis Laboratory contained lines with T-DNA insertions in \textit{AtUPF1}. We obtained three lines (SALK_4606, SALK_081178 and SALK_022721) with T-DNA insertions in different locations of \textit{AtUPF1} (Fig. 1).

Table 1  \textit{lbai} mutation affects seed size and shape

<table>
<thead>
<tr>
<th>Seeds</th>
<th>(N)</th>
<th>Length ((\mu)m) (^{\text{a}}) (%)</th>
<th>Width ((\mu)m) (^{\text{a}}) (%)</th>
<th>Weight ((\mu)g) (^{\text{b}}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>75</td>
<td>469 ± 33 (100)</td>
<td>299 ± 17 (100)</td>
<td>20.0 ± 1.7 (100)</td>
</tr>
<tr>
<td>\textit{lba1}</td>
<td>119</td>
<td>571 ± 56 (122)</td>
<td>299 ± 23 (100)</td>
<td>27.0 ± 0.0 (135)</td>
</tr>
<tr>
<td>\textit{35S:AtUPF1/lba1}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>72</td>
<td>462 ± 48 (98)</td>
<td>280 ± 19 (94)</td>
<td>19.0 ± 1.0 (95)</td>
</tr>
<tr>
<td>L4</td>
<td>85</td>
<td>508 ± 54 (108)</td>
<td>295 ± 26 (99)</td>
<td>22.7 ± 0.6 (95)</td>
</tr>
<tr>
<td>L6</td>
<td>101</td>
<td>506 ± 30 (108)</td>
<td>306 ± 21 (102)</td>
<td>21.0 ± 1.7 (105)</td>
</tr>
<tr>
<td>L7</td>
<td>129</td>
<td>496 ± 31 (106)</td>
<td>308 ± 22 (103)</td>
<td>19.7 ± 1.2 (98)</td>
</tr>
<tr>
<td>L10</td>
<td>108</td>
<td>511 ± 44 (109)</td>
<td>311 ± 32 (104)</td>
<td>21.3 ± 0.6 (107)</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) Seeds were photographed, and seed length and width were determined from the indicated number of seeds (\(n\)). Means ± SD are shown. The numbers in parentheses indicate the percentage compared with the value for Col seeds.

\(^{\text{b}}\) Seed weight was calculated from the weight of 100 seeds and represents the mean of three independent measurements from the same batch. Similar results were obtained with other batches.
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In addition to various phenotypes described previously for the lba1 mutant (Mita et al. 1997, Yoine et al. 2006), we noticed that seeds of the lba1 mutant plants grown under continuous light were larger than those of Col at similar stages (Fig. 3C; compare 6, 7 and 8 with 1, 2 and 3); however, the size of embryos during these stages were not significantly different between lba1 and Col. In contrast, during the curled cotyledon stage, lba1 developed an elongated embryo in the seed sac, composed of integument and endosperm, that was larger than Col (Fig. 3C; compare 4 and 5 with 9 and 10). These results suggest that elongation of lba1 seeds compared with that of Col seeds is primarily due to larger seed sacs.

Elongated seed phenotype of the lba1 mutant is rescued by 35S:AtUPF1

We next examined seeds of the lba1 mutant that had been transformed with 35S:AtUPF1, which contains the wild-type AtUPF1 coding region and the 3'-untranslated region (3'-UTR) of the nos gene. The transformed plants express the wild-type AtUPF1 mRNA at levels similar to that in Col, in addition to the lba1 mutant form of AtUPF1 mRNA (Yoine et al. 2006). Reduced sugar-induced expression of Atβ-Amy and Dinll as well as phenotypes such as early flowering and altered sensitivities of seedling development to glucose, abscisic acid and mannose were rescued in the transformants (Yoine et al. 2006). In addition, the length of the long axis and weight of seeds from these transformed lines were similar to those of Col seeds (Table 1). These results indicate that the elongated seed phenotype of the lba1 mutant is due to missense mutation of AtUPF1.

Development of lba1 seeds

Silique of lba1 and Col plants were similar in size and shape. When fully developed young siliques of similar sizes on primary shoots were opened, the lba1 siliques contained seeds that were larger and longer than in the Col siliques (Fig. 3A). The lba1 siliques also contained fewer seeds per unit length of siliques than Col siliques. The number of seeds contained per 5 mm of lba1 siliques was on average 84% of that in Col siliques (Fig. 3B).

To compare the development of lba1 and Col seeds, we examined cleared seeds at different stages of development. The developing lba1 seeds of globular to torpedo stage embryos were already larger and mostly longer than seeds of Col at similar stages (Fig. 3C; compare 6, 7 and 8 with 1, 2 and 3); however, the size of embryos during these stages were not significantly different between lba1 and Col. In contrast, during the curled cotyledon stage, lba1 developed an elongated embryo in the seed sac, composed of integument and endosperm, that was larger than Col (Fig. 3C; compare 4 and 5 with 9 and 10). These results suggest that elongation of lba1 seeds compared with that of Col seeds is primarily due to larger seed sacs.

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Mutants with T-DNA insertions in the 5'-UTR and the C-terminal coding region of AtUPF1

The positions of T-DNA insertion in SALK lines (Fig. 1) were determined by sequencing PCR products obtained with a T-DNA-specific primer and primers derived from the sequence of AtUPF1. In SALK_4606 and SALK_022721, T-DNA was inserted in the 5'-UTR and in the 28th exon covering the C-terminal coding region of AtUPF1, respectively. Mutations in AtUPF1 of SALK_4606 and SALK_022721 were designated atupf1-2 and atupf1-4, respectively (Fig. 1). The lba1 mutation is counted as atupf1-1. By screening progeny of SALK_4606 and SALK_022721 lines by genomic PCR, we identified plants homozygous for atupf1-2 and atupf1-4, respectively. These

Fig. 2 Comparison of the size of Col and lba1 seeds. (A) Pictures of the mature dried seeds from Col (left) and lba1 (right). Bar = 500 µm. (B) Relationship between the length and width of Col (black circles) and lba1 (red circles) seeds. Seeds were photographed, and seed length and seed width were measured using ImageJ software (http://rsb.info.nih.gov/ij/).

lba1 mutant plants produce elongated seeds

In addition to various phenotypes described previously for the lba1 mutant (Mita et al. 1997, Yoine et al. 2006), we noticed that seeds of the lba1 mutant plants grown under continuous light were larger than seeds of the wild-type Col (Fig. 2A). The lba1 seeds were on average 22% longer in their long axis than Col seeds, but their widths or short axes were not significantly different (Table 1). Also, the lba1 seeds were on average 35% heavier than Col seeds (Table 1). The size distributions of Col and lba1 seeds (Fig. 2B) suggest that the mutation of AtUPF1 in lba1 causes elongation of the seed primarily along the long axis.
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homozygous plants grew normally (Fig. 4A). These two mutants contained AtUPF1 mRNA at levels lower than in Col (Fig. 4B). In particular, the level of AtUPF1 mRNA in atupf1-4(−/−) was only about 20% of that in Col. The lba1 mutant showed a significant increase in the level of transcript 2 of AtTFIIIA, which is generated by an alternative splicing event and contains a PTC in the third exon (Yoine et al. 2006). In contrast to the lba1 mutant, the levels of transcript 2 of AtTFIIIA in homozygous atupf1-2 and atupf1-4 plants were similar to that in Col (Fig. 4C). Unlike Col seeds (Pego et al. 1999), germination of lba1 seeds on agar medium containing 1% sucrose was not inhibited by 10 mM mannose (Yoine et al. 2006). Similarly to Col seeds, germination of seeds of homozygous atupf1-2 and atupf1-4 plants was strongly inhibited by mannose (Fig. 4D). These results indicate that the reduced expression of AtUPF1 and the loss of the C-terminal region of AtUPF1 do not severely affect the function of AtUPF1 in planta.

Disruptant of AtUPF1 is seedling lethal

The SALK_081178 line contained T-DNA within the 17th exon of AtUPF1 (designated atupf1-3; Fig. 1). When seeds from self-pollinated heterozygous atupf1-3(+−) plants were germinated on agar plates under continuous light, 24% of them stopped growing after the emergence of a radicle from the seed coat (Fig. 5A). These abortive infant seedlings showed neither growth of roots nor expansion of cotyledons. The rest of the seeds yielded seedlings that were indistinguishable from wild-type seedlings. DNA was extracted from each of the wild-type as well as the abortive seedlings and used for PCR-based detection of the wild-type AtUPF1 genomic sequence and T-DNA insertion in atupf1-3. For this purpose, downstream primer a or the T-DNA-specific primer b were used along with an upstream primer to amplify the wild-type or atupf1-3 genomic sequence, respectively (Fig. 5B). Results of some representative samples are shown in Fig. 5C. DNA from abortive seedlings yielded a
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Fig. 4 Reduced expression of AtUPF1 in *atupf1-2* and *atupf1-4* mutants. (A) Seeds of Col, *atupf1-2*(-/-) and *atupf1-4*(-/-) were germinated and grown for 3 weeks on MS-agar plates containing 3% (w/v) sucrose. (B and C) Relative levels of AtUPF1 mRNA (B) and transcript 2 of AtTFIIFIA (C) in Col, *iba1*, *atupf1-2*(-/-) and *atupf1-4*(-/-). Total RNA was isolated from the aboveground tissues of 3-week-old seedlings, and quantitative real-time RT-PCR was performed with primers specific for AtUPF1 and transcript 2 of AtTFIIFIA using ACT2 mRNA as an internal standard. Average levels of mRNA relative to the level in Col from two independent experiments ± SD. (D) Mannose sensitivity of seed germination. Seeds of Col, *iba1*, *atupf1-2*(-/-) and *atupf1-4*(-/-) were germinated on minimal salt medium containing 1% sucrose and 5 mM mannose. Eight days after imbibition, the number of seedlings showing open and green cotyledons was counted. The results represent the average from three independent experiments with >25 seeds for each plant ± 5D.

Fig. 5 Homozygous *atupf1-3*(-/-) seeds are seedling lethal. (A) Growth of seeds of self-pollinated *atupf1-3* (+/-) heterozygous plants on agar medium 14 d after imbibition. Arrows in the left photograph indicate abortive seedlings that are shown in the right photograph with asterisks (*). Bar = 1 mm. (B) Positions of PCR primers to determine the genotypes of *atupf1-3* mutants. (C) DNA isolated from individual offspring was analyzed for the insertion of T-DNA by PCR using primer sets a and b indicated in (B). The results of genomic PCR from representative samples are shown. ‘Wild type’ and asterisk (*) indicate DNAs from the wild-type and abortive seedlings, respectively. The number of seedlings that showed each genotype is indicated in the bottom. The $\chi^2$ value for a 1 : 2 : 1 ratio was 1.79 ($P > 0.05$). (D) The heterozygous *atupf1-3*(+/-) plant was crossed with the homozygous *iba1* mutant transformed with 35S:AtUPF1, and hygromycin-resistant F1 plants with *iba1* on one chromosome and *atupf1-3* on the other chromosome were selected. Seeds obtained from the *iba1/atupf1-3* heterozygote were sown on plates containing hygromycin, and hygromycin-resistant F2 seedlings that grew for 2 weeks were subjected to genotyping for *iba1* mutation with dCAPS primers. The right photograph shows one of the *atupf1-3*(-/-) mutants with 35S:AtUPF1 2 weeks after imbibition on plates containing hygromycin.
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578 bp DNA fragment when primer set b was used, whereas PCR with primer a did not yield a band, indicating that the abortive seedlings were homozygous for atupf1-3(–/–). On the other hand, DNA from the wild-type seedlings produced either only a 784 bp band with primer set a for the wild-type AtUPF1 (+/+) or both the wild-type band and the atupf1-3-specific band (+/–). None of the wild-type seedlings showed a –/– genotype. Analysis of DNA from all of the 117 seedlings germinated in two agar plates showed a segregation of +/+ : +/– : –/– = 31 : 63 : 23 (χ² for a ratio of 1 : 2 : 1 = 1.79; P > 0.05; Fig. 5C), and the seedling-lethal phenotype co-segregated with the T-DNA insertion as a single Mendelian recessive locus. These results suggest that the seedling-lethal phenotype is genetically linked to a T-DNA insertion in atupf1-3.

To examine further that seedling lethality is due to atupf1-3(–/–), a heterozygous atupf1-3(+/–) plant was crossed with line L1 of the homozygous lba1 mutant transformed with 35S:AtUPF1 (Yoine et al. 2006), and hygromycin-resistant F₁ plants with lba1 on one chromosome and atupf1-3 on the other chromosome were selected by genotyping with primer sets for atupf1-3 (Fig. 5B) and derived cleaved amplified polymorphic sequence (dCAPS) primers that can distinguish lba1 from Col sequence (Yoine et al. 2006). Seeds obtained from self-pollinated lba1/atupf1-3 heterozygote were sown on plates containing hygromycin, and all of the 97 seedlings grown for 2 weeks on two plates were subjected to genotyping with lba1 dCAPS primers. Approximately 30% of them showed the genotype of atupf1-3(–/–) (Fig. 5D). These results further indicate that seedling lethality is due to atupf1-3(–/–). The 35S:AtUPF1, however, did not fully complement the knockout of AtUPF1, since atupf1-3(–/–) plants with 35S:AtUPF1 grew abnormally with pale leaves on plates containing hygromycin (Fig. 5D), which could be due to a difference in the expression pattern of 35S:AtUPF1 from that of the wild-type AtUPF1.

Young siliques of self-pollinated heterozygous atupf1-3(+/–) plants did not contain dead or abnormal seeds (Fig. 6A). Variation in seed size, in particular the seed length, was not significantly different from that of Col seeds (Fig. 6B). These results indicate that atupf1-3(–/–) seeds develop normally in siliques of heterozygous atupf1-3(+/–) plants at least with respect to size and shape.

Fig. 6 Seeds produced by heterozygous atupf1-3(+/–) plants. (A) Open siliques of Col and atupf1-3(+/–) plants. Bar = 1 mm. (B) Variations in length among mature dried seeds of Col, lba1 and offspring of self-pollinated atupf1-3(+/–) plants. Shown is the relationship between the length and width of seeds from Col, lba1 and atupf1-3(+/–) plants. Open circles represent the means, and green lines indicate the SD.

Discussion

Elongated seeds of the lba1 mutant

In addition to the previously described growth and developmental phenotypes of the lba1 mutant (Mita et al. 1997, Yoine et al. 2006), we found that mature lba1 seeds are 22% longer and 35% heavier than Col seeds. This seed phenotype of lba1 was rescued by the expression of 35S:AtUPF1.

During early seed development in Arabidopsis, seed growth is driven by rapid divisions of the triploid endosperm accompanied by elongation of the integuments, and growth of the diploid embryo lags behind these processes. In the later stages, the embryo grows to fill the seed sac by replacing most of the endosperm, and the integuments differentiate into the seed coat (Mansfield and Bowman 1993). Three different growth programs for the embryo, endosperm and diploid maternal ovule control the size of the seed, and a cross-talk between maternal and zygotic tissues participates in coordination of the seed size (Sundaresan 2005). In addition, the size of an individual seed may be inversely controlled by the number of seeds, so that a reduction in seed number can cause an increase in seed size (Alonso-Blanco et al. 1999, Jofuku et al. 2005, Ohto et al. 2005).
In the early stages of \( \text{lba1} \) seed development, globular to torpedo stage embryos were contained in seed sacs that were larger than those of Col seeds of similar developmental stages, suggesting that the elongation of \( \text{lba1} \) seeds is mostly due to elongation of the seed sac. When Col pollens were crossed with the \( \text{lba1} \) pistils, the resulting \( F_1 \) seeds were all elongated (data not shown). Taken together, these results suggest that the elongated seeds are due to a maternal effect of the \( \text{lba1} \) mutation on the development of seed sacs. That \( \text{atupf1-3}(–/–) \) seeds develop normally, at least with respect to their size and shape, in siliques of self-pollinated \( \text{atupf1-3}(+/-) \) plants is consistent with this view.

Several other \( \text{Arabidopsis} \) mutants that produce seeds larger than the wild type have been described recently. Mutations in the floral homeotic gene \( \text{APETALA2} \) result in the production of seeds that are 30–70% heavier than the wild-type seeds (Jofuku et al. 2005, Ohto et al. 2005). The \( \text{ap2} \) mutant embryos are larger and exhibit increased cell numbers and cell sizes; however, the increased seed size is determined maternally by the genotypes of integument and endosperm rather than the genotype of the embryo. The \text{auxin response factor 2 (arf2)} mutant also produces seeds that are larger and 16–46% heavier than the wild-type seeds (Schruff et al. 2005). The increased seed size in \( \text{arf2} \) mutants appears to be driven by integuments, and the seed sacs of \( \text{arf2} \) mutants maintain their large size during seed development. \( \text{ARF2} \) is thought to be a repressor of cell division and organ growth, and mutation of \( \text{arf2} \) causes extra cell division and expansion in many organs. Triple mutants of the cytokinin receptors \( \text{AHK2}, \text{AHK3} \) and \( \text{CRE}/\text{AHK4} \) produce seeds that are ~30% larger than those of wild-type seeds (Riefler et al. 2006). Genetic analysis indicated that this phenotype is primarily due to a loss of receptors in the integument and/or endosperm of developing seeds. Thus, seed development in \( \text{lba1} \) and these three mutants seems to be characterized by maternally controlled enlarged seed sacs during the early developmental stages, and altered development of integument and/or endosperm participates in the production of large seeds. Unlike \( \text{lba1} \) seeds, however, seeds of \( \text{ap2} \) and \( \text{arf2} \) mutants as well as the triple mutant of cytokinin receptors are irregular in shape (Ohto et al. 2005) or are both longer and wider (Schruff et al. 2005, Riefler et al. 2006).

The \( \text{ap2} \) and \( \text{arf2} \) mutants as well as the triple mutant of cytokinin receptors set fewer seeds compared with the wild type due to reduced fertility, although the seed number effect alone does not account for the observed increase in seed size (Jofuku et al. 2005, Ohto et al. 2005, Schruff et al. 2005, Riefler et al. 2006). Siliques of the \( \text{lba1} \) mutant did not differ significantly from those of Col in size and shape, whereas the \( \text{lba1} \) mutant siliques contained fewer seeds per unit length of sique, and the distance between seeds was ~19% greater than in Col siliques. Whether the \( \text{lba1} \) mutation affects ovule development in pistils remains to be determined.

**Disruptant of AtUPF1 is seedling lethal**

Our results indicate that plants homozygous for \( \text{atupf1-3} \) with a T-DNA insertion in the middle of \( \text{ATUPF1} \) are seedling lethal. In yeast, mutants deficient in \( \text{UPF1} \) or other NMD factors accumulate PTC-containing mRNAs, yet they exhibit only a minor respiratory defect (Leeds et al. 1992). Similarly, \( \text{C. elegans} \) mutants deficient in \( \text{UPF1} \) or other NMD factors show minor morphological abnormalities in their genitalia, yet they are otherwise viable (Pulak and Anderson 1993). In contrast, \( \text{UPF1} \) is essential for the growth of mice, and \( \text{upf1}(–/–) \) homozygotes die at the embryo blastocyst stage (Medghalchi et al. 2001). In \( \text{Drosophila} \) cells, transient targeted depletion of \( \text{UPF1} \) by RNA interference (RNAi) impairs cell proliferation, and cells are arrested at the G2/M phase of the cell cycle (Rehwinkel et al. 2005).

There are several possible explanations for the difference in the importance of \( \text{UPF1} \) in the growth of various eukaryotes (Medghalchi et al. 2001, Rehwinkel et al. 2005). First, there could be a difference in the relative importance of NMD among various eukaryotes. In humans, an estimated 64% of genes undergo alternative pre-mRNA splicing, and >35% of mRNAs derived from alternative splicing events are estimated to contain PTCs (Lewis et al. 2003). Defects in NMD might cause accumulation of PTC-containing mRNAs, which could give rise to the production of potentially harmful truncated proteins (Frischmeyer and Dietz 1999). In \( \text{Arabidopsis} \), 11.6% of transcription units are estimated to produce alternatively spliced transcripts (Iida et al. 2004), and alternatively spliced transcripts containing PTCs are targets of plant NMD (Hori and Watanabe 2005, Yoine et al. 2006). In contrast, alternative splicing is a relatively minor event in yeast.

Secondly, a difference in phenotypes of NMD-deficient mutants among various organisms could be due to a difference in natural targets of NMD. The mechanisms of how PTCs in transcripts are recognized and are marked to recruit NMD factors for degradation are different among yeast, \( \text{Drosophila} \) and human (reviewed in Culbertson and Leeds 2003, Lejeune and Maquat 2005), and these organisms do not share common orthologous genes as NMD targets (Rehwinkel et al. 2005). Thirdly, \( \text{UPF1} \) in mammals and plants may have acquired novel non-NMD functions that are essential for growth. In mammals, NMD factors are involved in the efficient translation initiation of mRNAs that have undergone intron splicing (Nott et al. 2004), and \( \text{UPF1} \) is required for Staufen1-mediated mRNA decay, which does not require intron splicing and occurs when \( \text{UPF2} \) or \( \text{UPF3} \) is down-regulated (Kim et al. 2005).

**Role of UPF1 and NMD in seed development**

Because \( \text{atupf1-3} \) is seedling lethal, the \( \text{lba1} \) mutant provides a good tool for studying the mechanisms of NMD and the role of NMD or \( \text{UPF1} \) in the growth and development of plants. To our knowledge, the \( \text{lba1} \) mutant is the first example of a missense mutant of \( \text{UPF1} \) identified in multicellular higher
eukaryotes. Because NMD is a pathway that targets PTC-containing mRNAs for degradation, phenotypes of the *iba1* mutant are likely to be due to altered post-transcriptional regulation of NMD-targeted genes that play important roles in the phenomenon of interest. In our previous study, we detected transcripts of 74 genes, corresponding to 0.4% of the total genes studied, that showed a 2-fold increase in sucrose-treated *iba1* plants compared with sucrose-treated Col plants (Yoine et al. 2006). Comparative transcriptome analysis of mRNAs between young siliques of *iba1* and Col might help identify candidate NMD-targeted genes that are involved in the control of seed size.

### Materials and Methods

#### Plant materials and growth conditions

*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col) was used as the wild-type plant. The *iba1* mutant (Mitsu et al. 1997) used in the current studies had been backcrossed eight times with Col (Yoine et al. 2006). The T-DNA insertion lines SALK 4606, SALK 81178 and SALK 22721 were obtained from the Salk Institute Genomic Analysis Laboratory (La Jolla, CA, USA). The *iba1* mutant transformed with 3SS-AtUPF1, which contains the full-length AtUPF1 cDNA and the 3′-UTR of the nos gene downstream of the cauliflower mosaic virus 35S promoter, has been described (Yoine et al. 2006). Transformed lines L1, L3, L4, L6, L7 and L10 were used in this study.

Unless otherwise indicated, seeds were sterilized in sterile water, stored at 4°C for 3 d, and sown on 0.3% (w/v) gellan gum plates containing Murashige and Skoog (MS) medium (Wako, Tokyo, Japan), pH 5.8, supplemented with 100 mg liter\(^{-1}\) myoinositol, 10 mg liter\(^{-1}\) thiamine-HCl, 1 mg liter\(^{-1}\) nicotinic acid, 1 mg liter\(^{-1}\) pyridoxine HCl and 3% (w/v) sucrose. Plates were incubated in a growth chamber at 22°C under continuous light at an intensity of 50 µmol m\(^{-2}\) s\(^{-1}\). After 3 weeks, plants were transferred to soil and grown under continuous light (50 µmol m\(^{-2}\) s\(^{-1}\)). For selection of plants with 35S-AtUPF1, hygromycin-resistant seedlings were selected on MS agar medium containing 20 mg liter\(^{-1}\) hygromycin.

#### Analysis of mature dried seeds

Mature dried seeds were imaged using a SZX12 microscope (Olympus, Tokyo, Japan) and photographed. The seed length and width were determined using ImageJ software (http://rsb.info.nih.gov/ij/). Seed mass was determined by weighing seeds in batches of 100 using a HA-180M microbalance (A&D, Tokyo, Japan).

#### Microscopy

Seeds were dissected from siliques of primary inflorescences and cleared in a small amount of clearing solution (8 g of chloral hydrate, 2 ml of water and 1 ml of glycerol) on a glass slide. Cleared seeds were photographed under a BX60 microscope (Olympus) equipped with Nomarski optics.

#### Seed germination assay

To examine the mannose sensitivity of seed germination, seeds were sown on 0.7% (w/v) agar plates containing minimal salt medium with 1% (w/v) sucrose and with or without 5 mM mannose. Germination (cotyledon opening and greening) was scored 8 d after imbibition.

#### Genotyping of atupf1-2, atupf1-3, atupf1-4 and iba1 mutations

For genotyping of the T-DNA insertion lines, genomic PCR was carried out with a pair of gene-specific primer and a T-DNA border-specific primer as follows: for atupf1-2 mutation, the primers were At5g47010_1F (5′-AAATGAG-AGGAGGCCCTGGTG-3′) and At5g47010_1R (5′-GCAGAAC- CACTTTCACAAGAGG-3′); for AtTFIIIA cDNA and the 3′-UTR of the nos gene downstream of the cauliflower mosaic virus 35S promoter, he has been described (Yoine et al. 2006). Transformed lines L1, L3, L4, L6, L7 and L10 were used in this study.

Unless otherwise indicated, seeds were sterilized in sterile water, stored at 4°C for 3 d, and sown on 0.3% (w/v) gellan gum plates containing Murashige and Skoog (MS) medium (Wako, Tokyo, Japan), pH 5.8, supplemented with 100 mg liter\(^{-1}\) myoinositol, 10 mg liter\(^{-1}\) thiamine-HCl, 1 mg liter\(^{-1}\) nicotinic acid, 1 mg liter\(^{-1}\) pyridoxine HCl and 3% (w/v) sucrose. Plates were incubated in a growth chamber at 22°C under continuous light at an intensity of 50 µmol m\(^{-2}\) s\(^{-1}\). After 3 weeks, plants were transferred to soil and grown under continuous light (50 µmol m\(^{-2}\) s\(^{-1}\)). For selection of plants with 35S-AtUPF1, hygromycin-resistant seedlings were selected on MS agar medium containing 20 mg liter\(^{-1}\) hygromycin.

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For genotyping of the *iba1* mutation, dCAPS primers that can distinguish *iba1* from the Col sequence were used as described (Yoine et al. 2006).

Preparation of RNA and quantitative real-time RT–PCR

Total RNA was isolated from the upper green parts of 3-week-old plants using an RNEasy plant mini kit (Qiagen, Valencia, CA, USA), and the levels of various mRNAs were determined by quantitative real-time reverse transcription–PCR (RT–PCR) using an iCycler iQ with iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) as described previously (Yoine et al. 2006). The following pairs of primers were used: for ACT2 mRNA, 5′-CTGTGTCGACCTGGAGCAG-GAGATGGA-3′ and 5′-GACTTCTGCGCATCTGACTCTCA-3′; for AtTFIIIA transcript 2, 5′-AACTCCCAAATGCGGACCT-3′ and 5′-TGAAAATCTGCCTGATGCG-3′; and for AtUPF1 mRNA, 5′-ACGAGGGCCTAATGGTGACG-3′ and 5′-CAACATCCATCTCACAAGAAGG-3′. For each pair of primers, melting curve analysis was performed to confirm that the PCR produced only a single fragment. The cycle at which the fluorescence of the PCR product–SYBR® Green complex first exceeded the background level was measured, and the relative template concentration compared with control was determined based on a standard curve for each transcript. The level of each mRNA was normalized by the level of ACT2 mRNA.

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References


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