Localization and targeting of the VP14 epoxy-carotenoid dioxygenase to chloroplast membranes

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Received 23 February 2001; revised 4 June 2001; accepted 5 June 2001
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Summary

Abscisic acid (ABA) is a key regulator of seed dormancy and plant responses to environmental challenges. ABA is synthesized via an oxidative cleavage of 9-cis epoxy-carotenoids, the first committed and key regulatory step in the ABA biosynthetic pathway. Vp14 of maize encodes an epoxy-carotenoid dioxygenase that is soluble when expressed in E. coli. An important goal has been to determine how the soluble VP14 protein is targeted to epoxy-carotenoid substrates that are located in the thylakoid and envelope membranes of chloroplasts and other plastids. Using an in vitro chloroplast import assay, we have shown that VP14 is imported into chloroplasts with cleavage of a short stroma-targeting domain. The mature VP14 exists in two forms, one which is soluble in stroma and the other bound to thylakoid membranes. Analysis of a series of truncated VP14 mutants mapped the membrane targeting signal to the 160 amino acid N-terminal sequence. A putative amphipathic α-helix within this region is essential, but not sufficient, for the membrane targeting. Either deletion of or insertion of helix breaking residues into this region abolished the membrane binding, whereas a chimeric protein carrying just the amphipathic region fused with bacterial glutathione S-transferase failed to associate with the thylakoid membrane. The membrane-bound VP14 was partially resistant to chaotropic washes such as 0.1 M Na2CO3 (pH 11.5) and 6 M urea. Unlabelled recombinant VP14 inhibited the tight binding of imported VP14, suggesting that VP14 is associated with specific components of the thylakoid membrane.

Keywords: abscisic acid, epoxy-carotenoid dioxygenase, VP14, chloroplast, membrane, maize.

Introduction

The plant hormone abscisic acid (ABA) is a key regulator in seed development, maturation, dormancy and plant adaptation to a variety of environmental stresses such as drought and cold (Zeevaart and Creelman, 1988). In higher plants, ABA is synthesized from an oxidative cleavage of the epoxy-carotenoids 9-cis neoxanthin and 9-cis violaxanthin, to produce xanthoxin that is subsequently converted to ABA. The epoxycarotenoid cleavage is the first committed step in the pathway (Walton and Li, 1995; Zeevaart and Creelman, 1988), and is believed to be the key regulatory step in the ABA biosynthetic pathway (Qin and Zeevaart, 1999). In maize, the cleavage is catalysed by a specific novel epoxy-carotenoid dioxygenase, VIVIPAROUS14 (VP14) (Schwartz et al., 1997; Tan et al., 1997).

The initial steps of ABA biosynthesis most likely occur inside plastids because hydrophobic carotenoids, the precursors of ABA, reside exclusively in the membranes of chloroplasts and other plastids (Walton and Li, 1995; Zeevaart and Creelman, 1988). Several carotogenic enzymes, such as phytoene synthase, phytoene desaturase and lycopene cyclase, have been localized to plastids (Al-Babili et al., 1996; Bonk et al., 1997). The zeaxanthin epoxidase of tobacco, catalysing conversion of zeaxanthin to all-trans violaxanthin, is targeted into chloroplasts in an in vitro import assay (Marin et al., 1996). Carotenoid-associated proteins have been isolated from plastid membranes (Chen et al., 1998; Markwell et al., 1992), indicating that carotenoids interact with specific binding proteins located in the membrane.

Enzymatically active recombinant VP14 expressed from E. coli is water-soluble (Schwartz et al., 1997). Thus, an important question arises as to how the soluble VP14 gains access to its substrates. Membrane interaction and access to substrates are potential steps for post-translational regulation of ABA biosynthesis. The cleavage of...
epoxycarotenoids is suggested to take place in plastids, while the later steps take place in the cytosol (Cutler and Krochko, 1999). Consistent with this scenario, conversion of xanthoxin to ABA appears to be cytosolic (Sinhu and Walton, 1987). The substrates 9-cis violaxanthin and 9-cis neoxanthin are found in both chloroplast envelope membranes and thylakoid membranes (Neill et al., 1996; Parry and Horgan, 1991). In fact, PvNCED1, a parologue of VP14 from bean, is located exclusively in the insoluble fraction of the chloroplasts (Qin and Zeevaart, 1999).

Membrane association is frequently mediated by amphipathic helical domains (Biggin and Sansom, 1999; Car et al., 1991; Dunne et al., 1996). Membrane binding of CTP:phosphocholine cytidylyltransferase (CT), a regulatory enzyme in the synthesis of the phosphotidylcholine phospholipids of animal cells, is mediated through an amphipathic α-helix of approximately 60 amino acids located at the N-terminus (Craig et al., 1994; Yang et al., 1997). An interconversion between the membrane-bound (active) and cytoplasmic (inactive) forms plays a key role in regulation of enzymatic activity (Tronchere et al., 1994). In plant cells, the carotenoid biosynthetic enzyme phytoene desaturase exhibits similar behaviour. The enzymatically active form is bound to the thylakoid membrane, while the inactive form remains soluble in the stroma (Al-Babili et al., 1996). In light of the evidence that carotenoids are bound to membranes by specific proteins (Chen et al., 1998; Markwell et al., 1992), we hypothesize that VP14 interacts with a specific carotenoid-binding protein or protein complex.

Using a chloroplast import assay and immunoblot analysis, we show that VP14 exists in both a soluble stromal form and a thylakoid membrane-bound form, and differs from PvNCED1 in this respect. We provide evidence that a putative amphipathic α-helix at the N-terminus of the mature VP14 is essential, but not sufficient, to target VP14 to thylakoid membranes. The thylakoid membrane targeting domain was mapped to approximately 160 amino acid residues in the N-terminal sequence of the mature VP14. In addition, we present evidence that tight binding of VP14 to the thylakoid membrane is saturable, suggesting a specific interaction with a membrane component.

Results

VP14 is targeted into chloroplasts

Nuclear-encoded chloroplast proteins are synthesized on cytoplasmic ribosomes as larger precursors with a cleavable stroma-targeting domain (STD) at the N-terminus. There is no conserved sequence among known STDs except for enrichment in hydroxylated residues and the absence of acidic residues (Cline and Henry, 1996). The N-terminal sequence of VP14 has characteristics of an STD. The N-terminal 40 amino acid residues contain eight serine residues (20% compared to 5% for the whole protein), eight positively charged residues, and no acidic residues. As a result, this region has a calculated pI of 12, in contrast to a pI of 5.72 for the full protein. Similar features were also observed in the N-terminus of ABA2 in tobacco, an epoxidase that is upstream of VP14 in the ABA biosynthetic pathway (Marin et al., 1996). To test for the presence of an STD, in vitro translated and tritium-labelled VP14 protein was incubated with fresh isolated chloroplasts under import conditions as described previously (Cline et al., 1993). Imported chloroplasts were purified and treated with thermolysin to degrade proteins outside or adhering to the chloroplast outer envelope. When VP14 was incubated with pea chloroplasts, VP14 protein was imported into chloroplasts such that it is protected from thermolysin degradation (Figure 1a). In addition, precursor VP14 was processed to a smaller molecular weight with the loss of approximately 4.5 kDa. This is consistent with proteolytic cleavage and removal of an N-terminal STD of approximately 45 amino acids. As expected, import of VP14 required either ATP or light (Figure 1b). A pea light-harvesting chlorophyll α/β-binding protein (LHCP) was tested as a control. LHCP is a well-characterized thylakoid protein (Cline and Henry, 1996; Keegstra and Cline, 1999). These data demonstrate that VP14 is a nuclear-encoded chloroplast-localized protein.

Localization of VP14 inside chloroplasts

The chloroplast contains six different compartments to which a protein may be targeted. These include the outer and inner envelope membranes, envelope interspace, stroma, thylakoid membranes and thylakoid lumen (Cline and Henry, 1996). After import into stroma, targeting of mature proteins depends on intrinsic signals that determine its final destination. To further localize VP14, chloroplasts were sub-fractionated following import into three fractions: a soluble fraction that contained mainly the stromal proteins plus envelope interspace proteins, a thylakoid fraction that included thylakoid membrane and lumen proteins, and an envelope fraction that included outer and inner envelope proteins. VP14 was found predominantly in the soluble fraction, suggesting that mature VP14 remained in stroma as a soluble protein (Figure 2). However, approximately 35% of the imported VP14 was found associated with thylakoids. When an equal amount of the intact thylakoids was treated by limited thermolysin digestion, a partial degradation of the associated VP14 resulted as indicated by the smaller and much weaker band. Complete disappearance of this band was observed with an increased amount of thermolysin or extension of incubation time. The accessibility by protease

suggested that VP14 was bound to thylakoid membranes facing the stroma. Limited protease digestion showed that one terminus of VP14 was more exposed to stroma, while the other structure might be tightly folded or imbedded into the membrane. In contrast, mature LHCP was found only in the thylakoid fraction, and part of the protein was protected from thermolysin digestion because it is embedded in the membrane potentially via three hydrophobic transmembrane helices (Kuhlbrandt et al., 1994; Schnell, 1998). Sequence analysis of VP14 did not reveal any potential putative transmembrane domains. In addition, the thylakoid membrane-bound VP14 appeared to have the same molecular weight as the stromal form, suggesting that VP14 does not have a cleavable thylakoid-targeting domain.

The stromal and thylakoid localization of VP14 is evidently different from the strict membrane localization of PvNCED1 in bean (Qin and Zeevaart, 1999), although both catalyse the oxidative cleavage of 9-cis epoxycarotenoids. To rule out the possibility that VP14 localization may be an artefact of the heterologous pea chloroplast environment, maize chloroplasts were also used to determine localization. The distribution of VP14 between stroma and thylakoids in maize chloroplasts was consistent with the pea chloroplast results (Figure 2a). To further determine whether the partitioning of imported VP14 is representative of endogenous VP14, we used Western blotting as an alternative approach to measure the distribution of endogenous VP14. Because it is known that Vp14 expression is highly inducible in desiccated leaves (Tan et al., 1997), chloroplasts were isolated from stressed leaves and sub-fractionated as in the import assay. A monoclonal antibody (3H12) raised against purified recombinant VP14 was used to detect the endogenous protein. It was confirmed that 3H12 detected VP14 in wild-type leaves, but not in either of the two vp14 mutant alleles (vp14-2274 and vp14-3250, data not shown). As shown in Figure 2(c), the localization of endogenous VP14 is consistent with imported VP14. Thus, this confirmed that VP14 has a distinctive localization that differs from that of bean PvNCED1.

A putative amphipathic α-helix is essential in VP14 association with thylakoid membranes

Analysis of the VP14 sequence revealed a putative amphipathic α-helix at the N-terminus between residues...
Lys^{81} and Asp^{100} of the precursor VP14. Secondary structure analysis using the PHD program (http://www.public.iastate.edu/~pedro/pprotein_query.html; Rost and Sander, 1994) indicated a strong probability that \(\alpha\)-helix is formed in this region. A helical wheel plot showed strong characteristics of amphipathy, one side with highly charged residues (Lys\(^{+}\), Arg\(^{+}\), Asp\(^{-}\), Glu\(^{-}\)) and the opposite side with hydrophobic residues (Figure 3a). Although the amphipathic region is not present in closely related bacterial proteins, a similar amphipathic region is conserved in NCED proteins of maize, tomato (Burbridge et al., 1999), bean (Qin and Zeevaart, 1999) and Arabidopsis (Deng and McCarty, unpublished data). Amphipathic \(\alpha\)-helices are potentially involved in protein–protein interactions or anchoring of the protein to membranes (Biggin and Sansom, 1999; Car et al., 1991; Dunne et al., 1996).

Considering that substrates of VP14 are membrane-bound, we tested whether this region is involved in targeting VP14 to membrane. To dissect the function, several mutated forms of VP14 were created. VP14-Amp\(^{D}\) is a concise deletion of the amphipathic region. VP14-GGG and VP14-PPP contain insertions of triple glycine or triple proline residues between Ala\(^{90}\) and Ala\(^{91}\) (Figure 3b), respectively. Proline and glycine residues are expected to disrupt \(\alpha\)-helices. These proteins were \textit{in vitro} translated in the presence of \(^{3}\text{H}\)-leucine and imported into chloroplasts. Chloroplasts were sub-fractionated into envelope, stromal and thylakoid sub-fractions. Cross-contamination between fractions was monitored using imported ssRBS (Rubisco small subunit), LHCP and P36 proteins as markers for stroma, thylakoid and envelope, respectively. Thylakoid contamination of the stroma fraction and envelope contamination of the thylakoid fraction were minimal. Contamination of thylakoid fractions by stromal and envelope proteins is more common. Thus, ssRBS was used as an internal monitor for possible contamination of thylakoid with stromal fraction. As shown in Figure 3(b), either deletion of the amphipathic \(\alpha\)-helix or insertion of helix breakers abolished the binding of VP14 to thylakoid membranes. This indicates that this amphipathic \(\alpha\)-helix is essential for VP14 thylakoid membrane association.

\textit{The putative amphipathic \(\alpha\)-helix is not sufficient for VP14 association with thylakoid membranes}

To further test whether this amphipathic region is sufficient for targeting of VP14 or a foreign protein to thylakoid membranes, the amphipathic region was fused to the N-terminus of the bacterial glutathione \(S\)-transferase (GST). In order to deliver this chimeric protein to the stroma, the VP14 stroma-targeting domain was also included at the N-terminus. This protein was designated Std-Amp-GST (Figure 4a). A deletion derivative lacking the amphipathic

\[\text{MQLQAPTQV SIWBLPAPV RRAAGNVPF STRAYGVPK ASCLQPAPK PVADLPAPG KVAAJAVPG AAAPKASCG KGGLNLYMA AAAALADFG GRVANY\ldots\]
sequence (STD-GST) was used as a control to monitor thylakoid-binding activity by GST itself. When 3H-labelled Std-Amp-GST and Std-GST were incubated with chloroplasts, both chimeric proteins were imported into chloroplasts. The VP14 amphipathic region failed to target the chimeric GST to thylakoid membranes (Figure 4a). In both cases, GST fusions remained soluble in the stromal fraction. A weak signal observed in the thylakoid fraction after a long exposure evidently resulted from stromal cross-contamination because the ratios of radioactivity between stroma and thylakoids for both proteins matched that for the internal control, ssRBS. This indicates that the putative amphipathic α-helix is necessary, but not sufficient for targeting of VP14 to the thylakoid membrane.

To define the minimal sequence that is sufficient for membrane targeting, VP14 N-terminal sequences of varying lengths were fused with GST to generate a series of VP14–GST chimeric proteins, as shown in Figure 4(b). These proteins were radiolabelled and imported into pea chloroplasts. After fluorography of the gel, the bands were excised and the radioactivity was measured in a scintillation counter as described in Experimental procedures. The binding activity of these proteins to thylakoids was determined by the percentage of radioactivity in the thylakoid fraction, normalized with the internal control ssRBS. As numbered from the first methionine including the approximately 45 amino acid STD, inclusion of 166 amino acids of the N-terminus sequence of VP14. All these proteins were imported into chloroplasts which were fractionated into stromal and thylakoid fractions. The radioactivity of the bands was determined and the percentage of radioactivity in the thylakoids is given. S, stroma; T, thylakoid; TP, in vitro translation precursor.

Figure 4. Binding of chimeric VP14-GST proteins to thylakoid membranes. Schematic diagrams of the chimeric protein structures are given on the left.
(a) The putative amphipathic α-helix is not sufficient for targeting GST to thylakoid membranes. The 106 amino acid N-terminus sequence of VP14, which includes the putative amphipathic α-helix, was fused to GST (STD-Amp-GST). A deletion of the amphipathic helix of this protein was designated as STD-GST. Import of these two proteins is shown in the right panel. Pea ssRBS was imported as a control to monitor the cross-contamination.
(b) Thylakoid targeting of chimeric VP14-GSTs with different lengths of VP14 N-terminus sequence. VP14<sup>106</sup> indicates that the protein included 106 amino acids of the N-terminus sequence of VP14. All these proteins was imported into chloroplasts which were fractionated into stromal and thylakoid fractions. The radioactivity of the bands was determined and the percentage of radioactivity in the thylakoids is given. S, stroma; T, thylakoid; TP, in vitro translation precursor.
VP14 is tightly bound to thylakoid membranes

Because carotenoids are found bound to specific membrane proteins (Chen et al., 1998; Markwell et al., 1992), VP14 binding to thylakoid membranes is potentially mediated through interaction with other proteins. Thermolysin digestion of thylakoids indicates that the location of bound VP14 is on the outer surface facing the stroma (Figure 2). To distinguish peripheral and integral membrane proteins, 6 M urea and 0.1 M Na₂CO₃ (pH 11.5) wash treatments were used. Proteins that cannot be removed by such chaotropic agents are considered to be membrane-integral proteins, whereas those removed are peripheral proteins (Fujiki et al., 1982; Li et al., 1994).

Purified thylakoids after import were resuspended in a 50-fold volume of import buffer, 0.1 M Na₂CO₃ (pH 11.5) or 6 M urea, and incubated on ice for 40 min. This wash was repeated after centrifugation. The majority of VP14 was removed from thylakoids by washes with 0.1 M Na₂CO₃ (pH 11.5) or 6 M urea (Figure 5a). However, as determined by the radioactivity contained in the excised bands, approximately 20% of the protein still remained bound to thylakoids after extensive chaotropic washes. This fraction did not result from contamination by stroma carry-over because VP14-AmpΔ was not detected in the thylakoid fraction (Figure 4). As an internal control, pea LHCP was imported and the chloroplasts mixed with VP14-loaded chloroplasts prior to fractionation. As expected, LHCP was integrated into the membrane and was not removed by either wash treatment. In addition, the association of VP14 with the thylakoid membrane was not affected by incubation with 10 mM EDTA in the import buffer (data not shown), indicating that the non-haem iron, which was required for its activity in vitro (Schwartz et al., 1997), was not required for membrane association. These data indicate that a significant fraction of VP14 associated with thylakoid membranes is very tightly bound.

VP14 interacts specifically with components in thylakoid membranes

To test whether the stromal form of VP14 retains the competency to bind to thylakoids, a reconstitution assay was carried out in which stromal fraction containing mature VP14 was incubated with fresh isolated thylakoids. Similar chaotropic washes were applied to the thylakoids using VP14-AmpΔ as a control. The results indicated that the stromal form of VP14 retains the competency for tight binding to thylakoids as shown in Figure 6(b). This allowed the possibility of performing a competition experiment.

Existence of a tightly bound form of VP14 suggests that it may associate specifically to a partner or partners, possibly the carotenoid-binding proteins in thylakoid membrane. Hence, an unlabelled recombinant VP14 truncated to remove the transit peptide was tested for its ability to compete with ³H-leucine-labelled VP14 (³H-VP14). The unlabelled mature VP14 was expressed in E. coli as a GST fusion and the GST was removed by thrombin cleavage. This protein was enzymatically active in cleaving 9-cis neoxanthin to produce xanthoxin (data not shown). In the competition assay, thylakoids were pre-incubated with cold VP14 in the presence of stroma extract, then incubated with stroma faction that contains the imported and processed ³H-VP14, which retains competency for membrane association. Figure 6 shows the binding of ³H-VP14 to thylakoids after extensive washes with 6 M urea. The ³H-VP14 binding to thylakoids was inhibited in a concentration-dependent manner by unlabelled VP14, indicating that the unlabelled recombinant VP14 was able to displace imported ³H-VP14 from the thylakoid membrane.

Discussion

We have shown that VP14, the key regulatory enzyme in the ABA biosynthetic pathway, is targeted to the chloroplasts by a stroma-targeting domain of approximately 4.5 kDa. Mature VP14 exists in a soluble stromal form and a thylakoid membrane-bound form. Protease susceptibility experiments indicate that VP14 is bound to the stromal face of the thylakoid membrane.
forms may have different roles in the developmental and environmental regulation of ABA biosynthesis. At least five putative NCEDs are found in the newly completed Arabidopsis genome sequence. In fact, one of the AtNCEDs exists only in the thylakoid membrane like PvNCED1, whereas another resembles VP14 (Deng et al., unpublished data). In vitro reconstitution experiments (Figure 5) demonstrate that the stroma-localized VP14 retains competency for tight binding to isolated thylakoids. Hence, the partitioning observed in intact chloroplasts cannot be attributed to incomplete or improper folding of imported VP14. We suggest two possible explanations that are not mutually exclusive: (1) the thylakoids may have a limited capacity for VP14 binding, or (2) the degree of thylakoid association may be regulated in intact chloroplasts. The competition experiments (Figure 6) confirm that tight binding of VP14 is saturable, but it is unclear whether thylakoid capacity alone would account for limited binding of stromal VP14. A similar mode of regulation of membrane association has been proposed for the carotegenic enzyme phytoene desaturase. Phytoene desaturase is predominantly soluble in the stroma (Bonk et al., 1997), but, similarly to VP14, is also found in a membrane-bound form (Al-Babili et al., 1996). Furthermore, the soluble form is enzymatically inactive and the membrane-bound form is active as assayed by incubation with radiolabelled substrates (Al-Babili et al., 1996). The stromal form of phytoene desaturase is apparently in a complex with Hsp70, which may play a role in the folding and targeting of the protein. Thus, there is reason to speculate that the two forms of VP14 may reflect enzymatically active and inactive forms. This is a potential mode of enzymatic regulation. Unfortunately, it is technically difficult to determine the activity of VP14 in stroma and thylakoid membranes. The tobacco zeaxanthin epoxidase, an enzyme upstream of the VP14-catalysed step in the ABA biosynthetic pathway, is also imported into chloroplasts (Marin et al., 1996), but further localization has not been determined.

The putative amphipathic α-helix at the N-terminus of mature VP14 is necessary, but not sufficient for membrane association. Complete deletion or interruption of this sequence with helix-breaking residues (proline and glycine) abolished the membrane association. This sequence itself did not target a heterologous protein (GST) to thylakoids. Hence the minimal thylakoid targeting signal involves approximately 160 N-terminus amino acid residues of the mature VP14, and includes part of the catalytic domain that is conserved in related proteins of bacteria and mammals. This suggests that the tertiary protein structure is involved in membrane localization. The remainder of the conserved catalytic domain of VP14 consisting of approximately 400 amino acids at the C-terminus is not involved in membrane localization. This region includes four invariant His residues that are likely to
be involved in coordination of the non-haem iron co-factor (Tan et al., 1997). For this reason, we suggest that the conserved 9-cis epoxycarotenoid dioxygenases (NCED) and related carotenoid dioxygenases contain two structural subdomains, a C-terminal iron-binding domain and an N-terminus domain involved in co-localization and/or binding with substrates.

It is unclear whether VP14 binding to thylakoid membranes is mediated by protein–protein interactions or through a hydrophobic interaction with the lipid bilayer. Competition experiments show that cold VP14 can completely displace tightly bound 3H-labelled VP14 on thylakoids, indicating that the binding is saturable and therefore specific. That unlabelled VP14 incompletely displaced labelled protein from buffer-washed thylakoids indicates that the fraction removed by mild wash conditions is non-specifically bound to thylakoids. Because the concentration of cold recombinant VP14 that is competent for tight binding is unknown, the relative affinity of tight binding cannot be determined. Amphipathic proteins have been implicated in protein–protein interaction among many metabolic enzymes (Biggin and Sansom, 1999; Car et al., 1991; Dunne et al., 1996). One of the most extensively studied enzymes, CTP:phosphocholine cytidylyltransferase (CT), is a regulatory enzyme in the synthesis of phosphotidylcholine, the major phospholipid of animal cells. An amphipathic α-helix of approximately 60 amino acid residues at its N-terminus mediates reversible binding of CT to membranes (Craig et al., 1994; Yang et al., 1997). The principal mode for regulation of CT is via an interconversion between the membrane (active) and cytoplasmic (inactive) forms (Tronchere et al., 1994), which is reminiscent of the two forms of plant enzyme phytoene desaturase (Al-Babili et al., 1996). The association with the membrane increases the affinity of the enzyme for CTP (Yang et al., 1995). Conceivably, the partitioning between soluble and membrane-bound forms mediated by the amphipathic region may regulate activity of VP14 in a similar fashion. Carotenoids in chloroplast membranes are bound to protein complexes (Green and Durnford, 1996). A carotenoid–protein complex isolated from spinach chloroplasts contains violaxanthin (Markwell et al., 1992), the substrate of ABA biosynthesis. It remains to be determined whether VP14 interacts specifically with carotenoid-binding protein complexes.

### Experimental procedures

#### Plasmid constructs

The efficiency of in vitro transcription and translation of VP14 was optimized by shortening the space between the translation start code and the SP6 promoter (Tranel et al., 1995). To remove the 5′ untranslated sequence of VP14, a fragment of the cDNA was amplified from the first ATG using a primer incorporating a BamHI site to facilitate the cloning (forward primer 5′-TGGGATCCATGGGAGGGTCTGCCCAGG, reverse primer 5′; the translation start codon is underlined). The resulting product was ligated into pGem-3Z (Promega) to place VP14 under the control of the SP6 promoter. This plasmid, named pSP6-VP14, was used to generate mutant VP14s. VP14-GGG and VP14-PPP, mutant VP14s that have an insertion of triple glycine or proline residues in the centre of the putative amphipathic α-helix, were created by ligating annealed oligonucleotides 5′-GGCCCCCCCCC-3′ and 5′-GGCAGGGGAGG-3′, respectively, into the Not I site in pSP6-VP14. VP14-Amp, a deletion of the amphipathic helix residues from Ala77 to Phe102, was generated by PCR amplification of sequence located 5′ of the amphipathic helix using primer 5′-CCACTTTCTCCGCGCCCGG and SP6 with template pSP6-VP14, then ligating this fragment into the Ball position at codons Val103 and Ala104 of VP14.

To generate chimeric constructs that fuse varying lengths of N-terminal sequence of VP14 to the N-terminus of bacterial glutathione S-transferase (GST), GST coding sequence was amplified from pGex-2T (Pharmacia) using PCR with primers 5′-TCATGTCCTCATATAGGT and 5′-GATCTAGACCGGGAGCT-3′ (the codon for the first Met is underlined). This GST fragment was digested with Xbal and cloned into pSP6-VP14 digested with Xbal, and a second restriction enzyme to remove varying amounts of VP14 C-terminal sequences. Ball was used for VP14(106)-GST and VP14(206)-GST, Thr111 for VP14(151)-GST, and PvuI for VP14(205)-GST. VP14(166)-GST was generated by PCR amplification of VP14 N-terminal sequences (primer 5′-CGGCCGATCCCGTCAAG-3′ and SP6) and ligating this fragment to GST. All the resulting plasmids were sequenced to confirm the reading frame and fusion junctions.

In vitro transcription and translation

Plasmids were linearized by digestion in the 3′ linker region with EcoRI, and the DNA purified by phenol/chloroform extraction. The in vitro transcription reaction was carried out as described by Cline et al. (1993). The mRNA was quantified and tested for in vitro translation efficiency in a wheat germ cell-free system or rabbit reticulocyte lysate system (Promega).

For a 50 μl translation reaction, 50 μl 1H-leucine (50 μCi, 3000 Ci mmol−1; NEN) was dried in a Speed-Vac, and 7 μl RNAase-free water, 36 μl rabbit reticulocyte lysate, 1 μl RNAase inhibitor (Promega), 1 μl amino acids mix (without leucine) and 5 μl mRNA (approximately 100 ng) were added. The reaction was incubated at 30°C for 1.5 h and kept on ice until use. Immediately before the import assay, translation product was diluted with an equal volume of 60 mM leucine in 2 × import buffer (IB) (1 × IB = 50 mM HEPES/KOH pH 8.0, 0.33 mM sorbitol).

In vitro protein import into pea chloroplasts

Chloroplasts were isolated from 9–10-day-old seedlings of pea (Laxton’s Progress 9) and the protein import assay was carried out as described previously (Cline et al., 1993). All the import reactions were carried at 25°C under light with 5 mM Mg-ATP except where ATP- and light-dependent import was tested. The unimported proteins were removed by washing the chloroplasts with import buffer, and the surface-adhering protein was removed by incubation with 0.1 mg ml−1 thermolysin on ice for 40 min. The chloroplasts were re-purified through a 35% Percoll cushion, and rinsed twice with 1 × import buffer containing 5 mM...
EDTA. The chloroplasts were lysed by suspension in a hypertonic solution containing 20 mM EDTA.

For sub-fractionation of membrane and soluble compartments, chloroplasts were lysed in 10 mM HEPES-KOH (pH 8.0) containing 10 mM MgCl₂ on ice for 5–10 min. Complete lysis was monitored by using 39% Percoll cushion centrifugation which separates the unlysed chloroplasts from the lysed. The lysed chloroplasts were centrifuged at 3200 g for 8 min. The pellet fraction that contains thylakoids was rinsed twice with 1 x import buffer and centrifuged at 3200 g before lysis for protein analysis. The supernatant fraction was further centrifuged at 18 000 g for 1 h at 4°C to pellet the envelope membrane fraction; the supernatant fraction was collected as the stroma fraction. Sub-fractions of the envelope membranes and thylakoids were lysed in 20 mM EDTA. The gel was treated with DMSO and 20% 2,5-diphenyloxazole (PPO) in DMSO, then dried. The radioactive protein was detected by fluorography (Cline et al., 1993).

For the binding strength assay of VP14 to thylakoid membrane, purified thylakoids (100 µg chlorophyll) after import were incubated with 1 ml 6 M urea or 0.1 M Na₂CO₃ (pH 11.5) on ice for 40 min. The thylakoids were recovered by centrifugation at 30 000 g and washed twice with import buffer for 10 min on ice for each time.

**Determination of radioactivity in the gel**

After fluorography of the dry gel, bands of interest were cut out and dissolved in 1.1 ml of 80% TS-2 (Research Products International Corp.) at 50°C for at least 8 h. This solution was mixed with 10 ml scintillation cocktail containing 49.5% toluene, 49.5% Triton X-100, 0.5% acetic acid, 0.6% PPO and 0.025% 1,4-bis(2-phenyloxazolyl)benzene (POPOP). The radioactivity was counted in a scintillation counter.

**Western blot analysis**

Maize chloroplasts were isolated from dehydrated leaves 10 days after germination and fractionated into three sub-fractions in the same way as in the import assay. The proteins were resolved in a 12.5% SDS–polyacrylamide gels (Laemmli, 1970). The gel was treated with DMSO and 20% 2,5-diphenyloxazole (PPO) in DMSO, then dried. The radioactive protein was detected by fluorography (Cline et al., 1993).

**Competitive binding of VP14 to thylakoids**

A truncated version of VP14 that did not contain the transit peptide was expressed in a GST fusion construct in JM109 according to manufacturer’s protocol (Pharmacia). The purified truncated VP14 was checked for purity by SDS–PAGE and its concentration was determined using Coomassie assay (Bradford, 1976). To check whether the truncated protein was enzymatically active, it was assayed for cleavage of 9-cis neoanthaxin as described by Schwartz et al. (1997). The competitive binding assays were carried out in a 150 µl volume of import buffer. Purified thylakoids containing 50 µg chlorophyll were incubated with different concentrations (0–160 µg/ml) of cold VP14 in the presence of estramet (equivalent to 50 µg chlorophyll) at 25°C for 30 min. Then the thylakoids were incubated under identical conditions for 30 min with the stromal fraction which contained ³H-labelled VP14 that had been imported and processed. The thylakoids were purified by centrifugation at 3200 g and washed three times with import buffer. The washed thylakoids were resuspended in 6 M urea and incubated on ice for 40 min. The thylakoids were repurified by centrifugation at 300 000 g for 30 min and washed again with import buffer. The ³H-VP14 bound to thylakoids was analysed by SDS–PAGE and fluorography (Cline et al., 1993).

**Acknowledgements**

We thank Xian-Yue Ma and Mike McCaffery for technical assistance, and Shan Wu for isolation of chloroplasts. This work was supported by a grant from the US Department of Energy to D.R.M. This paper is Florida Agricultural Experiment Station journal series number R-08258.

**References**


