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Use of chimaeric genes harbouring small subunit transit peptide sequences to study transport in chloroplasts

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A sequence coding for the transit peptide part of the precursor of the ribulose bisphosphate carboxylase small subunit can be used to construct chimaeric genes coding for easily detectable marker proteins. Chloroplasts of transgenic plants containing such chimaeric genes were found to contain the marker proteins. In addition, measurements of uptake by isolated chloroplasts of various precursor proteins, synthesized *in vitro*, demonstrated that most if not all of the transit peptide sequence was necessary and sufficient to obtain transport into chloroplasts. However, quantitative measurements of uptake indicated that high efficiency of transport required domains from both the transit peptide and the mature small subunit protein.

Introduction

The small (S) subunit of Rubisco is synthesized in the cytoplasm as a 20 kDa precursor protein, which is then imported into the chloroplast (Highfield & Ellis 1978; Grossman *et al.* 1980, 1982). The S subunit precursor contains a transit peptide extension at its amino-terminal end, which is removed during, or immediately after, import into the chloroplast (Dobberstein *et al.* 1977; Cashmore *et al.* 1978; Highfield & Ellis 1978). Several authors have described the precise amino acid sequences of the precursor protein coded by S subunit genes of different origin. A high degree of variation was found. Notwithstanding that, the precursor proteins of one species are taken up and processed by chloroplasts of other species (Coruzzi *et al.* 1984; Broglie *et al.* 1984; Mishkind *et al.* 1985). To study in detail the amino acid sequences specifically involved and important for the transfer of precursor proteins into chloroplasts, both *in vivo* and *in vitro*, chimaeric genes were constructed, linking the whole or subsegments of the transit peptide coding sequence of S subunits to DNA sequences coding for a foreign reporter protein. Initially two different fusion genes were constructed, one containing the transit peptide sequence merged directly to the neomycin phosphotransferase II coding sequence (nptII) by a linker molecule, providing the first amino acid derived from the mature small subunit (tp–nptII) (Van den Broeck *et al.* 1985). The other fusion was designed to test the assumption that, in addition to the transit peptide, the conserved region situated next to the proposed cleavage site in the amino-terminal part of the mature polypeptide would be required for efficient transport and processing. This region indeed shows a markedly higher degree of homology in a comparison of several small subunit polypeptides from different plant species (Broglie *et al.* 1983) and *Chlamydomonas* (Mishkind *et al.* 1985) than the transit peptide itself.

A DNA fragment containing the 5′ flanking region as well as the sequence coding for the transit peptide and for 23 amino-terminal amino acids of a small subunit structural gene from pea (Cashmore 1983) was fused to the nptII coding sequence (tp–ss–nptII) (Schreier *et al.*

1985). Both chimaeric genes were transformed with the help of *Agrobacterium tumefaciens* Ti plasmid vectors (Zambryski *et al.* 1983).

Transformed plantlets were regenerated and analysed for the presence in leaf tissue of neomycin phosphotransferase II (NPTII) activity (Reiss *et al.* 1984; Schreier *et al.* 1985). The translation products of both types of fusion (tp–nptII and tp–ss–nptII) were shown to be transported to the chloroplasts. Moreoever, both were apparently processed to yield either the NPTII protein (tp–nptII) or a fusion protein consisting of the amino-terminal end of the small subunit and NPTII (tp–ss–nptII) and found to be located in the stroma of the chloroplasts. The NPTII protein itself weakly associates with the chloroplast envelope, but cannot be translocated.

These findings indicate that the transit peptide alone is sufficient to facilitate the transport into and the processing of 'novel' precursor proteins in chloroplasts.

In vitro uptake

Since *in vivo* analysis reflects a steady-state situation and does not allow the detection of differences in the relative efficiency with which different fusion polypeptides can be translocated into chloroplasts, *in vitro* uptake experiments were used to make such a comparative study. Van den Broeck *et al.* (1985) used the *lac* UV5 promoter to express the tp–nptII precursor protein in *E. coli* and showed in crude extracts that this precursor protein was taken up *in vitro* by isolated chloroplasts. L. Szabo and A. Cashmore (personal communication) used the SP6 system to transcribe *in vitro* the wild-type S subunit precursor and the tp–nptII construct (Van den Broeck *et al.* 1985) and translated the resulting RNAs in a wheatgerm system. When they compared the efficiency of uptake of the TP–NPTII protein to the uptake of the wild-type precursor protein, it was found that the NPTII protein was imported very inefficiently by comparison with the S subunit protein.

In contrast, C. C. Wasmann (personal communication), who used the SP6 and wheatgerm system to study the *in vitro* uptake of the TP–SS–NPTII protein (this precursor protein links the transit peptide and 23 amino acid residues of the amino-terminal region of the S subunit to NPTII (Schreier *et al.* 1985), found that in this case the SS–NPTII protein was imported in the chloroplasts with an efficiency which was 60–80% of that observed for the wild-type S subunit. It is therefore obvious that domains within the mature part of the imported S subunit protein contribute to the efficiency of import, as suggested on theoretical grounds by Ellis (1984). This conclusion is further strengthened by the recent observation of K. Keegstra (Madison, Wisconsin, personal communication to A. Cashmore) that a deletion at the C-terminal end of SS also reduces the efficiency of *in vitro* uptake.

A further set of experiments was designed to study the role of various domains of the transit peptide for binding to the envelope and for import into chloroplasts. L. Szabo & A. Cashmore (personal communication) expressed *in vitro* a number of deletions, which gradually removed amino acid residues 6 to 59 of the amino-terminal end of the S subunit precursor and found that import got progressively worse, down to no import at all. Analogous experiments were performed by C. C. Wasmann (personal communication) with similar results. None of these deletions allowed us to differentiate between binding and import. In addition, however, these authors found that a deletion removing amino acid residues 42 to 57 from the S subunit transit peptide abolished import completely. Remarkably, an internal deletion removing amino acid

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residues 6 to 25 was found to bind and be imported far less well than a similar, but larger, deletion removing amino acid residues 6 to 29. Finally, and contrary to what might have been expected, the removal of the methionine residue at position 58 of precursor S subunit, which modifies the presumed cleavage site from Cys–Met to a Cys–Gly, did not significantly affect import and processing.

In vivo translocation and processing of fusion proteins with various S subunit transit peptide sequences

Two of the deletion mutants in the tp–ss–nptII or tp–nptII fusion genes were also transformed into plants and tested *in vivo* (M. Kuntz, unpublished). The first one contains a deletion of the two codons for cysteine and methionine at the junction between the transit peptide sequence and the sequence coding for the mature small subunit. Chloroplasts isolated from plant leaves showed the presence of a processed fusion protein in the stromal fraction. This observation indicates that the two amino acid residues at the junction between transit peptide sequence and mature small subunit do not have a decisive influence on processing. Indeed, no processing intermediate was visible in these experiments. One might have expected to see an intermediate, since the modification of the cysteine residue by iodoacetate (Robinson & Ellis 1984) showed the accumulation of such a processing intermediate *in vitro*.

A second mutant, which eliminates amino acid residues 42 to 57 of a subunit transit peptide sequence linked by three amino acid residues to the NPTII reporter protein, produced a fusion protein of molecular mass 34 kDa, which was apparently not processed and not imported into chloroplasts.

To study the possible role of transit peptide domains in the specific compartmentation of proteins imported in chloroplasts, M. Hand & A. Cashmore (personal communication) constructed chimaeric genes mixing transit peptide sequences from the S subunit gene with those of the chlorophyll *a/b* binding protein gene linked to a sequence coding for mature chlorophyll *a/b* binding protein. *In vitro* uptake studies indicated that this precursor protein was imported and that the chlorophyll *a/b* binding protein was membrane-associated.

Finally, P. Winter & R. Herrmann (personal communication) constructed chimaeric genes with the purpose of testing whether chloroplast-encoded proteins can also be taken up through chloroplast membranes *in vitro* and *in vivo*. The S subunit transit peptide sequence of pea was fused to an *Oenothera* chloroplast DNA fragment coding for the large subunit (LS) of Rubisco. This construct was expressed by the SP6–wheatgerm system and also transformed into *Oenothera* plants by Ti plasmid vectors. Uptake and processing was observed both *in vivo* and *in vitro*.

The results discussed here demonstrated that the mechanism underlying binding, import and processing of precursor proteins in chloroplasts is complex and involves domains from both the transit peptide and the mature protein. However, in several cases, transit peptide sequences linked to proteins that are not normally transported into chloroplasts were found to be able to direct the import of these proteins into chloroplasts both *in vivo* and *in vitro*.

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