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Targeting of Nuclear Encoded Proteins to Chloroplasts: a New Insight into the Mechanism

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ABSTRACT Outer envelope membrane proteins of chloroplasts encoded by the nuclear genome are transported without the N-terminal transit peptide. Here, we investigated the targeting mechanism of AtOEP7, an Arabidopsis homolog of small outer envelope membrane proteins in vivo. AtOEP7 was expressed transiently in protoplasts or stably in transgenic plants as fusion proteins with GFP. In both cases AtOEP7:GFP was targeted to the outer envelope membrane when assayed under a fluorescent microscope or by Western blot analysis. Except the transmembrane domain, deletions of the N- or C-terminal regions of AtOEP7 did not affect targeting although a region closed to the C-terminal side of the transmembrane domain affected the targeting efficiency. Targeting experiments with various hybrid transmembrane mutants revealed that the amino acid sequence of the transmembrane domain determines the targeting specificity. The targeting mechanism was further studied using a fusion protein, AtOEP7:NLS:GFP, that had a nuclear localization signal. AtOEP7:NLS:GFP was efficiently targeted to the chloroplast envelope despite the presence of the nuclear localization signal. Taken together, these results suggest that the transmembrane domain of AtOEP7 functions as the sole determinant of targeting specificity and that AtOEP7 may be associated with a cytosolic component during translocation to the chloroplast envelope membrane.

Results and Discussion

Isolation and expression of an Arabidopsis homolog of pea OEP14

Pea OEP14 is one of small molecular weight outer envelope membrane proteins (Li et al. 1991). An OEP14 homolog is present in the Arabidopsis genome (accession number CAB43440). However, the molecular weight of the Arabidopsis homolog was approximately 7 kDa. Thus, we named the Arabidopsis homolog AtOEP7. To isolate and characterize AtOEP7 we isolated the gene by PCR amplification using gene-specific primers. The amino acid sequence showed 68 % and 38 % amino acid sequence identity with OEP14 of pea and spinach OEP6.7 (Salomon et al. 1990), respectively (Figure 1). Since we isolated AtOEP7 from the genomic DNA by PCR amplification we investigated whether AtOEP7 is indeed expressed in plant tissues. Northern blot analysis was carried out with total RNA obtained from various tissues using AtOEP7 as a hybridization probe. AtOEP7 clearly detected a hybridizing band from RNA samples obtained from green tissues such as leaves, siliques and flowers but not from root tissues. Thus, this result is in a good agreement with expectation if the gene product plays a role in the chloroplast.

AtOEP7:GFP fusion protein is targeted to the chloroplast envelope membranes in protoplasts

Previously it has been shown by the in vitro import assay that pea OEP14 is targeted to the outer envelope membrane by a novel pathway that did not require ATP and the N-terminal transit peptide (Li et al. 1991). In this study we wanted to investigate the targeting mechanism in vivo in the intact cell to better understand the exact mechanism in the cell. To assay the targeting in vivo in the intact cell, green fluorescent protein (GFP)

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AtOEP7    : -------MGRTSGAKQATVVVAAMALGWLAIEIAFKPFLDKFRSSSIDKSDPTKDPDDFDTAATATTSKEGL---- : 64
OEP14     : -------MGR---AKEAVVVAGALAFVWLAIELAFKPFLSQTPDSSIDKSDPTRDPDDAPPPPPPETDAGDADKDD : 65
SpOEP6.7  : MESVAKPATREGSAKQAAIVVGVLALGWFAIEVAFIPLFNEVF----GGGSDEKDDLVNAFTPDT---------- : 61
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Figure 1. Amino acid sequence comparison between AtOEP7 homologs; The deduced amino acid sequence of AtOEP7 was used to search the public databases using Blastp of the NCBI e-mail server. The gaps were introduced to maximize the sequence alignment. The accession numbers for AtOEP7, Pea OEP14, and SpOEP6.7 (spinach OEP6.7) were CAB43440, AAA63414, and AAA34035, respectively.

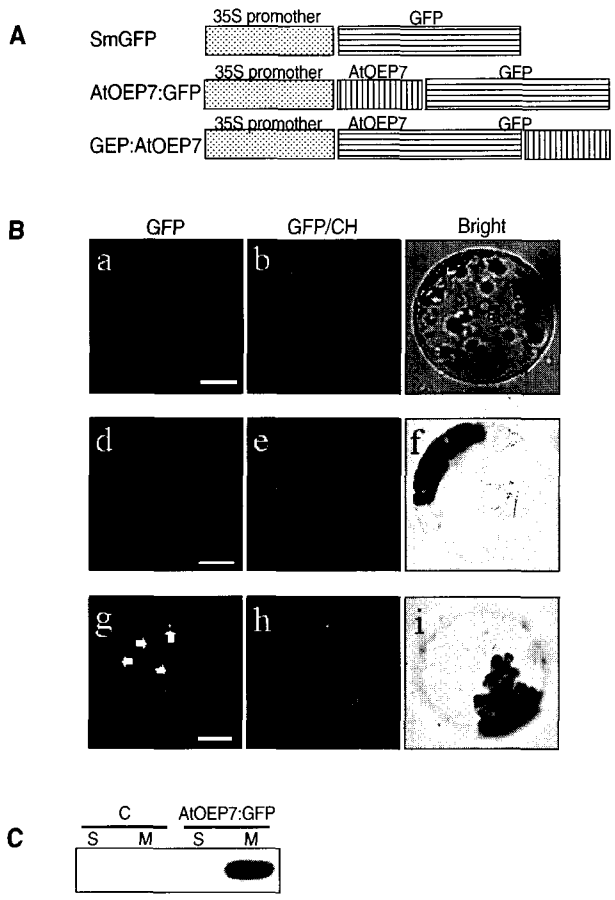

Figure 2. In vivo targeting of AtOEP7:GFP in protoplasts
A. Schematic presentation of fusion constructs between AtOEP7 and GFP; **B.** In vivo targeting of AtOEP7:GFP in protoplasts. Protoplasts preprared from Arabidopsis leaf tissues were transformed with the fusion constructs and examined at various time points after transformation. Panels a, d and g are green fluorescent signals of GFP for smGFP, AtOEP7:GFP, and GFP:AtOEP7, respectively. Panels b, e, and h are the overlaps of green fluorescent signals of GFP and red fluorescent signals of chlorophyll for GFP for smGFP, AtOEP7:GFP, and GFP:AtOEP7, respectively. Panels c, f, and i are bright field images. CH indicates chloroplasts. The pictures are representative of protoplasts expressing these constructs at 24 hr after transformation. At least three independent transformation experiments were carried out for each condition; **C.** Western blot analysis of AtOEP7:GFP. Total proteins were isolated from the transformed protoplasts and fractionated into soluble and membrane fractions by ultracentrifugation. These fractions were then analyzed by Western blot analysis using a mouse monoclonal anti-GFP antibody (Clonetech, Inc, USA). S and M indicate the soluble and membrane fractions. The bars indicate 20 mm.

was fused to the N- or C-termini of AtOEP7 (Figure 2A). The fusion constructs were introduced into protoplasts prepared from Arabidopsis tissues by the PEG transformation method (Kang et al. 1998). As shown in Figure 2B, AtOEP7 was targeted to chloroplasts when GFP was fused to the C-terminus of AtOEP7 (Figure 2B, panels d and e). The green fluorescent signals were present as rings, indicating that AtOEP7:GFP may be localized at the envelope membrane. However, when GFP was fused to the N-terminus it gave a punctate staining pattern, indicating that it may be mistargeted to other organelle (Figure 2B, panels g and h). In the case of pea OEP14, the N-terminus has shown to be inserted into the intermembrane space (Li and Chen, 1996). Thus, the C-terminus exposed to the cytosol may allow GFP fusion without affecting targeting whereas the bulky GFP domain at the N-terminus may not across the outer envelope membrane into the intermembrane space. Next we investigated whether the fusion protein is indeed inserted into the chloroplast envelop membrane. Total protoplast extracts were fractionated into membrane and soluble fractions by ultracentrifugation (Li et al. 1991). The presence of AtOEP7:GFP in these fractions were probed by Western blot analysis using a polyclonal anti-GFP antibody. The anti-GFP antibody detected a band at a position of 35 kDa, an expected size of AtOEP7:GFP in the membrane fraction, but not in the soluble fraction (Figure 2C). These results strongly suggest that the fusion protein is targeted to the chloroplast and inserted into the outer envelope membrane.

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