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The Omp85-Related Chloroplast Outer Envelope Protein, OEP80, is Essential for Viability in Arabidopsis

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ABSTRACT

Beta-barrel proteins of the Omp85 (Outer membrane protein, 85 kD) superfamily exist in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts. Prominent Omp85 proteins in bacteria and mitochondria mediate biogenesis of other β-barrel proteins, and are indispensable for viability. In Arabidopsis (Arabidopsis thaliana) chloroplasts, there are two distinct types of Omp85-related protein: Toc75 (Translocon at the outer envelope membrane of chloroplasts, 75 kD) and OEP80 (Outer Envelope Protein, 80 kD). Toc75 functions as a preprotein translocation channel during chloroplast import, but the role of OEP80 remains elusive. We characterized three T-DNA mutants of the Arabidopsis OEP80 (AtOEP80) gene. Selectable markers associated with the oep80-1 and oep80-2 insertions segregated abnormally, suggesting embryo-lethality of the homozygous genotypes. Indeed, no homozygotes were identified amongst >100 individuals, and heterozygotes of both mutants produced ~25% aborted seeds upon self-pollination. Embryo arrest occurred at a relatively late stage (globular embryo-proper), as revealed by analysis using Nomarski optics microscopy. This is substantially later than arrest caused by loss of the principal Toc75 isoform, atToc75-III (two-cell stage), suggesting a more specialized role for AtOEP80. Surprisingly, the oep80-3 T-DNA (located in exon 1, between the first and second ATG codons of the open reading frame) did not cause any detectable developmental defects, or affect the size of the AtOEP80 protein in chloroplasts. This indicates that the N-terminal region of AtOEP80 is not essential for the targeting, biogenesis or functionality of the protein, in contrast with atToc75-III which requires a bipartite targeting sequence.
INTRODUCTION

Chloroplasts and mitochondria evolved from bacteria through endosymbiosis. Recently, a family of β-barrel proteins related to Omp85 (Outer membrane protein, 85 kD) from Neisseria meningitidis was found in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts (Yen et al., 2002; Gentle et al., 2005). Some bacteria contain multiple Omp85-related proteins, with the different homologs playing distinct roles in protein secretion (Jacob-Dubuisson et al., 2004) or the sorting of β-barrel proteins to the outer membrane (Voulhoux et al., 2003; Wu et al., 2005). By contrast, there appears to be just a single functional Omp85 homolog in mitochondria (Sam50 [Sorting and assembly machinery, 50 kD]; alternatively, Tob55 [Topogenesis of β-barrel proteins, 55 kD]), and this mediates β-barrel insertion into the membrane (Kozjak et al., 2003; Paschen et al., 2003; Gentle et al., 2004), as well as the insertion of other outer membrane proteins (Stojanovski et al., 2007).

Unlike mitochondria, chloroplasts contain at least two distinct types of Omp85 homolog, namely Toc75 (Translocon at the outer envelope membrane of chloroplasts, 75 kD) and OEP80 (Outer Envelope Protein, 80 kD). Because homologs exist in extant cyanobacteria (one of which was shown to be essential for viability), these proteins are postulated to be derived from a common ancestor in the original endosymbiont ( Bölter et al., 1998; Reumann et al., 1999; Reumann et al., 2005). Detailed phylogenetic analyses suggested that Toc75 and OEP80 diverged early in the evolution of chloroplasts (Inoue and Potter, 2004).

Pea Toc75 (psToc75) is one of the most abundant proteins in the chloroplast outer envelope membrane (Cline et al., 1981). It associates with precursor proteins in vitro (Perry and Keegstra, 1994; Schnell et al., 1994), and was reconstituted as a cation-selective ion channel in artificial liposomes (Hinnah et al., 1997), suggesting that it forms a major component of the preprotein translocation channel (Bédard and Jarvis, 2005; Kessler and Schnell, 2006; Smith, 2006). Unlike other outer membrane proteins, Toc75 is synthesized as a larger precursor with a bipartite targeting signal (Tranel et al., 1995; Tranel and Keegstra, 1996); the first part is a standard transit peptide for chloroplast import (Inoue et al., 2001), and the second part acts as an intraorganellar targeting signal that is cleaved by an envelope-bound type I signal peptidase (Inoue and Keegstra, 2003; Inoue et al., 2005; Baldwin and Inoue, 2006).

Arabidopsis (Arabidopsis thaliana) possesses four genomic sequences with homology to psToc75, on chromosomes I, III, IV and V; these are termed atTOC75-I, -III, -IV and -V, respectively (Jackson-Constan and Keegstra, 2001; Eckart et al., 2002). Among them, atTOC75-I, -III and -IV are highly homologous to one another, and to the pea gene, with predicted amino acid sequence identities ranging from 60% to 75%. Previous work demonstrated that atTOC75-I is a pseudogene, and that atTOC75-IV encodes a truncated protein which lacks a transit peptide and is expressed at very low levels (Baldwin et al., 2005). By contrast, atToc75-III is synthesized as a larger precursor with a bipartite transit peptide, like psToc75, and is highly expressed (Inoue and Keegstra, 2003; Baldwin et
Disruption of \textit{atTOC75-III} with T-DNA insertions arrested embryo development at a very early stage (the two-cell stage) (Baldwin et al., 2005; Hust and Gutensohn, 2006). These results indicate that atToc75-III is the most direct functional ortholog of psToc75.

The fourth Omp85 homolog of Arabidopsis chloroplasts, atToc75-V, is more distantly related to psToc75, sharing only 22\% identity (Eckart et al., 2002). A putative pea ortholog of atToc75-V was not associated with the protein translocation machinery, suggesting that its role is not directly related to chloroplast protein import (Eckart et al., 2002). The pea protein appeared to be smaller (apparent size \textasciitilde 66 kD) than the conceptual translation of \textit{atTOC75-V} (~80 kD), while the latter was predicted to carry a transit peptide. Thus, it was proposed that atToc75-V has an 11 kD targeting sequence that is removed to yield a mature protein of 69 kD (Eckart et al., 2002). However, subsequent work suggested that atToc75-V (a polypeptide of 732 residues, as encoded by the originally annotated open reading frame) was targeted to isolated chloroplasts without undergoing any change in size (Inoue and Potter, 2004). Additionally, the imported protein was similar in size to a native chloroplast protein recognized by an atToc75-V antibody (~80 kD), and was significantly larger than atToc75-III (~75 kD). Based on these observations, and on the absence of evidence supporting a role in protein translocation, the protein was renamed with the more general designation, AtOEP80 (Inoue and Potter, 2004).

The Toc75 and OEP80 subfamilies are both widely distributed in different plant species (Inoue and Potter, 2004). However, in contrast with Toc75, the function of OEP80 remains elusive. Here, we demonstrate that AtOEP80 is essential for viability, and reveal that the N-terminal part of the protein (corresponding to the region between the first and second AUG codons of the annotated open reading frame) is not required for its biogenesis or function.
RESULTS AND DISCUSSION

The AtOEP80 Gene is Expressed Throughout Development

To gain initial insight into the in vivo role of AtOEP80, we examined its mRNA expression using publicly available microarray data and the Genevestigator V3 analysis tool (https://www.genevestigator.ethz.ch/) (Zimmermann et al., 2004; Grennan, 2006). For comparison, we also analysed the other expressed Toc75-related Arabidopsis genes (atTOC75-III and atTOC75-IV), as well as the gene for a major component of the TIC (Translocon at the Inner envelope membrane of Chloroplasts) complex, atTIC110. A developmental time-course revealed that all four genes are expressed throughout the life-cycle (Supplemental Fig. S1A). The atTOC75-III and atTIC110 genes were expressed most strongly, while atTOC75-IV was expressed at very low levels. The AtOEP80 gene exhibited an intermediate expression level, equivalent to ~25% of that observed for atTOC75-III. Interestingly, developmental fluctuations in the expression of atTOC75-III and atTIC110 were paralleled in the AtOEP80 expression pattern, albeit with reduced amplitude, which is consistent with the hypothesis that the AtOEP80 function is related to plastid biogenesis (Inoue and Potter, 2004).

When expression of the four genes was considered on an anatomical basis (Supplemental Fig. S1B), a similar trend was observed: i.e., in most cases, atTOC75-III and atTIC110 displayed the highest levels, AtOEP80 an intermediate level, and atTOC75-IV the lowest level. Interestingly, there were some notable exceptions to this rule, in pollen, mature embryos and the endosperm. In embryos, AtOEP80 expression was ~40% higher than that of atTOC75-III. Given that atToc75-III, like atTic110, is essential during embryogenesis (Baldwin et al., 2005; Inaba et al., 2005; Kovacheva et al., 2005; Hust and Gutensohn, 2006), this observation suggests that AtOEP80 may also be important during seed development.

Inactivation of AtOEP80 Causes Seed Abortion

To assess the importance of OEP80 in plastids, we identified three different Arabidopsis lines with T-DNA insertions in the AtOEP80 gene (Fig. 1A). These mutants carry insertions in the tenth intron (oep80-1), the fifth intron (oep80-2), and the first exon (oep80-3) of the gene. With one exception, all T-DNA junction sequences were amplified and sequenced to obtain precise positional information (Fig. 1A); the 3’ side of the oep80-2 T-DNA insertion could not be amplified, presumably because it is incomplete and lacks an LB or RB sequence. Interestingly, analysis of the T-DNA-associated selectable marker in segregating populations of oep80-1 and oep80-2 revealed significant deviations from standard Mendelian inheritance: only two antibiotic-resistant plants were observed for every one antibiotic-sensitive plant (Supplemental Table SI). These data imply that the homozygous
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genotype is lethal in each case; they also indicate that both mutants carry just a single T-DNA
insertion locus. Consistent with the first conclusion, when we analyzed 78 antibiotic-resistant oep80-1
plants and 41 antibiotic-resistant oep80-2 plants, by either progeny testing on selective medium or
PCR analysis using gene- and T-DNA-specific primers, all 119 individuals were found to be
heterozygous for the mutation. Examples of the PCR-based genotyping experiments we conducted are
shown in Figure 1B: PCR reactions (“T”) utilizing one T-DNA border primer and one AtOEP80
primer gave a clear amplification product in both mutants, as did additional reactions (“G”) utilizing
two AtOEP80 gene-specific primers flanking the insertion site.

The absence of homozygous individuals from segregating populations suggested that the
mutations might be lethal. To assess the possibility of embryo lethality, we inspected mature siliques
of heterozygous oep80-1 and oep80-2 plants. In both cases, aborted seeds were observed, and these
occurred with a frequency of almost exactly 25% (Fig. 2, A and B), strongly supporting the notion that
the homozygous genotypes were responsible for seed abortion. Identification of two independent
mutant alleles that give rise to the same phenotype is widely accepted as proof of a causal relationship
(Sjögren et al., 2004; Baldwin et al., 2005; Puyaubert et al., 2008). Thus, our data provide very strong
evidence of an essential role for AtOEP80. Consistent with this conclusion, we observed that the
transgenic overexpression of an AtOEP80 cDNA could efficiently complement the distorted
segregation and seed abortion defects of oep80-1, enabling the identification of apparently normal
homozygous mutants at Mendelian frequency (unpublished observations).

There are numerous precedents for embryo lethality caused by lesions in chloroplast proteins
(Uwer et al., 1998; Apuya et al., 2001). In fact, in a large-scale screen for mutations affecting seed
development, >25% of the identified loci were predicted to encode plastidic proteins (McElver et al.,
2001). Most relevant amongst these previous studies are those pertaining to two major components of
the chloroplast protein import machinery: atToc75-III (Baldwin et al., 2005; Hust and Guttensohn,
2006) and atTic110 (Inaba et al., 2005; Kovacheva et al., 2005). Interestingly, the aborted seeds in
oep80 plants (Fig. 2A) appeared to be significantly larger than those observed previously in toc75-III
mutants, and were more similar in size to those in the tic110 mutant (Baldwin et al., 2005; Kovacheva
et al., 2005). This suggested that the oep80 mutation, like tic110, may act at a later stage in
embryogenesis than toc75-III.

While the toc75-III mutations appeared to be completely recessive (Baldwin et al., 2005),
heterozygous tic110 plants were visibly pale and exhibited quantifiable defects in chloroplast
biogenesis (Kovacheva et al., 2005). In this regard, the oep80 mutations are more similar to the toc75-
III mutations, since heterozygotes could not be distinguished from the wild type, not only at a
macroscopic level (Fig. 3A) but also in relation to chlorophyll content (Fig. 3B) and photosynthetic
performance (Supplemental Table SII). This indicates that a single copy of the AtOEP80 gene is able
to produce sufficient quantities of the protein for normal growth under standard, controlled conditions.
The greater dosage dependency of tic110 may reflect a higher rate of turnover of the atTic110 protein, or the absence of excess expression capacity for atTic110 in the wild type.

**Homozygous oep80-1 Embryos Arrest at the Globular Stage**

To determine more precisely the stage at which developmental arrest occurs, we conducted a thorough examination of developing embryos in wild-type and mutant plants using Nomarski optics. Figure 2C shows equivalent developmental series for normal (i to iv) and mutant (v to viii) embryos within immature siliques of self-pollinated oep80-1 heterozygotes. When normal embryos were at the globular stage (Fig. 2C, i and ii), mutant embryos (equivalent to 26% of the total number; Table I) were retarded at the proembryo stage (Fig. 2C, v and vi). As normal embryos progressed to the heart stage (Fig. 2C, iii), mutant embryos (equivalent to ~24-29% of the total number; Table I) developed to the globular stage (Fig. 2C, vii), but began to take on an abnormal, “raspberry-like” appearance, with protuberances on the surface of the embryo-proper (Yadegari et al., 1994; Apuya et al., 2002). By the time the normal embryos had reached the torpedo stage (Fig. 2C, iv), the “raspberry-like” phenotype of the mutant embryos had become even more pronounced (Fig. 2C, viii). The mutant embryos (equivalent to ~22% of the total number; Table I) did not develop beyond the globular stage. In contrast with the situation in oep80-1 siliques, where two clear, distinct classes of embryos could be observed (normal and mutant), embryos within individual wild-type siliques rarely spanned more than two consecutive developmental stages (Table I).

A very similar “raspberry-like”, globular-stage arrest phenotype was reported for the atTic110 knockout mutant, tic110 (Kovacheva et al., 2005). The protuberances on the surface of the embryo proper, responsible for the “raspberry-like” appearance of the oep80 and tic110 mutant embryos, are in fact a characteristic feature of many mutants that arrest during this stage of embryogenesis. It is thought that they are caused by cellular maturation processes that normally occur during late embryogenesis, and which continue to proceed in the mutants in spite of the block in embryo growth and morphogenesis (Yadegari et al., 1994; Apuya et al., 2002). Unlike oep80, the tic110 mutation was also associated with abnormal, asymmetric or unsynchronized cell divisions at earlier developmental stages in some embryos (Kovacheva et al., 2005). Since no such early defects were observed in the AtOEP80 knockout, the oep80 mutant phenotype can be considered somewhat less severe, and later acting, than tic110. Nevertheless, the oep80-mediated block in growth occurs considerably earlier than the stage during which photosynthetic establishment normally commences (heart stage; Apuya et al., 2001), and so the data indicate that the role of AtOEP80 is not directly associated with photosynthesis.

The late-acting effect of oep80 contrasts with the much earlier defect reported for the atToc75-III knockout mutation, toc75-III (Baldwin et al., 2005). In toc75-III, embryo arrest was observed to occur when the embryo-proper was composed of just two cells. Since the two genes are expressed at
comparable levels in embryos (Supplemental Fig. S1B), this difference in phenotype severity between
\textit{toc75-III} and \textit{oep80} may reflect differing roles of the proteins. While \textit{atToc75-III} is believed to be the
channel responsible for the import of most proteins (Bédard and Jarvis, 2005; Kessler and Schnell,
2006; Smith, 2006), \textit{AtOEP80} has been proposed to play a more specialized role in the biogenesis of
outer envelope $\beta$-barrel proteins, like certain bacterial and mitochondrial Omp85 proteins (Inoue and
Potter, 2004).

\section*{Homozygous \textit{oep80-3} Plants Are Phenotypically Normal}

In contrast with the situation for \textit{oep80-1} and \textit{oep80-2}, the selectable marker associated with
the \textit{oep80-3} T-DNA segregated normally, exhibiting standard Mendelian inheritance: three antibiotic-
resistant plants were observed for every one antibiotic-sensitive plant (Supplemental Table SI). This
implies that the homozygous \textit{oep80-3} genotype is not lethal, which is surprising given the location of
the T-DNA in the first exon (Fig. 1A). Families containing only antibiotic-resistant individuals were
identified, and these were confirmed as \textit{oep80-3} homozygotes by PCR analysis (Fig. 1B).
Remarkably, homozygous \textit{oep80-3} mutants were indistinguishable from wild type. The mutant was of
a similar size and colour to wild type throughout development (Fig. 3A), and contained normal levels
of chlorophyll (Fig. 3B). Chlorophyll fluorescence measurements did not reveal any differences in
photosynthetic performance between \textit{oep80-3} homozygotes and wild type (Fig. 3C; Supplemental
Table SII), nor did assays of non-photosynthetic development (root length, hypocotyl length in
etiolated plants, and de-etiolation efficiency; Supplemental Table SII).

The aforementioned data provide strong evidence that there are no phenotypic consequences
associated with the \textit{oep80-3} T-DNA insertion. This initially suggested that the mutant may encode a
truncated form of \textit{AtOEP80}, and that the missing N-terminal region is not essential. To investigate this
possibility, we first of all analyzed \textit{AtOEP80} expression by RT-PCR. Using the “RT” pair of
amplification primers (which flank the \textit{oep80-3} T-DNA insertion site; Fig. 1A), an amplicon of the
expected size was obtained for wild type, but no expression was detected in \textit{oep80-3} (Fig. 4A).
However, when the forward amplification primer was replaced with the “RTa” primer (located
downstream of the T-DNA; Fig. 1A), we observed clear evidence of \textit{AtOEP80} mRNA expression in
the mutant (Fig. 4A). In fact, the detected transcript was overexpressed in the mutant, relative to wild
type, presumably as a consequence of CaMV 35S enhancer sequences in the T-DNA construct. These
data indicate that a truncated \textit{AtOEP80} message is indeed produced in the \textit{oep80-3} mutant.
The N-Terminus of “Full-Length” AtOEP80 Is Dispensable

To determine precisely the nature of the oep80-3 transcript, we amplified its 5’ end by RACE-PCR, and sequenced the resulting product. The mutant mRNA was found to comprise ~86-90 nucleotides encoded by the T-DNA left border, fused to AtOEP80-encoded sequence at the expected position based on the previously-determined T-DNA-gene junction sequence (Fig. 1A; Supplemental Fig. S2). This transcript lacks the first AUG codon (AUG1) of the wild-type message, and so is predicted to encode a truncated, ~74 kD protein of 680 residues starting from the second, in-frame AUG (AUG2); an ~80 kD polypeptide of 732 residues is encoded by initiation at AUG1. To test for the presence of this smaller protein, we analyzed isolated chloroplasts from wild-type and oep80-3 plants by immunoblotting. Surprisingly, the mutant chloroplasts contained an AtOEP80 protein of the same size as that in wild type, and this migrated at a position just above atToc75-III (Fig. 4B; see also Supplemental Fig. S3A); the atToc75-III protein has a calculated molecular weight of ~75 kD, and yet runs significantly faster than the 75 kD standard on a 7.5% SDS-PAGE gel. Assuming that AtOEP80 is ~74 kD in size, its slower migration than atToc75-III may be due to post-translational modification of one of the proteins, since there are no obvious differences in amino acid composition.

In an attempt to explain the above data, we used SDS-PAGE and immunoblotting to compare the sizes of different, in vitro-translated AtOEP80 proteins, imported into chloroplasts, with that of the endogenous protein. We previously took a similar approach to show that the “full-length” protein of 732 residues, following import, migrates in similar fashion to the endogenous protein as recognized by an AtOEP80-specific antibody (Inoue and Potter, 2004). At that time, we had been focusing on determining whether AtOEP80 is synthesized with a cleavable, ~11 kD transit peptide, and so might not have detected more subtle mobility differences between the proteins. Here, we used an affinity-purified sample of the antibody (Supplemental Fig. S3B) to improve sensitivity and clarity, and extended the gel electrophoresis time to thoroughly scrutinize any small mobility differences.

We prepared two translation reactions: one utilizing the full-length AtOEP80 cDNA as template (“AtOEP80 [AUG1]”), and another utilizing a truncated cDNA lacking the first 156 nucleotides of the coding sequence and starting from the second AUG codon (“AtOEP80 [AUG2]”) as template (Fig. 5A, lanes 4 and 10). While the former reaction contained a single, major product of the expected size (~80 kD), the latter contained a number of smaller proteins (presumably corresponding to initiation at downstream AUG codons; predicted sizes: 71, 70, 59, 54 and 48 kD) in addition to the expected product of 74 kD. The prominence of alternative initiation products in the second translation reaction most likely reflects the sub-optimal context of AUG2 in the translation system used (see Supplemental Appendix). In import experiments conducted in vitro, both proteins were recovered in chloroplasts (Fig. 5A, lanes 5 and 11). Proper membrane integration of the 680-residue protein was confirmed in a high-pH wash experiment: the longest translation product was recovered almost exclusively in the membrane fraction following alkaline treatment, whereas the most abundant shorter
product was substantially released to the supernatant (Fig. 5B, compare lanes 6 and 7). These data confirm that the first 52 residues of the “full-length” 732-residue protein are not essential for import or membrane integration. Interestingly, while AtOEP80 translated from AUG1 (the 732-residue protein) migrated more slowly than endogenous AtOEP80, that translated from AUG2 (the 680-residue protein) migrated in a very similar position to the endogenous protein (Fig. 5A; compare lanes 5 and 2, and lanes 11 and 8; Fig. 5B, compare lanes 5 and 1, and 7 and 3).

These results may be explained in two different ways. One possibility is that the AtOEP80 protein is normally translated from a non-canonical, downstream initiation codon, even in wild type, with AUG2 being one candidate (see Supplemental Appendix; Supplemental Fig. S4). This hypothesis is supported by the fact that the *oep80-3* mutant expresses a protein of the same size as that in wild type (Fig. 4; Supplemental Fig. S3), and by the co-migration of the 680-residue protein translated from AUG2 with the endogenous protein in chloroplasts (Fig. 5). It is also noteworthy that the two most similar sequences present in the protein databases (OsI_006101 [EAY84868] and OsJ_005573 [EAZ22090]; both from rice) align with AtOEP80 only at positions downstream of the “second” methionine. Such non-canonical initiation might have developmental or regulatory significance.

However, in two different in vitro translation systems (wheat germ, Fig. 5A; rabbit reticulocyte, Fig. 5B), initiation at AUG2 appeared to be rather inefficient. Moreover, a recent proteomic study indicated that translation from AUG1 can occur in vivo; of 89 AtOEP80 peptides identified by Dunkley et al. (2006), one (FSSSSIR; positions 10-16 relative to Met1/AUG1) was found to correspond to sequence between AUG1 and AUG2 (K. Lilley, personal communication). Thus, an alternative to the non-canonical initiation possibility outlined above is that translation from AUG1 leads to the formation of a 732-residue precursor protein, which is processed to a lower molecular weight form during targeting or membrane insertion. Because the targeting, accumulation and functionality of AtOEP80 were not detectably altered in the *oep80-3* mutant (Figs. 3 and 4), one may conclude that any cleavable, N-terminal targeting sequence that is present is dispensable. It is noteworthy that neither of the AtOEP80 translation products changed in size upon import (Fig. 4). However, this may simply indicate that proteolytic processing is inefficient in the context of an in vitro protein import assay, as has been observed previously for Tic22 and Toc75 (Kouranov et al., 1999; Inoue and Keegstra, 2003).
CONCLUSION

Our aim was to assess the importance of the plastidic protein, AtOEP80, for plant growth and development. As a first step, we analyzed the expression of AtOEP80 using publicly available microarray data, relative to well-known components of the protein translocation machinery of the plastid envelope. Expression levels of AtOEP80 paralleled those of atTOC75-III and atTIC110 throughout development, but at substantially lower levels (~25% of the level of atTOC75-III). Interestingly, a different trend was observed in embryos, with AtOEP80 expression being ~40% higher than atTOC75-III expression, hinting at an important role for AtOEP80 during embryogenesis. Consistent with this notion, the knockout mutations, oep80-1 and oep80-2, were embryo-lethal in the homozygous state, demonstrating that AtOEP80 plays an essential role during early stages of plastid development. Developmental arrest occurred at a relatively late stage in oep80 (globular stage embryo-proper), which contrasts with the early defect (two-cell stage) caused by loss of atToc75-III (Baldwin et al., 2005). This difference in phenotypic severity may reflect differences in the roles of the proteins: atToc75-III playing a wide-ranging role in the import of many proteins, and AtOEP80 a more specialized role, perhaps in the biogenesis of a relatively small subset of proteins. Further experimentation will be required to determine the exact function of AtOEP80. Surprisingly, a third AtOEP80 mutant, oep80-3, which carries a T-DNA insertion in the first exon, was found to be viable and indistinguishable from wild type in the homozygous state. In spite of the fact that oep80-3 expresses a truncated transcript lacking the first AUG codon, the mutant was found to express an AtOEP80 protein of the same size as that in wild type. Together with data from in vitro translation, import and immunoblotting experiments, this observation led to the conclusion that the N-terminal region of the putative “full-length” AtOEP80 protein of 732 residues is not required for targeting, membrane insertion, or functionality. This contrasts with the situation for atToc75-III, which requires a bipartite targeting sequence for proper biogenesis (Tranel and Keegstra, 1996; Inoue and Keegstra, 2003). Thus, there are two essential, Omp85-related β-barrel proteins in the outer envelope membrane of chloroplasts, but it would appear that these proteins have quite different requirements for membrane insertion.
EXPERIMENTAL PROCEDURES

Plant Growth Conditions

All Arabidopsis thaliana plants were of the Columbia-0 ecotype. For in vitro growth, seeds were surface sterilized, sown on Murashige-Skoog (MS) agar medium in petri plates, cold-treated at 4°C, and thereafter kept in a growth chamber, as described previously (Aronsson and Jarvis, 2002). To select for the presence of T-DNA insertions, the following antibiotics were added to the medium: hygromycin B, 15-30 µg/mL (oep80-1); sulfadiazine, 11.25 µg/mL (oep80-2 and oep80-3). All plants were grown under a long-day cycle (16 hours light, 8 hours dark).

Root length measurements were conducted as described previously (Constan et al., 2004), using plants grown on vertically-oriented MS agar plates under standard conditions for ten days. Hypocotyl length measurements were done using plants germinated on soil, and grown in sealed propagators in the dark for five days, using a published method (Salter et al., 2003). De-etiolation experiments were conducted according to a previous report (Baldwin et al., 2005) and as described below, using plants grown on MS medium lacking sucrose. Following cold treatment, plates were exposed to standard light for four hours, to promote germination, and then kept in darkness for six days; then, plates were transferred to continuous light for a further period of two days prior to scoring.

Identification of the oep80 Mutants

The T-DNA insertion lines were obtained from the following sources: oep80-1 was from the Csaba Koncz laboratory (pool 894, line 89350) (Ríos et al., 2002); oep80-2 and oep80-3 were from Genomanalyse im Biologischen System Pflanze-Kölner Arabidopsis T-DNA (GABI-Kat; lines 429H12 and 430F02, respectively) (Rosso et al., 2003).

Mutant genotypes were assessed by PCR (Fig. 1B). Genomic DNA was extracted from Arabidopsis plants using a published protocol (Edwards et al., 1991), and PCR was conducted using standard procedures. The primers used were as follows: oep80-1 forward, 5’-CAT GGA TTG AAG GAG ATG ACA AGA G -3’; oep80-1 reverse, 5’-GAA ACG AGC TGG TCC AAT GTG TAT G-3’; oep80-1 T-DNA right border (RB), 5’-CAG TCA TAG CCG AAT AGC CTC TCC A-3’; oep80-2 forward, 5’-AGT AAG AAC GAA AGA TGG TGA GGA-3’; oep80-2 reverse, 5’-TCA CTT TCC CTA CAC AGC TTG A-3’; oep80-2 and oep80-3 T-DNA left border (LB), 5’-CCC ATT TGG ACG TGA ATG TAG ACA G-3’; oep80-3 forward, 5’-TCC TTG TTT GTT GTT CAT GTG GTG TGT GA-3’; oep80-3 reverse, 5’-TCC TCA CCA TCT TCG TGT CTT ACT-3’; oep80-3 T-DNA RB, 5’-GCA AGT GGA TTG ATG TGA TAT CTC CAC-3’. The amplification products were analyzed by agarose gel electrophoresis, and stained with ethidium bromide. The location of each T-DNA insertion was...
determined precisely by the sequencing of PCR products spanning both junctions (except in the case of oep80-2, where only one junction was identified).

**Chlorophyll Quantification and Photosynthetic Measurements**

Chlorophyll was extracted from 14-day-old plants grown *in vitro*, and determined photometrically as described previously (Porra et al., 1989; Aronsson et al., 2003). Photosynthetic electron transport rates (Fig. 3C), as well as the photochemical efficiency of PSII ($F_v/F_m$) and photosynthetic performance index (PI) (Supplemental Table SII), were determined by measuring chlorophyll fluorescence using a continuous excitation fluorimeter (Handy PEA; Hansatech Instruments Ltd., King’s Lynn, Norfolk, UK), according to the manufacturer’s instructions and as described previously (Meyer et al., 1997; Strasser et al., 2004). The plants used for these chlorophyll fluorescence assays were grown on selective medium for eight days (where appropriate), rescued to non-selective medium, and then transferred to soil after two weeks.

**Isolation of RNA and RT-PCR**

Total RNA was isolated from Arabidopsis seedlings with an RNeasy Plant Mini Kit (Qiagen, Valencia, CA), and used to prepare cDNA with Superscript III and random primers (Invitrogen, Carlsbad, CA). Using the resultant cDNA as a template, PCR amplifications were performed using the following *AtOEP80* gene-specific primers (Figs. 1A and 4A): RT forward, 5’-ATG CAT TGT CAC AAC GAT GA-3’; RTa forward, 5’-ATG CTC CAG TCG CTA AAG AAT C-3’; RT reverse, 5’-TCT ACA TCC CTC TTC CCT TGA A-3’. Control amplification of a sequence derived from 18S rRNA was performed according to the manufacturer’s instructions (Ambion, Foster City, CA).

**Chloroplast Isolation, Import and Immunoblotting**

Isolation of chloroplasts from plate-grown Arabidopsis seedlings and in vitro chloroplast protein import assays were performed essentially as described previously (Fitzpatrick and Keegstra, 2001; Inoue and Potter, 2004). For the preparation of radiolabeled precursor proteins, TNT coupled systems containing wheat germ extract (Fig. 5A) or rabbit reticulocyte lysate (Fig. 5B) were used (Promega, Madison, WI). The cDNA construct encoding the short form of the *AtOEP80* protein (*AtOEP80* [AUG2]; 680 residues) was prepared by subcloning a PCR product (forward primer, 5’-ATG CTC CAG TCG CTA AAG AAT C-3’; reverse primer, 5’-CTC GAG TTA GTT CCG CAG...
ACC AAC-3’), amplified using pGEMT-AtOEP80 (Inoue and Potter, 2004) as template, into the pGEM-T Easy vector (Promega). Samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Imported proteins were detected by autoradiography. For immunodetection, membranes were incubated with antisera raised against psToc75 or AtOEP80 (see below). Immunoreactive proteins were detected using a secondary antibody conjugated with alkaline phosphatase and a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate mixture (Bio-Rad Laboratories).

A previously described antiserum against residues 325-337 of AtOEP80 was employed (Inoue and Potter, 2004). Its specificity was confirmed in a competition experiment, and then the crude serum was purified by affinity chromatography for use in Figures 4 and 5 (Supplemental Fig. S3). Briefly, a 200 µg sample of the antigen peptide (Inoue and Potter, 2004) was coupled to 100 µl UltraLink Iodoacetyl Gel (MicroLink Peptide Coupling Kit, Pierce, Rockford, IL) in the provided mini-column according to the manufacturer’s instructions. The crude serum (300 µl) was applied to the column and incubated at room temperature for 2 h. The unbound fraction was collected, and then the column was washed 15 times with 300 µl wash buffer (0.7 M NaCl, 0.05% [v/v] Tween20), and a further three times with 300 µl phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). Bound antibodies were eluted with 0.1 M glycine, pH 2.8, immediately neutralized with a 1/20th volume of 1M Tris-HCl, pH 9, and stored at 4 °C until further use.

Embryo Analysis by Light Microscopy

The analysis of cleared wild-type and oep80-1 mutant embryos using Nomarski optics (Fig. 2C) was performed as described previously (Goubet et al., 2003; Baldwin et al., 2005; Kovacheva et al., 2005). A microscope (model BHS; Olympus) equipped for differential interference contrast (model BH2-NIC; Olympus) was employed for these studies.
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression patterns of the AtOEP80 gene relative to genes for other envelope proteins.

Supplemental Figure S2. Annotated T-DNA left border junction sequence for the oep80-3 mutant, illustrating the structure of the 5’ end of the expressed transcript.

Supplemental Figure S3. Specificity confirmation and affinity purification of the AtOEP80 antibody used in the immunoblotting experiments shown in Figures 4 and 5.

Supplemental Figure S4. In silico analysis of the 5’ region of the AtOEP80 mRNA.

Supplemental Table SI. Segregation of the T-DNA-associated selectable marker in each of the oep80 mutants.

Supplemental Table SII. Phenotypic analysis of the oep80 mutants, including photosynthetic measurements as well as studies on non-photosynthetic growth.

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FIGURE LEGENDS

Figure 1. Basic characterization of the AtOEP80 T-DNA insertion mutants.
A, Schematic showing the structure of the AtOEP80 gene and the location of each T-DNA insertion. Protein-coding exons are represented by black boxes, and untranslated regions by white boxes; introns are represented by thin lines between the boxes. The gray area at the 5’ end of the exon 1 represents a putative untranslated region, or alternatively encodes a non-essential cleavable peptide. Locations of primers used for RT-PCR analysis (“RT”; Fig. 4A) are shown. T-DNA insertion sites are indicated precisely, but the insertion sizes are not to scale. ATG, potential translation initiation codons; Stop, translation termination codon; p(A), polyadenylation site; LB, T-DNA left border; RB, T-DNA right border.
B, Analysis of mutant genotypes by PCR. Genomic DNA extracted from wild-type and mutant plants (oep80-1, “80-1”; oep80-2, “80-2”; and oep80-3, “80-3”) was analyzed by PCR. Appropriate T-DNA- and AtOEP80-gene-specific primers were employed. Two different primer combinations were used: the first (“T”) comprised one T-DNA primer (RB for oep80-1; LB for oep80-2 and oep80-3) and one gene-specific primer (“reverse” in the case of oep80-1 and oep80-3; “forward” in the case of oep80-2); the second (“G”) comprised two gene-specific primers flanking the T-DNA insertion site. The results shown for oep80-1 and oep80-2 are representative of those obtained for all antibiotic-resistant plants tested; amplification using both “T” and “G” indicated the presence of both mutant and wild-type alleles, respectively, and demonstrated that the plants were heterozygous. Results shown for oep80-3 are representative of those obtained for all homozygotes tested; absence of amplification using the “G” primers indicated that the wild type allele was not present. Sizes of the amplicons are indicated at right (in kb).

Figure 2. Embryo lethality of the oep80-1 and oep80-2 mutations.
A, Appearance of aborted seeds within mature siliques of oep80-1 heterozygous plants. The aborted seeds are smaller in size than the normal seeds, and have a darker, shrivelled appearance.
B, Frequency of aborted seeds within mature siliques of wild-type, oep80-1 and oep80-2 plants. The data shown are means (±SD) derived from analyses of three or four siliques from each of three to six independent plants per genotype.
C, Analysis of embryo development in oep80-1 using Nomarski optics. Equivalent developmental series for normal (i to iv) and mutant (v to viii) embryos within immature oep80-1 heterozygous siliques. Normal embryos: (i) early globular stage; (ii) late globular stage; (iii) heart stage; (iv) torpedo stage. Corresponding mutant embryos from the same siliques: (v) proembryo stage; (vi) early globular stage; (vii and viii) “raspberry-like” globular stage. Embryo cell stage names refer to the morphology of the embryo proper. All images are at the same magnification. Bar = 50 µm.
Figure 3. Phenotypic analysis of the oep80 mutants.
A, Plants of the indicated genotypes were grown on selective medium (except for the wild type) in vitro for eight days, rescued to non-selective medium, and then photographed on day 14 (top panel). Additional similar plants were transferred to soil on day 14 and then allowed to grow for further period of ten days prior to photography (bottom panel). Representative plants are shown in both cases.
B, Chlorophyll concentrations in 14-day-old plants grown as described in A above were determined photometrically. Values shown are means (± SE) derived from 16 independent samples per genotype, each one containing six plants. Units are nmol chlorophyll $a + b$ per plant.
C, Analysis of photosynthesis in the oep80-3 mutant. Light response curves of photosynthetic electron transport rates in wild-type and mutant plants were determined by measuring chlorophyll fluorescence. Values were recorded at different irradiances of photosynthetically active radiation (PAR), ranging from 0 to 1,200 µmol photons m$^{-2}$ s$^{-1}$. Units for the data shown are µmol electrons m$^{-2}$ s$^{-1}$, assuming that 84% of the incident light is absorbed, and that the transport of each electron utilizes two photons (Meyer et al., 1997; Aronsson et al., 2007). Measurements were done on fully-grown leaves from ten different 29-day-old plants per genotype grown under identical conditions. Values shown are means (± SD).

Figure 4. Analyses of mRNA and protein expression in the oep80-3 mutant.
A, Analysis of AtOEP80 mRNA expression. Total RNA extracted from wild-type and homozygous oep80-3 mutant plants was analyzed by RT-PCR. Each reaction contained two primer pairs: the first specifically amplified a 1.1 kb fragment from the wild-type AtOEP80 transcript (locations of the “RT” primers used are indicated in Fig. 1A), or a 0.9 kb fragment from the wild-type and oep80-3 mutant transcripts (the forward “RT” primer was replaced with “RTa” in this case; see Fig. 1A); the second amplified a 315 bp fragment derived from 18S rRNA, and served as a positive control. Reactions lacking reverse transcriptase (-RT) were included as negative controls. Images from different portions of the same gel are separated by vertical lines. Sizes of the amplicons are indicated at left (in kb).
B, Analysis of AtOEP80 protein expression. Isolated chloroplast samples (equivalent to 20 µg [left panel] or 10 µg [right panel] chlorophyll) were separated by SDS-PAGE, and then analyzed by immunoblotting using antiserum against AtOEP80 only (left panel), or a mixture of antisera against AtOEP80 and psToc75 (right panel). Protein bands corresponding to AtOEP80 and atToc75-III are indicated at right. Positions of molecular weight standards are indicated at left (sizes in kD); note that the 75 kD standard migrates more slowly than atToc75-III, and at approximately the same speed as endogenous AtOEP80. A 40 kD protein band that was non-specifically recognized by the psToc75 antiserum is indicated with an asterisk. Images from different portions of the same gel are separated by a vertical line.
**Figure 5.** Electrophoretic mobility comparisons between proteins imported into Arabidopsis chloroplasts in vitro, and endogenous AtOEP80.

A. Radiolabeled long ("AtOEP80 [AUG1]"; 732 residues) and short ("AtOEP80 [AUG2]"; 680 residues) forms of the AtOEP80 protein were generated by in vitro translation using different cDNA templates. These were incubated with Arabidopsis chloroplasts under import conditions, and then the chloroplasts were recovered. In vitro translation products equivalent to 10% of the amount added to each import assay ("IVT+/Chl-"), Arabidopsis chloroplasts containing imported, radiolabeled proteins ("IVT+/Chl+"), and equivalent chloroplast samples lacking imported, radiolabeled protein ("IVT-/Chl+") were resolved side-by-side using SDS-PAGE, blotted onto the same membrane, and then analyzed either by probing with AtOEP80 and psToc75 antiserum ("Immunoblot"), or by autoradiography ("Autorad"). The positions of endogenous AtOEP80 and atToc75-III proteins are indicated at right ("'80'" and "'75'", respectively). Positions of molecular weight standards are indicated at left (sizes in kD). Under the conditions used, the endogenous AtOEP80 protein migrated slower than atToc75-III, while both proteins ran faster than the 75 kD marker protein.

B. In vitro translated, radiolabeled AtOEP80 ("AtOEP80 [AUG2]"; 680 residues) was incubated with Arabidopsis chloroplasts under import conditions. Half of the recovered chloroplast sample was subjected to alkaline extraction using 0.1 M Na₂CO₃, and separated into soluble and membrane fractions as described (Inoue and Potter, 2004). In vitro translation products equivalent to 5% of the amount used for the import assay ("IVT"), unfractionated Arabidopsis chloroplasts containing imported, radiolabeled protein ("Imp"), and the supernatant ("Sup") and membrane ("Mem") fractions obtained after alkaline extraction were resolved side-by-side using SDS-PAGE, blotted onto the same membrane, and then analyzed either by probing with AtOEP80 antiserum ("Immunoblot"), or by autoradiography ("Autorad"). The position of imported AtOEP80 is indicated at right. Positions of molecular weight standards are indicated at left (sizes in kD); note that the 75 kD marker runs significantly slower than atToc75-III on a 7.5% SDS-PAGE gel.

**TABLES**

**Table I.** Distribution of embryo phenotypes in single siliques of oep80-1 heterozygotes
SUPPLEMENTAL APPENDIX

Most plant cytosolic mRNAs have a 5’-terminal cap comprising a 7-methylguanosine residue joined by a 5’-to-5’ triphosphate linkage. A cap-binding complex recruits the 40S ribosomal subunit to the 5’ end of the message, which then scans along the transcript until the first AUG codon is encountered, at which point the 60S ribosomal subunit also binds and translation commences (Kozak, 1989). While this “ribosome scanning” mechanism of translation initiation is used with most transcripts, exceptional modes of cap-dependent and cap-independent initiation do operate, especially in conjunction with viral mRNAs (Touriol et al., 2003). One mechanism allowing for the use of an alternative, downstream AUG codon is “leaky scanning”. In this case, the ribosome passes over the first AUG, because it is in an unfavourable context, and instead utilizes the next, more favourable AUG codon for translation initiation. In the case of AtOEP80, the first AUG codon is actually more similar (8/11 matches) with the dicot consensus (5’-aaA(A/C)aAUGGCU-3’; Joshi et al., 1997) than the second AUG (6/11 matches).

Secondary structural elements in transcript leader sequences can also influence the position of initiation (Chabregas et al., 2003; Touriol et al., 2003). To assess whether such factors might play a role in AtOEP80 translation, we examined the 5’ end of the transcript in silico (using DNASTAR GeneQuest software, version 7.2). A stem-loop structure was predicted to occur adjacent to AUG1, while no such structures were predicted in the vicinity of AUG2. A more thorough analysis using specialized software (mfold version 3.2; Mathews et al., 1999; Zuker, 2003) supported the initial AUG1 stem-loop prediction, and revealed that it may be sufficiently stable to interfere with translation (Supplemental Fig. S4A). If the AUG1 stem-loop does indeed impede canonical initiation, there should be a mechanism for internal ribosome initiation further downstream. Previous reports indicated that complementarity between leader sequences and the central region of the 18S rRNA can act to recruit ribosomes in cap-independent fashion (Akbergenov et al., 2004; Dresios et al., 2006). We examined the AtOEP80 leader for such an internal ribosome entry site (IRES), and found a 17 nucleotide stretch, just upstream of AUG2, that is complementary to the Arabidopsis 18S rRNA (Supplemental Fig. S4B). Interestingly, this region of complementarity overlaps well with a sequence (“1105-1114”; Supplemental Fig. S4C) shown previously to strongly promote cap-independent binding and enhance translational efficiency (Akbergenov et al., 2004).

Use of a non-canonical, downstream initiation might have developmental or regulatory significance, enabling functional differentiation or optimization of the protein. It is interesting to note that in Neurospora crassa, a mitochondrial Omp85 homologue, Tob55 (Topogenesis of β-barrel proteins, 55 kD; an alternative name for Sam50 [Sorting and assembly machinery, 50 kD]), exists in three different forms due to alternative splicing, and that these exhibit functional differences (Hoppins et al., 2007). Another possibility is that downstream initiation might optimize the organelar targeting properties of AtOEP80 in vivo. Analysis of the long (732 residues) and short (680 residues) forms of
the protein using some localization prediction programs supported this hypothesis (Schein et al., 2001; Bannai et al., 2002; Small et al., 2004; Horton et al., 2007), although in vitro import data are inconclusive in this regard (Fig. 5A).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. Expression patterns of the \textit{AtOEP80} gene relative to genes for other envelope proteins.

Publicly available Affymetrix GeneChip microarray data were analyzed and retrieved using the Genevestigator V3 analysis tool (https://www.genevestigator.ethz.ch/) (Zimmermann et al., 2004; Grennan, 2006). Presented data were prepared using the Meta-Profile Analysis tool, using either the Development (A) or Anatomy (B) representations in scatter-plot format. Data from all high-quality, ATH1(22k) arrays were analyzed; this amounted to a total of 3110 arrays. Values shown are means (± SE). The total number of arrays used to derive each data point shown is indicated in each panel. The software differentiates between signals that are significantly above background noise, and those which are close to background: probe sets represented by closed circles are those which are called “present”, while those represented by open circles (none in this case) are called “absent”. Data representations were exported from Genevestigator in Encapsulated PostScript format, and then compiled and annotated using appropriate graphics software. The genes analyzed were as follows: \textit{atTOC75-III} (At3g46740; red); \textit{atTIC110} (At1g06950; blue); \textit{AtOEP80} (At5g19620; yellow); \textit{atTOC75-IV} (At4g09080; green).

Supplemental Figure S2. Annotated T-DNA left border junction sequence for the \textit{oep80-3} mutant. Sequence flanking the LB junction of the \textit{oep80-3} T-DNA insertion was amplified as illustrated in Figure 2B. The composition of the amplified fragment was analysed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) provided by the National Center for Biotechnology Information (NCBI), and then annotated accordingly. Orange bars show the location of DNA sequences corresponding to the LB region of the T-DNA, and to the 5’ region of the \textit{AtOEP80} gene. The green bar shows the conceptual position of the \textit{AtOEP80} transcript synthesized in the \textit{oep80-3} mutant, as determined by RACE-PCR; the blue bar shows the position of the open reading frame within the transcript, and the red bar shows the sequence of the putative \textit{AtOEP80} protein, initiating from the second AUG codon of the wild-type \textit{AtOEP80} transcript (nucleotides 276 to 278 of cDNA NM_121967).
Supplemental Figure S3. Specificity and affinity purification of the AtOEP80 antibody.

A, Confirmation of the specificity of the AtOEP80 antiserum by competition assay. Protein samples from chloroplasts (equivalent to 10 µg chlorophyll) of either wild-type (“WT”) or homozygous oep80-3 mutant (“80-3”) Arabidopsis seedlings, were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with crude antiserum against AtOEP80 (Inoue and Potter, 2004; left panel), or the same antiserum pre-incubated with 10 µg/mL of the antigen peptide (right panel). Protein bands corresponding to AtOEP80 are visible in the left panel, as indicated, but not in the right panel. Positions of molecular weight standards are indicated at left (sizes in kD).

B, Affinity purification of the AtOEP80 antibody. The crude anti-peptide antiserum described in A above, and previously (Inoue and Potter, 2004), was purified using UltraLink Iodoacetyl Gel coupled with the antigen peptide. Arabidopsis chloroplast protein samples (equivalent to 10 µg chlorophyll) were separated by SDS-PAGE, and then analyzed by immunoblotting using the crude serum, as well as unbound and eluted fractions from the purification column. The position of the bands corresponding to AtOEP80 are indicated at right. Positions of molecular weight standards are indicated at left (sizes in kD).

Supplemental Figure S4. In silico analysis of the 5’ region of the AtOEP80 mRNA.

A, Stable stem-loop structure at the first AUG codon. Sequence spanning the first AUG codon of the native AtOEP80 transcript (nucleotides 118 to 144 of cDNA NM_121967) was analyzed using the mfold version 3.2 web server (http://www.bioinfo.rpi.edu/applications/mfold/) (Mathews et al., 1999; Zuker, 2003). Hybridization of bases is indicated as follows: red, CG or GC base pairing; blue, AU or UA base pairing; green, GU or UG wobble base pairing. Nucleotides are annotated with the results of ss-count analysis (see key): this measures the propensity of a base to be single stranded, as determined by the number of times it is single stranded in a group of predicted foldings. The calculated minimum free energy associated with the folded structure, $\Delta G$, is indicated.

B, Complementarity between the 5’ end of the AtOEP80 transcript and 18S rRNA. Sequence between AUG1 and AUG2 of AtOEP80 (nucleotides 120 to 278 of cDNA NM_121967) and nucleotides 1001 to 1200 of Arabidopsis 18S rRNA (At3G41768) were analysed together using mfold. This identified the region of complementarity shown, which was then re-analysed using shorter sequences, together with an appropriate linker (LLL), in order to calculate the free energy associated with the hybridized structure. Hybridization of bases is indicated as in A.

C, Overlap of the 18S-rRNA-complementary region with sequence motifs known to promote cap-independent binding and enhance translation efficiency. The rRNA-complementary region of AtOEP80 shown in B is aligned with reverse complements (RC) of 18S rRNA sequences from Arabidopsis (At18S) and rice (Os18S); numbers at right refer to positions in the forward complement in each case. Complementarity between AtOEP80 and the 18S sequences is indicated by shading,
using the same colour scheme as in A and B: red, CG or GC base pairing; blue, AU or UA base pairing; green, GU or UG wobble base pairing. The positions of translation enhancer motifs identified by Akbergenov et al. (2004) are indicated below the alignment.

SUPPLEMENTAL TABLES

Supplemental Table SI. Segregation of the T-DNA-associated selectable marker in each of the oep80 mutants

Supplemental Table SII. Phenotypic analysis of the oep80 mutants
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| Genotype | Siliquem | Proembryo\(^{c}\) | Globular (raspberry-like) | Globular | Heart | Torpedo | N.D.\(^{d}\) | Total scored | Proportion delayed or abnormal |
|----------|---------|-----------------|-------------------------|----------|--------|---------|-------------|--------------|--------------------------------|
| WT       | 4       | 54              | -                       | -        | -      | -       | 9           | 54           | -                             |
|          | 6       | 1               | -                       | 58       | -      | -       | 6           | 59           | -                             |
|          | 8       | -               | 14                      | 46       | -      | -       | 1           | 60           | -                             |
|          | 10      | -               | 1                       | 48       | 7      | -       | 0           | 56           | -                             |
|          | 12      | -               | -                       | 9        | 48     | 10      | 57          | -            | -                             |
| oep80-1  | 4       | 54              | -                       | -        | -      | -       | 6           | 54           | -                             |
|          | 6       | 17              | -                       | 49       | -      | -       | 1           | 66           | 0.26                          |
|          | 8       | -               | 17                      | 41       | 12     | -       | 1           | 70           | 0.24                          |
|          | 10      | -               | 16                      | -        | 39     | 1       | -           | 56           | 0.29                          |
|          | 12      | -               | 14                      | -        | 2      | 47      | 1           | 63           | 0.22                          |

\(^{a}\)Embryo developmental stage names refer to the morphology of the embryo proper.

\(^{b}\)Siliques were numbered consecutively from the top of the inflorescence, such that the oldest siliques have the highest numbers.

\(^{c}\)Proembryo stage includes one-cell to 16-cell stage embryos.

\(^{d}\)Not determined; seeds that were not classified for technical reasons.
A

\textbf{AtOEP80} / \textit{atTOC75-V}

B

\begin{tabular}{ccc}
\textbf{T} & \textbf{G} & \\
80-1 WT & 80-1 WT & \\
\end{tabular}

\begin{tabular}{ccc}
\textbf{T} & \textbf{G} & \\
80-2 WT & 80-2 WT & \\
\end{tabular}

\begin{tabular}{ccc}
\textbf{T} & \textbf{G} & \\
80-3 WT & 80-3 WT & \\
\end{tabular}
