Functional Characterization of Sequence Motifs in the Transit Peptide of Arabidopsis Small Subunit of Rubisco

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The transit peptides of nuclear-encoded chloroplast proteins are necessary and sufficient for targeting and import of proteins into chloroplasts. However, the sequence information encoded by transit peptides is not fully understood. In this study, we investigated sequence motifs in the transit peptide of the small subunit of the Rubisco complex by examining the ability of various mutant transit peptides to target green fluorescent protein reporter proteins to chloroplasts in Arabidopsis (Arabidopsis thaliana) leaf protoplasts. We divided the transit peptide into eight blocks (T1 through T8), each consisting of eight or 10 amino acids, and generated mutants that had the original sequence partially restored in single- or double-T-block Ala (A) substitution mutants. Analysis of chloroplast import of these mutants revealed several interesting observations. Single-T-block mutations did not noticeably affect targeting efficiency, except in T1 and T4 mutations. However, double-T-mutants, T2A/T4A, T3A/T6A, T3A/T7A, T4A/T6A, and T4A/T7A, caused a 50% to 100% loss in targeting ability. T3A/T6A and T4A/T6A mutants produced only precursor proteins, whereas T2A/T4A and T4A/T7A mutants produced only a 37-kD protein. Detailed analyses revealed that sequence motifs ML in T1, LKSSA in T3, FP and RK in T4, CMQVW in T6, and KKFET in T7 play important roles in chloroplast targeting. In T1, the hydrophobicity of ML is important for targeting. LKSSA in T3 is functionally equivalent to CMQVW in T6 and KKFET in T7. Furthermore, subcellular fractionation revealed that Ala substitution in T1, T3, and T6 produced soluble precursors, whereas Ala substitution in T4 and T7 produced intermediates that were tightly associated with membranes. These results demonstrate that the transit peptide contains multiple motifs and that some of them act in concert or synergistically.

The majority of chloroplast proteins are encoded by the nuclear genome and synthesized in the cytoplasm. Considerable effort has been devoted to elucidating the molecular mechanisms of protein import into various parts of chloroplasts (Keegstra and Froehlich, 1999; Bauer et al., 2000; Jarvis and Robinson, 2004; Kessler and Schnell, 2004; Soll and Schleiff, 2004; Hofmann and Theg, 2005). Nuclear-encoded plastid proteins can be divided into two groups depending on whether or not they possess a cleavable transit peptide. In most cases, nuclear-encoded plastid proteins destined for chloroplast localization have a cleavable N-terminal transit peptide that is both necessary and sufficient for targeting to chloroplasts (Keegstra and Cline, 1999; Vothknecht and Soll, 2000; Bruce, 2001), although plastid proteins that lack the classical transit peptide have been recently identified (Rathinasabapathi et al., 1994; Miras et al., 2002). For proteins with a transit peptide, the N-terminal cleavable transit peptide is necessary and sufficient. In contrast, a majority of outer envelope membrane proteins are targeted without a cleavable transit peptide (Lee et al., 2001, 2004; Tu et al., 2004; Hofmann and Theg, 2005).

Despite numerous studies, the information in the chloroplast-targeted transit peptide (cTP) is not clearly understood. cTPs are highly divergent in amino acid sequence, length, and organization (von Heijne et al., 1989; Bruce, 2001; Zhang and Glaser, 2002). However, two trends have emerged: cTPs tend to have a high content of hydroxylated amino acid residues such as Ser, Thr, and Pro, and only a few acidic amino acid residues such as Asp and Glu (von Heijne et al., 1989; Zhang and Glaser, 2002). One of the most prominent features of the cTP is its unusually long length. Thus, it has been suggested that the cTP is composed of multiple domains that may have different roles and/or overlapping functions that are required for import across the two chloroplast membranes (von Heijne et al., 1989; Plion et al., 1995; Rensink et al., 1998, 2000). However, the exact nature of these domains remains elusive.

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Previous studies have identified or suggested that components of chloroplast import receptors such as Toc (translocon at the outer envelope of chloroplasts) 159, Toc75, and Toc34 bind to domains or sites in the N-terminal region of the cTP in vitro (Gutensohn et al., 2000; Hinnah et al., 2002; Jarvis and Soll, 2002; Becker et al., 2004; Kessler and Schnell, 2004; Smith et al., 2004). In addition, the cTP contains other domains or motifs such as the cleavage site for the stromal processing peptidase (Richter and Lamppa, 1999, 2002) and the DnaK-/HSP70-binding site (Rial et al., 2000). Sequence analysis has revealed that 75% of 727 plastid precursor proteins contain at least one DnaK-binding site (Rial et al., 2000). In vitro and in vivo analyses have shown that DnaK/HSP70 binds to its predicted binding sites in the cTP of the small subunit of the Rubisco complex (RbcS) and the ferredoxin-NADP reductase precursor (Ivey and Bruce, 2000; Ivey et al., 2000; Rial et al., 2000). The cTP also has a tendency to bind to membranes containing chloroplast envelope-specific phospholipids in vitro (Cline et al., 1985; van’t Hof et al., 1993; van’t Hof and de Kruijff, 1995; Pinnaduwage and Bruce, 1996; Chen and Li, 1998). The lipid-binding domain of the precursor form (Pr) of RbcS has been mapped to the C-terminal 20 amino acids of the cTP (Pinnaduwage and Bruce, 1996). This interaction may be important for initial binding of the preprotein to chloroplasts, which subsequently leads to diffusion of the preprotein to the receptor complex (van’t Hof and de Kruijff, 1995; Chen and Li, 1998).

In addition, it has been proposed that the cTP contains a 14-3-3 protein-binding site (May and Soll, 2000; Bruce, 2001). The binding of 14-3-3 to the cTP appears to require phosphorylation of a Ser within the binding site and, together with HSP70, may induce formation of the proposed guidance complex in the cytoplasm (May and Soll, 2000). In vitro import experiments have shown that preprotein in the guidance complex is imported into chloroplasts much more efficiently. However, other evidence has shown that phosphorylation is not important for protein targeting to chloroplasts (Nakrieko et al., 2004).

In this study, we identified the sequence motifs in the cTP of RbcS that are critical for chloroplast targeting and investigated the functional relationships among these motifs during protein import. Here we demonstrate that the cTP of RbcS consists of a complex set of sequence motifs and that some of these sequence motifs display complex functional relationships during protein targeting to chloroplasts.

**RESULTS**

**Ala Substitution of 10-Amino Acid Blocks Identifies Functional Sequences in the RbcS Transit Peptide**

To investigate the sequence motifs in the cTPs, Ala substitution mutants of the Arabidopsis (*Arabidopsis thaliana*) RbcS transit peptide were generated. In this study, the RbcS transit peptide was divided into eight blocks of eight or 10 amino acids, T1 through T8, and each block was replaced with the corresponding number of Ala residues to generate the mutants T1A through T8A (Fig. 1A). These mutant transit peptides were fused to green fluorescent protein (GFP) and introduced into protoplasts by polyethylene glycol-mediated transformation (Jin et al., 2001; Chew et al., 2003; Lee et al., 2003). The subcellular localization of the reporter proteins in protoplasts showed that most mutants (T2A, T3A, T5A, T6A, T7A, and T8A) had GFP patterns that were nearly identical to that of the wild type (Fig. 1B, a and b), indicating that these mutant transit peptides delivered proteins into chloroplasts as efficiently as the wild type. However, the staining pattern of T1A:GFP was clearly different from that of the wild type (Fig. 1B, c and d). At 12 h after transformation (AT), T1A:GFP exhibited a diffuse GFP pattern in the cytoplasm, in addition to the GFP signal in chloroplasts (Fig. 1B, c). However, at 24 h AT, the diffuse cytoplasmic pattern was no longer observed and GFP signals were detected only in the chloroplasts (Fig. 1B, d). These data indicated that T1A:GFP was imported into chloroplasts slowly in comparison to the wild type. T4A:GFP displayed a different staining pattern. A strong GFP signal was detected in the chloroplasts (Fig. 1B, c). However, the GFP signal also produced a ring pattern that appeared to surround the red autofluorescent chlorophyll signal (see arrow in the enlarged image in Fig. 1B, i and j; compare with the enlarged images of the wild type in a and b), indicating that a portion of the reporter protein was not imported into the chloroplast, but rather accumulated at the chloroplast surface. Again, GFP signals in the chloroplasts were much stronger at 24 h AT than at 12 h AT, indicating that T4A:GFP was imported slowly.

To confirm the results obtained from image analysis of GFP signals in protoplasts, western-blot analysis was performed using protein extracts from transformed protoplasts. Previously we have shown that RbcS-nGFP in protoplasts produced a proteolytically processed protein species at 31 kD, corresponding to the mature form (M) within the chloroplast, whereas the Pr at 39 kD was almost undetectable (Lee et al., 2003). Most of the mutants, with the exceptions of T1A:GFP and T4A:GFP, had western-blot patterns that were nearly identical to the wild type (Fig. 1C), indicating that these mutant transit peptides were able to deliver reporter proteins into chloroplasts.

T1A:GFP and T4A:GFP had different western-blot patterns that were in agreement with the image analyses. Approximately 20% of T1A:GFP accumulated as the Pr at 12 h AT (Fig. 1C, b). However, Pr was no longer observed 24 or 36 h AT, and, concomitantly, the intensity of M increased. These results suggested that the Pr of T1A:GFP was imported into chloroplasts more slowly than the wild type. T4A:GFP showed a western-blot pattern that differed from the other mutants, as well as from the wild type. T4A:GFP produced two major protein species of 31 and 37 kD.
Figure 1. In vivo targeting and western-blot analysis of individual-T block Ala substitution mutants in protoplasts. A, Sequences of individual-T block Ala substitution mutations. B, Localization of reporter proteins. Protoplasts from Arabidopsis leaf tissues were transformed with the indicated constructs and GFP patterns were observed 12 and 24 h AT. At least three independent
tracts were treated with Na₂CO₃ or Triton X-100 and association of the T₄A:GFP intermediate, protein exceeded between the supernatant and pellet fractions, whereas more of the M was found in the supernatant (Fig. 1D, b), suggesting that Pr was present in the cytoplasm and M in the stroma. In the case of T₁A:GFP, both Pr and M were detected in the supernatant and pellet fractions, respectively (Fig. 1D; Ahmed et al., 2000; Inoue and Keegstra, 2003). RbcS-nt:GFP that was analyzed by western blotting using anti-GFP antibody. Under these conditions, reporter proteins present in the cytoplasm and the stroma of chloroplasts should be detected in the supernatant, whereas the reporter proteins accumulated in envelope membranes via interaction with lipid membranes or receptor components, and trapped in the import channels should be detected in the pellet. In accordance with this expectation, aleurain in the vacuolar lumen and Toc75 in the chloroplast envelope membrane were detected in the supernatant and pellet fractions, respectively (Fig. 1D; Ahmed et al., 2000; Inoue and Keegstra, 2003). RbcS-nt:GFP that was imported efficiently into chloroplasts was detected in the supernatant as M. In the case of T₁A:GFP, both Pr and M of T₁A:GFP were detected primarily in the soluble fraction, with only a small amount of M in the pellet (Fig. 1D, b), suggesting that Pr was present in the cytoplasm and M in the stroma. In the case of T₄A:GFP, the 37-kD form was nearly equally distributed between the supernatant and pellet fractions, whereas more of the M was found in the supernatant than in the pellet (Fig. 1D, c). To examine membrane association of the T₄A:GFP intermediate, protein extracts were treated with Na₂CO₃ or Triton X-100 and fractionated by ultracentrifugation. Both treatments only slightly increased the amount of the 37-kD form detected in the supernatant (Fig. 1E). One possible explanation for this was that the 37-kD form was trapped in the import channel. These results strongly suggested that in the absence of the T₄ motif, the reporter protein had difficulty passing through the import channel. As controls for the fractionation and chemical treatments, aleurain, γ-COP (coat protein), and AtVSR (Arabidopsis vacuolar sorting receptor) were detected using anti-aleurain, anti-γ-COP, and anti-VSR antibodies, respectively (Ahmed et al., 1997; Pimpl et al., 2000; Sohn et al., 2003). γ-COP, a component of the COPI vesicle, is peripherally associated with the Golgi membrane, and AtVSR₁, a vacuolar sorting receptor, is an integral membrane protein. As expected, aleurain was detected in the supernatant, whereas γ-COP and AtVSR were detected in the pellet fraction. Furthermore, γ-COP and AtVSR₁ were solubilized by 0.1 M Na₂CO₃ and 1.0% Triton X-100, respectively, confirming that the fractionation experiments had been performed properly.

To further confirm the localization of T₁A:GFP and T₄A:GFP, protein extracts from the transformed protoplasts were separated into supernatant and pellet fractions by ultracentrifugation and these fractions were analyzed by western blotting using anti-GFP antibody. Under these conditions, reporter proteins present in the cytoplasm and the stroma of chloroplasts should be detected in the supernatant, whereas the reporter proteins accumulated in envelope membranes via interaction with lipid membranes or receptor components, and trapped in the import channels should be detected in the pellet. In accordance with this expectation, aleurain in the vacuolar lumen and Toc75 in the chloroplast envelope membrane were detected in the supernatant and pellet fractions, respectively (Fig. 1D; Ahmed et al., 2000; Inoue and Keegstra, 2003). RbcS-nt:GFP that was imported efficiently into chloroplasts was detected in the supernatant as M. In the case of T₁A:GFP, both Pr and M of T₁A:GFP were detected primarily in the soluble fraction, with only a small amount of M in the pellet (Fig. 1D, b), suggesting that Pr was present in the cytoplasm and M in the stroma. In the case of T₄A:GFP, the 37-kD form was nearly equally distributed between the supernatant and pellet fractions, whereas more of the M was found in the supernatant than in the pellet (Fig. 1D, c). To examine membrane association of the T₄A:GFP intermediate, protein extracts were treated with Na₂CO₃ or Triton X-100 and fractionated by ultracentrifugation. Both treatments only slightly increased the amount of the 37-kD form detected in the supernatant (Fig. 1E). One possible explanation for this was that the 37-kD form was trapped in the import channel. These results strongly suggested that in the absence of the T₄ motif, the reporter protein had difficulty passing through the import channel. As controls for the fractionation and chemical treatments, aleurain, γ-COP (coat protein), and AtVSR (Arabidopsis vacuolar sorting receptor) were detected using anti-aleurain, anti-γ-COP, and anti-VSR antibodies, respectively (Ahmed et al., 1997; Pimpl et al., 2000; Sohn et al., 2003). γ-COP, a component of the COPI vesicle, is peripherally associated with the Golgi membrane, and AtVSR₁, a vacuolar sorting receptor, is an integral membrane protein. As expected, aleurain was detected in the supernatant, whereas γ-COP and AtVSR were detected in the pellet fraction. Furthermore, γ-COP and AtVSR₁ were solubilized by 0.1 M Na₂CO₃ and 1.0% Triton X-100, respectively, confirming that the fractionation experiments had been performed properly.

The ML Motif and Hydrophobicity of T₁ Are Critical for Efficient Targeting to Chloroplasts

To determine which amino acid residues in T₁ played a critical role in chloroplast targeting, T₁ was subdivided into two regions of five amino acids each, D₁ and D₂, and each region was replaced with five Ala residues (Fig. 2A). Both mutant transit peptides translocated the reporter proteins into chloroplasts as efficiently as the wild type (Fig. 2, B, c–f, C, b and c) when examined by image and western-blot analyses, indicating that D₁ and D₂ of T₁ were functionally redundant. This was not surprising since D₁ and D₂ both included two Ser, one Met, and one Ala. T₁ included 50% hydroxylated amino acid residues. Hydroxylated amino acid residues such as Ser and Thr are highly represented in the transit peptide as a whole and are thought to be important for function (Zhang and Glaser, 2002).

To examine the importance of hydroxylated amino acids in T₁, one, two, or three Ser residues were introduced into the T₁A background to generate T₁A + 1S, T₁A + 2S, and T₁A + 3S, respectively (Fig. 2A). T₁A + 3C, T₁A + ML, and T₁A + LL mutants, which...
Figure 2. Hydrophobic amino acid residues in T1 are critical for efficient protein targeting to chloroplasts. A, Sequences of T1 substitution mutations. B, Localization of reporter proteins. Protoplasts were transformed with the indicated constructs and GFP patterns were observed 12 and 24 h AT. Green, red, and yellow signals indicate GFP, autofluorescence of chlorophyll, and the overlap between green and red fluorescent signals, respectively. Arrows indicate the GFP signals in the cytoplasm. Bar = 20 μm. C, Western-blot patterns of reporter proteins. Protein extracts from protoplasts were analyzed by western blotting using anti-GFP antibody. Pr, Precursor form; M, mature forms; and h, hour.
had three Cys, one Met and one Leu, and two Leu, respectively, also were generated in the T1A background (Fig. 2A). Protein targeting to chloroplasts with these mutant transit peptides was examined by image and western-blot analyses. The increased number of Ser residues in T1A adversely affected the chloroplast-targeting efficiency, although T1A + 1S appeared to be slightly better targeted than T1A (Fig. 2, B, g–l, C, d–f). These data indicated that a large number of Ser residues in T1 were detrimental for targeting. Surprisingly, the targeting efficiency of T1A + 3C to chloroplasts was similar to that of the wild type (Fig. 2, B, m and n, C, g). Cys is similar in structure to Ser, with a sulphydryl group instead of a hydroxyl group. Thus, Cys is more hydrophobic than Ser. Finally, targeting of T1A + ML:GFP and T1A + LL:GFP to chloroplasts was as efficient as the wild type (Fig. 2, B, o–r, C, h and i), indicating that the Met and Leu residues in T1 were critical for targeting and that Leu could replace Met. To further examine the importance of these hydrophobic residues in the targeting, we generated two additional mutants, T1ML/AA and T1MLM/AAA, which had Ala substitutions of two and three hydrophobic residues in T1 in the wild-type background, respectively (Fig. 2A). Both mutants displayed green fluorescent signals in the cytoplasm and the chloroplast at both time points, indicating that the targeting of these two mutants was less efficient than the wild type (arrows in Fig. 2B, s–v). When examined by western-blot analysis using anti-GFP antibody, a significant amount of the reporter protein was detected in Pr throughout the time course of the experiment, and the pattern resembled that of T1A + 3S (Fig. 2C, j and k). In addition, T1MLM/AAA accumulated more precursor protein than T1ML/AA. These results strongly suggested that hydrophobicity in T1 was critical for protein targeting to chloroplasts. Furthermore, T1ML/AA and T1MLM/AAA, which had four Ser residues at the original positions, yielded more precursors than T1A. This result is consistent with the notion that Ser residues are detrimental for targeting to chloroplasts.

FP and RK Residues in T4 Are Critical for Chloroplast Targeting

Among the 10-amino acid block mutants, Ala substitution in T4 was the most detrimental for targeting proteins to chloroplasts. T4 contained part of a sequence that was similar to the proposed 14-3-3 binding site (May and Soll, 2000; Bruce, 2001). To define critical amino acid residues in T4, two mutants, T4A + FP and T4A + RK, were generated by restoring FP and RK in the first and second halves of T4, respectively (Fig. 3A). These mutant transit peptides fused to GFP were transiently expressed in protoplasts and their localization was examined. Both mutants produced GFP signals in chloroplasts at 24 h AT (Fig. 3B). In addition, T4A + RK:GFP produced small speckles attached to chloroplasts (indicated by arrows in Fig. 3B, d). The nature of these speckles was unclear and was not further pursued. These results indicated that either FP or RK was almost sufficient for restoring the targeting efficiency of T4A to the wild-type level.

To determine the targeting efficiency at the biochemical level, as well as the form of the reporter proteins, western-blot analysis was performed with protein extracts from the transformed protoplasts. T4A + FP:GFP was imported into chloroplasts as efficiently as the wild type (Fig. 3C, c). T4A + RK:GFP also yielded over 90% of the total reporter protein in M (Fig. 3C, d), indicating that restoration of RK to T4A improved the targeting efficiency to near the wild-type level. However, T4A + RK:GFP produced three additional protein species in minor amounts, migrating at 36, 37, and 65 kD. The 36- and 37-kD protein species that migrated faster than the precursor may have been intermediate forms. However, the protein species at 65 kD (asterisk in Fig. 3C, d), which was much larger than Pr, may have been T4A + RK:GFP that was associated tightly with a protein complex, even under SDS/PAGE conditions. The nature of this putative complex was unclear.

To determine the subcellular distribution of the various protein species derived from the reporter protein, protein extracts were separated into supernatant and pellet fractions by ultracentrifugation and the fractions were analyzed by western blotting using anti-GFP antibody. All of the 36-, 37-, and 65-kD forms, together with approximately 50% of the M, were detected in the pellet fraction (Fig. 3D, a). These proteins were not made soluble by Na2CO3 or Triton X-100 treatments (Fig. 3D, b). As controls for the fractionation and chemical treatments, we detected aequorin, γ-COP, and AtVSR1 using anti-aequorin, anti-γ-COP, and anti-VSR antibodies, respectively. Soluble aequorin was detected only in the supernatant, whereas a large fraction of γ-COP and all of AtVSR1 were detected in the pellet fraction, confirming that there was no cross contamination of the pellet and supernatant fractions by soluble and membrane proteins, respectively. Furthermore, as expected, membrane-associated γ-COP was completely solubilized by 0.1 M Na2CO3 and the integral membrane protein AtVSR1 could only be completely solubilized by 1.0% Triton X-100. These results suggested that the restoration of RK to T4A rescued T4A just enough to translocate the reporter protein to the point where the stromal processing peptidase could cleave the transit peptide, but the M still remained in the channel. One possibility was that RK was part of a larger motif and that additional neighboring amino acid residues were necessary for full activity.

To further localize the reporter proteins, intact chloroplasts purified from gently lysed protoplasts were treated with thermolysin and analyzed by western blotting using anti-GFP antibody. As a control for chloroplast fractionation, RFP (red fluorescent protein) was cotransformed into the protoplasts together with T4A + RK:GFP. Coexpressed RFP was not detected in the purified chloroplast fraction, confirming that it
Figure 3. FP and RK in T4 are critical for protein targeting to chloroplasts. A, Sequences of T4 substitution mutations. B, Localization of reporter proteins. Protoplasts were transformed with the indicated constructs and GFP patterns were observed 24 h AT. Green, red, and yellow signals indicate GFP, autofluorescence of chlorophyll, and the overlap between green and red.
was not contaminated by cytoplasmic proteins. The 36-, 37-, and 65-kD forms, but not the 31-kD form, were sensitive to thermolysin (Fig. 3E), indicating that the GFP moiety of these three protein species was exposed to the cytoplasm. As controls for thermolysin treatment, Toc75 and the large subunit of Rubisco (RbcL) were detected by anti-Toc75 antibody and Coomassie Blue staining, respectively. In agreement with previous observations (Inoue and Potter, 2004), both proteins were resistant to thermolysin. These results suggested that both the FP and RK motifs in T4 were critical for targeting a protein to the chloroplast and that at least one of the motifs was required for efficient targeting.

Double-T Mutants Reveal Functional Redundancy in Transit Peptide Motifs

The results obtained from the import experiments with single-T mutants strongly suggested that the absence of a sequence motif was tolerated or was compensated for by other sequence motifs in the transit peptide. To address this question, double-T mutants were generated in which two 10-amino acid blocks in the transit peptide were substituted with the corresponding number of Ala residues (Fig. 4A). These double-T mutants were fused to GFP and transiently expressed in protoplasts to examine their targeting to chloroplasts. Mutants with Ala substitutions in neighboring two T blocks, resulting in 20 consecutive Ala residues, were almost undetectable by either GFP imaging or western-blot analysis. In addition, GFP fusion constructs with T1A in combination with each of the domains from T3A through T8A also were poorly expressed. Thus, these double-T mutants could not be evaluated.

The other combinations of double-T mutants were examined for their ability to target the reporter protein to chloroplasts. First, the targeting of double-T mutants with combinations of T2A and each domain from T4A through T8A, T2A/T4A to T2A/T8A, was examined by image analysis of GFP fluorescence. The GFP patterns of T2A/T5A:GFP, T2A/T6A:GFP, and T2A/T8A:GFP were nearly identical to that of the wild type (data not shown). In addition, the GFP patterns of T2A/T4A:GFP and T2A/T7A:GFP were nearly identical to those of T4A:GFP and T7A:GFP, respectively (Fig. 4B, a, b, e, and f). To examine the targeting of these mutants to chloroplasts at the biochemical level, protein extracts were prepared from transformed protoplasts and analyzed by western blotting using an anti-GFP antibody. In agreement with the image analyses, the western-blot patterns of T2A/T5A:GFP, T2A/T6A:GFP, and T2A/T8A:GFP were nearly identical to that of the wild type (data not shown).

However, the western-blot pattern of T2A/T4A:GFP and T2A/T7A:GFP were different from those of the individual T-block Ala substitution mutants. T2A/T4A:GFP yielded only the 37-kD form with no detectable M throughout the time course (Fig. 4B, c). In contrast, T2A/T7A:GFP yielded two protein species at 31 and 37 kD (Fig. 4B, g). At 12 AT, 40% to 50% of the total reporter protein accumulated as the 37-kD form. Although the amount of the 37-kD form declined gradually over time, it persisted until 36 h AT. This was quite similar to the western-blot pattern of T4A:GFP (Fig. 1C, e), although T4A:GFP yielded slightly more of the 37-kD form than T2A/T7A:GFP. These data indicated that the sequence motifs in T2, T4, and T7 were important for crossing the chloroplast envelope membrane. To further characterize the distribution of T2A/T4A:GFP and T2A/T7A:GFP, protein extracts were separated into supernatant and pellet fractions by ultracentrifugation. The M of T2A/T7A:GFP was detected in the supernatant, whereas the 37-kD form of both T2A/T4A:GFP and T2A/T7A:GFP was in the pellet (Fig. 4B, d and h), strongly suggesting that the 37-kD form was trapped in the import channel. As a control for the fractionation, we detected aleurain and anti-Toc75 using anti-aleurain and anti-Toc75 antibodies, respectively, and found that aleurain and Toc75 were detected only in the supernatant and pellet fractions, respectively. The 37-kD form was thermodysin sensitive (data not shown), consistent with the hypothesis that T2A/T7A:GFP was trapped in the import channel with the GFP moiety exposed to the cytoplasm.

Among the mutants T3A/T5A:GFP through T3A/T8A:GFP, T3A/T5A:GFP had strong GFP signals in chloroplasts at both 12 and 24 h AT, indicating that T3A/T5A:GFP was imported efficiently into chloroplasts (data not shown). Western-blot analysis of protein extracts obtained from T3A/T5A:GFP-transformed protoplasts confirmed this (data not shown). T3A/T6A:GFP produced diffuse GFP signals in the cytoplasm, but not in the chloroplasts, at both 12 and 24 h AT (Fig. 4C, a and b), indicating that motifs in T3 and T6 were important for the binding to chloroplasts. In agreement with data from image analysis,
Figure 4. In vivo targeting and western-blot analysis of double-T block Ala substitution mutations in protoplasts. A, Sequences of double-T Ala substitution mutations. B and C, Localization and western-blot analysis of reporter proteins. Protoplasts were
T3A/T6A:GFP primarily yielded the Pr with only a small portion of M at 12 h AT (Fig. 4C, c). The amount of Pr was greatly reduced at 24 h AT and barely detectable at 36 h AT. However surprisingly, the M increased only slightly with time, suggesting that Pr was degraded rapidly in the cytoplasm. To confirm that this putative proteolytic degradation was specific for T3A/T6A:GFP, RFP and T3A/T6A:GFP were co-transformed into protoplasts and RFP was detected by western-blot analysis using anti-RFP antibody. The amount of RFP increased gradually with time (Fig. 4C, d), confirming that the proteolysis was specific for T3A/T6A:GFP. The reason for the proteolysis was unclear. It is possible that Ala substitutions in T3 and T6 render the reporter protein susceptible to a protease. Protein extracts from the T3A/T6A:GFP-transformed protoplasts were separated into supernatant and pellet fractions by ultracentrifugation and these fractions were analyzed by western blotting using anti-GFP antibody. In addition, aleurain and Toc75 were detected by anti-aleurain and anti-Toc75 antibodies, respectively. In agreement with the image analysis, most of Pr was detected in the supernatant (Fig. 4C, e). These results suggested that the motifs in T3 and T6 may be necessary for initial binding of the reporter protein to the components of the receptor complex. However, we cannot rule out the possibility that these regions are involved in binding to the lipid membranes of chloroplasts (Pilon et al., 1995; Pinnaduwage and Bruce, 1996), as well as to the guidance complex (May and Soll, 2000).

Transformation with T3A/T7A:GFP produced no GFP signal (data not shown) even though the GFP protein level was comparable to that of the wild type (see western-blot data below). Proteins are unfolded during translocation across the chloroplast envelope membrane (Guera et al., 1993; Walker et al., 1996). Thus, the lack of a GFP signal from T3A/T7A:GFP may have been due to the unfolding of the GFP domain of T3A/T7A:GFP. Immunohistochemistry with the anti-GFP antibody was used to localize T3A/T7A:GFP. As a control, protoplasts transformed with GFP were immunostained with anti-GFP antibody. Interestingly, in contrast to the diffuse pattern of GFP proteins in the cytoplasm (Fig. 4C, n), the reporter protein formed a ring pattern that surrounded, but did not overlap with, the red autofluorescent signal of chlorophyll at both 12 and 24 h AT (Fig. 4C, f and g), indicating that T3A/T7A:GFP accumulated at the chloroplast envelope membrane but was not imported into the chloroplast. These results indicated that the T7 motif was involved in a later step than the T6 motif and may play a role in crossing the envelope membrane. Western-blot analysis showed that approximately 80% of the T3A/T7A:GFP was in the intermediate form at 12 h AT, with the remaining protein in M (Fig. 4C, h). The 37-kD form of T3A/T7A:GFP persisted until 36 h AT, but was reduced significantly as the M increased, indicating that T3A/T7A:GFP was imported into chloroplasts very slowly. The M of T3A/T7A:GFP migrated at 30 kD, as compared with the wild-type protein at 31 kD. These results indicated that the processing of T3A/T7A:GFP occurred downstream of the normal processing site. The processing site is located in T6 (Gavel and von Heijne, 1990) and mutations at or near that site may cause processing to occur at alternative sites. To obtain independent evidence for the localization of T3A/T7A:GFP, samples taken at 12 h AT were fractionated and the supernatant and pellet fractions were analyzed by western blotting using anti-GFP antibody. In agreement with the image analysis, the intermediate form was detected primarily in the pellet (Fig. 4C, i), indicating that T3A/T7A:GFP accumulated at the envelope membrane as the intermediate form. These results suggested that motifs in T3 and T7 were necessary for translocation across the envelope membrane. T7 corresponded to the N-terminal region of the mature RbcS and these data support previous results showing that the N-terminal region of the mature RbcS increases the efficiency of protein import into chloroplasts (Comai et al., 1988).

With T3A/T8A:GFP at 12 h AT, GFP signals accumulated at discrete sites on or along the surface of the chloroplasts (Fig. 4C, j). At 24 h AT, the GFP signals were present in the chloroplasts (Fig. 4C, k). These results suggested that T3A/T8A:GFP accumulated at the chloroplast envelope membrane at early time points and was imported into chloroplasts over time, at a slower rate than the wild-type protein. The import of T3A/T8A:GFP was analyzed by western blotting using anti-GFP antibody. At 12 h AT, approximately 60% of the total T3A/T8A:GFP protein was detected as M, with the remainder of the protein in the 37-kD form (Fig. 4C, l). The intermediate of T3A/T8A:GFP was reduced gradually over time as M increased, indicating that import occurred slowly. Samples taken at 12 h AT were fractionated by ultracentrifugation and the supernatant and pellet were analyzed by western blotting using anti-GFP antibody. Both the 37-kD form and the M were detected in the pellet but not in the chloroplast. These results indicated that the T7 motif was involved in a later step than the T6 motif and may play a role in crossing the envelope membrane. Western-blot analysis showed that approximately 80% of the T3A/T7A:GFP was in the intermediate form at 12 h AT, with the remaining protein in M (Fig. 4C, h). The 37-kD form of T3A/T7A:GFP persisted until 36 h AT, but was reduced significantly as the M increased, indicating that T3A/T7A:GFP was imported into chloroplasts very slowly. The M of T3A/T7A:GFP migrated at 30 kD, as compared with the wild-type protein at 31 kD. These results indicated that the processing of T3A/T7A:GFP occurred downstream of the normal processing site. The processing site is located in T6 (Gavel and von Heijne, 1990) and mutations at or near that site may cause processing to occur at alternative sites. To obtain independent evidence for the localization of T3A/T7A:GFP, samples taken at 12 h AT were fractionated and the supernatant and pellet fractions were analyzed by western blotting using anti-GFP antibody. In agreement with the image analysis, the intermediate form was detected primarily in the pellet (Fig. 4C, i), indicating that T3A/T7A:GFP accumulated at the envelope membrane as the intermediate form. These results suggested that motifs in T3 and T7 were necessary for translocation across the envelope membrane. T7 corresponded to the N-terminal region of the mature RbcS and these data support previous results showing that the N-terminal region of the mature RbcS increases the efficiency of protein import into chloroplasts (Comai et al., 1988).

Figure 4. (Continued.)

transformed with the indicated constructs and GFP patterns were observed 12 and 24 h AT. In certain cases, RFP was cotransformed into protoplasts together with GFP constructs. Protein extracts from protoplasts were analyzed by western blotting using anti-GFP antibody. RFP was detected with an anti-RFP antibody. In addition, protein extracts were fractionated and detected as described for Figure 1D. In the cases of T3A/T7A:GFP and GFP alone, protoplasts were fixed and immunostained with anti-GFP antibody followed by FITC-labeled anti-rabbit IgG. Green, red, and yellow signals indicate GFP, autofluorescence of chlorophyll, and the overlap between green and red fluorescent signals, respectively. Bar = 20 μm. Pr, Precursor form; M, mature form; T, total; P, pellet fraction; and S, supernatant fraction.
the supernatant (Fig. 4C, m), indicating that both forms remained associated with envelope membranes. Again, these results suggested that T8, which is the N-terminal region of the mature RbcS, played a role in translocation across the envelope membrane, as observed previously (Comai et al., 1988).

The Sequence Motif in T4 Displays Functional Redundancy with Motifs in T6, T7, and T8 Regions

Next, double-T mutants T4A/T6A, T4A/T7A, and T4A/T8A were generated and fused to GFP (Fig. 5A).

Figure 5. The sequence motif in T4 displays functional redundancy with motifs in T6, T7, and T8 regions. A, Sequences of double-T Ala substitution mutations. B, Localization and western-blot analysis of reporter proteins. Protoplasts were transformed with the indicated constructs and GFP patterns were observed 12 and 24 h AT. In certain cases, RFP was cotransformed into protoplasts together with GFP constructs. The patterns of T4A/T7A:GFP and GFP alone, protoplasts were fixed and immunostained with anti-GFP antibody followed by FITC-labeled anti-rabbit IgG. Protein extracts from protoplasts were analyzed by western blotting using anti-GFP antibody. RFP was detected with an anti-RFP antibody. In addition, protein extracts were fractionated and detected as described for Figure 1D. Green, red, and yellow signals indicate GFP, autofluorescence of chlorophyll, and the overlap between green and red fluorescent signals, respectively. Bar = 20 μm. Pr, Precursor form; M, mature form; I, intermediate form; T, total; P, pellet fraction; and S, supernatant fraction. C, Protein extracts from protoplasts transformed with T4A/T7A:GFP were treated with 1.0% Triton X-100 treatment on ice and separated into soluble (S) and membrane (P) fractions by ultracentrifugation. These fractions were analyzed by western blotting using anti-GFP, anti-aleurain, and anti-VSR antibodies. D, Thermolysin sensitivity of reporter proteins. Protoplasts were transformed with either T4A/T7A:GFP or GFP alone. Thermolysin sensitivity was determined as described for Figure 3E. In the case of GFP alone, gently lysed protoplasts were treated with thermolysin.

These constructs were transiently expressed in protoplasts to examine their targeting to chloroplasts. The targeting efficiency of another group of mutants, T4A/T6A:GFP, T4A/T7A:GFP, and T4A/T8A:GFP, was examined. T4A/T6A:GFP produced a diffuse GFP pattern in the cytoplasm, together with a ring pattern around the chloroplasts, at 12 and 24 h AT (Fig. 5B, a and b). The GFP pattern of T4A/T6A:GFP was quite similar to that of T3A/T6:GFP (Fig. 5B, a and b). Consistent with the image analysis, western-blot analysis using anti-GFP antibody showed that T4A/T6A:GFP was present in the 39-kD Pr form with barely
detectable amounts of M, up to 36 h AT (Fig. 5B, c). These results indicated that Ala substitution in T4 and T6 resulted in complete inhibition of protein import at very early stages. However, in contrast to T3A/T6A:GFP, the amount of T4A/T6A:GFP was not reduced over time, suggesting that T4A/T6A:GFP was not susceptible to protease despite its localization in the cytoplasm. When protein extracts were fractionated, Pr was equally distributed in the supernatant and pellet fractions (Fig. 5B, d), indicating that the proteolysis was specific for T4A/T7A:GFP. When protein extracts that had been treated with thermolysin were fractionated, the 37-kD form of T4A/T7A:GFP was detected in the pellet (Fig. 5B, i). To further examine the membrane association of T4A/T7A:GFP, protein extracts that had been treated with Triton X-100 were fractionated into soluble and membrane fractions. The 37-kD form of T4A/T7A:GFP was resistant to solubilization by Triton X-100 (Fig. 5C, a). Under the same conditions, the integral membrane AtVSR1 protein was completely solubilized (Fig. 5C, c). These results raised the possibility that T4A/T7A is trapped in the import channel, although we cannot completely exclude the possibility that it may form a high M₈ complex. Next, we examined accessibility of T4A/T7A:GFP to thermolysin and found that the 37-kD form of T4A/T7A:GFP was thermolysin sensitive (Fig. 5D, a). In the presence of thermolysin, the 37-kD form was converted to a 31-kD form, which may have been due to the resistance of the GFP domain to thermolysin (Fig. 5D, b). Under the same conditions, RbcL was also resistant to thermolysin (Fig. 5D, c).

GFP signals from T4A/T8A:GFP overlapped with the red autofluorescent signal of chlorophyll at 12 and 24 h AT (Fig. 5B, j and k), indicating that it was imported efficiently into chloroplasts. Western-blot analysis of protein extracts using an anti-GFP antibody showed that 50% to 60% of the total reporter protein was in the M and the remainder was in the intermediate (Fig. 5B, l). The western-blot pattern of T4A/T8A:GFP was quite similar to that of T4A:GFP, suggesting that the motif in T8 may not have been necessary for translocation when the transit peptide had an intact T4 region. When protein extracts obtained at 12 h AT were fractionated, the 37-kD form of T4A/T8A:GFP was detected primarily in the supernatant. This was slightly different from the fractionation pattern of T4A:GFP that yielded 50% of the 37-kD form in the pellet.

**LKSSA, CMQVW, and KKFET in T3, T6, and T7, Respectively, Are Critical Motifs That Are Functionally Equivalent for Protein Targeting to Chloroplasts**

To determine the critical amino acid residues in T3, T6, and T7, the mutants shown in Figure 6A were generated. The original sequences of subregions of T3, T6, or T7 were restored in each of the double-T mutants. The targeting efficiency of T3A + APFNG/T6A:GFP, T3A + LKSSA/T6A:GFP, T3A/T6A + GGRVN:GFP, and T3A/T6A + CMQVW:GFP was examined in protoplasts. Protein extracts were analyzed by western blotting using an anti-GFP antibody. The targeting efficiency of T3A/LKSSA/T6A improved greatly and was comparable to that of T3A or T6A (Fig. 6B, b), whereas the targeting efficiency of T3A + APFNG/T6A improved moderately, to 40% of that of T3A (Fig. 6B, c). These data indicated that LKSSA was the critical motif in T3. In addition, the targeting efficiency of T3A/T6A + CMQVW was equivalent to that of T3A or T6A (Fig. 6B, e), whereas the targeting efficiency of T3A/T6A + GGRVN improved marginally, to 30% to 40% of that of T3A (Fig. 6B, d), indicating that CMQVW was the critical motif in T6. Together, these results suggested that the transit peptide with either LKSSA in T3 or CMQVW in T6 was nearly sufficient for targeting of a protein to chloroplasts and that these motifs either were functionally redundant or could compensate for each other.

The targeting efficiency of T3A + APFNG/T7A, T3A + LKSSA/T7A, T3A/T7A + PPIGK, and T3A/T7A + KKFET was examined by western-blot analysis (Fig. 6C). The targeting efficiencies of T3A + LKSSA/T7A and T3A/T7A + KKFET were similar to those of T3A and T7A (Fig. 6C, b and e), indicating that LKSSA in T3 or KKFET in T7 completely rescued the targeting deficiency of T3A/T7A. In addition, the targeting efficiencies of T3A + APFNG/T7A and T3A/T7A + PPIGK were improved over that of T3A/T7A, although not to the extent that T3A + LKSSA/T7A or T3A/T7A + KKFET improved targeting (Fig. 6C, c and d). These results suggested that the KKFET motif in T7 was critical for targeting a protein to chloroplasts. Furthermore, these results suggested that the KKFET motif in T7 was functionally redundant or compensatory to the LKSSA motif in T3 for protein targeting to chloroplasts.
Among Single 10-Amino Acid Blocks, Only the T4 Deletion Affects Targeting Efficiency; However, Deletion of Double-T Blocks Strongly Inhibits Targeting

Ala substitutions inevitably introduced a new secondary structure into the transit peptide that could affect targeting efficiency. Therefore, as an alternative approach, serial deletion mutants, ΔT2 through ΔT7, with a single-T deletion were generated (Fig. 7A). In addition, the double-T deletion mutants, ΔT2/ΔT3, ΔT3/ΔT4, ΔT3/ΔT6, ΔT4/ΔT7, and ΔT6/ΔT7 were generated (Fig. 7A). These deletion mutants were fused to GFP and transiently expressed in protoplasts to examine their targeting efficiency. The single-T deletion mutants, with the exception of ΔT4, primarily yielded the M (Fig. 7B), as observed with the wild type. The major M from the ΔT2, ΔT3, and ΔT7 mutants migrated at 31 kD, as did the wild-type protein. In contrast, ΔT5 and ΔT6 yielded a 32-kD protein as the major M (Fig. 7B, d and e), raising the possibility that these proteins were processed at an alternative cleavage site, due to deletions at or near the original processing site. Similarly, an alternative processing site is used when transit peptides have mutations in the carboxy-terminal region of the transit peptide (Archer and Keegstra, 1993). However, we did not further pursue this matter in this study. These results were quite similar to those obtained with the individual-T Ala substitution mutants. In the case of ΔT4, the reporter proteins displayed a complex pattern with four protein species at 31, 32, 34, and 35 kD (Fig. 7B, c). When gently lysed protoplasts were treated with thermolysin, the 34- and 35-kD forms of ΔT4/GFP were thermolysin sensitive, whereas the 31- and 32-kD forms were thermolysin resistant (data not shown), indicating that the 34- and 35-kD forms were intermediates and that the 31- and 32-kD forms were M within the chloroplast. The two intermediate forms accounted for approximately 30% of the total reporter protein at 12 h.
Figure 7. In vivo targeting of deletion mutants in protoplasts. A, Sequences of deletion mutations. B to D, Western-blot analyses of reporter proteins. Protein extracts from protoplasts transformed with the indicated constructs at the indicated time points were used for western-blot analysis. Pr, Precursor form; and M, mature form. E, Subcellular distribution of reporter proteins. Protein extracts were fractionated and detected as described for Figure 1D. T, P, and S indicate total, pellet, and supernatant fractions, respectively.
Sequence Motifs Found in the Transit Peptide of RbcS
Are Also Found in the Transit Peptides of Other Proteins

Next, we examined whether the sequence motifs identified in the transit peptide of RbcS are also present in the transit peptides of other proteins. Sequence alignments revealed that identical or similar sequence motifs are present in the transit peptides of ferredoxin-2 and Asp carbamoyltransferase (ATCase; Fig. 8; Williamson and Slocum, 1994). In particular, the FP motif in T4 and the KKFET motif in T7 are present in the transit peptide of ATCase. In addition, the KK motif in the transit peptide of ATCase may be equivalent to the RK motif in the transit peptide of RbcS. Furthermore, the transit peptide of ferredoxin-2 was found to contain a copy of the LKSS motif of T3 present in the RbcS transit peptide. The positions of these sequence motifs in the transit peptides of ATCase and ferredoxin-2 were different from those in the RbcS transit peptide. However, in this study, we did not examine whether the motifs in the transit peptides of ATCase and ferredoxin-2 play a critical role in the targeting of these proteins to the chloroplast. In a previous study, the LKSS motif in the transit peptide of ferredoxin-2 was shown to be part of the binding site of a 14-3-3 protein (Pilon et al., 1995).

DISCUSSION

In vivo targeting experiments using various Ala substitution mutants show that sequence motifs for protein targeting to chloroplasts are distributed throughout the entire transit peptide. The relationships among the eight domains of the transit peptide are very complex. Under the conditions used in these experiments, only Ala substitution of T4 significantly affects the targeting efficiency. However, among the various double-T Ala substitution mutants, many exhibit 50% to 100% loss of targeting efficiency. The data herein clearly demonstrate that the transit peptides have multiple sequence elements with complex relationships. In addition, the data obtained with Ala substitution mutants were confirmed with individual- or double-T deletion mutants.

In T1 of the transit peptide, the hydrophobic nature of the ML motif is important for the initial rate of targeting. The hydrophobic N-terminal region may be necessary for binding of the transit peptide to the chloroplast through the interaction between the hydrophobic region and chloroplast lipid membranes (Ferry et al., 1991; van’t Hof et al., 1991, 1993; Pilon et al., 1995; Pinnaduwage and Bruce, 1996; Rensink et al., 1998). Consistent with our data, a deletion (Δ6-14) in the N-terminal region of ferredoxin that has uncharged amino acids causes the transit peptide to lose its ability to penetrate into the monogalactosyldiacylglycerol monolayer (Pilon et al., 1995).

Ala substitution of T2 alone does not affect targeting. However, when T2A is combined with T4A, the reporter proteins accumulate at the envelope membrane as intermediate forms, indicating that T2 and T4 act together to translocate a protein into chloroplasts. In addition, Ala substitution of both T2 and T7 causes 40% to 50% of the total reporter protein to remain in the intermediate form, almost identical to the result with T4A alone. These results strongly suggest that T2, T4, and T7 are important for translocation of protein across the envelope membrane, possibly through interaction with components of the receptor complexes. The N-terminal region of pSSU (precursor of Rubisco small subunit) interacts with Toc159 with high affinity.
Critical Sequence Motifs in Transit Peptide

The T4 region is the most critical for targeting to chloroplasts. Ala substitution or deletion of T4 causes targeting efficiency to drop to 40% to 50% of the wild-type level. T4 has two essential sequences, FP and RK. In addition, as indicated above, FP is part of the sequence that is similar to the proposed binding site of 14-3-3 (May and Soll, 2000; Bruce, 2001). The western-blot patterns obtained with T4A + RK:GFP and T4A + FP:GFP differ slightly: T4A + FP:GFP yields only M, whereas T4A + RK:GFP yields a small amount of 36- and 37-kD intermediates together with a very slowly migrating protein species of 65 kD, in addition to M. These protein species are tightly associated with the particulate fraction and are resistant to solubilization by Triton X-100 or Na2CO3. Based on these data, we favor the hypothesis that these sequences function at different steps during translocation.

T4 also has a functional relationship to T6. When T4A is combined with T6A, the mutant transit peptide completely loses its ability to target a protein to chloroplasts and primarily yields Pr, along with a minor amount of the 37-kD form, that are detected both in the cytoplasm and at the chloroplast envelope membrane. It is possible that the presence of the T3 motif may allow a portion of the precursor proteins to bind to the chloroplasts, but the absence of T4 motifs may prevent the mutant transit peptide from translocating through the import channel.

As described above, among the individual-T Ala substitutions and deletions, only the T4 substitution or deletion noticeably reduces the targeting efficiency. One possible explanation for this is redundancy in the transit peptide motifs. Consistent with this idea, the T2 motif is functionally equivalent to or compensates for the T7 motif. In addition, the LKSSA sequence in T3 is functionally equivalent to or compensates for the CMQVW sequence in T6 and the KKFET sequence in T7. However, the motifs that are functionally equivalent or compensatory do not necessarily have any sequence similarity. These motifs likely serve as binding sites for proteins or membranes at various steps during translocation (Richter and Lamppa, 1999; Gutensohn et al., 2000; Rial et al., 2000; Hinahah et al., 2002; Zhang and Glaser, 2002; Becker et al., 2004; Smith et al., 2004). The proteins may include the components of Toc and Tic complexes in the chloroplast envelope membrane and ones involved in the guidance complex in the cytoplasm. Each of these proteins may have multiple binding sites within the transit peptide. This is consistent with a previous result showing that both the C- and N-terminal regions of pea pSSU bind to Toc159, albeit with different binding affinities (Becker et al., 2004).

However, without knowing which factors bind to these motifs in the transit peptide, it is difficult to understand the exact roles of these motifs and their functional relationships to each other. The next step will be to identify factors that bind to these motifs. Components of the Toc and Tic complexes have been identified and characterized at the molecular level.
Gartensohn et al. (2000; Hinnah et al., 2002; Becker et al., 2004; Smith et al., 2004). However, the identities of cytoplasmic factors that interact with the transit peptide remain elusive. HSP70 or 14-3-3 may bind to motifs in the transit peptide, as proposed previously (Ivey et al., 2000; May and Soll, 2000; Rial et al., 2000). Further studies will be necessary to define the exact role of these motifs and to identify the binding factors that may mediate their functions.

MATERIALS AND METHODS

Growth of Plants

Arabidopsis (Arabidopsis thaliana) was grown in soil at 20°C to 25°C in a greenhouse with a 16-h-light/8-h-dark cycle, or on Murashige and Skoog plates in a growth chamber at 20°C. Leaf tissues were harvested from 2-week-old plants and used immediately for protoplast isolation.

PCR-Based Mutagenesis and Construction of Plasmids

Construction of RbcS-ntGFP was described previously (Lee et al., 2002). A PCR approach was used to introduce mutations in the transit peptide. The primers are shown in the supplemental material. For each mutant, two complementary upper and lower primers were generated. The upper primer consisted of two or three different regions: a 5′ region of 18 bases flanking the 5′ side of the mutated region, a central region of a variable number of bases depending on the replaced amino acid residues, and a 3′ region of 18 bases flanking the 3′ side of the mutated region. The central region was absent in the deletion mutants primers. The lower primers were exactly complementary to the upper primers. With these primers, the first round of PCR was performed to generate 5′ and 3′ fragments, using as the template RbcS-ntGFP in an expression vector. The 5′ fragments were PCR amplified using the lower primers and the common cauliflower mosaic virus (CaMV)-5′ primer, and thus contained the CaMV 3′S promoter, the transit peptide 5′ of the mutated region, the mutated region, and the 18 bps 3′ to the mutated region. The 3′ fragments were amplified using the upper primers and the common nos-terminator primer, and thus contained the 18 bps 5′ to the mutated region, the mutated region, the GFP coding region, and the nos terminator. The second round of PCR was performed with the two 5′ and 3′ PCR products obtained from the first round as templates, and the primers CaMV-5′ and nos-terminator primer. For Ala substitution and deletion mutations in two 10-amino acid blocks, the appropriate individual 10-amino acid block mutants were used as the templates and mutations were introduced as described above for the individual 10-amino acid block mutants. The PCR products were subcloned into a vector and sequenced. These constructs were introduced into a pUC-based expression vector.

Transient Expression and in Vivo Targeting of Reporter Proteins

All plasmids were purified with Qiagen (Basel) columns, according to the manufacturer’s protocol. The plasmids were introduced into Arabidopsis protoplasts prepared from leaf tissues by polyethylene glycol-mediated transformation (Jin et al., 2001). Images were taken with a cooled CCD camera and a Zeiss Axioplan fluorescence microscope (Zeiss), and presented in pseudocolor format (Jin et al., 2001; Kim et al., 2001).

Preparation of Protein Extracts and Western-Blot Analysis

Protein extracts were prepared from the transformed protoplasts as described previously (Jin et al., 2001). Western-blot analysis was carried out as described previously (Jin et al., 2001).

For subcellular fractionation, cell extracts were separated into soluble and membrane fractions by ultracentrifugation at 100,000g for 30 min. For Na2CO3 or Triton X-100 treatment, protein extracts were supplemented with Na2CO3 or Triton X-100 to a final concentration of 0.1 M or 1%, respectively, incubated on ice for 30 min, and subjected to ultracentrifugation at 100,000g for 30 min. These fractions were analyzed by western blotting. The protein blots were developed with an ECL kit (Amersham Pharmacia Biotech) and images were obtained using a LAS3000 image capture system (FUJIFILM). The amount of protein on the immunoblots was determined by measuring the intensity of the bands with LAS3000 software.

To examine the thermolysin sensitivity of the reporter proteins, intact chloroplasts were purified from gently lysed protoplasts and were treated with thermolysin, as described previously (Li et al., 1991). Proteins were prepared from the thermolysin-treated samples and analyzed by western blotting an anti-GFP antibody.

Immunohistochemistry

Transformed protoplasts were placed on poly-L-Lys-coated glass slides and fixed as described previously (Lee et al., 2003). The fixed cells were incubated with an anti-GFP antibody in TSW buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.02% SDS, and 0.1% Triton X-100) at 4°C overnight and washed three times with TSW buffer. The cells then were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) in TSW buffer and washed three times with TSW buffer.

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LITERATURE CITED


Inoue K, Keegstra K (2003) A polyglycine stretch is necessary for proper targeting of the protein translocation channel precursor to the outer envelope membrane of chloroplasts. Plant J 34: 661–669


