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**In vivo import of plastocyanin and a fusion protein into developmentally different plastids of transgenic plants**

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Transgenic tomato plants that constitutively express a foreign plastocyanin gene were used to study protein transport in different tissues. Normally expression of endogenous plastocyanin genes in plants is restricted to photosynthetic tissues only, whereas this foreign plastocyanin protein is found to be present in all tissues examined. The protein is transported into the local plastids in these tissues and it is processed to the mature size. We conclude that plastids of developmentally different tissues are capable of importing precursor proteins that are normally not found in these tissues. Most likely such plastids, though functionally and morphologically differentiated, have similar or identical protein import mechanisms when compared to the chloroplasts in green tissue.

Key words: in vivo protein transport/transgenic tomato plants/non-leaf tissue

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**Introduction**

Due to the limited size of their genome, plastid functioning depends largely on nuclear encoded proteins imported from the cytoplasm. Such proteins are synthesized as larger precursors with an amino-terminal transit peptide and transported into the organelle in a post-translational way (Dobberstein et al., 1977). The transit peptide is cleaved off by a specific stromal processing protease (Robinson and Ellis, 1984) during or shortly after the translocation across the envelope membranes. This translocation is ATP dependent (Grossman et al., 1980; Pain and Blobel, 1987), whereas binding to the outer membrane can occur in the absence of ATP (Cline et al., 1985). Evidence for the presence of a receptor protein has been obtained recently (Pain et al., 1988). Protein import into chloroplasts has recently been reviewed extensively (Schmidt and Mishkind, 1986).

Proteins that function in the chloroplast lumen have to cross the thylakoid membrane in addition to the envelope membranes. In vitro experiments with the lumen protein, plastocyanin, show that this transport process occurs in two steps. First, the plastocyanin precursor is imported into the chloroplast stroma where part of the transit peptide is removed by a specific stromal protease. Next, this stromal intermediate is translocated across the thylakoid membrane into the lumen where it is processed to its mature size by a recently discovered thylakoid-associated processing enzyme (Hageman et al., 1986). The plastocyanin transit sequence, therefore, was proposed to consist of two distinct domains (Smeekens et al., 1986).

To study the in vivo protein-import mechanisms of plastids of photosynthetic and several non-photosynthetic tissues, we transformed tomato plants with the pre-plastocyanin coding region of *Silene pratensis* (white campion) fused to the cauliflower mosaic virus (CaMV) 35S promoter. This strong promoter constitutively directs the expression of the genes that are under its control (Odell et al., 1985; Sanders et al., 1987). A fusion gene, in which the coding sequence for the ferredoxin transit peptide is linked to the coding region of the plastocyanin mature protein (FDPC) (Smeekens et al., 1986), was also placed under the control of the 35S promoter. The targeting and processing of these precursor proteins in leaves, petals and roots of transformed plants was analysed and compared.

**Results**

**Vector construction and plant transformation**

The Silene plastocyanin gene (pPC) and the Silene ferredoxin/plastocyanin fusion gene (FDPC) were individually cloned behind the CaMV 35S promoter in the binary vector

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pBIN19 (Bevan, 1984) (Figure 1). The 35S promoter was isolated as an EcoRI–HindIII fragment from a 35S-cat vector described in detail by Morelli et al. (1985). The pPC gene was isolated from the vector pSPPC74 (Smeekens et al., 1986) by using the HindIII site in front of the gene and the EcoRI site behind the gene (both restriction sites are part of the original multiple cloning site with the plastocyanin sequence present in the Smal site). The FDPC fusion gene was also isolated as an HindIII–EcoRI fragment but from the vector pSFTP81 (Smeekens et al., 1986). The promoter was fused at the HindIII site with the pPC sequence or with the FDPC sequence and cloned in the EcoRI site of the vector pBIN19. The 35S promoter fragment contains all the 5′ sequences necessary for transcription (from −941 up to the transcription initiation site) and the first nine bases of the 35S RNA. The plastocyanin sequence contains part of the RNA leader, the complete coding region of the precursor and the 3′ end of the gene (Smeekens et al., 1985b, 1986). The FDPC fusion is similar to the plastocyanin construct, except that the plastocyanin transit peptide part and the 5′ untranslated region is replaced by that of ferredoxin (Smeekens et al., 1985a, 1986).

Tomato plants were transformed with the constructs as described in Materials and methods. Individual regenerated plants transformed with pPC received a number between 200 and 299 and regenerated plants transformed with FDPC received a number between 300 and 399.

Expression of transformed genes

Transcription of the Silene genes in transformed tomato plants was measured by Northern blot analysis (Figure 2). Total RNA isolated from leaf tissue was probed with the transit peptide sequence of the Silene plastocyanin or ferredoxin gene. The hybridization conditions were such (see Materials and methods) that no background hybridization with endogenous plastocyanin RNA was observed. Hybridization of the probe with RNAs of several transformed tomato plants shows that the introduced constructs are transcribed. The hybridizing RNA is comparable in length with normal (Silene) PC and FD RNA, indicating that the RNA was properly initiated and terminated. The observed differences in the expression level of the constructs between the different transformed plants probably resulted both from the number of copies integrated and from position effects.

The above plants were analysed for the presence of the Silene plastocyanin protein (Figure 3). We were able to discriminate between the Silene and the tomato plastocyanin protein on a Laemmli system due to differences in electrophoretic mobility, which most likely result from minor differences in the amino acid compositions. Figure 3 shows that the same plants that express the foreign plastocyanin genes on the level of RNA synthesize Silene-plastocyanin-specific protein in addition to endogenous tomato plastocyanin protein. In some plants the Silene protein accumulated to the same level as that found in Silene leaves, e.g. A202a, A204a and A205a. The amount of the endogenous tomato plastocyanin seems not be affected by the Silene protein in the transformed plants. Phenotypic effects, like an altered leaf morphology, a different growth response or a different protein pattern on Coomasie-stained gels, that might have been caused by the expression of the Silene plastocyanin or the FDPC fusion protein (see below) were not observed.

The Silene plastocyanin that is present in transgenic tomato leaf tissue comigrates with control mature plastocyanin from Silene leaves (Figure 3a), therefore their molecular weights must be comparable. Experiments with Silene plastocyanin synthesized in vitro showed that precursor, intermediate and mature plastocyanin can clearly be separated on this gel system (Hageman et al., 1986; Smeekens et al., 1986). The Silene protein is therefore processed to its mature size and considered to be transported into the chloroplast.

Tomato plants transformed with the FDPC fusion protein only show a faint band at the position of the Silene protein (Figure 3b), even though the corresponding expression at the RNA level was comparable to that of plastocyanin (Figure 2b). One explanation for this difference in the level of expression could be that this hybrid protein is degraded very rapidly in the cytoplasm due to a possibly aberrant conformation. This would result in a low amount of protein reaching the chloroplast. The FDPC fusion protein migrates with approximately the same velocity as mature Silene plastocyanin (Figure 2). This protein is therefore also
processed to its mature size and most likely transported into the chloroplast.

Analysis of protein transport in leaf tissue
To determine whether the Silene mature plastocyanin protein is indeed present inside the chloroplasts, tomato chloroplasts were isolated and protease treated (Figure 4a). This analysis shows that both Silene plastocyanin and tomato plastocyanin are protected against degradation. We conclude that both proteins are present inside the chloroplast. Further analysis of the chloroplast by fractionation experiments combined with protease treatment of the isolated thylakoids shows that both Silene and tomato plastocyanin were protected against degradation (Figure 4b). The activity of the protease was monitored by the characteristic shift to a lower molecular weight of the light harvesting complex protein on Coomassie-stained gels (not shown). Both plastocyanins could be released from the thylakoids after breaking them by freezing and thawing followed by sonication. The released proteins were isolated by pelleting the thylakoidal membranes (Figure 4b, lanes tl and tm).

We also analysed leaf tissue with transmission electron microscopy in combination with immunogold labelling. Although the plastocyanin antibodies react with both foreign and endogenous plastocyanin, the amount of foreign protein was high enough (Figure 3a) to reveal a possibly aberrant transport. However all gold label was found inside the chloroplast where it was associated with the thylakoid membrane system only. No significant label was observed in the chloroplast stroma, other organelles or the cytoplasm (data not shown). These combined data show that the foreign Silene plastocyanin is properly routed to the tomato chloroplast where it is found in the thylakoid lumen.

A similar analysis as described above for the plastocyanin localization was performed for tomato plants transformed with the FDPC fusion gene. In in vitro experiments with isolated pea chloroplasts this protein was transported to the chloroplast stroma only (Smeekens et al., 1986). Surprisingly, the mature plastocyanin of the FDPC fusion was found in the thylakoid lumen by all criteria applied above, again in this respect indistinguishable from the endogenous tomato plastocyanin (Figure 4a,b). Therefore this fusion protein is imported into the chloroplast and ends up in the thylakoid lumen despite the absence of a proper thylakoid transfer domain in the transit sequence, which was found to be essential for in vitro thylakoid membrane transfer (Smeekens et al., 1986; J.Hageman et al., in preparation).

Analysis of protein transport in petals and root
The transgenic plants that constitutively synthesize plastocyanin in all tissues allowed us to probe the plastids of petals and roots for their transport properties and in this way to analyse the effect of tissue differentiation on the import mechanism. Western blots of total protein isolated from petals and roots of non-transformed tomato plants (Figure 5a,b) shows that tomato plastocyanin is only weakly, if at all expressed in these tissues (the low level of plastocyanin expression in petals probably results from the veins that are still slightly green). Tomato plants transformed with the Silene plastocyanin precursor gene show a high expression of the new protein in petals and a somewhat lower expression in roots. In both tissues the protein has the same apparent molecular weight as the mature protein from Silene leaves and therefore processing of the precursor to the mature size must have occurred. This indicates that the protein is transported into the plastids present in these tissues. The localization of the processed protein in petal tissue was confirmed with electron microscopy (Figure 6). This analysis shows that the majority of the plastocyanin-specific label is located inside the chromoplasts whereas no significant labelling could be detected in chromoplasts of non-transformed tomato petals.

The FDPC fusion protein though not visible on the Western blots shown in Figure 5a and b can be detected in very low amounts when a larger quantity of total protein is electrophoresed (not shown). This protein is most likely also transported into chromoplasts and root plastids, where it is then processed to its mature size. The amount of protein
expressed in these tissues was too low to be detected with electron microscopy.

Discussion

The in vivo analysis of plastocyanin transport in transgenic tomato plants shows that the Silene plastocyanin precursor is expressed in different tissues and that subsequently it is imported into the plastids of these tissues.

In leaf tissue the Silene plastocyanin protein behaves just as the endogenous tomato protein. It is targeted towards the chloroplast, where it is found exclusively in the lumen as the mature-sized protein. This clearly demonstrates that the targeting information present in the Silene plastocyanin precursor is fully recognized and used by the protein translocation mechanism of the tomato chloroplasts.

The FDPC fusion protein is also transported into the chloroplast and mature plastocyanin is found in the thylakoid lumen. This result differs from the in vitro results with intact pea chloroplasts that were obtained with the same fusion protein (Smeekens et al., 1986). The reason for this difference is not clear. It is possible, however, that the mature protein also contains information for uptake into the lumen. This is in line with observations that plastocyanin transit by itself is not capable of directing foreign proteins to the thylakoid lumen in vitro (Smeekens et al., 1987; unpublished results). The absence of in vitro accumulation of the mature protein can be explained by the limited time of an in vitro import experiment as compared with the in vivo situation. At present we are testing whether information for thylakoid transport is indeed present in the mature plastocyanin protein.

In non-green tissue endogenous tomato plastocyanin is not expressed as has been found for many proteins active in photosynthesis (Coruzzi et al., 1984; Lamppa et al., 1985). In petals and roots of transformed tomato plants the Silene protein is found processed to its mature size and therefore it is most likely transported into the local plastids. The presence of the Silene protein in chromoplasts of petal tissue was confirmed using electron microscopy in combination with immunogold labelling. The plastids in non-green tissue are related to chloroplasts, but they become functionally and morphologically distinct during development (Thomson and Whatley, 1980). In all types of plastids examined the plastocyanin found is present in its mature processed form. Previous in vitro experiments have shown that the processing enzyme required for complete maturation of plastocyanin in the chloroplast resides in the thylakoids, from which it can be liberated by Triton X-100 treatment (Hageman et al., 1986). In this respect processing beyond the intermediate form in plastids other than chloroplasts is surprising, because no thylakoid-like structures were observed by us. The presence and location of the second processing enzyme is currently under investigation.

Processing in non-green tissue was also observed in tomato plants transformed with the FDPC fusion protein, although the amount of protein detected was much lower. This protein is likely to be transported into the local plastids also. Similar results were obtained when tomato plants transformed with the intact precursor gene of ferredoxin or tobacco plants transformed with the intact plastocyanin gene were used instead (unpublished results).

Experiments with isolated pea etioplasts have shown that these organelles are capable of importing and processing of the small subunit precursor of ribulosebisphosphate carboxylase (Schindler and Soll, 1986).
Our results show that chloroplasts and leucoplasts can import proteins specific for the chloroplast. We conclude that the import mechanism of different plastid types is very similar or even identical. Although chloroplast proteins are not normally expressed in non-leaf tissue, they are nevertheless recognized, imported and processed by the plastids present in these tissues. Also, it is most likely that other proteins can be imported into plastids of non-green tissues when fused to a chloroplast transit peptide and expressed under the control of an appropriate promoter. The observation that chloroplast proteins are imported by other plastids as well suggests that the number of different receptors found on a plastid is limited. It is unlikely that plastids contain specific receptors for the uptake of proteins that are not needed.

Materials and methods

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Maniatis et al., 1982).

Plant transformation

pBIN19 constructs were conjugated to Agrobacterium tumefaciens LBA4404 containing the modified Ti-plasmid pAL4404 (Hoekema et al., 1983) in a triparental mating event using the helper plasmid pRK2013 (Lam et al., 1985). All constructs were introduced into the hybrid tomato strain MsK93 using the leaf disc transformation method (Horsch et al., 1985; Koorneef et al., 1986). Transformed calli were regenerated and selected for kanamycin resistance (Koorneef et al., 1986).

RNA analysis

RNA was isolated from plant tissue using guanidine thiocyanate extraction (Chomczynski, 1997) after grinding the tissue in liquid nitrogen. RNA concentrations were determined by measuring the absorbance at 260 nm. RNA isolated from leaf tissue was electrophoretically separated on a 1.5% formaldehyde agarose gel system (Meinkoth and Wahl, 1984) and subsequently blotted onto Hybond-N filters (Amersham). DNA fragments were radioactively labelled using random calf thymus primers (Feinberg and Vogelstein, 1983). Hybridization was performed at 42°C for 20 h in 50% formamide, 5× SSC, 5× Denhardt’s, 25 mM sodium phosphate pH 7.5, 0.1% SDS and 100 μg/ml denatured DNA. The filter was subsequently washed at room temperature in 1× SSC and 0.1% SDS. Probes were removed by boiling several times in 0.1× SSC and 0.1% SDS.

Protein analysis

Protein was isolated by grinding plant tissue in liquid nitrogen and extracting the powder with 30 mM potassium phosphate pH 7.5, 400 mM NaCl, 2 mM 2-mercaptoethanol and 1% Triton X-100. The amount of protein was determined by the method of Bradford (1976). Samples were electrophoretically separated on a 15% SDS polyacrylamide gel system (Laemmli and Favie, 1973) and blotted onto a nitrocellulose membrane after which plastocyanin was specifically immunostained (Towbin et al., 1979) using antibodies raised against spinach plastocyanin (Smeekens et al., 1985b) and a second antibody coupled to a peroxidase with 3,3',5,5'-tetramethylbenzidine as a substrate.

Chloroplast isolation and fractionation

Chloroplasts were isolated from tomato leaves as described for pea leaves (Cline et al., 1985). Chloroplasts were incubated with or without 0.1 mg/ml thermoslysin for 30 min at 4°C. The reaction was stopped by adding EDTA to 10 mM and re-isolation of the intact chloroplasts by centrifugation through a 40% Percoll cushion (Cline et al., 1985). Chloroplasts were lysed in 50 mM Hepes pH 8 and thylakoids were pelleted by centrifugation (10 min at 30000 g) and washed once. Thylakoids were incubated with or without 0.4 mg/ml thermoslysin for 30 min at 4°C and the reaction was stopped by adding EDTA to 10 mM and pelleting the thylakoids. Thylakoids were opened by freezing and thawing followed by sonication. Thylakoid membranes were pelleted by centrifugation (30 min 40000 g). Protein was isolated from chloroplast fractions by dilution with an equal amount of two times sample buffer and boiling for 5 min. Laemmli and Favie, 1973). The amount of protein was determined on intact chloroplast samples by the method of Bradford (1976) and the amount of fractionated chloroplast proteins loaded on a protein gel was comparable to 20 μg of total chloroplast protein.

Transmission electron microscopical analysis

Ultrathin cryosections (Boonstra et al., 1985) of leaf or petal tissue of a tomato plant transformed with the Silene plastocyanin gene (AZ05a) were incubated with antibodies against spinach plastocyanin (Smeekens et al., 1985b) and labelled with 7 nm protein-A gold particles.

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