A Novel Chloroplastic Outer Membrane-targeting Signal That Functions at Both Termini of Passenger Polypeptides*

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Several components in the machinery mediating the import of nuclear-encoded chloroplastic precursor proteins have been identified. One of the components, OEP34, is an outer membrane protein and is synthesized at its mature size in the cytosol without a distinguishable chloroplast-targeting signal. To address the question of how components in the transport machinery are imported to chloroplasts themselves, we first identified the chloroplastic outer membrane-targeting signal of OEP34. Using an Arabidopsis homologue of the originally isolated pea OEP34, we show that the outer membrane-targeting signal of OEP34 is located within a 10-amino acid hydrophobic core of the C-terminal membrane anchor. Interestingly, this signal can target a passenger protein to the chloroplastic outer membrane no matter whether it is placed at the N or C terminus of a passenger protein. Proper insertion of fusion proteins into the outer membrane requires in addition the C-terminal hydrophilic region following the hydrophobic core. Furthermore, passenger proteins fused to the C terminus of the targeting/insertion signal were most likely imported into the intermembrane space of the envelope.

Most proteins in chloroplasts are nuclear-encoded and post-translationally imported into chloroplasts. With the exception of most outer membrane proteins, nuclear-encoded chloroplastic proteins are synthesized as higher molecular weight precursors with N-terminal extensions called transit peptides. Protein import into chloroplasts involves specific interactions between the transit peptides and a set of transport machinery in the envelope (1) and requires the hydrolysis of ATP and GTP. No consensus sequence has been found for the transit peptides, but they generally have a net positive charge, are devoid of negative charges, and are rich in serine and threonine (2, 3).

Outer envelope membrane proteins represent a unique branch of nuclear-encoded chloroplastic proteins. Most outer membrane proteins are synthesized at their mature size in the cytosol without cleavable transit peptides (4–9). Their insertion into the outer membrane does not require ATP (4–9). Most of them also do not require thermolysin-sensitive components on the chloroplasmic surface for their targeting to chloroplasts (4–8). Targeting signals from two of these outer membrane proteins have been identified. The first 48 amino acids of a hydrophilic peripheral membrane protein SCE/Com70 (10), and the first 30 amino acids of an integral membrane protein OEP14 (11), have been shown to be necessary and sufficient for chloroplastic outer membrane targeting. The first 30 amino acids of OEP14 is the membrane anchor of the protein (11). There is no similarity between the targeting signals of SCE/Com70 and OEP14 except that both are located at the N terminus of the respective protein.

Several components in the machinery responsible for the import of transit peptide-bearing precursor proteins have been identified (1, 12). cDNAs for some of the identified components have also been isolated and three of them encode outer membrane proteins (4, 9, 12–15). According to their molecular weight, they are named OEP/IAP (outer envelope membrane protein or import intermediate-associated protein) 86, 75, and 34. OEP86 and OEP34 are GTPase proteins (4, 9, 13) and may function as receptor and regulator of the transport machinery, respectively. OEP75 may function as a transport channel across the outer membrane (14, 15). In contrast to all other outer membrane proteins identified, OEP86 and OEP75 are synthesized as higher molecular weight precursors with cleavable targeting sequences at their N termini. OEP75 has a bipartite targeting sequence (16). The first part functions as a regular transit peptide (16) and OEP75 competes with the import of a stroma-targeting precursor protein (15), indicating that OEP75 uses the same, or at least part of the same, import pathway used by most precursor proteins. OEP86, on the other hand, does not compete with the import of a stroma-targeting precursor protein (13). Its cleavable targeting sequence is unusually long and highly negatively charged (13). This signal is also not sufficient for chloroplast targeting. Proper targeting and insertion of OEP86 to the outer membrane requires, in addition, a C-terminal portion of the mature protein (17).

OEP34 is synthesized at its mature size in the cytosol like the majority of outer membrane proteins (4, 9). However, although it is an integral membrane protein like OEP14, OEP34 does not have a membrane-anchoring domain at its N terminus. Therefore it is not clear what kind of signal directs the targeting of OEP34 to chloroplasts. Furthermore, while one report describes insertion of OEP34 into the outer membrane as being independent of ATP and thermolysin-sensitive components (4), another report has shown that insertion of OEP34 into the outer membrane is stimulated by ATP and is greatly impaired by thermolysin pretreatment of chloroplasts (9). Therefore, it is possible that OEP34 does not use an import pathway shared by other outer membrane proteins without

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1 The abbreviations used are: OEP, outer envelope membrane protein; IAP, import intermediate-associated protein; PCR, polymerase chain reaction; GST, glutathione S-transferase; SS, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; DHFR, dihydrofolate reductase; PAVE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MES, 4-morpholineethanesulfonic acid.
cleavable transit peptides, but instead uses a novel pathway that is yet to be described.

Because most chloroplastic proteins are imported from the cytosol, the synthesis and assembly of the protein transport machinery are some of the most important parts of chloroplast biogenesis. To address the interesting question of how components in the transport machinery are targeted to chloroplasts, we have started to investigate the import pathway used by one of the components, OEP34. Here we report the localization of its chloroplastic outer membrane-targeting signal and describe the unique features of this signal. These findings provide insight into the import mechanism of OEP34.

EXPERIMENTAL PROCEDURES

Constructs Encoding Deletion and Fusion Proteins—The AtOEP34 cDNA from the Arabidopsis Biological Resource Center at the Ohio State University (stock number 167B21T7, named by us as pLOX-AtOEP34) was excised with SalI and XbaI and subcloned into the SalI/XbaI site of the pSP64 (Promega, Madison, WI) vector, creating the plasmid pSP64-AtOEP34. ΔN43 was constructed by excising the coding region with ScaI, which cut at amino acid 37 of AtOEP34, and BamHI. The insert was subcloned into the ScaI/BamHI site of pSP65 (Promega). ΔN272 was constructed by cutting pSP64-AtOEP34 with PstI, which cut once in front of the AtOEP34 coding region in the pSP64 vector and once between amino acids 65 and 66 of AtOEP34. The plasmid was then blunt-ended with T4 DNA polymerase and self-religated, creating the plasmid pAOTE34ΔN116. ΔN206 was constructed by amplifying the corresponding coding region from pLOX-AtOEP34 by polymerase chain reaction (PCR) with an N-terminal primer, which changes amino acid 207 from isoleucine to methionine and creating an SpaI site in the process, and the Sp6 primer as the C-terminal primer. The amplified fragment was then digested with Sphi and XbaI and cloned into the Sp6I/XbaI site of plasmid pSP72 (Promega). ΔN272 was constructed by cutting pSP64-AtOEP34 with HindII, which cut once in the vector in front of the AtOEP34 coding region and once at amino acid 253 of AtOEP34 and then self-religated. The C-terminal deletion mutants At1–271 and At1–282 were constructed by amplifying the corresponding coding regions by PCR from pSP64-AtOEP34 using the Sp6 and the SP primer that is present in the process, and the SP6 primer as the C-terminal primer. The amplified fragment was then digested with SpaI and XbaI and cloned into the Sp6I/XbaI site of plasmid pSP72 (Promega).

The fusion proteins with glutathione S-transferase (GST) were constructed as follows. The GST coding region, which also contains a factor Xa processing site and a multiple cloning site at the C terminus, was excised from the plasmid pGEX-5X-1 (Pharmacia Biotech Inc., Uppsala, Sweden) with HindIII and EcoRI and subcloned into the EcoRI/EcoRI site of pBlueScript SK+ (Stratagene, La Jolla, CA), creating the plasmid pBlueScript-GST. The coding region of amino acids 250–313 of AtOEP34 was excised from pAOTE34ΔN116 with HindII and XbaI and cloned into the Smal/XbaI site plasmid pBlueScript-GST. The plasmid encoding the fusion protein GST (272–282) was constructed by inserting an EcoRI/PstI site of pBlueScript-GST linker sequence encoding an EcoRI site, the coding sequence for amino acid 272–282, and a PstI site.

The fusion proteins with dihydrofolate reductase (DHFR) and the carboxyl terminal targeting signal of dihydrofolate reductase (DHFR)-encoding (273–282) was constructed by inserting a linker sequence encoding (273–282) into the EcoRI/PstI site of pBlueScript-GST, creating the plasmid pAtOEP34(250–313)X-100 had been added, was reisolated by centrifugation at 125,000 × g for 45 min in a Beckman TLA 45 rotor.

Samples were analyzed by SDS-PAGE on 10–20% gradient Tricine gels or 10% NuPAGE gels with MES running buffer purchased from Novex (San Diego, CA). Quantitation of samples were performed using the PhosphorImager SP (Molecular Dynamics, Sunnyvale, CA) with dried gels.

RESULTS

An Arabidopsis Homologue of Pea OEP34—Through sequence comparison with the Arabidopsis EST data base (20), we identified a putative Arabidopsis homologue of the pea OEP34 (21). The Arabidopsis cDNA encodes a protein of 313 amino acids, which has about 65% identity and 78% similarity to the pea OEP34 (Fig. 1). Several regions in the sequence, e.g. the G1 to G3 potential GTP-binding motifs (22), are almost totally conserved between the two proteins. Results of charge distribution and α and β secondary structure-forming tendency analyses of the Arabidopsis protein were also very similar to those of pea OEP34 (data not shown). Hydrophathy analysis

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indicated that, like pea OEP34, the Arabidopsis protein also has only one potential membrane-spanning domain close to the C terminus of the protein (amino acids 269–283, Fig. 1, underline). These data indicate that the two proteins are likely to have very similar, if not identical, structures and the Arabidopsis protein most likely performs the same function as pea OEP34. We thus named the protein encoded by the Arabidopsis cDNA “AtOEP34” for Arabidopsis thaliana OEP34.

In view of the potential use of Arabidopsis to study the in vivo function of proteins identified in the transport machinery, we decided to further characterize AtOEP34. Another feature of AtOEP34 that proved useful to us was the distribution of methionine residues along the sequence of AtOEP34. Pea OEP34 has been predicted to insert into the outer membrane using the C-terminal hydrophobic domain, because thermolysin digestion of in vitro imported pea OEP34 results in a 6-kDa thermolysin-resistant fragment (9). One line of evidence supporting this fragment being the C-terminal portion is that pea OEP34 lacks methionine residues at its C-terminal half, and the 6-kDa fragment can only be seen when the protein is labeled with [35S]methionine. As discussed in the previous section, this suggests that the 6-kDa fragment is the C-terminal region of AtOEP34.

To locate the chloroplastic outer membrane-targeting signal within AtOEP34, we made N- and C-terminal deletions of AtOEP34. Mutant proteins with the N-terminal 43, 116, 206, or 272 amino acids or the C-terminal 31 or 42 amino acids deleted were constructed (Figs. 1 and 3A). They were named ΔN43, ΔN116, ΔN206, ΔN272, At(1–282), and At(1–271), respectively. As shown in Fig. 2, amino acids preceding number 272 could be entirely deleted without affecting the chloroplastic import of AtOEP34. However, the import efficiency varied with individual mutants (Fig. 4). While ΔN43 and ΔN116 retained less than 20% of the import efficiency of full-length AtOEP34, the import efficiency of ΔN206 was around 60% of that of AtOEP34. Interestingly, the mutant that has the largest deletion, ΔN272, had an import efficiency comparable with that of AtOEP34.

Thermolysin treatment of imported ΔN43, ΔN116, and ΔN206 all produced the same 6-kDa fragment as that of AtOEP34 (Fig. 2, lanes 4, 8, 12, and 16). ΔN272 has a length indistinguishable from the 6-kDa fragment in our gel system (Fig. 2, lane 17, arrow) and ΔN272 was basically thermolysin-resistant after import (Fig. 2, lane 20). These data further support that the 6-kDa fragment is the C-terminal portion of AtOEP34, most likely the portion from amino acids 273–313. These data also indicate that the N-terminal deletion mutants had inserted into the outer membrane in the same orientation as the full-length AtOEP34.

The mutant protein with the C-terminal 31 amino acids deleted, At(1–282), was still targeted to chloroplasts (Fig. 2, lane 23). Thermolysin treatment of imported At(1–282) resulted in a fragment (lane 24, arrow) smaller than the 6-kDa fragment of AtOEP34. This fragment further supports that the 6-kDa fragment of AtOEP34 resulted from thermolysin cleavage at around amino acid 273, so a C-terminal deletion mutant like At(1–282) would still have the cleavage site but resulted in a smaller sized fragment. This result also indicates that At(1–282) had inserted into the outer membrane the same way as AtOEP34 did. However the import efficiency of At(1–282) was much lower than that of AtOEP34 (Fig. 4). This suggests that amino acids following number 282 are not necessary for targeting but are important for import efficiency. Alkaline extraction of all the imported N-terminal deletion mutants and At(1–282) indicated that most of the imported proteins were integral membrane proteins (data not shown).

The deletion in the mutant protein At(1–271) extends into the hydrophobic core of the AtOEP34 membrane-anchoring domain (Fig. 3A). This mutant could no longer associate with [35S]methionine. As discussed in the previous section, this suggests that the 6-kDa fragment is the C-terminal region of AtOEP34.
Fig. 3. A, a schematic representation of deletion and fusion constructs. The first drawing on the top represents full-length ATOEP34, in which the three small open boxes represent the GTP-binding motifs, and the hatched box represents the hydrophobic core region of amino acid 273–282. In the fusion constructs, the shaded oval boxes represent the passenger protein GST. The black square boxes represent the factor Xa cleavage site. The rectangle boxes represent the passenger protein DHFR. The open oval boxes represent the passenger protein SS. The + and − signs at the right indicate whether the mutant protein can (+) or cannot (−) be imported to or inserted into the outer membrane of plastids. B, amino acid sequences at the junction regions of fusion proteins. The numbers on top indicate amino acid numbering of ATOEP34. Amino acids 286–309 are omitted and represented by a black square box. The first fusion protein contained only the hydrophobic core of amino acids 272–282 compared with ΔN272 (Fig. 3). This result indicates that amino acids 272–282 of ATOEP34 can lead to association with chloroplasts, proper insertion of a fusion protein into the outer membrane requires the entire C-terminal portion of ATOEP34, not only was the import efficiency higher (Fig. 4), but also when GST was fused to amino acids 253–313 of ATOEP34, proper insertion of a fusion protein into the outer membrane requires the entire C-terminal portion of ATOEP34, starting at least from amino acid 253. The C-terminal membrane-anchoring domain can also function as a targeting signal when located at the N terminus of a passenger protein.—An inspection of the deletion mutant ΔN272 revealed that the necessary targeting signal, amino acids 273–282, was located at the extreme N terminus of chloroplasts (Fig. 2, lanes 25–28). Compared with At(1–282) (Fig. 3A), this result indicates that amino acids 272–282 (or 273–282 compared with ΔN272) are necessary for targeting and insertion of ATOEP34 to the chloroplastic outer membrane.

The Entire C-terminal Domain Is Sufficient for Chloroplastic Outer Membrane Targeting and Insertion—To investigate if the hydrophobic core of amino acids 272–282 is also sufficient for chloroplastic outer membrane targeting, we made two fusion proteins in which the C-terminal portion of ATOEP34 was fused to the C terminus of Schistosoma japonicum GST. GST was chosen as the passenger protein, because many fusion proteins with foreign polypeptides fused at the C terminus of GST remain soluble and actively bind glutathione (29), indicating the GST portion has folded into its active conformation and the foreign polypeptide at the C terminus is not likely to be buried by GST. The first fusion protein contained only the hydrophobic core of ATOEP34 and was named GST×(272–282) (Fig. 3). To ensure better import efficiency and for cloning convenience, we also fused amino acids 253–313 of ATOEP34 to the C terminus of GST and created the fusion protein GST×(253–313) (Fig. 3). The results of their import to chloroplasts are shown in Fig. 5. GST by itself could not associate with chloroplasts (lanes 1–4). When amino acids 272–282 of ATOEP34 were fused at its C terminus, the fusion protein could associate with chloroplasts. However, the import efficiency was very low (Fig. 4), and few imported molecules had inserted into the outer membrane, since almost all the imported molecules remained thermolysin-sensitive (Fig. 5, lane 8). On the other hand, when GST was fused to amino acids 253–313 of ATOEP34, not only was the import efficiency higher (Fig. 4), thermolysin digestion of the imported molecules produced the same 6-kDa fragment as that of full-length ATOEP34 (Fig. 5, lane 12, arrow). This indicates that fusion protein GST×(253–313) had inserted into the outer membrane in the same orientation as ATOEP34. These data also show that although amino acids 273–282 of ATOEP34 can lead to association with chloroplasts, proper insertion of a fusion protein into the outer membrane requires the entire C-terminal portion of ATOEP34, starting at least from amino acid 253.
the protein (Fig. 3A). This prompted us to ask the question whether the targeting signal could still function if it was placed at the N terminus of a passenger protein as opposed to its normal C-terminal location. To answer this question, we made four fusion proteins in which various lengths of AtOEP34 C-terminal portion were fused to the passenger protein DHFR or the mature protein region of the SS (Fig. 3). These two proteins, when fused at the C termini of other polypeptides, have been used successfully to assay polypeptides with a potential function as targeting signals (11, 16, 24, 25), indicating polypeptides fused at their N termini are properly exposed. We first fused amino acids 273–282 and 273–313 to SS and created the fusion proteins (273–282)×SS and (273–313)×SS (Fig. 3). To make sure any effect we see with these fusion proteins is not due to the passenger protein we chose, we made another two fusion proteins in which the C-terminal portion of AtOEP34 from amino acid 250 or 273 was fused to another passenger protein, DHFR. These two fusion proteins were named (250–313)×DHFR and (273–313)×DHFR (Fig. 3). When synthesized in an in vitro translation system, the plasmid encoding (250–313)×DHFR yielded three major products (Fig. 6, lane 1). The two lower molecular weight products (lane 1, arrows) probably resulted from internal initiations from the methionine residue of amino acid 273 of AtOEP34 and the initiation methionine of DHFR. This is supported by the fact that the plasmid encoding (273–313)×DHFR yielded two bands identical to these two lower molecular weight bands of (250–313)×DHFR (Fig. 6, lane 5). The construct (273–313)×SS yielded two products in an in vitro translation system (Fig. 6, lane 13). The lower molecular weight product (lane 13, arrow) has the same molecular weight as SS and is most likely an internal initiation from the first residue of SS, which happens to be a methionine (Fig. 3B).

Import competency of these fusion proteins was tested with isolated chloroplasts, and all of them could associate with chloroplasts (Fig. 6, lanes 3, 7, 11, and 15). All of the imported (273–282)×SS molecules remained thermolysin-sensitive (Fig. 6, lane 12), indicating no insertion had occurred. These data show that the necessary signal, amino acids 273–282, could lead to association with chloroplasts even when placed at the N terminus of a passenger protein. However, as with the GST fusions, amino acids following number 282 are critical for import efficiency and insertion (see below).

Passenger Proteins at the C Terminus of the AtOEP34-targeting Signal Were Translocated into the Intermembrane Space—Most (273–313)×SS fusion protein molecules were thermolysin-resistant after import (Fig. 6, lane 16), indicating that (273–313)×SS had been translocated into or across the outer membrane. In addition, import of (250–313)×DHFR and (273–313)×DHFR resulted in a distinct population of lower molecular weight proteins (Fig. 6, brackets by lanes 4 and 8). These lower molecular weight proteins were almost totally thermolysin-resistant after import, indicating they were internal to the outer membrane. It is possible that the DHFR portion of the fusion proteins was translocated into the intermembrane space and was degraded by some unknown protease located there.

To confirm the location of thermolysin-resistant population of the imported fusion proteins, chloroplasts after import of (250–313)×DHFR and (273–313)×SS were treated with thermolysin, then fractionated into the outer and inner envelope membrane, the stroma, and the thylakoid fractions (Fig. 7). OEP34, SS, and chlorophyll a/b-binding protein were used as markers for the outer envelope membrane, the stroma, and the thylakoid, respectively. Chloroplasts containing imported ΔN272 were also fractionated to confirm its outer membrane location. As shown in Fig. 7, most of the thermolysin-resistant (250–313)×DHFR and (273–313)×SS fusion proteins were still located at the outer membrane. The portion in the inner membrane most likely arose from contamination by the outer membrane (Ref. 26, see also the OEP34 control). Interestingly, some of the lower molecular weight proteins generated after import of (250–313)×DHFR were predominantly located in the “stroma” fraction. Our fractionation method cannot separate the
content of the intermembrane space from that of the stroma. It is possible that these lower molecular weight products were in the intermembrane space. This would agree with our hypothesis that these lower molecular weight products were generated from degradation and release of imported (250–313)×DHFR by some protease in the intermembrane space. However, we could not exclude the possibility that these lower molecular weight products were in the stroma.

To further confirm that the passenger proteins at the C terminus of the AtOEP34-targeting signal have been translocated across or into the outer membrane, we employed another more specific protease, factor Xa. The cleavage site for factor Xa has been engineered into the junctions of all fusion proteins between AtOEP34 and the various passenger proteins (Fig. 3). If the prediction about the membrane topology of AtOEP34 in the outer membrane is correct, then a fusion protein with the passenger protein located at the N terminus of the AtOEP34-targeting signal, e.g. GST×(253–313), should have the factor Xa cleavage site exposed in the cytosol, and imported GST×(253–313) should be sensitive to exogenous factor Xa. On the other hand, for a fusion protein with the passenger protein located at the C terminus of the AtOEP34-targeting signal, e.g. (273–313)×SS, the factor Xa cleavage site should be buried in the outer membrane or translocated into the intermembrane space, and imported (273–313)×SS should be factor Xa-resistant.

As shown in Fig. 8A, in vitro translated GST×(253–313) was cleaved by factor Xa into two major bands, the GST portion and the AtOEP34 membrane-anchoring portion (Fig. 8A, lane 2, arrows). When imported GST×(253–313) was treated with factor Xa, all of the imported proteins were factor Xa-sensitive (lane 4). The GST portion was released into the supernatant (lane 5) and the membrane-anchoring region of AtOEP34 remained associated with the chloroplasts (lane 4). In contrast, although in vitro translated (273–313)×SS was also cleaved by factor Xa into two major bands, the SS region and the membrane-anchoring region of AtOEP34 (lane 7, arrows), most imported (273–313)×SS molecules were factor Xa-resistant (lane 9), indicating the factor Xa cleavage site had been translocated into a location that was inaccessible to factor Xa. The supernatant still contained some digested fragments, but both the SS and the AtOEP34 membrane-anchoring fragments were there (lane 10). These fragments probably arose from those (273–313)×SS molecules that were only bound to, but had not inserted into, the outer membrane. The small amount of SS remained associated with chloroplasts after digestion could be due to the tendency of SS to stick to the envelope membranes of chloroplasts (27).

In an effort to find out if the factor Xa site in (272–313)×SS was buried in the outer membrane or exposed in the intermembrane space, outer membrane vesicles were isolated after import of GST×(253–313) and (273–313)×SS and subjected to various treatments. When these outer membrane vesicles were treated with factor Xa directly, most of the imported GST×(253–313) was sensitive (Fig. 8B, lane 3), and most of the imported (273–313)×SS was resistant (lane 7), confirming the right-side-out orientation of these vesicles (7, 28). Imported (272–313)×SS could be fully digested if the membranes were permeabilized by 1% Triton X-100 (Fig. 8B, lane 8), indicating the factor Xa resistance of imported (273–313)×SS was not due to the possibility that (273–313)×SS had folded into a conformation that rendered the factor Xa site inaccessible. It has been reported that when membrane vesicles are subjected to a cycle of freezing and thawing, contents from the surrounding solution, e.g. exogenous proteases, can be enclosed into the vesicles during thawing (19). We tried this treatment on the isolated chloroplastic outer membrane vesicles to enclose factor Xa into the vesicles. While this treatment had no additional effect on the digestion of imported GST×(253–313) (Fig. 8B, lane 2), the treatment increased the amount of (273–313)×SS digested by factor Xa (lane 6). Although the amount of increase was low, it supports that the factor Xa cleavage site in (273–313)×SS was exposed in the intermembrane space.

**FIG. 8.** The factor Xa cleavage site of imported GST×(253–313) is exposed to the cytosol and that of (273–313)×SS is exposed to the intermembrane space. A, treatment of imported GST×(253–313) and (273–313)×SS in intact chloroplasts. Proteins obtained by in vitro translation (TR) were either directly treated with factor Xa (lanes 2 and 7) or incubated with chloroplasts under import conditions (Chpt). After import (lanes 3 and 8), half of the chloroplast sample was further treated with factor Xa (lanes 4 and 9) as indicated by the + sign above the line number. Lanes 5 and 10 are the supernatant fractions (S) of the digestion shown in lanes 4 and 8, respectively. B, treatment of imported GST×(253–313) and (273–313)×SS in isolated outer membrane vesicles (OMV). Outer membrane vesicles from chloroplasts after import of GST×(253–313) and (273–313)×SS were isolated and divided into five portions. Each portion either received no further treatment (lanes 1 and 5) or received treatment of factor Xa digestion (lanes 3 and 7) or a combination of digestion plus freezing and thawing (lanes 2 and 6) or 1% Triton X-100 (lanes 4 and 8).

**DISCUSSION**

AtOEP34 is the first protein reported to have a C-terminally located chloroplast-targeting signal. Uniquely, this signal can function at both termini of passenger proteins. Although the signal is a signal-anchor sequence as in the case of OEP14, the two signals not only are located at opposite ends of their respective polypeptide, but also insert into the outer membrane in opposite orientations. The signal of OEP14 has its C-terminal end facing the cytosol (11), while the signal of OEP34 has its N-terminal end facing the cytosol. These data suggest that AtOEP34 may use a different import pathway from the one used by OEP14 or other outer membranes proteins without
cleavable transit peptides.

Fusion protein studies also suggest that AtOEP34 uses an unusual mechanism for its insertion into the outer membrane. Passenger proteins fused to the C terminus of the AtOEP34-targeting signal are translocated into the intermembrane space of the envelope. This means the mechanism AtOEP34 uses for integration can translocate a polypeptide of at least 26 kDa (the molecular mass of DHFR) across the outer membrane. This supports the existence of a facilitated transport system, e.g. a proteinaceous channel, rather than a spontaneous insertion. Indeed, one report has shown that the insertion of pea OEP34 into the outer membrane requires some thermolysin-sensitive components and is stimulated by ATP. Our data also indicate that association of AtOEP34 and (273–313)SS with chloroplasts is sensitive to thermolysin pretreatment of chloroplasts. Insertion of both AtOEP34 and (273–313)SS is stimulated by ATP. These data suggest that AtOEP34 uses an import pathway that has a proteinaceous receptor that recognizes a C-terminal signal-anchor sequence, different from all chloroplast protein import pathways described so far.

It therefore seems the three components in the transport machinery of the chloroplastic outer membrane, OEP86, OEP75, and OEP34, may use three different pathways for their targeting to chloroplasts. OEP75 uses the general transit peptide-dependent targeting pathway at least for the beginning of its import (15). OEP86 does not use the general transit peptide-dependent pathway, but possesses a negatively charged cleavable targeting sequence at its N terminus (13). OEP34 is targeted to chloroplasts by a C-terminal signal-anchor sequence. Unfortunately, plant mutants defective in individual components of the transport machinery are not available. Therefore, it is not clear whether the three components really use three different pathways or each uses other components in the complex to facilitate its own import and therefore bypass part of the pathway, similar to the situation of the yeast mitochondrial OEP34-dependent pathway, but possesses a negatively charged cleavable transit peptides.

Inner Membrane-targeting Signal

Deletion of 58 amino acids from the C terminus of pea OEP34 also abolishes the association of pea OEP34 with chloroplasts (9). It is likely that the chloroplastic outer membrane-targeting signal of pea OEP34 is also located at the C-terminal hydrophobic-core region (Fig. 1, underlined). At least two other clones that show a high degree of similarity to OEP34 have been found in the Arabidopsis EST data base (20). If they are also homologues of OEP34, sequence comparison with these two additional clones and site-directed mutagenesis will help to identify the critical residues or structure(s) of the OEP34-targeting signal.

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A Novel Chloroplastic Outer Membrane-targeting Signal That Functions at Both Termini of Passenger Polypeptides

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