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Published in:
Gene

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1988

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Boer, D. D., Cremers, F., Teertstra, R., Smits, L., Hille, J., Smeekens, S., & Weisbeek, P. (1988). In vivo import of plastocyanin and a fusion protein into developmentally different plastids of transgenic plants. *Gene*, 65(1).

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

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Communicated by L.L.M.van Deenen

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

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 (Smeeckens *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) (Smeeckens *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

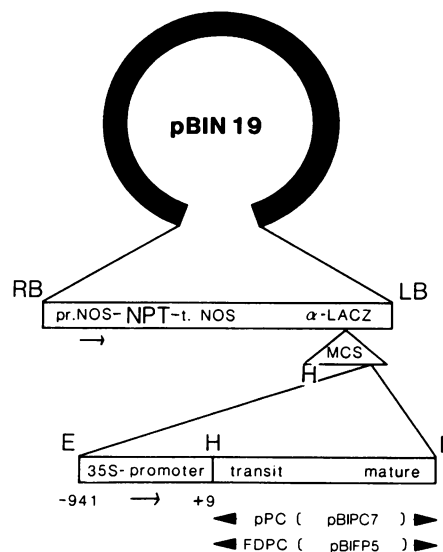


Fig. 1. pBIN19 constructs used to transform tomato plants. pBIPC7 represents the pBIN19 construct with the 35S promoter in front of the plastocyanin precursor gene and pBIFP5 represents a similar construct with the FDPC fusion gene instead of the plastocyanin gene. LB and RB indicate left and right borders of T-DNA, respectively. pr.NOS, nopaline synthetase promoter; t.NOS, nopaline synthetase terminator; NPT, neomycin phosphotransferase gene; MCS, multiple cloning site; α LACZ, α complementary region of the β -galactosidase gene; E, *EcoRI*; H, *HindIII*.

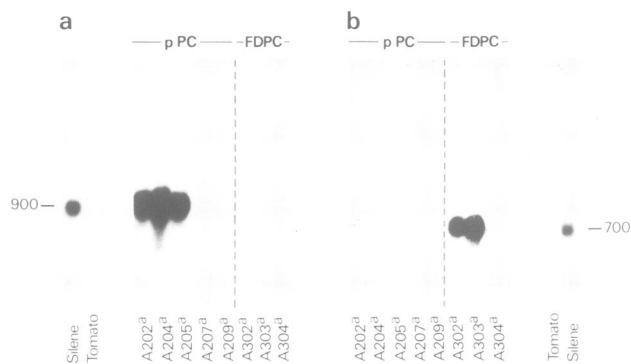


Fig. 2. Northern blot of leaf RNA. (a) 40 μ g samples of total RNA isolated from tomato plants transformed with the pPC or FDPC constructs were used and probed with a *HindIII*–*NcoI* fragment from pSPPC74 (Smeekens *et al.*, 1986) containing the transit sequence of the Silene pPC gene. RNA isolated from Silene and non-transformed tomato leaf tissue was used as positive and negative control respectively. (b) The same blot as described in (a) was used, but after removal of the first probe reprobed with a *HindIII*–*BstEII* fragment from pSPFD22 (Smeekens *et al.*, 1986) containing the transit sequence of the Silene ferredoxin gene.

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 *SmaI* 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

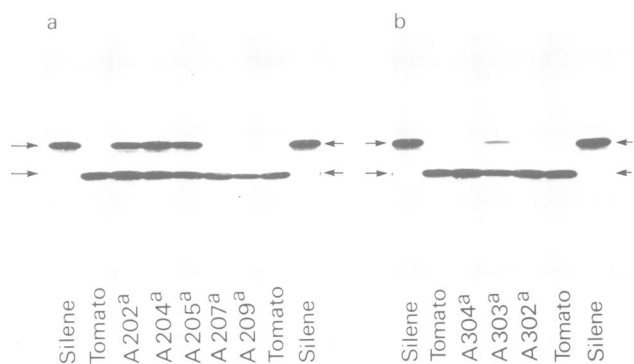


Fig. 3. Western blot of protein isolated from leaf tissue. (a) 20 μ g of total protein isolated from tomato plants transformed with the pPC construct were used. Protein isolated from leaf tissue of Silene and non-transformed tomato was used as control. The blot was reacted with antibodies raised against spinach plastocyanin (Smeekens *et al.*, 1985b). Arrows point at the position of mature Silene and mature tomato plastocyanin. (b) 20 μ g of total protein isolated from plants transformed with the FDPC construct were used. Arrows, controls and antibodies are the same as in (a).

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 Coomassie-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

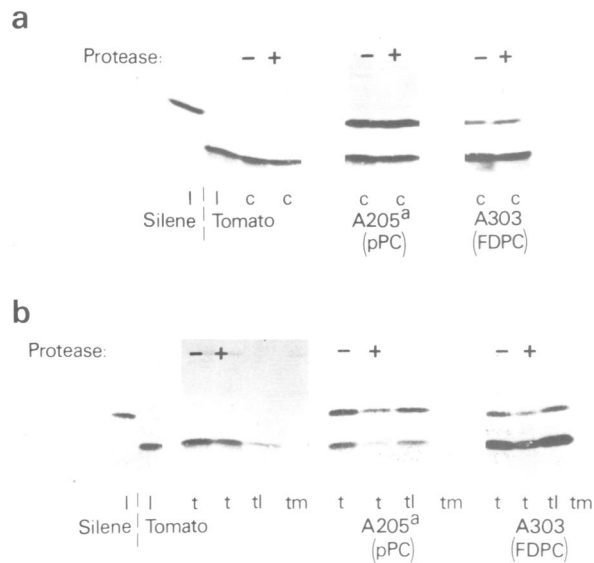


Fig. 4. Localization of Silene proteins in green tissue of tomato plants. (a) Western blot of 20- μ g samples of total protein isolated from chloroplasts treated with or without a protease. Protein isolated from Silene and non-transformed tomato leaves was used as a control. l, leaf protein; c, chloroplast protein. The blot was reacted with antibodies raised against spinach plastocyanin (Smeekens *et al.*, 1985b). (b) Western blot of protein isolated from fractionated chloroplasts. The amount of protein from the different fractions is directly comparable to 20 μ g total chloroplast protein used in (a). l, leaf protein; t, thylakoid protein; tl, lumen protein; tm, thylakoid membrane protein. The antibodies used were the same as in (a).

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.

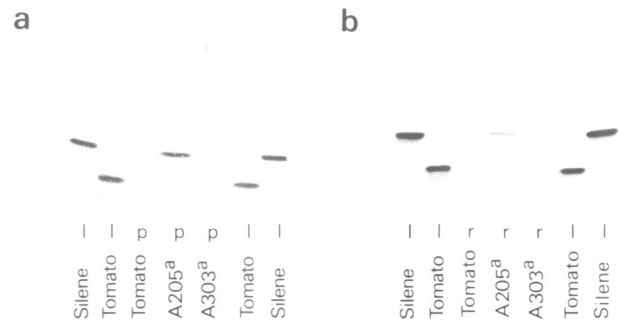


Fig. 5. Detection of Silene proteins in non-green tissue. (a) Western blot of 20- μ g samples of total protein isolated from petal tissue. Silene and non-transformed tomato leaf proteins were used as controls. l, leaf protein; p, petal protein. (b) Western blot of 20- μ g samples of total protein isolated from root tissue. Controls were the same as in (a). l, leaf protein; r, root protein.

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 labelling 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

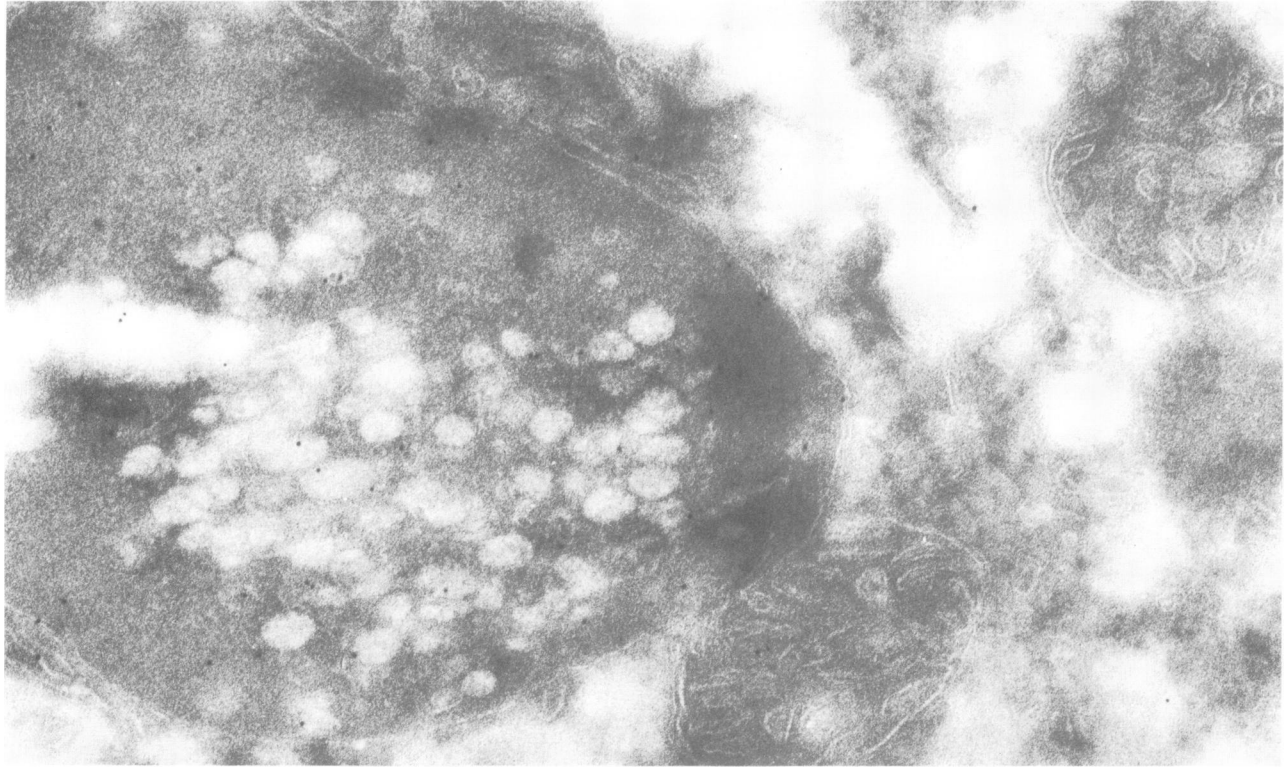


Fig. 6. Transmission electron microscopical analysis of the localization of the Silene plastocyanin in petal tissue of transformed tomato plants. Petal tissue of a tomato plant (A205a) transformed with the Silene plastocyanin gene was used with antibodies raised against spinach plastocyanin. Magnification $\times 72\,000$.

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 the 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 ribulosebiphosphate carboxylase (Schindler and Soll, 1986).

Our results show that chromoplasts 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 (Chirgwin, 1979) 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 (Berger 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. Probe was 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 thermolysine 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 4000 g) and washed once. Thylakoids were incubated with or without 0.4 mg/ml thermolysine 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 40 000 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 (A205a) were incubated with antibodies against spinach plastocyanin (Smeekens *et al.*, 1985b) and labelled with 7 nm protein-A gold particles.

Acknowledgements

We thank Dr Nam-Hai Chua for providing us with the CaMV 35S promoter plasmid, Dr Paul Hooykaas for making pBIN19 available to us and Mr Harry Bettenbroek for technical assistance during the initial parts of this work. This work was supported in part by the Netherlands Organization for Advancement of Pure Science (SON/ZWO).

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Received on March 11, 1988; revised on May 27, 1988