Description

The invention relates to means, particular recombinant vectors, and to processes for the controlled introduction of foreign gene products in plant chloroplasts.

The disclosure which follows contains reference numbers in the form of exponents. They refer to bibliographic references relative to literature referred to at the end of this specification. Other literature is also referred to in the course of this description by the name of the first author and date of publications. All the articles as well as the patent applications, patents, etc., which shall be referred to throughout this specification are incorporated herein by references.

It is well known that the cells of eukaryotic organisms, and more particularly plant cells, contain distinct subcellular compartments, or organelles, delimited by characteristic membrane systems and performing specialized functions within the cell. In photosynthetic leaf cells of higher plants the most conspicuous organelles are the chloroplasts, which exist in a semi-autonomous fashion within the cell, containing their own genetic system and protein synthesis machinery, but relying upon a close cooperation with the nucleo-cytoplasmic system in their development and biosynthetic activities[1].

The most essential function of chloroplasts is the performance of the light-driven reactions of photosynthesis. But chloroplasts also carry out many other biosynthetic processes of importance to the plant cell. For example, all of the cell's fatty acids are made by enzymes located in the chloroplast stroma, using the ATP, NADPH, and carbohydrates readily available there. Moreover, the reducing power of light activated electrons drives the reduction of nitrite (NO^{-}_{2}) to ammonia (NH_3) in the chloroplast ; this ammonia provides the plant with nitrogen required for the synthesis of aminoacids and nucleotides.

The chloroplast also takes part in processes of particular concerns to the agrochemical industry.

Particularly it is known that many herbicides act by blocking functions which are performed within the chloroplast. Recent studies have identified the specific target of several herbicides. For instance, triazine derived herbicides inhibit photosynthesis by displacing a plastoquinone molecule from its binding site in the 32 Kd polypeptide of the photosystem II. This 32 Kd polypeptide is encoded for in the chloroplast genome and synthesized by the organelle machinery. Mutant plants have been obtained which are resistant to triazine herbicides. These plants contain a mutant 32 Kd protein from which the plastoquinone cannot longer be displaced by triazine herbicides.

Several other herbicides are known to block specific steps in aminoacid synthesis. Sulfonyl-urea are known to inhibit acetolactate synthase. This enzyme is involved in isoleucine and valine synthesis. Glyphosate inhibits the function of 5-enol pyruvyl-3-phosphoshikimate synthase, which is an enzyme involved in the synthesis of aromatic aminoacids. All these enzymes are encoded by the nuclear genome, but they are translocated into the chloroplast where the actual aminoacid synthesis takes place.

Enzymes responsible for the same functions are also present in prokaryotes. It should be easy to obtain bacterial mutants in which the enzyme of interest is no longer sensitive to the herbicide. Such a strategy was used with success to isolate Salmonella typhimurium mutants with an altered aro A gene product, which confers resistance to glyphosate (Comai et al Science 221, 370 (1983).

Thus the use of chloroplastic or bacterial genes to confer herbicide resistance to plant cells could be successfull if their gene products were efficiently transported into the chloroplast where they function.

Chloroplasts are also involved in the complex mechanisms which regulate the levels of aminoacid synthesis. One of the most important regulatory mechanisms is the so-called retroregulation. This mechanism involves the inhibition of the key enzyme of a given pathway by the end product(s) of this pathway. When a key enzyme is no longer subjected to such regulation the organism overproduces the corresponding end product (e.g. an aminoacid).

Isolation of mutant genes encoding for enzymes that are insensitive to inhibition by the corresponding end product is well documented in bacteria. Similar mutants in plant cells are difficult to obtain and only a few examples have been reported. Furthermore the isolation of genes from plant cells is a very complex task when compared to the isolation of bacterial genes.

As mentioned earlier, most aminoacid synthesis takes place inside the chloroplast.

Thus there is a great interest for the development of a technique for transforming plant cells with bacterial genes encoding an enzyme insensitive to inhibition by the abovesaid end product in a way such that the result of this transformation process would be the introduction of said enzyme in the plant chloroplasts. The ultimate result of this process would be an over-production of aminoacid.

These are but a few examples (additional examples will be mentioned later) of the prospects of considerable development of plant genetic engineering which will be at hand of the specialists as soon as practical techniques suitable for the introduction of determined foreign polypeptides or proteins in chloroplasts will become available.

Indeed many techniques have been proposed for the transfer of DNA to plants such as direct DNA uptake, micro-injection of pure DNA and the use of viral or plasmid vectors. Plasmid vectors which have proven particularly efficient are those derived from tumor-inducing (Ti) plasmids of the microorganism Agrobacterium tumefaciens which is the agent of crown gall disease in dicotyledonous plants. Those plasmids can be modified by removal the tumor-causing genes from their T-DNA. The so modified plasmids then no longer interfer with normal plant growth and differentiation and, upon insertion of a determined foreign gene at an appropriate site of said plasmids, can be used for promoting the expression of the protein encoded by said determined gene in plant cells. Particularly, the foreign gene may be inserted close to one of the border sequences or between the two border sequences which surround the T-DNA in intact Ti-plasmids. Reference can be made by way of examples to the articles of :

- A. CAPLAN et al. titled "Introduction of Genetic Material into Plant Cells", Science, 18 November 1983, volume 222, pp. 815-821 ;
- L. HERRERA-ESTRELLA et al. titled "Expression of chimaeric genes transferred into plant genes using a Ti-plasmid-derived vectors", Nature, vol. 303, No. 5914, pp. 209-213, 19 May 1983 ;
- L. HERRERA-ESTRELLA et al., titled "Light-inducible and chloroplast associated expression of a chimaeric gene introduced into Nicotiana tabacum using a Ti-plasmid vector", Nature, vol. 310, n° 5973, pp. 115-120, 12 July 1984 ;
- or to European Patent Application N° 0 116 718 (or to US Application n° 570,646) or to the International Application WO 84/02913 published under the PCT ; all of these articles or patent applications being incorporated herein by reference.

Yet all of these techniques do not provide for an efficient and relatively easy method of transformation of chloroplasts, despite the considerable work which has been devoted to the subject and the already large amount of knowledge which has been acquired, particularly concerning the production of proteins in plant cells and transfer into the chloroplasts. As a matter of fact vectors for the direct transformation of chloroplasts are unavailable at this time. Furthermore mature proteins, including those normally encoded in the plant cells including said chloroplasts and ultimately isolatable naturally from said chloroplasts, cannot as such be caused to penetrate in the chloroplasts if supplied thereto from outside.

Most chloroplast proteins are coded for in the nuclear DNA and are the products of protein synthesis on cytoplasmic ribosomes, many as soluble higher molecular weight precursors[2-9]. These precursors are then translocated through either one or both of the plastid envelope membranes, processed, and assembled into their final organellar compartment or holoenzyme complex. In vitro reconstitution experiments using isolated chloroplasts, have demonstrated that the uptake and processing of over a hundred nuclear-encoded, cytoplasmically synthesized precursors by chloroplasts occurs by an energy-dependent[17], post-translational mechanism[6], [10-17].

The most extensively characterized of these nuclear-encoded chloroplast proteins is the small subunit of ribulose-1,5-bisphosphate (RuBP) carboxylase. This polypeptide is synthesized on free cytoplasmic ribosomes as a precursor of 20,000 daltons containing an amino terminal extension or transit peptide of approximately 5-6,000 daltons [6-7], [9]. During or immediately after import of the precursor into the chloroplast, the transit peptide is proteolytically removed in two steps by a soluble protease[18], yielding the mature small subunit polypeptide of 15,000 daltons. This polypeptide is then assembled with endogenous large subunit into the functional RuBP carboxylase holoenzyme[11,12].

Similar observations were made with the chlorophyl a/b binding proteins. These polypeptides are synthesized as soluble precursors on cytoplasmid ribosomes (Apel and Kloppstech, 1978 ; Schmidt et al., 1981) and are post-translationally translocated into chloroplats. During or after translocation the NH_2-terminal transit peptides are proteolytically cleaved (Schmidt et al., 1981) to yield the mature polypeptides. The mature A and B polypeptides associated with chlorophyl a and b are integrated into the thylacoid membrane. The transit peptides of post-translationally transported chloroplast proteins are characterized by a preponderance of basic aminoacids, a feature which has been proposed as important in the interaction of the transit peptide with the chloroplast envelope[19]. Comparison of transit peptides of small sub-unit precursors form various plant species show a variation in aminoacid sequence, but a relatively strong conservation in the position of prolines and charged aminoacid residues[20-22] and a substantial homology in a region surrounding the cleavage site of the precursors, as observed in soybean (Berry-Lowe et al.; 1982) pea (Cashmore, 1983), duck weed (Stiekema et al., 1983) and wheat (Broglie et al. ; 1983). These common properties may be of functional significance since both in vitro[11-12] and in vivo[23], the small subunit precursors from one plant species can be imported and correctly processed by the chloroplasts of others and vice-versa.

The molecular basis of how the post-translational translocation of polypeptides into chloroplasts occurs and which signals are involved in this process, more particularly the relative contributions of the transit

peptide and the mature protein to the uptake and processing mechanism are still not fully understood, even though it was already presumed that the transit peptide is required for the translocation of the mature protein. Consistent with this is the observation that the mature small subunit protein is not translocated into chloroplasts[24].

The invention stems from several discoveries which have been made by Applicants, as a result of further studies of the translocation mechanisms through the chloroplast membranes of chloroplast-protein precursors encoded by the nuclear DNA of plant cells. It seems that no cytoplasmic factor is required for the translocation mechanism itself as a result of further studies carried out on RuBP.

Further it has been found that all the sequence information required for translocation and transport of the mature protein or of a subunit thereof through the chloroplast membranes seems to reside within the precursor subunits and even within the sole transit peptides normally associated therewith. It further appeared that transit peptides not only mediate translocation, but also include information necessary for site-specific processing of the corresponding proteins.

These different properties of the transit peptides are useful in recombinant DNAs, particularly recombinant vectors including a DNA sequence coding for a determined protein or polypeptide, particularly a foreign protein, sought to be introduced and processed in chloroplasts, as well as in processes for the introduction of such a foreign polypeptide or protein into the chloroplasts, for instance in the tylacoid membranes or, preferably, in the stroma thereof. As a matter of fact, an essential element of such recombinant vectors consists of a DNA sequence coding for a transit peptide, it being understood that this expression, as used throughout this specification and claims attached thereto, designates the aminoacid sequence contained in any chloroplast protein precursor which, upon import of the precursor, is proteolytically removed during or immediately after import of the precursor into the chloroplast to yield either the corresponding functional mature protein or a subunit thereof, particularly when, like in the case of RuBP, the final processing of the mature protein takes place within the chloroplast. Such final processing comprises for instance the assembling of said subunit with another endogenous subunit to yield the final functional protein.

In accordance with this invention, a process for providing a foreign protein or polypeptide in a chloroplast of a cell of a plant comprises the steps of:

A) expressing, in the cytoplasm of said cell, a chimaeric precursor of said foreign protein or polypeptide; said precursor being encoded by a chimaeric DNA sequence in the genome of said cell; said chimaeric DNA sequence comprising: i) a first nucleic acid sequence that codes for a transit peptide of a cytoplasmic precursor of a chloroplast protein or polypeptide of a plant species and ii) a second nucleic acid sequence that codes for said foreign protein or polypeptide, that is heterologous to said first nucleic acid sequence and that is downstream of, and in the same transcriptional unit as, said first nucleic acid sequence; and then B) transporting said foreign protein or polypeptide from the cytoplasm of said cell into said chloroplast, with removal of said transit peptide from said foreign protein or polypeptide.

Also in accordance with the invention are provided: plant cells, plant cell cultures and plants in which the process of the invention has been carried out; and seeds of such plants.

The recombinant DNA according to the invention which can be introduced into plant cells is characterized by the presence therein of a chimaeric gene comprising a first nucleic acid and a second nucleic acid recombined with each other, said first nucleic acid and said second nucleic acid being of different origins, particularly being foreign to each other, wherein said first nucleic acid contains a first coding sequence which has essential sequence homology with a natural gene sequence coding for a transit peptide belonging to a precursor comprising at least the N-terminal subunit of a chloroplast protein capable of being transported into or processed within the chloroplast of said plant cells and wherein said second nucleic acid contains a second coding sequence distinct of the gene sequence coding for said chloroplast protein or chloroplast protein sub-unit, said second nucleic acid being located downstream of said first nucleic acid in the direction of the transcription of their first and second sequences respectively.

In joint efforts of the inventors to solve the problem sought, i.e. of providing methods and means for transporting a protein into chloroplasts, the inventors have devised two main approaches which have in common the use of DNA recombinants all including a sequence coding for a transit peptide. These two approaches resulted in the two preferred embodiments which will be examplified hereafter and which both proved to be effective.

Example I is illustrative of the first approach which took into account the possibility that a larger part of the nuclear genes, including the entire region of high homology around the cleavage site of the precursors would be a necessary requirement for transport and processing of proteins, particularly foreign proteins into chloroplasts. This resulted in a first series of preferred recombinant DNAs of this invention more particularly characterized in that the first nucleic acid as defined hereabove contains a third sequence corresponding to

at least part of a nucleic acid encoding the N-terminal cytoplasmic subunit of a chloroplast protein downstream of said first sequence and in that the extremity of said third sequence is directly contiguous to substantially with the extremity of said first nucleic acid.

Preferably the third sequence does not extend beyond the nucleotides encoding the C-terminal extremity of the cytoplasmic subunit of said chloroplast protein, yet comprises an intron, particularly that which initially belonged to the same gene as the exons encoding the peptidic portions which will ultimately provide the precursor subunit of the corresponding natural chloroplast protein.

It will be seen that a preferred embodiment of a DNA recombinant corresponding to the abovesaid first approach included a first sequence encoding the transit peptide and a third sequence which initially belonged to the same gene and which encoded the first 22 aminoacids of the small subunit gene (rbcS) from Pisium sativum (Cashmore, 1983), said third gene being then fused to the coding region of a foreign protein, such as the nptII gene which codes for neomycine phosphotransferase II (npt(II) gene obtained from a Tn5 transposon).

Example II is illustrative of the construction which can be made upon taking the second approach to the same problems to be solved. In this construction, the nptII coding sequence was fused directly to the transit peptide coding sequence such that the potential protein cleavage site did not contain any aminoacids derived from the mature small subunit protein except the methionine following the last amino acid of the transit peptide. More generally, and preferably, the first codon of the second sequence (encoding the protein, particularly a foreign protein, the translocation of which in the chloroplasts is sought) is immediately adjacent to the last codon of the said first DNA sequence coding for said transit peptide. Thus, particularly when the abovesaid second sequence encodes a polypeptide or protein different from the chloroplast protein normally associated with the transit peptide encoded by said first sequence, the nucleotide sequence next to said first sequence in said chimaeric gene will generally (possibly except for a first codon coding for methionine) be free of sequence homology with the nucleotide sequence encoding the N-terminal part of the normal chloroplast protein. Yet, the last codon of the first sequence and the first codon of the second sequence may be separated by any number of nucleotide triplets, preferably in the absence of any intron or stop codon. For instance, a "third sequence" encoding the first aminoacids of the mature protein normally associated with the transit peptide concerned in the corresponding natural precursor (particularly those encoded by the exon containing the first sequence encoding said transit peptide) may be present between said first and second sequences. This "third sequence" may consist of the region of high homology which the N-terminal parts of cytoplasmic precursor-subunits of chloroplast proteins from soybean, pea, duck-weed and wheat have in common. For instance such "third sequence" (be it in the constructions resulting from the "first approach" or those from the "second approach" considered hereabove) encodes the pentapeptide sequence M-Q-V-W-P. These letters correspond to the standard one-letter-abbreviated designations of the natural aminoacids. Obviously other "third sequences" of nucleotide-sequences can be contemplated upstream or/and also downstream of the above-defined second sequence) to the extent where the aminoacid sequences encoded are not likely to alter significantly the biological properties of the hybrid protein then formed and translocated into the chloroplasts. Yet in most preferred constructions according to that type the second sequence is preferably fused in phase-register with the first sequence directly contiguous thereto as mentioned higher or separated therefrom by no more than a short peptide sequence, such as that encoded by a synthetic nucleotide-linker possibly used for achieving the fusion. As shown by Example II such a construction is then capable of ensuring the translocation into the chloroplasts of any protein or protein fragment of controlled aminoacid sequence, for instance a bacterial protein or protein fragment or a synthetic polypeptide free of hybridisation with any determined peptidic sequence also possessed by a chloroplast protein or precursor.

It must be understood that in the preceding definitions "transit peptide" has the broad meaning indicated hereabove. The transit peptide may further be selected depending upon the plant species which is to be transformed, although, as mentioned earlier, transit peptides or smaller sub-unit precursors containing such transit peptides are often not plant-specific. Sub-unit precursor from one plant species can often be imported and directly processed by the chloroplasts of another.

Preferred DNA sequences encoding a transit peptide for use in the DNA-recombinants of this invention correspond to any of those encoding a transit peptide associated with the small sub-unit of RuBP of pea cells, or also of wheat or soybean cells.

Preferred "first nucleotide sequences" coding for transit peptides are defined hereafter, merely by way of examples. It must be understood that the letters above the lines refering to the nucleotide sequence per se designate the successive aminoacids encoded by the successive triplets of the nucleotide sequence. The letters below said line correspond to designations of nucleotides which can be substituted for those designated immediately above them in the nucleotide sequence :

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 M   A   S   M   I   S   S   S   A   V   T   T   V   S
ATG GCT TCT ATG ATA TCC TCT TCC GCT GTG ACA ACA GTC AGC

 R   A   S   R   G   Q   S   A   A   V   A   P   F   G
CGT GCC TCT AGG GGG CAA TCC GCC GCA GTG GCT CCA TTC GGC
    T       T               G

 G   L   K   S   M   T   G   F   P   V   K   K   V   N
GGC CTC AAA TCC ATG ACT GGA TTC CCA GTG AAG AAG GTC AAC
    G

 T   D   I   T   S   I   T   S   N   G   G   R   V   K
ACT GAC ATT ACT TCC ATT ACA AGC AAT GGT GGA AGA GTA AAG

 C
TGC
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Of course DNA sequences coding for other transit peptides can also be used for the construction of the chimaeric gene of this invention. For instance "first sequences" within the meaning of this application may consist of a sequence encoding the transit peptide of the light-harvesting chlorophyll a/b-protein complex, normally located in thylakoid membranes, such as :

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 M   A   A   S   S   S   S   S   M   A   L   S   S   P
ATG GCC GCA TCA TCA TCA TCA TCC ATG GCT CTC TCT TCT CCA

 T   L   A   G   K   Q   L   K   L   N   P   S   S   Q
ACC TTG GCT GGC AAG CAA CTC AAG CTG AAC CCA TCA AGC CAA

 E   I   G   A   A   R   P   T
GAA TTG GGA GCT GCA AGG TTC ACC
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The DNA sequence coding for the transit peptide is advantageously the natural nuclear DNA gene portion or a cDNA obtained from the corresponding mRNA.

Needless to say that any other DNA sequence encoding similar aminoacid sequences can be substituted therefor. It may for instance be contemplated to use a synthetically produced DNA sequence in which some of the codons differ from corresponding codons in the natural DNA sequence, while nevertheless coding for the same corresponding aminoacids. In that respect the expression "transit peptide" should also be understood as extending to any peptide which could differ from a natural transit peptide at the level of some of the aminoacids, to the extent where the substitutions contemplated would not alter the operability of the resulting peptide to promote the translocation into the chloroplast of the foreign polypeptide or protein encoded by the DNA sequence associated with or adjacent to the sequence encoding such peptide. Thus, the chimaeric genes of the invention may be constructed with any "first sequence" having substantial sequence homology with a natural DNA sequence encoding a natural transit

peptide.

As concerns the protein or polypeptide encoded by the abovesaid "second sequence", it should also be understood that it may consist of any protein or polypeptide sought to be introduced into or processed within the chloroplasts of determined plants. Therefore the DNA sequence encoding it is usually foreign to or heterologous with respect to the DNA sequence encoding the polypeptide or protein normally associated with the chosen transit peptide. In other words the first and second DNA sequences will usually originate from different sources. Particularly the second sequence will encode a foreign protein or polypeptide, for instance, of bacterial origin. But, the invention also extends to proteins that are naturally endogenous to chloroplasts of plants other than the "determined plant" considered hereabove or even to chloroplast proteins corresponding to natural chloroplast proteins of the same plant, yet differing therefrom but by a few amino-acids ("mutated" protein). Techniques for directing such mutation (whether in the first or the second sequences) are examplified in a recent paper of S. Gutteridge et al. titled "A site specific mutation within the active site of ribulose-1,5-biphosphate carboxylase of "Rhodospirillum rubrum" (1984).

Furthermore the chimaeric gene of a preferred DNA recombinant according to the invention comprises a promoter region upstream of the above mentioned fused sequences, under such manner that, when said chimaeric gene is inserted within an appropriate vector, the transcription of both the abovesaid first and second sequences are under the control of said promoter. The promoter region contemplated hereabove should of course be selected among those which are recognized by the polymerases endogenous to the plant sought to be transformed. Of particular advantage are the promoters effective in pea, wheat, soybean or tobacco cells. The promoter may be that normally associated with the sequence encoding the chosen transit peptide. It may however also be different. An example of construction using another promoter will be illustrated later in the examples. For instance, suitable promoters are those belonging to the genes of plastocyanin, ferredoxin-$NADP^+$ oxydoreductase, etc.. Other suitable promoters are examplified in the litterature referred to in this application.

Preferably the sequence coding for the transit peptide is under the direct control of the selected promoter. This means that the first nucleotide triplet transcribed and expressed under the control of said promoter is preferably that of the sequence encoding the transit peptide. This of course is not critical, for instance as evidenced by the first example.

Finally the invention also relates to recombinant vectors, particularly plasmids which can be introduced and maintained in plant cells and containing the abovesaid chimaeric gene, including the above-defined promoter region.

Preferred vectors of this type are those derived from the Ti-plasmids referred to hereabove. More particularly, a preferred vector of this type comprises in addition to said chimaeric gene a DNA fragment suitably positioned with respect to said foreign gene and having essential sequence homology with the DNA of a Ti plasmid including a T-DNA fragment, said vector further comprising the sequences encoding the essential functions capable of causing the transfer of said T-DNA fragment and said chi-meric gene into said plant cells. Particularly, a preferred vector according to the invention contains a T-DNA border sequence and said chimaeric gene is positioned close thereto. Even more preferred vectors of this type comprise two border sequences, the chimaeric gene then being positioned between these two border sequences. As concerns general methods for inserting the chimaeric gene in Ti-plasmids, reference is made to the patents referred to above by way of examples.

Advantageously the DNA recombinant (be it the chimaeric gene as such or the vector which contains it) should preferably include the appropriate site for the initiation of the corresponding RNA transcription upstream of the first codon to be translated, in most cases an ATG codon. It is also of advantage that the DNA recombinant comprises downstream of the foreign gene to be expressed appropriate transcription termination and polyadenylation signals.

The invention also concerns a process for achieving and controlling the introduction of a determined protein or polypeptide (or fragment of said protein or polypeptide) into the chloroplasts of determined plant cells. Any suitable process for performing this introduction can be resorted to. Advantageously use is made of vectors of the type examplified and modified by a chimaeric gene according to the invention and comprising a "second coding sequence" encoding and protein or polypeptide. But any other process can be resorted to. For instance a chimaeric gene according to the invention could be inserted in plant cells simply by the calcium chloride polyethylenglycol precipitation methods or also by microinjection into the plant cells.

Additional features of the invention will appear in the course of the following disclosure of the conditions under which the structural requirements of vectors capable of transforming plant cells for the sake of ultimately causing a determined foreign gene product to be inserted in chloroplasts were determined. Reference will be made to the drawings in which :

- fig. 1A diagrammatically represents the successive steps of the construction of preferred DNA recombinants, including a vector suitable for the transformation of plant cells, containing a chimaeric gene according to a first preferred embodiment of this invention ;
- fig. 1B represents the structure of the characteristic portion of the chimaeric gene according to this invention and included in the abovesaid DNA recombinants ;
- fig. 2 shows the results obtained in Southern hybridization experiments with DNA recombinants according to the invention, in relation to the detection of the incorporation of the abovesaid chimaeric gene in the genome of plant cells ;
- fig. 3 is a schematic representation of the organization of the gene fusion and the plant-vector sequences of the vector of fig. 1A, as modified by said chimaeric gene ;
- fig. 4 shows the results obtained in RNA-hybridization experiments carried out in relation to the detection of the transcriptional activity of the promoter included in the chimaeric gene of the invention ;
- fig. 5A and 5B show comparative results of transcription experiments under the control of a light-dependent promoter in plant materials transformed by the DNA recombinants of the invention ;
- fig. 6A is representative of the results obtained in experiments purporting to demonstrate the transport of the products encoded by the aboive said chimaeric gene into the chloroplasts of plant cells ;
- fig. 6B is a graphic display of the relative mobility of the different activities of the gene-product detected in fig. 6A ;
- fig. 7 illustrates results obtained in assays (to show the light-dependent expression of the fusion protein encoded by the chimaeric gene) ;
- fig. 8A diagrammatically represents successive steps of the construction of preferred DNA recombinants according to a second preferred embodiment of the invention, as well as of other DNA recombinants for study purposes ;
- fig. 8B represents the aminoacid sequences encoded by a portion of a chimaeric gene diagrammatically shown in fig. 8a particularly at the junction of the DNA sequence coding for the selected transit peptide of a gene encoding the amino terminus of the bacterial neomycine phosphotransferase II (NPT(II)) used as a model of protein of bacterial origin transportable into the chloroplasts ;
- fig. 9 is a diagrammatical representation of another examplified chimaeric gene according to the invention ;
- fig. 10-13 are autoradiograms of the observations made in series of gel-separation assays which are disclosed hereafter.

In the examples which follow the approach taken has been to construct a chimaeric gene encoding a fusion protein containing the transit peptide of the precursor to the small sub-unit of RuBP carboxylase from pea and a coding sequence of the bacterial neomycin phosphotransferase (II)(abbrevited as NPT(II)).

The NPT(II) protein was chosen because NPT(II) protein confers resistance for kanamycin to plants (HERRERA-ESTRELLA et al., 1983 ; FRALEY et al., 1983 ; BEVAN et al., 1983), fusion proteins are biologically active (Reiss et al, 1984b) and an enzymatic assay for in situ detection of NPT(II) or NPT(II) fusion proteins in non-denaturing polyacrylamide gels has been described recently[47]. This method is particularly useful to distinguish processed from unprocessed forms of the fusion protein.

EXAMPLE I

GENERAL OUTLINE

Construction of plasmids pSNIP and pSNIF containing the chimaeric gene (tp-ss-nptII

A genomic clone for one of the rbcS genes from pea was isolated, sequenced and made available by Dr. A. CASHMORE, Rockefeller University, New York (pPSR6). From this clone the promoter signals, (CASHMORE, 1983 ; HERRERA-ESTRELLA et al., 1984), the first exon coding for the rbcS transit peptide and the first two codons of the mature small subunit protein, followed by the first intron (83pb) and part of the second exon (66bp) coding for the amino terminus of the mature small subunit protein were fused via a Sau3A restriction endonuclease recognition site with the BamHI site of the plasmid pKm109/9 (REISS et al., 1984b) which contains the coding region for the nptII gene from Tn5 (BECK et al., 1982).

The fusion gene which was obtained and which contained the transit sequence (56 codons) and 22 codons from the mature rbcS gene linked via seven artificial codons with the second codon from the nptII gene (figure 1B) were found to be similarily active. The size of the coding region of the nptII gene is 1130 bp. The fusion junction was verified (data not shown) by DNA sequencing (MAXAM and GILBERT, 1977).

The chimaeric protein should have a size of Mr 38,023 in the unprocessed and of Mr 32,298 in the processed form. Southern type (SOUTHERN, 1975) hybridisation data (figure 2) established that transformed plant tissues contained the chimaeric gene constructs in the nuclear DNA and that no detectable DNA rearrangements had occured during integration. A schematic representation of the results is given in figure 3.

A more detailed disclosure of the construction will be given hereafter, more particularly in relation to fig. 1A, 1B and 3.

Production of vectors capable of transforming plants

To introduce the chimaeric genes in the nuclear genome of plants, the plasmid was inserted into the T-DNA of pGV3851 and of pGV3850, both derivatives of the Ti plasmid pTIC58, in which parts of the T-DNA where substituted by pBR322 (ZAMBRYSKI et al., 1983 ; 1984). The T-DNA of pGV3851 still contains the genes coding for transcripts 4, 6a and 6b (WILLMITZER et al., 1983) which results in a teratoma-like growth of the transformed tissue (JOOS et al., 1983), whereas all tumor controlling genes have been eliminated in pGV3850 with the result that plant cells transformed with this vector can differentiate and grow as normal plants (ZAMBRYSKI et al., 1983 ; DE BLOCK et al., 1984). The gene constructions were introduced into pGV3850 and pGV3851 Ti-plasmids by homologous recombination after mobilization from E. coli to Agrobacterium with the help of plasmids R64drd11 and J G28 (VAN HAUTE et al., 1983).

Cointegrates were selected on spectinomycin and streptomycin containing plates and their structure verified by Southern blot hybridization (SOUTHERN, 1975) using various parts of the constructions as probes (data not shown).

Plant transformation

The chimaeric genes were introduced into Nicotiana tabacum cv. Wisconsin 38 or SR1 by inoculation of a wounded plantlet or by co-cultivation of protoplats with Agrobacterium. Transformed material obtained by wounding was screened for the presence of nopaline synthase activity (OTTEN, 1982), a cotransferred marker. Transformants (pGV3851::pSNIP) grew on 250ug/ml kanamycin as green teratoma tissue, suggesting that a functional chimaeric gene was present and transcribed. In co-cultivation experiments N. tabacum SR1 protoplasts were incubated with Agrobacterium containing (PGV3850::SNIF) and selected after two weeks with 100 ug/ml kanamycin. From 9 individual colonies which were positive when tested for NPTII activity, one was chosen and regenerated under constant selected pressure to a fully normal looking plant. Genetic analysis shows inheritance of the NPTII marker in a classical mendelian fashion. These results suggested that transcripts from the chimaeric genes were properly processed, transported out of the nucleus and translated into a functionally active protein.

Light induction of the chimaeric gene

Poly(A)+ and poly(A)-RNA from wild type and from transformed tissues (pGV3851::pSNIP) was isolated and analysed by so called "Northern" gel hybridizations. When the coding region of the nptII gene (BamHI-SmaI fragment from pKM109/9) was used as a probe a complex hybridization pattern was observed with RNAs ranging between 5,500 nucleotides and 8,000 nucleotides in size. These RNAs were detected in light grown teratomas only. Four days of darkness after a day:night rhythm of twelve hours resulted in a marked decrease of the signals (fig. 4). The very large size of these transcripts probably results from the fact that no proper polyadenylation and transcription termination site was introduced near the translation termination signal. No signals of comparable size or strength were observed in wild type Wisconsin 38 tobacco or in material obtained from a plant transformed with the pGV3850 vector only (fig. 4). In order to compare the light dependent transcription of the chimaeric gene with that of both the endogenous rbcS gene and the chloroplast gene coding for the large subunit of Rubisco (rbcL), poly(A)+ and poly(A)- RNA from light- and dark-grown teratoma were hybridized to specific probes for each of these genes. The results are illustrated in fig. 5A. Signals of the endogenous rbcS transcripts (850 nucleotides) were observed at the expected position. Similarly, a transcript of 1750 nucleotides was observed when a rbcL specific probe was used (ZURAWSKI et al. ; 1981). The results suggest that the promotor of the rbcL gene, which resides in the chloroplasts, is less sensitive to light stimuli than both the endogenous rbcS and the newly introduced chimaeric gene. Dot blot experiments were included to quantify these results (fig. 5B). The ame probes were used as mentioned before. Individual dots were cut out and the radioactivity counted. A difference of about 25-fold was measured between poly(A)+ RNA from light- and dark-grown teratoma shoots probed

with either rbcS or nptII sequences. In contrast, the difference is only 5-fold for poly(A)- RNA specific for rbcL sequences. These results support the Northern experiments indicating that the transcripts of the chloroplast gene coding for the large subunit is less sensitive to influence of light in comparison with the nuclear gene for the small subunit. In addition, it seems that the pea rbcS promotor of the introduced chimaeric gene has a sensitivity to different light regimes which is comparable to that of the endogenous promotor or promotors measured in the tobacco teratoma tissue.

Features of fusion proteins

In order to detect the fusion protein formed between the transit peptide, the NH_2-terminal region of the mature small subunit and the NPTII protein in plants, an assay detecting the phosphotransferase-II activity in crude extracts of plants was developed. The method was adapted from published procedures (REISS et al., 1984a) and eliminates most of the endogenous self-phosphorylating proteins which interfere with the assay by proteinase K treatment. The results presented in fig. 6 demonstrate that NPTII activity is detected in a crude extract (lane 4) of leaves of tobacco plants containing the pGV3850:: pSNIF construct. The activity migrates in the gel assay with a mobility which is intermediate between that of the TP-NPTII fusion protein (35.5 kd) and that of the normal NPTII PROTEIN (29 kd) from extracts of E. coli (lane 1). The relative mobility of the NPTII activity in lane 4 is consistent with a conclusion that it represents the processed form of the precursor protein (SS-NPTII) which has a theoretical molecular weight of 32,298. Since the polarity index (CAPALDI and VANDERKOOI ; 1972) of the three proteins is 41 for NPTII, 40 for SS-NPTII and 41 for TP-NPTII, it is legitimate to compare the three proteins by their mobility on native polyacrylamide gels (see fig. 6B). Indeed the unprocessed TP-SS-NPTII protein has a molecular weight of about 38,000 and would therefore presumably migrate more slowly than the TP-NPTII marker. the SS-NPTII fusion protein is degraded in vitro after isolation yielding active subfragments with a mobility which approaches that of the normal NPTII enzyme. That the lower molecular weight spots seen in fig. 6A and 7 are due to unspecific degradation was shown by demonstrating that this and other NPTII fusion proteins are actually degraded in vitro in both bacterial and plant extracts (data not shown). Incubation in the presence of protease inhibitors could not completely prevent this degradation. No activity was detected in control extracts from tobacco lacking the TP-SS-NPTII chimaeric gene (lane 3). The SS-NPTII activity observed in crude extracts can also be detected in isolated chloroplasts (lane 2). the relative amount of activity detected in the chloroplats is significantly less than the activity observed in crude extracts. This is probably due to leakage of the activity out of the chloroplasts during chloroplast isolation. Indeed the procedure used to isolate chloroplasts led and with this particular plant material, to a substantial damage of the chloroplasts. More than 90 % of the chloroplast material is either visibly damaged or runs at a reduced density in the percoll gradients. Further manipulations during recovery and concentration prior to the NPTII assay could contribute to further minor damage leading to significant loss of the protein by leakage. These observations do not exclude the possibility that although all of the precursor TP-SS-NPTII protein is processed to the SS-NPTII form, it is not actually all transported in vivo into the stroma of the chloroplasts. However the data obtained clearly demonstrate that at least some of the processed SS-NPTII protein is within the stromal fraction of the chloroplasts. Indeed the activity associated with the chloroplasts was shown to be located within the stroma by demonstrating that broken chloroplasts did not contain any detectable NPTII activity and that the NPTII activity in intact chloroplasts could not be eliminated by trypsin treatment (data not shown). Further evidence that the detected SS-NPTII activity was derived from the introduced light inducible chimaeric gene was obtained by demonstrating that the activity was significantly reduced when tobacco plants containing the pGV3850::pSNIF construct and grown in the green house in a 12 hour light/dark regime (fig. 7. lane 3) were transferred for 96 hours to complete darkness (fig. 7, lane 2).

The details concerning the conditions under which the constructions of DNA recombinants were obtained and the methods used for appreciating the results asserted hereabove, inasmuch as they are not ascertainable from the previous discussion will be recalled hereafter.

MATERIALS AND METHODS

Strains and plasmids

E. coli DH1 was used for in vitro transformation. Agrobacterium C58ClRif was the receptor strain in all bacterial conjugations. The conjugation followed the protocol described by Van Haute et al. (1983) and ZAMBRYSKI et al. (1984).

DNA techniques

Restriction endonucleases and other DNA modifying enzymes were used as recommended by the manufacturers. Other techniques were used as described by MANIATIS et al. (1982).

Nopaline assay

The presence or synthesis of nopaline due to expression of the nos gene in transformed calli and regenerating shoots from these calli was monitored according to OTTEN (1982).

Plant transformation

Small axenically growing plants were kept in 1/2 M+S medium (MURASHIGE and SKOOG, 1962) in jars and were inoculated after decapitation with Agrobacterium strains as described (ZAMBRYSKI et al., 1984). Wound calli were removed and put on medium containing 0.2 mg/l benzaminopurine and 0.6 mg/l indolacetic acid and 0.5 mg/ml cefotaxime (HOECHST). After ca. 4 weeks the callus material was transferred to hormone free medium and emerging shoots were tested for nopaline production. Nopaline synthase positive shoots were propagated and tested on 100 to 500 ug/ml kanamycin. Teratoma shoots which grew on concentrations of 100 ug/ml or higher were used for analysis. Protoplast were kept in coculture with Agrobacteria according to MARTON et al. (1979) with modifications described by HAIN et al. (1985).

Analysis of DNA and RNA

DNA was isolated according to BEDBROOK (1981) from preparations of nuclei. The DNA was digested with restriction endonucleases (10-30 ug/lane, overnight digestion with a 3-fold excess of enzymes), separated on agarose gels according to size and transferred to nitrocellulose filters (THOMAS, 1983). Hybridization with radioactive probes was performed in 50 % formamide, 4 times SSC, 10 times Denhardt's solution, 0.2 SDS and 0.1 mg/ml calf thymus DNA at 50 °C for 48 hours (BOHNERT et al., 1982). The filters were washed twice for 15 minutes each in 50 % formamide, 4 times SSC at the hybridization temperature, followed by washing in 50 % formamide, 3 times SSC at room temperature (1-4 hours) and 2 times SSC at room temperature (1 hour). Dot blot hybridizations were performed according to THOMAS (1983) with DNA amounts covering a range equivalent from 1000 to 0.1 gene copies per sample. Hybridization was as described above. RNA was isolated according to CHIRGWIN et al. (1979), and separated into poly(A)+ and poly(A)-RNA by passage over oligo d(T)-cellulose (Collaborative Research, type III) following the procedure of AVIV and LEDER (1972). RNAs were separated according to size in 1 % agarose gels containing 5 mM methylmercury hydroxide (BAILEY and DAVIDSON, 1976). Hybridizations with ^{32}P-labelled, nick-translated probes were carried out as described (BOHNERT et al., 1982) ; between 2 and 3 x 10^5 cpm/lane were used.

Neomycin phosphotransferase activity assay

The assay was adapted for plant extracts from a procedure worked out for bacterial and animal cell lysates (REISS et al., 1984a). Between 20 and 100 mg of tissue from transformed plants was crushed in 0.1 ml buffer (10 % glycerol, 5 % ∞-mercaptoethanol, 62.5 mM Tris/HCl, pH 6.8, 50 ug/ml bromophenol blue and 0.1 % SDS). Several protease inhibitors were used in an attempt to inhibit specific and unspecific proteases. Aprotinin (Trade name Trasylol) was used at a final concentration of 100 ug/ml in water. p-hydroxy-mercuri-benzoate (PHMB) was used at a concentration of 1 mM, ε-amino-n-caproic-acid and 1-10-phenantroline were added to a final concentration of 5 mM. Protease inhibitors were used according to Gray (1982). Cristalline phenylmethylsulfonylfluoride (PMSF) was added immediately before use at a concentration of 100 ug/ml. The cleared homogenate (5 min., 13,000 rpm, Eppendorf centrifuge) was loaded onto 10 % non-denaturing polyacrylamide gels (Laemmli, 1970 ; without SDS). After electrophoresis the buffer in the gel was exchanged against 67 mM Tris/maleate, 42 mM MgCl2, 400 mM NH4Cl, pH 7.1, and the acrylamide gel was covered by an agarose gel (1 %) containing kanamycin-sulfate (1 mg/ml) and γ^{32}P-ATP (5 uCi/um pf a specific activity of 2000-3000 Ci/mMol) in the same buffer as the polyacrylamide gel. The gel-sandwich was covered by Whatman P81 paper, Whatman 3MM paper, and paper towels. After 3 hours the P81 paper was incubated for 30 minutes in a solution containing 1 % SDS and 1 mg/ml proteinase K in water at 60 °C and subsequently washed several times in 10 mM phosphate buffer (pH 7.5) at 80 °C, dried

and exposed to Kodak XR5 film for up to 48 hours. The principle of this method is the binding of kanamycin to the phosphorylated DEAE paper by which the positions in the gel are revealed where a kanamycin phosphorylating activity migrated. The additional proteinase treatment suppresses signals of plant activities which after phosphorylation bind to P81 paper but do not phosphorylate kanamycin.

Isolation of chloroplats.

Chloroplasts were isolated from 1-2 g of leaves of transformed plants. Structurally intact chloroplats were collected from Percoll (Pharmacia) gradients (Ortiz et al., 1980). The washed chloroplasts were concentrated by centrifugation, lysed and than used for the in situ demonstration of NPTII activity as described above. Trypsinisaton of chloroplasts was performed according to BARTLETT et al. (1982).

CONSTRUCTIONAL DETAILS AND METHOD EMBODIMENTS IN RELATION TO THE DRAWINGS.

1) Construction of the chimaeric rbcS-npt-II genes pSNIP and pSNIF (fig. 1A).

A BamHI-SalI fragment from pKM109/9 (REISS et al., 1984b) containing the entire coding region from a modified nptII gene from Tn5 (BECK et al., 1982) was inserted in plasmid pPSR6 Δ-RV next to a 950 bp DNA fragment (EcoRI-EcoRV) containing the promotor region and the 5'-end of the rbcS gene resulting in plasmid I-22. In this plasmid the HindIII-BamHI fragment was replaced by a HindIII-Sau3A fragment (53 bp) from the original rbcS clone (pPSR6) to form the plasmid II-4 containing the fusion gene. The pBR derived region in II-4 was exchanged against an EcoRI-SalI fragment from pGV710 in order to introduce streptomycin and spectinomycin resistance to be used as a marker to select for cointegration of this final plasmid (pSNIP (10.4 kbp)) with the Ti-plasmid in Agrobacterium. Plasmid pSNIF (12.3 kbp) was constructed by replacement of the SmaI-SalI fragment of pSNIP with an PvuII-XhoI fragment from the octopin synthase gene from plasmid pAGV40 (HERRERA-ESTRELLA et al. 1983 ; DE GREVE et al., 1983) harboring the polyadenylation site of that gene next to a BamHI restriction site of that gene next to a BamHI restriction site.

2) Structure of the rbcS-npt-II chimaeric gene (fig. 1B).

The black bar represents the transit-peptide sequence with the first ATG, the white area (two codons in exon 1 and 22 codons in exon 2) is interrupted by the first intron and represents the mature rbcS sequence. The hatched part represents the nptII sequence.

3) Southern hybridization experiments (fig. 2).

Hybridization of different probes to nuclear DNA from transformed (pGV3851::pSNIP) (a, c and e) and untransformed (b and d) tobacco. In Southern hybridization experiments (Southern, 1975) lane a and b resolve bands of different size resembling the small subunit gene family when a 661 bp EcoRV-AvaIII DNA fragment from the genomic small subunit clone was used as probe (Cashmore, 1983). An additional band of 10.4 kbp reveals the chimaeric gene fragment in lane a. In lanes c, d and e DNA was digested with PstI and EcoRI and either the promoter region of the small subunit gene (972 bp EcoRI/HindIII fragment) (lane c and d) or the coding region of the nptII gene (1000 bp BamHI/SmaI fragment from plasmid pKM109/9) were used as probes. In lane c a strong signal is detected from untransformed material (lane d). Weak signals in lane c are most likely due to crosshybridization of endogenous rbcS sequences or incomplete digestion of the DNA. In lane e a band of 0.9 kbp lights up the internal PstI fragment of the nptII gene and the weaker band shows again the 1.5 kbp fragment seen in lane c, due to a small overlap between the probe and the promotor region of the chimaera.

4) Schematic representation of the organization of the fusion and the flanking vector sequences (fig. 3).

Sizes are indicated in kbp. the chimaeric rbcS-nptII coding region is indicated by an open bar, the 5'-flanking sequence by a closed bar. EcoRI and PstI indicate restriction endonuclease sites. SpR and ApR represent antibiotic resistance markers against spectinomycin and ampicillin. Numbers indicate the size of fragments obtained in the Southern experiments (fig. 2). The DNA fragments between the gene fusion and the right part of the T-DNA represent the pBR322 sequences present in the vector pGV3851.

5) Transcriptional activity of rbcS promotor (fig. 4).

RNA was separated in denaturing 1 % agarose gels and transferred to nitrocellulose filters which were probed with different parts of the construction. The coding region of the nptII gene (BamHI- SmaI fragment from pKM109/9) was used as a probe. Lane 1:RNAs from light grown teratoma shoots. Lane 2: RNAs from plant material kept in darkness for four days after a day/night rhythm of twelve hours. Lane 3 : RNAs from plant leaves transformed with pGV3850. Lane 4 : RNAs from wild type Wisconsin 38. Weak signals in the latter are probably due to contaminating material in the probe which hybridizes to mRNA which is transcribed through the pBR322 sequences from a promotor active in the T-DNA or near the position of insertion in the plant chromosome. Numbers of the left indicate size in nucleotides, numbers on the the right refer to the Svedberg values of RNA markers.

6) Comparison of light dependence of rbcS and rbcL promotors (fig. 5A).

Poly(A)+ RNA from teratoma shoots grown in a daily rhythm of 12 hours light/dark (L) and material kept subsequently for four days in the dark (D) were hybridized to an nptII specific probe (see figure 4) and to a rbcS specific probe (see fig. 2). The endogenous rbcS transcripts are observed at the position of 850 nucleotides. poly(A)-RNA was analysed with the same technique probed with a 1750 bp fragment from a rbcL gene (ZURAWSKI et al., 1981). Numbers on the left refer to Svedberg values of RNA markers or to the size of the mRNA (right).

7) Dot blot hybridization to RNA from transformed (pGV3851::pSNIP) plant material (fig. 5B).

L indicates light grown material in 12 hour light/dark cycle. D indicates subsequent growth in the dark for four days. Single dots where cut out and radioactivity measured.

8) Demonstration of transport of TP-SS-NPTII precursor in chloroplasts of tobacco plants containing the pGV3850::pSNIF construct (fig. 6A).

The results obtained in each lane are commented hereafter :
Lane 1 : extracts from E. coli pGLT neoI expressing a TP-NPTII protein (VAN DEN BROECK et al., Nature in press) and E. coli pKM2 containing the Tn5 encoded NPTII enzyme.
Lane 2 : Neomycinphosphotransferase activity in chloroplasts purified from leaves of tobacco plants containing the chimaeric tp-ss-nptII gene.
Lane 3 : Crude extract from leaves of a control SR1 tobacco plant.
Lane 4 : Crude extract from leaves of tobacco plants containing the chimaeric tp-ss-nptII gene. The P.K. band is presumed to be due to a cytoplasmic self-phosphorylating protein and C.P.K. is presumed to be due to a chloroplast self-phosphorylating protein.

9) Graphic display of the relative mobility of the different NPTII activities detected in fig. 6A (fig. 6B.

As described hereabove it is legitimate to make the assumption that these proteins are separated according to molecular weight on these native gels because of their very similar polarity index (CAPALDI and VANDERKOOI ; 1972).

10) Light dependant expression of the SS-NPTII fusion protein (fig. 7).

Lane 1 : Idem as for fig. 6A.
Lane 2 : Idem as for fig. 6A lane 4 except for the fact that the plants were kept in complete darkness for 96 hours before extraction. Lane 3 : Idem as for fig. 6A. lane 4.

The results obtained demonstrate that the use of Agrobacterium vectors to transfer and express genes in plant cells (amply documented by CAPLAN et al., 1983 ; ZAMBRYSKI et al., 1983 ; 1984 ; HERRERA-ESTRELLA et al, 1983 ; 1984) can be extended to target a foreign protein for a specific cell compartment, namely the chloroplast. The results further demonstrate

(i) that the gene fusion is integrated in the nuclear DNA of tobacco without rearrangement of the DNA
(ii) that the transcription of this chimaeric gene (which contained a light inducible promoter sequence) is regulated by light.

It is important to note that the induced transcription of this introduced gene is as efficient as that of the endogenous small subunit gene(s) and rather more efficient than previously observed in tobacco with another chimaeric gene using the same pea small subunit promotor (HERRERA-ESTRELLA et al., 1984). Possibly the higher level of induced steady state mRNA in these tissues is due to improved mRNA stability. The presence of one intron in the transcript derived from this transit peptide small subunit neomycin phosphotransferase chimaeric gene (tp-ss-nptII) and the absence of any intron in the construction described by HERRERA-ESTRELLA et al. (1984), might explain an increased stability of this RNA (Hamer and Leder, 1978). Our observations also demonstrate that the pea small sub-unit promotor can be active in leaves of normal tobacco plants. This is in contrast to previous observations in several laboratories which indicated that the pea small subunit promotor while active in tobacco tissue cultures and teratomas, was inactive in leaves of normal plants. Possibly a position effect is involved in this phenomenon. the chimaeric tp-ss-nptII gene in (pGV3851::pSNIP) did not contain a polyadenylation or a transcription termination signal, which probably explains the observed very large transcripts. It will be shown in Example II that the provision of a suitable polyadenylation or a transcription termination signal at the appropriate location after the nptII gene results in the production of transcripts having substantially the same lengths as the transcripts of the nptII in its natural environment.

The data obtained hereabove demonstrate that the chimaeric tp-ss-nptII gene, which upon expression yields a fusion protein with a transit peptide and the conserved amino acid sequence flanking the processing site, is indeed translocated to the chloroplasts and is processed to yield a fusion protein located in the stroma, consisting of the NH_2-terminal end of the small subunit protein and an active NPTII protein. This SS-NPTII fusion protein migrates in the gel NPTII-assay with an electrophoretic mobility which is intermediate between the TP-NPTII (35.5 kd) and that of the original NPTII activity (29 kd). This mobility is in very good agreement with the molecular weight (32,298) of the SS-NPTII fusion protein. The results obtained indicate that this fusion protein, which confers kanamycin resistance to the transformed tobacco plants, is located within the chloroplasts and might leak out when the chloroplasts are broken.

However, the results obtained with the construction described in Example II hereafter demonstrate that the NPTII component of a precursor protein which contains only the transit peptide sequence immediately fused to the NPTII protein and thus missing part of the conserved aminoacid sequence flanking the processing site, is equally translocated across the chloroplast envelope and apparently properly processed. The latter results indicate that the transit peptide sequence alone is sufficient to both transport and process precursor proteins into chloroplats.

EXAMPLE II

In this example a chimaeric gene encoding a fusion protein consisting of the transit peptide of the precursor to the small subunit of RuBP carboxylase from pea[44] directly linked to the amino-terminus of NPT(II) was constructed.

In other words the bacterial enzyme into a novel "precursor" polypeptide was tested for its ability to be post-translationally imported and processed by chloroplasts both under in vivo and in vitro conditions.

General outline of the plasmids construction :

Two plasmids have been constructed which contain chimaeric genes encoding TP-NPT(II) (figure 8A). In the first plasmid, pGSSTneo3, the coding sequence for TP-NPT(II) is under control of the pea ss3.6 promoter which directs expression of chimaeric genes in plant cells[42,45].This construction has been used to study the fate of the fusion protein in vivo in transformed tobacco cells. Another plasmid, pGLTneoI, was constructed to direct the synthesis of TP-NPT(II) in E. coli under control of the lacUV5 promoter[45] in order to obtain sufficient quantities of the fusion protein for use in in vitro reconstitution experiments with isolated chloroplasts. The fusion protein encoded in both plasmids consists of the 57 aminoacid transit peptide and the first methionine of the mature small sub-unit polypeptide encoded by the pea ss3.6 gene[44], a 7-aminoacid linker fragment, and the NPT(II) devoid of the first methionine[45] (263 aminoacids). The amino acid sequences in the authentic small sub-unit precursor encoded by ss3.6 and the fusion protein are compared in fig. 8B. It can be seen that the Cys/Met cleavage site of the precursor to the small sub-unit is left intact in the TP-NPT(II) fusion protein.

To study the fate of the TP-NPT(II) fusion protein in vivo. it was necessary to first obtain transformed plant cells expressing the tp-npt(II) gene product.

The tp-npt(II) gene of pGSSTneo3 was brought into the genome of plant cells by means of the vector pGV3851, a derivative of the Agrobacterium Ti-plasmid pTiC58[48]. The plasmid pGV3851 contains a deletion

which removes several of the T-DNA-encoded transcripts, including those involved in auxin production, but retains the gene involved in cytokinin synthesis. The result of this modification is that Agrobacterium harbouring pGV3851 induces shoot-forming tumours. In pGV3851, the deleted portion of the T-DNA has been replaced by pBR322. pGSSTneo3 was inserted into the T-DNA of pGV3851 by recombination through the pBR322 homology[49].

The T-DNA of several Agrobaterium exconjugants obtained on kanamycin-containing plates was examined by Southern hybridization analysis[50] to confirm that the proper cointegration between pGSSTneo3 and the T-DNA of pGV3851 had occurred. The results obtained for one of these pGV3851::pGSSTneo3 exconjugants is shown in fig.9A.

Stems of sterile tobacco seedlings were inoculated with this strain after wounding with a needle below the first internode. After 2-3 weeks, green, shoot-forming tumours appeared. Axenic tissue was obtained by growing the transformed tissue in vitro on Murashige and Skoog (MS) medium[52] containing 500 µg/ml of cefotaximum, an antibiotic to which ampicillin-resistant agrobacteria are sensitive. During propagation of the tissue, the sucrose concentration of the MS medium was reduced from 3 % to 1 % to improve greening. The green tissues were able to grow on medium containing 200 µg/ml of kanamycin, indicating that the tp-npt(II) gene was present and functionally expressed. The presence of the tp-npt(II) gene was confirmed by Southern hybridization analysis[50] of genomic DNA obtained from the transformed callus tissue (fig. 8B).

A parallel series of cointegration and transformation experiments (data not shown) provided tobacco tumours containing a second chimaeric gene, nos-npt(II)[ref.40] coding for the unaltered NPT(II) protein under control of the promoter from the nopaline synthase gene[35,43]. This allowed the study the fate of NPT(II) itself in transformed cells.

Fate of the tp-npt(II) gene product in plant cells.

Since the TP-NPT(II) fusion protein is not a normal component of plant cells, it was of interest to determine the final location of the fusion protein in transformed cells. Specifically, we wished to know whether the transit peptide alone is capable of directing the uptake and processing of the TP-NPT(II) fusion protein by chloroplasts in vivo. Therefore, the following series of experiments were performed to determine the fate of both TP-NPT(II) fusion protein and unaltered NPT(II) in transformed tobacco cells.

The presence of NPT(II) or active NPT(II) fusion proteins in a given extract can be determined using an in situ enzymatic assay for phosphotransferase activity after gel electrophoresis (fig. 10). The positions of the original NPT(II) and the TP-NPT(II) fusion protein were determined by assaying extracts of E. coli harbouring either pBR322::Tn5 or pGLTneo1, prepared as described[47]. As shown (lane 3, fig. 10), the enzymatic assay on extracts of plant tissue that does not contain the NPT(II) coding sequence in its genome reveals two bands of phosphotransferase or kinase acrtivity (these are noted by P.K., plant kinase). These bands do not represent NPT(II) activity since they can also be observed when no kanamycin is included as substrate in the enzymatic reaction (data not shown). The faster migrating band is also found with chloroplast preparations from the same tissue (lane 4, fig. 10). When a bacterial extract containing the TP-NPT(II) fusion protein encoded by pGLTneo1 is mixed with plant extract, a new major band of NPT activity appears (lane 2, fig. 10). This band migrates more slowly than NPT(II) encoded by Tn5 (lane 1, fig. 10), and probaby corresponds to the bona fide TP-NPT(II). The change in mobility is due to a change in both molecular weight and charge as a result of the addition of the transit peptide. In lane 2 (fig. 10), also minor bands with higher mobility can be observed. These likely correspond either to degradation products of the fusion polypeptide, or to smaller polypeptides translated from an internal ATG of the TP-NPT(II) coding sequence.

Crude extracts obtained from transformed tissue containing a nos-npt(II) chimaeric gene contain NPT(II) activity (lane 5, fig. 10). However, intact chloroplasts isolated from the same tissue do not have detectable NPT(II) activity associated with them (lane 5, fig. 10). This observation suggests that the product of this chimaeric gene lacks the information necessary to mediate its translocation into chloroplasts. Crude extracts from tissue containing the tp-npt(II) chimaeric gene also contain considerable NPT(II) activity (lane 7), fig. 10). When intact chloroplasts are isolated from this tissue, considerable levels of NPT(II) activity are found to be associated with them (lane 8, fig. 11). Moreover, the one neomycin-phosphorylating protein observed in both the crude extract and the isolated chloroplats, migrates with the same mobililty as the Tn5 authentic protein, and differs from the NPT(II) fusion protein from E. coli harbouring the tp-npt(II) chimaeric gene (see also fig. 11, lanes 1, 2, 3). Even after longer exposure of the auto-radiogram there was no indication of the presence of this NPT(II) fusion protein. These observations show that the NPT(II) activity is concentrated in the chloroplast fraction, and that the TP-NPT(II) fusion protein is cleaved very efficiently close to the fusion site, removing the transit peptide.

Since the mature SS polypeptide is part of a soluble protein present in the stroma, it was of interest to determine whether the NPT(II) activity associated with the isolated chloroplast fraction is also sequestered in the same suborganellar compartment. Therefore, chloroplasts from pGV3851::pGSSTneo3-transformed tissue were lysed by resuspension in a hypo-osmotic buffer, and fractionated into stromal and membrane fractions. The membrane fraction was further washed to eliminate stromal contamination. Aliquots from these fractions were then subjected to electrophoresis on non-denaturing gels and assayed in situ for NPT-(II) activity. The results of this analysis (fig. 11) clearly demonstrate that all of the enzyme activity associated with the chloroplast fraction isolated from transformed tissue is located in the stromal (lane 3, fig. 11) rather than membrane (lane 4, fig. 11) fraction of the plastids. To ensure that these findings represent uptake of the fusion protein by the chloroplasts and not non-specific binding to the plastid envelope and release during organelle fractionation, aliquots of isolated chloroplasts were subject to protease treatment[53]. Equal amounts of chloroplasts from protease-treated and non-treated preparations were then fractionated as described above, and stromal fraction assayed for NPT(II) activity. A large percentage of the NPT(II) activity present in non-treated chloroplasts (lane 3, fig. 12) remains present in protease-treated chloroplasts (lane 4, fig. 12) until these chloroplasts are broken (lane 2, fig. 12). the slight decrease in activity observed is likely the result of losses from plastid lysis rather than the lack of sequestering of the processed fusion protein within the chloroplast.

These results, clearly demonstrate that the TP-NPT (II) fusion protein is targeted to the chloroplast, translocated into the stroma, and processed in a fashion similar to that of the small subunit polypeptide.

In vitro uptake and processing of the fusion protein by isolated chloroplasts.

As an alternative approach to determine whether the transit peptide alone is sufficient to direct post-translational uptake of proteins other than the mature small subunit polypeptide into chloroplasts and to test whether chloroplasts can recognize and proteolytically remove the transit peptide of a fusion protein, a series of in vitro reconstitution experiments were carried out with isolated intact chloroplasts. The in vitro approach has previously been shown to be useful in the analysis of chloroplast translocation processes[6,10-17]. Here, we have adapted this method for use with fusion proteins produced by E. coli.

Bacterial extracts containing the TP-NPT(II) fusion protein were prepared by sonication of exponentially growing liquid cultures of Escherichia coli harbouring pGLTneo1. Aliquots of the TP-NPT(II) containing cleared bacterial extracts were incubated for 1 hour with chloroplasts isolated from pea leaves[53]. Following incubation, the chloroplasts were reisolated form the incubation mix and washed several times an isosmotic buffer until no TP-NPT(II) activity was detected in the supernatant.

This preparation was used to determine whether there was NPT(II) activity associated with the stroma or membrane fraction of these chloroplasts. Lanes 1 and 2 of fig. 13 show respectively the position of NPT(II) and TP-NPT(II) present in bacterial extracts. Lane 3 (fig. 13) shows that prior to incubation with E. coli extracts containing TP-NPT(II), the stroma of chloroplasts isolated from pea does not contain any phosphotransferase or kinase activity comigrating with either the TP-NPT(II) fusion polypeptide or authentic NPT(II). .However, as observed earlier in tobacco, our assay conditions reveal an endogenous kinase activity (P.K.) associated with chloroplasts. After incubating these chloroplasts with bacterial extracts containing TP-NPT(II), the stromal fraction obtained from the isolated organelles contains a considerable level of NPT(II) activity (lane 4, fig. 13), whereas the membrane fractions does not (lane 6, fig. 13). This NPT(II) activity migrates like the original bacterial enzyme, which indicates processing. To confirm that the NPT(II) activity observed in the stromal fraction of chloroplasts incubated in the presence of the TP-NPT(II) fusion protein was the result of uptake and not the result of liberation during the fractionation procedure, of protein bound to the chloroplast envelope, chloroplasts were reisolated from the uptake incubation mixture, washed and subjected to limited proteolysis[53]. Following repurification, protease-treated chloroplasts were fractionated as above, and the NPT(II) activity was determined in both the stromal and membrane fractions. Most of the stromal NPT(II) activity appears to be protected against protease digestion since the amount of activity recovered (lane 5, fig. 13) is similar to that found in non-treated chloroplasts (lane 4, fig. 13). The membrane fraction of protease-treated chloroplasts was completely free of activity (lane 7, fig. 13). Similar results on in vitro uptake of the TP-NPT(II) fusion protein have been obtained using intact chloroplasts isolated from young expanding tobacco leaves (data not shown).

These results demonstrate that the transit peptide of the precursor to the small subunit of ribulose-1,5-bisphosphate is capable of mediating the uptake of polypeptides other than the mature small subunit by chloroplasts under in vitro assay conditions. that uptake of the NPT(II) protein by chloroplasts in vitro does not occur in the absence of the transit peptide (data not shown) is consistent with our in vivo observation that chloroplasts prepared from callus tissue transformed with nos-npt(II) do not contain activity. These

observationS further confirm the requirement for the transit peptide in the translocation process.

Unlike previous in vitro uptake studies[6], [11–17] which relied on the use of wheat germ extracts for the synthesis of precursor polypeptides, we have used an E. coli expression system for the preparation of our fusion protein. Since translocation of the fusion protein proceeds in this in vitro uptake system, this may be taken as an evidence for the lack of a requirement for additional cytoplasmic factors in the translocation mechanism. However, in contrast to translocation studies with microsomal membranes[54], it is not practical to wash chloroplast preparations with high salt buffers. Consequently, we cannot completely eliminate the possibility that the translocation of chloroplast proteins requires additional cytoplasmic factors which may be tightly bound to our chloroplast preparations.

The constructions of Example II and the conditions under which the results referred to herebefore were obtained, inasmuch as they are not ascertainable from the foregoing disclosure, will now be disclosed in a more detailed manner.

1 - Detailed description of the construction of plasmids containing chimaeric genes encoding the TP-NPT-(II) fusion protein (figure 8A) :

A 1-kb EcoRI-SphI restriction fragment from pPSR6, a pBR327 derivative containing the pea small subunit ss3.6 gene[44], was purified from a 1 % agarose gel. This fragment, which contains the promoter region, nucleotide sequences encoding the transit peptide and first methionine codon of the mature small subunit polypeptide, was ligated into EcoRI/BamHI-cut pKm109/9 to replace the small EcoRI/BamHI fragment in front of the NPT(II)-coding region. The plasmid pKm109/9 is a pBR322 derivative containing the npt(II) gene of Tn5 devoid of its 5'-untranslated region and the first methionine codon[45]. To fuse the 3'-overhanging end of the SphI restriction site with the 5'-overhanging end of the BamHI restriction site, a single-stranded oligonucleotide 5' GATCCATG 3', complementary to both protruding ends, was synthesized and added to the ligation mix[55]. After fusion, the SphI site is abolished, but the BamHI site remains. The resulting plasmid, pGSSTneo1, was restricted with SmaI and a 700-bp PvuII fragment, containing the transcription termination and polyadenylation signal from the ocs gene[56], was ligated into the site to ensure proper 3' transcription termination and processing. The intermediate pGSSTneo2 plasmid was then used in two different cloning steps.

(A) A 1,400 bp BamHI fragment from pUC4K[ref.57] encoding the kanamycin resistance gene from Tn903 was isolated and cloned into the unique BglII restriction site of pGSSTneo2 yielding the plasmid pGSSTneo3. Kanamycin resistance is used as a marker to select for the cointegration of pGSSTneo3 with the Ti-plasmid in Agrobacterium.

(B) A 200 bp EcoRI/HindIII fragment from pKm109/3[ref.45] containing the lacUV5 promoter region, was exchanged for the small EcoRI/HindIII fragment of pGSSTneo2. This allows for the expression of the TP-NPT(II) fusion protein in E. coli. The resulting plasmid is referred to as pGLTneo1. Abbreviations : Ap^R,ampicillin resistance ; Km^R, kanamycin resistance. Symbols :— , pBR322 sequence ; [[[neo▥, coding region for NPT(II) ; [→▥, lacUV5 promoter region ; Δ, 3' end. Representation of the octopine synthase gene : [Pocs▥, promoter region ; [ocs ▥, coding region. Representation of the gene for the small subunit of ribulose-1,5-bis-phosphate carboxylase : [= = =▥, promoter and 5'-untranslated region ; [///▥, sequence encoding the transit peptide ; [▥, exon ; [▥, intron.

2 - Partial aminoacid sequence comparison of the TP-NPT(II) fusion protein and the precursor to the ribulose-1,5-bisphosphate carboxylase small subunit polypeptide (fig. 8B).

Partial aminoacid sequences for the precursor to the small subunit polypeptide or ribulose-1,5-bisphosphate carboxylase encoded by the pea ss3.6 gene[44] (upper line) and the TP-NPT(II) fusion protein (lower line) are presented. The area near the processing site of the small subunit precursor and the fusion point for the TP-NPT(II) fusion protein are shown. The arrow indicates the proteolytic processing site defined for the small subunit precursor. The aminoacid residues derived from the original NPT(II) protein are underlined. Aminoacid residues are numbered above the sequences with the first methionine residue of the mature small subunit protein being taken as aminoacid number 1.

```
Met...Ser Asn Gly Gly Arg Val Lys Cys Met Gln Val Trp Pro
      Pro Ile Gly Lys Lys ....
Met...Ser Asn Gly Gly Arg Val Lys Cys Met Asp Pro Ala Asn
      Leu Ala Trp Iso Glu ...
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3) Incorporation of tp-npt(II) gene into the genome of plant cells.

To insert pGSSTneoIII in between the PGV3851 T-DNA borders, pGSSneoIII was first introduced into the E. coli strain Gj23 which harbours the helper plasmids R64drdII and Gj28. These last two plasmids provided the Tra and Mob functions required to mobilize pGSSTneo3 from E. coli to Agrobacterium tumefaciens (harfouring pGV3851). Thus, after conjugation between the corresponding E. coli and A. tumefaciens strains, Agrobacterium exconjugants the cointegrate between pGSSTneoIII and pGV3851 were selected on kanamycin containing plates.

The T-DNA of several kanamycin-resistant Agrobacterium exconjugants was examined by Southern hybridization analysis[50] to confirm that the proper cointegration between pGSSTneo3 and the T-DNA of pGV3851 had occured. the result obtained for one of these pGV3851::pGSSTneo3 exconjugants is shown in figure 3.

Reference is also made at the more detailed description of fig. 10 which appears hereafter.

4) Southern hybridization analysis of Agrobacterium and plant DNA (fig. 9).

The autoradiogram above shows the results of Southern hybridization[50] analysis confirming the presence and the structure of the tp-npt(II) chimaeric gene in both cointegrate pGV3851::pGSSTneo3 DNA and in genomic DNA from tmsformed tobacco cells. Lane 1, total Agrobacterium DNA from pGV3851::pGSSTneo3 ; lane 2, plant genomic DNA from tobacco callus transformed with pGV3851::pGSSTneo3. In both lanes two fragments hybridize with the specific probe: one fragment of 2.6 kb representing the EcoRI/BamHI fragment of pGSSTneo3 containing the Km^R gene of Tn903 and the SS promoter and transit peptide region ; a second fragment of 1.85 kb representing the BamHI/SalI fragment of pGSSTneo3 that contains the coding region of NPT(II) and the OCS 3' end.

Total Agrobacterium DNA[58] and plant genomic DNA from transformed callus tissue[59] were prepared and restricted with EcoRI, BamHI, and SalI. Digest products were fractionated on a 1 % agarose gel, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labelled probe specific for the promoter and the coding region of the TP-NPT(II) fusion protein (the probe was the smaller EcoRI/SalI fragment of pGSSTneo1 ; see fig. 8A).

5) Localization of NPT(II) activity in chloroplasts callus tissue (fig. 10).

The autoradiogram shows the presence and mobility of NPT(II) activity in bacterial extracts and cellular fractions following in situ localisation on a 10 % non-denaturing polyacrylamide gel[47]. Lane 1, E. coli extracts containing NPT(II), mixed with crude extract of green pGV3851-transformed tobacco tissue ; lane 2, E. coli extract containing TP-NPT(II), mixed with crude extract of green, pGV3851-transformed tobacco tissue ; lane 3, crude extract from green pGV3851-transformed tobacco tissue ; lane 4, intact chloroplasts from green pGV3851-transformed tobacco tissue ; lane 5, crude extract from green pGV3851::pLGV23neo-transformed tobacco tissue ; lane 6, intact chloroplasts from green pGV3851::pLGV23neo-transformed tobacco tissue ; lane 7, crude extract from green pGV3851::pGSSTneo3-transformed tobacco tissue ; lane 8, intact chloroplasts from green pGV3851::pGSSTneo3-transformed tobacco tissue. P.K. (?) : non-specific band present in untransformed plant tissue, probably due to the activity of plant kinase.

Methods : Three grams of green callus were homogenised by a few short bursts at low speed in a Waring Blendor in GR buffer (0.33 M sorbitol, 50 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$; 1 mM MnCl2 ; 1 mM $MnCl_2$; 1 mM Na_2-EDTA, 2 mM Na_2-EGTA, 1 mg/ml isoacorbate, 0.5 mg/ml BSA). The homogenate was filtered through two layers of Miracloth and the filrate was centrifuged from 0 to 4340 x g and braked in the shortest possible time. The crude chloroplasts pellet was resuspended in a few ml of GR buffer. Intact chloroplasts were prepared from crude chloroplasts pellets by sedimentation in Percoll density gradients[53]. Gradient-purified intact chloroplasts were washed with GR and lysed in 25 mM Tris-HCl (pH 7.5) containing 0.5 % β-mercaptoethanol.

Crude callus extracts were prepared by homogenizing 70 mg tissue in 70 µl extraction buffer (1 % β-mercaptoethanol ; 50 mM Tris ; pH 6.8 ; 0.13 mg/ml leupeptine) and clearing of the homogenate (2 minutes at 18,800 g). Crude extracts of E. coli were prepared by sonication in a buffer containing 10 mM Tris.HCl (pH 7.5) ; 10 mM $MgCl_2$; 25 mM NH_4Cl and 10 mM DTT[ref.60], followed by centrifugation to remove cellular debris. The assay for NPT(II) activity is a modification of the in situ detection method described[47]. Samples diluted with a 10 x loading buffer (50 % glycerol ; 0.5 % SDS ; 10 % β-mercaptoethanol ; 0.005 % bromophenol blue) were separated on a 10 % (w/v) nondenaturing polyacrylamide gel. After electrophoresis, the gel was washed twice for 10 minutes with distilled water and equilibrated for 30 minutes in 2x reaction buffer (100 mM Tris, pH 7.5 ; 50 mM $MgCl_2$; 400 mM NH_4Cl ; 1 mM DTT). The gel was then transferred onto a glass plate and overlaid with a 1 % agarose gel containing 30 µg/ml kanamycin sulphate and 200 µCi γ^{32}P-ATP in 1x reaction buffer. After 30 minutes at room temperature, the gel sandwich was covered with Whatman P81 phosphocellulose paper, two sheets of Whatman 3MM paper, and a stack of blotting paper pressed by weight (1 kg) to allow binding of the phosphorylated kanamycin to the P81 paper in a Southern-type transfer. After 3 hours the P81 paper was washed for 5 minutes with 500 ml hot water (80 °C), and for 3 hours several times with a 50 mM sodium phosphate buffer (pH 7.0). The p81 paper was dried and autoradiographed overnight using an intensifying screen to visualise the radio-labelled kanamycin formed at the position where the proteins with NPT(II) activity migrate in the polyacrylamide gel.

6) Localization of NPT(II) activity in the stromal fraction of chloroplasts isolated from pGV3851::pGSSTneo3-transformed tobacco tissue (fig. 11).

Intact chloroplasts were isolated from pGV3851::pGSSTneo3-transformed callus tissue and fractionated into stromal and membrane fractions. The NPT(II) activity associated with each of these fractions was assayed. Lane 1, E. coli extract containing NPT(II) ; lane 2, E. coli extract containing TP-NPT(II); lane 3, stromal fraction of chloroplasts isolated from green pGV3851::pGGSSTneo3-transformed tobacco tissue ; lane 4, membrane fraction of chloroplasts in lane 3 ; lane 5, was of the membrane fraction shown in lane 4. P.K.(?), see figure 10.

Intact chloroplasts were isolated from greened tobacco tissue as described in the legend to fig. 10. Chloroplasts washed twice with sorbitol-Hepes buffer and recovered by centrifugation were fractionated intro stroma and membrane portions by resuspending plastids in 25 mM Tris-HCl (pH 7.5) containing 0.5 % β-mercaptoethanol followed by centrifugation at 18,800 x g. Membrane fractions were twice washed and pelleted to remove residual stromal contamination. Wash fractions were routinely tested for residual NPT(II) activity.

7) Protection of the NPT(II) activity present within chloroplasts of pGV3851::pGSST-neo3-transformed tobacco cells to protease treatment (fig. 12).

Intact chloroplasts isolated from pGV3851::pGSST-neo3-transformed tobacco callus tissue were subjected to limited proteolytic digestion and then fractionated into the stromal and membrane components. The protease linsensitivity of NPT(II) activity associated with these fractions was assayed. Lane 1, E. coli extract containing NPT(II) ; lane 2, stromal fraction of intact chloroplasts isolated from green pGV3851::pGSSTneo3-transformed tobacco tissue and lysed before protease treatment ; lane 3, stromal fraction on non-protease-treated intact chloroplasts isolated from green pGV3851::pGSSTneo3-transformed tobacco tissue ; lane 4, stromal fraction of protease-treated intact chloroplasts isolated from green pGV3851::pGSSTneo3-transformed tobacco tissue ; P.K. (?), see fig. 3.

Intact chloroplasts were prepared from greened tobacco callus tissue as described in the legend to fig. 10. Protease treatment of isolated chloroplast was carried out as previouosly described[53]. protease-treated and untreated plastids were fractionated as described in the legend to fig. 5.

8) In vitro uptake of TP-NPT(II) fusion protein by isolated pea chloroplasts (fig. 13).

An autoradiogram showing the in situ localization of NPT(II) activity in bacterial and chloroplast fractions following fractionation on non-denaturing polyacrylamide gels is presented. Lane 1, extract from E. coli harbouring pBR322::Tn5 (NPT(II)) ; lane 2, extract from E. coli harbouring pGLTneoI (TP-NPT(II)) ; lane 3, stromal fraction of pea chloroplasts prior to incubation with bacterial extracts ; lane 4, stromal fraction of pea chloroplasts incubated with bacterial extracts containing the TP-NPT(II) fusion protein ; lane 5, stromal fractions of protease-treated pea chloroplasts (same amount as in lane 4) incubated with bacterial extracts containing the TP-NPT(II) fusion protein ; lane 6, washed membrane fractions of the same chloroplasts as in

lane 5 ; lane 7, washed membrane fraction of the same chloroplasts as in lane 4.

Methods : Intact chloroplasts were isolated from pea (Pisum sativum) leaves by sedimentation through Percoll density gradients[53]. Intact chloroplasts were washed and resuspended in sorbitol-Hepes buffer (50 mM Hepes-KOH, pH 7.5 ; 0.33 M sorbitol) and stored at 0 • C. In vitro uptake into isolated chloroplasts was carried out essentially as described[53] except the incubation mix was modified for use with bacterial extracts. Uptake reactions (300 µl final volume) contained intact chloroplasts (equivalent to 200-300 µg chlorophyll) and 50 µl of bacterial extract (as described in the legend to fig. 10) in buffer containing 0.33 M sorbitol, 50 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM Na_2-EDTA. Following incubation at 20-22 • C in the light with gentle shaking for 1 hour, chloroplats were diluted with sorbitol-Hepes buffer and intact chloroplasts recovered by centrifugation at 4340 x g. Chloroplasts washed twice with sorbitol-Hepes buffer and recovered by centrifugation were either fractioned immediately (see legend to fig. 11) or subject to porotease treatment as previously described[53]. Aliquots of samples were either assayed immediately for NPT(II), or stored at-80 • C and assayed at a later time.

The results presented in this example from both the in vivo and in vitro studies clearly demonstrate that the NPT(II) component of the TP-NPT(II) fusion protein is translocated across the chloroplast envelope and is finally located in the stroma. The requirement of the transit peptide for this process is shown by the failure to detect uptake of NPT(II) by chloroplasts, when the transit peptide has not been fused to NPT(II). The TP-NPT(II) fusion protein, however, bears no similarity in the aminoacid sequence to the small subunit precursor, particularly near to the processing site thereof immediately following the transit peptide. This suggests that all of the sequence information required for translocation resides within the transit peptide.

Under normal physiological growth conditions for plants, the small subunit precursor is rapidly taken up and processed by the chloroplasts, and a large free pool of unprocessed precursor is not observed[1, 10]. It has been shown here, that in tobacco cells transformed with pGV3851::pGSSTneo3, all of the NPT(II) activity observed in either crude cellular extracts or isolated chloroplast fractions migrates on the gel system[47] used with similar electrophoretic mobility to the original NPT(II). Processing of the TP-NPT(II) fusion protein is presumably carried out by the same soluble, chloroplast-associated protease[18] that is responsible for the processing of the small subunit precursor. It seems likely, therefore, that the processing of the TP-NPT(II) fusion protein occurs at the same Cys/Met site (figure 8B) used in the small subunit precursor. Thus it can be hypothesized that the transit peptide can mediate not only translocation, but also site-specific processing. Furthermore, both the translocation and processing steps apparently occur rather efficiently in pGV3851::pGSSTneo3-transformed tobacco cells, since within the detection limits of our assay system, all of the NPT(II) activity observed corresponds to the processed form of the TP-NPT(II) fusion protein.

The results presented here again clearly demonstrate the applicability of using Aarobacterium-mediated cell transformation to introduce foreign genes into plants.

EXAMPLE III.

Construction of a plasmid encoding a chimaeric gene encoding the TP-NPT(II) fusing protein and wherein the coding sequences are under the control of a foreign promotor (fig. 14)

The construction starts from pGSST neo3. This plasmid was then digested with EcoRI and Hind III. The staggered ends of the long fragments were filled in with the Klenow polymerase. The DNA obtained was then ligated to a SauIIIA fragment (270 bp) originating from plasmid pLGV 2382 published by This SauIIIA fragment contains the promoter of the nopaline synthase (HERRERA-ESTRELLA L. et al (1983) Embo. J., 2, 987-995. The latter fragment was also treated with the klenow fragment of the DNA polymerase. The SauIIIA fragment so repaired and the repaired fragment from pGSSTneo3 were then ligated with T4 ligase, whereby plasmid pLSSTneo1 was obtained. The plasmid containing the promoter region oriented in the proper direction was identified by restriction analysis with the SacII restriction enzyme and BamHI. The plasmid (pLSSTneo1) which proved to contain the biggest SacII-BamHI fragment was also the one which contained the promoter region and the TP-NPT II fragment in the proper orientation and under control of said promoter.

There is thus shown another plasmid having this time a constitutive promoter instead of the normal leaf specific light-inducible promoter. Consequently, a plasmid was obtained which can cause the protein located downstream of the promoter to be expressed also in the dark and also in other tissues of the plant. In such a manner one controls the level of production of metabolites of interest, for instance fatty acids or aminoacids.

It will be appreciated that the invention also makes it possible to put a gene normally expressed under photosynthetic conditions under the control of a promoter which is normally operative in a constant manner (day and night). In such a way and for instance one can obtain the constant production of a determined aminoacid under the control of a promoter operative in seeds.

The invention thus opens the way to important agricultural applications involving chloroplast functions. More particularly it enables the introduction of proteins of controlled structure in plant-cells chloroplast. These proteins can be introduced into the chloroplast either as such or as fusions with proteins or protein subunits which are coded for by natural genes and normally transported into the plant cell chloroplast. These proteins may either be proteins foreign to the plant cells to be transformed or be similar to endogenous proteins, yet different therefrom by controlled mutations. Particularly the invention now provides for the possibility of modifying at will genes including a determined protein, for instance for the sake of improving the activity of the enzyme encoded by the chloroplast genes. The invention also provides for the possibility of substituting another promoter for the endogenous promoter included in the natural gene to thereby regulate in a controlled manner the production of the chloroplast proteins.

The invention further provdes valuable tools for a better understanding of the role played by various domains of transported proteins interacting with chloroplast coded proteins. It also renders possible the study of whether determined chimaeric genes can direct the transfer of proteins normally encoded by the chloroplast back into this organelle. Model systems of chloroplast-encoded genes of importance for basic research and agricultural application are readily available, such as the large subunit of RuBP carboxylase, which contains the catalytic site of the holoenzyme, or the 32 K protein conferring resistance to certain herbicides. The similarity between the results obtained from in vivo and in vitro studies also suggests that the production in E. coli of fusion proteins composed of segments of nuclear-encoded organelle polypeptides and an enzymatic reporter is a powerful technique for the rapid analysis of the signals and processes involved in protein import by isolated organelles.

The invention further provides the means which enables chimaeric engineering of plants with a potential for aminoacid overproduction or improvement of plant productivity, and therefore meets needs which have already been recalled in the preamble of this application

The use of transit peptides for specifically targeting polypeptides in the chloroplast also provides the possibility of genetically engineering genes containing sequences encoding key enzymes of given pathways in such manner that said key enzymes are no longer subjected to the normal regulation systems included in the natural plant cells.

The invention also provides means for solving other problems that have been mentioned in the preamble i.e. the production of herbicide resistant plants. Actually the invention now provides a method for fusion of a "second sequence'" encoding the protein of interest with a first sequence encoding a transit peptide, the chimaeric gene so produced being capable after its insertion in the genetic DNA of the cells of the plant of interest to control the translocation of the protein of interest into the chloroplasts.

The invention opens the way to many other applications. A few additional examples are illustrated hereafter and in which the enzyme Ribulose biphosphate carboxylase (RuBPCase) can be brought into play.

a) Improvement of the carboxylase/oxidase ratio.

This enzyme catalyzes two enzymatic reactions :

1) The condensation of a molecule of Ribulose bisphosphate with a molecule of CO_2 to form two molecules of phosphoglyceric acid (Carboxylase reaction).

2) Reaction of a ribulose bisphosphate molecule with a molecule of oxygen to produce phosphoglycolate (oxidase reaction).

The latter is a competitive reaction with the carboxylation. Therefore it limits the efficiency of conversion of CO_2 into organic compounds.

The invention now provides a technique of site directed mutagenesis which allows the controlled alteration of a determined protein to be given full effect. For instance the modification of the RuBPCase in such a way that the carboxylase/oxidase ratio is much more favourable can now be contemplated. Another approach is to simply take a gene encoding for the RuBPCase from another plant or from another organism such as cyanobacteria which have a more favourable ratio, to fuse itwith a nucleic acid fragment containing a promoter and a transit peptide effective in the plant of interest and to introduce the chimaeric gene obtained into said plant.

b) Improvement of plant productivity.

There are several factors limiting plant productivity such as lack of nutrients and a low efficiency in light harvesting or CO_2 assimilation. Since the lack of nutrients can be solved using fertilizers, one of the main limiting factor for plant productivity becomes CO_2-assimilation. CO_2 uptake by a leaf depends mostly on two factors :

1) The physical diffusion of CO_2 along the plant cells and

2) the efficiency of CO_2 conversion to organic compounds.

Although different pathways for CO_2 assimilation exist in higher plants, they share the same limiting step, which is the efficiency of the RuBPCase enzyme. Here again the invention provides means for overcoming this problem at least in part, for instance upon introducing in the cells of the plant a chimaeric gene comprising sequences fused with one another and which respectively contain a promoter region and a fragment encoding a transit peptide which are particularly effective in that plant, on the one hand, and a sequence encoding a more efficient RuBPCase and originating from another plant, on the other hand.

Cultures comprising plasmids, intermediate cloning vectors, and microorganisms prepared by the processes of this invention are exelmplified by cultures deposited in the German Collection of Microorganisms (DSM), Gûttingen, Germany. These cultures are identified hereafter :

(1) E. coli HB101 (pSRP6)

(2) E. coli HB101 (pKM 109/9)

(3) E. coli HB101 (pGSST3)

These cultures were deposited on December 27, 1984.

These cultures were assigned accession numbers 3172 (1) 3171 (2) 3170 (3).

Other cultures referred to in this application have also been deposited on or before December 27th, i.e. on December 20th, 1984. Plasmids were maintained in the microorganisms identified in the left hand part of the table hereafter.

These cultures have been assigned the following accession numbers :

Internal Code/Taxonomic Designation	Plasmid in strain	DSM No.
AZ 1/E. coli K12 and VII	p PSR 6 delta R V	3161
AZ 2/E. coli K12 and VII	p I-22	3162
AZ 3/E. coli and VII	p II-4	3163
AZ 4/E. coli K12 and VII	pGV 710	3164
AZ 5/AGR. TUMEF. VII	pGV 3850::pSNIPP	3165
AZ 6/AGR. TUMEF.	pGV 3850::pSNIF	3166
AZ 7/AGR. TUMEF. VII	pGV 3850	3167

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Claims

1. A process for providing a foreign protein or polypeptide in a chloroplast of a cell of a plant; said process comprising the steps of:
   A. expressing, in the cytoplasm of said cell, a chimaeric precursor of said foreign protein or polypeptide; said precursor being encoded by a chimaeric DNA sequence in the genome of said cell; said chimaeric DNA sequence comprising: i) a first nucleic acid sequence that codes for a transit peptide of a cytoplasmic precursor of a chloroplast protein or polypeptide of a plant species and ii) a second nucleic acid sequence that codes for said foreign protein or polypeptide, that is heterologous to said first nucleic acid sequence and that is downstream of, and in the same transcriptional unit as, said first nucleic acid sequence; and then
   B. transporting said foreign protein or polypeptide from the cytoplasm of said cell into said chloroplast, with removal of said transit peptide from said foreign protein or polypeptide.

2. The process of claim 1, wherein said foreign protein or polypeptide, when present in chloroplasts of cells of said plant, confers resistance to an herbicide on said plant.

3. The process of claims 1 or 2, wherein said chimaeric DNA sequence also comprises a third nucleic acid sequence that encodes an N-terminal region of said chloroplast protein or polypeptide of said plant species and that is downstream of said first nucleic acid sequence; the 5' end of said third nucleic acid sequence being substantially contiguous to the 3' end of said first nucleic acid sequence.

4. The process of claim 3, wherein said third nucleic acid sequence encodes at least a part of a cytoplasmic subunit of said chloroplast protein or polypeptide of said plant species.

5. The process of claim 4, wherein said third nucleic acid sequence does not encode more than said cytoplasmic subunit of said chloroplast protein or polypeptide of said plant species.

6. The process of any one of claims 3-5, wherein said first and third nucleic acid sequences comprise an exon.

7. The process of claim 6, wherein said third nucleic acid sequence comprises an intron.

8. The process of any one of claims 3-7, wherein said third nucleic acid sequence consists essentially of a nucleic acid sequence with sequence homology to nucleic acid sequences which encode N-terminal

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Claims

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   A. expressing, in the cytoplasm of said cell, a chimaeric precursor of said foreign protein or polypeptide; said precursor being encoded by a chimaeric DNA sequence in the genome of said cell; said chimaeric DNA sequence comprising: i) a first nucleic acid sequence that codes for a transit peptide of a cytoplasmic precursor of a chloroplast protein or polypeptide of a plant species and ii) a second nucleic acid sequence that codes for said foreign protein or polypeptide, that is heterologous to said first nucleic acid sequence and that is downstream of, and in the same transcriptional unit as, said first nucleic acid sequence; and then
   B. transporting said foreign protein or polypeptide from the cytoplasm of said cell into said chloroplast, with removal of said transit peptide from said foreign protein or polypeptide.

2. The process of claim 1, wherein said foreign protein or polypeptide, when present in chloroplasts of cells of said plant, confers resistance to an herbicide on said plant.

3. The process of claims 1 or 2, wherein said chimaeric DNA sequence also comprises a third nucleic acid sequence that encodes an N-terminal region of said chloroplast protein or polypeptide of said plant species and that is downstream of said first nucleic acid sequence; the 5' end of said third nucleic acid sequence being substantially contiguous to the 3' end of said first nucleic acid sequence.

4. The process of claim 3, wherein said third nucleic acid sequence encodes at least a part of a cytoplasmic subunit of said chloroplast protein or polypeptide of said plant species.

5. The process of claim 4, wherein said third nucleic acid sequence does not encode more than said cytoplasmic subunit of said chloroplast protein or polypeptide of said plant species.

6. The process of any one of claims 3-5, wherein said first and third nucleic acid sequences comprise an exon.

7. The process of claim 6, wherein said third nucleic acid sequence comprises an intron.

8. The process of any one of claims 3-7, wherein said third nucleic acid sequence consists essentially of a nucleic acid sequence with sequence homology to nucleic acid sequences which encode N-terminal

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parts of cytoplasmic subunits of chloroplast proteins or polypeptides that are common to soybean, pea, duck-weed and wheat.

9. The process of claim 8, wherein said third nucleic acid sequence encodes the pentapeptide, M-Q-V-W-P.

10. The process of claims 1 or 2, wherein the 3' end of said first nucleic acid sequence is substantially contiguous to the 5' end of said second nucleic acid sequence.

11. The process of claim 10, wherein a nucleotide-linker is between said first and second nucleic acid sequences.

12. The process of claim 10 or 11, wherein said second nucleic acid sequence does not code for an N-terminal region of a chloroplast protein or polypeptide.

13. The process of claim 12, wherein said chimaeric DNA sequence is free of an intron between said first and second nucleic acid sequences.

14. The process of any one of claims 10-13, wherein the first codon of said second nucleic acid sequence codes for methionine and is adjacent to the last codon of said first nucleic acid sequence.

15. The process of any one of claims 1-14, wherein said chimaeric DNA sequence also comprises a promoter that is upstream of said first nucleic acid sequence so that both said first and second nucleic acid sequences are under transcriptional control of said promoter; and wherein said promoter contains a nucleic acid sequence which is recognized by polymerases of said cell.

16. The process of claim 15, wherein said promoter and said first nucleic acid sequence are heterologous.

17. The process of claim 15, wherein said promoter is from a plastocyanin gene, a ferredoxinNADP$^+$ oxydoreductase gene or a nopaline synthase gene or is normally associated with said first nucleic acid sequence.

18. The process of any one of claims 1-17, wherein said first nucleic acid sequence codes for a transit peptide of a cytoplasmic precursor of a small subunit of a ribulose-1,5-bisphosphate carboxylase-oxygenase.

19. The process of any one of claims 1-17, wherein said second nucleic acid sequence codes for a bacterial or plant protein or polypeptide.

20. The process of claim 19, wherein said second nucleic acid sequence codes for a chloroplast protein or polypeptide or a mutated chloroplast protein or polypeptide.

21. The cell obtainable by the process of any of one of claims 1-20 or a plant or plant cell culture containing of such cells; each such cell containing i) said chimaeric DNA sequence in its genome, preferably its nuclear genome, and ii) a chloroplast which contains said foreign protein or polypeptide, free of said transit peptide.

22. A seed of the plant of claim 21.

Patentansprüche

1. Verfahren zur Bereitstellung eines Fremdproteins oder -polypeptids in einem Chloroplasten einer Pflanzenzelle, das durch folgende Stufen gekennzeichnet ist:
   A. Exprimieren eines chimären Vorläufers des genannten Fremdproteins oder -polypeptids im Cytoplasma der genannten Zelle; dabei wird der Vorläufer durch eine chimäre DNA-Sequenz im Genom der genannten Zelle codiert, und die genannte chimäre DNA-Sequenz umfaßt:
      i) eine erste Nucleinsäuresequenz, die für ein Transitpeptid eines cytoplasmischen Vorläufers eines Chloroplastenproteins oder -peptids einer Pflanzenart codiert und

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ii) eine zweite Nucleinsäuresequenz, die für das genannte Fremdprotein oder -polypeptid codiert, das heterolog ist gegenüber der ersten Nucleinsäuresequenz und das sich stromabwärts von der ersten Nucleinsäuresequenz und in der gleichen Transkriptionseinheit befindet wie die erste Nucleinsäuresequenz; und darauf

B. Transportieren des genannten Fremdproteins oder -polypeptids vom Cytoplasma der genannten Zelle in den genannten Chloroplasten unter Entfernung des genannten Transitpeptids aus dem Fremdprotein oder -polypeptid.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Fremdprotein oder -polypeptid, wenn in Chloroplasten von Zellen der genannten Pflanze vorhanden, dieser Pflanze Widerstandsfähigkeit gegenüber einem Herbizid verleiht.

3. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß die genannte chimäre DNA-Sequenz auch eine dritte Nucleinsäuresequenz umfaßt, die eine N-terminale Region des genannten Chloroplastenproteins oder -polypeptids der genannten Pflanzenart codiert und die sich stromabwärts von der genannten ersten Nucleinsäuresequenz befindet; wobei das 5'-Ende der genannten dritten Nucleinsäuresequenz dem 3'-Ende der genannten ersten Nucleinsäuresequenz unmittelbar benachbart ist.

4. Verfahren nach Anspruch 3, dadurch gekennzeichnet, daß die dritte Nucleinsäuresequenz mindestens einen Teil einer Cytoplasma-Untereinheit des genannten Chloroplastenproteins oder -polypeptids der genannten Pflanzenart codiert.

5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, daß die dritte Nucleinsäureseqenz nicht mehr als die genannte Cytoplasma-Untereinheit des genannten Chloroplastenproteins oder -polypeptids der genannten Pflanzenart codiert.

6. Verfahren nach einem der Ansprüche 3 bis 5, dadurch gekennzeichnet, daS die erste und die dritte Nucleinsäuresequenz jeweils ein Exon enthalten.

7. Verfahren nach Anspruch 6, dadurch gekennzeichnet, daß die dritte Nucleinsäuresequenz ein Intron enthält.

8. Verfahren nach einem der Ansprüche 3 bis 7, dadurch gekennzeichnet, daß die dritte Nucleinsäuresequenz im wesentlichen aus einer Nucleinsäuresequenz mit Sequenzhomologie gegenüber Nucleinsäuresequenzen, die N-terminale Teile von Cytoplasma-Untereinheiten von Chloroplastenproteinen oder -polypeptiden, die der Sojabohne, der Erbse, der Wasserlinse und Weizen gemeinsam sind, codieren, besteht.

9. Verfahren nach Anspruch 8, dadurch gekennzeichnet, daß die dritte Nucleinsäuresequenz das Pentapeptid M-Q-V-W-P codiert.

10. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß das 3'-Ende der ersten Nucleinsäuresequenz im wesentlichen dem 5'-Ende der zweiten Nucleinsäuresequenz direkt anliegt.

11. Verfahren nach Anspruch 10, dadurch gekennzeichnet, daß ein Nucleotid-Linker zwischen der ersten und der zweiten Nucleinsäuresequenz vorhanden ist.

12. Verfahren nach Anspruch 10 oder 11, dadurch gekennzeichnet, daß die zweite Nucleinsäuresequenz nicht für eine N-terminale Region eines Chloroplastenproteins oder -polypeptids codiert.

13. Verfahren nach Anspruch 12, dadurch gekennzeichnet, daß die chimäre DNA-Sequenz frei ist von einem Intron zwischen der ersten und der zweiten Nucleinsäuresequenz.

14. Verfahren nach einem der Ansprüche 10 bis 13, dadurch gekennzeichnet, daß das erste Codon der zweiten Nucleinsäuresequenz für Methionin codiert und dem letzten Codon der ersten Nucleinsäuresequenz benachbart ist.

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15. Verfahren nach einem der Ansprüche 1 bis 14, dadurch gekennzeichnet, daß die chimäre DNA-Sequenz auch einen Promotor umfaßt, der sich stromaufwärts von der ersten Nucleinsäuresequenz befindet, so daß sowohl die erste als auch die zweite Nucleinsäuresequenz unter Transkriptionskontrolle des Promotors stehen; und worin der Promotor eine Nucleinsäuresequenz enthält, die von Polymerasen der Zelle erkannt wird.

16. Verfahren nach Anspruch 15, dadurch gekennzeichnet, daß der Promotor und die erste Nucleinsäuresequenz heterolog sind.

17. Verfahren nach Anspruch 15, dadurch gekennzeichnet, daß der Promotor aus einem Plastocyanin-Gen, einem Ferredoxin-NADP$^+$-Oxydereductase-Gen oder einem Nopalinsynthase-Gen stammt oder normalerweise mit der ersten Nucleinsäuresequenz assoziiert ist.

18. Verfahren nach einem der Ansprüche 1 bis 17, dadurch gekennzeichnet, daß die erste Nucleinsäuresequenz für ein Transitpeptid eines Cytoplasma-Verläufers einer kleinen Untereinheit einer Ribulose-1,5-bisphosphat-Carboxylase-Oxygenase codiert.

19. Verfahren nach einem der Ansprüche 1 bis 17, dadurch gekennzeichnet, daß die zweite Nucleinsäuresequenz für ein Bakterien- oder ein Pflanzenprotein oder -polypeptid codiert.

20. Verfahren nach Anspruch 19, dadurch gekennzeichnet, daß die zweite Nucleinsäuresequenz für ein Chloroplastenprotein oder -polypeptid oder für ein mutiertes Chloroplastenprotein oder -polypeptid codiert.

21. Zelle, erhältlich durch das Verfahren nach einem der Ansprüche 1 bis 20, oder Pflanze oder Pflanzenzellkultur, die solche Zellen enthält, wobei jede solche Zelle i) die chimäre DNA-Sequenz in ihrem Genom, vorzugsweise in ihrem Kerngenom, enthält, und ii) einen Chloroplasten enthält, der das Fremdprotein oder -polypeptid frei von dem Transitpeptid enthält.

22. Samen der Pflanze nach Anspruch 21.

Revendications

1. Procédé d'obtention d'une protéine ou d'un polypeptide étrangers dans un chloroplaste d'une cellule d'un végétal; ledit procédé comprenant les étapes:
   A. d'expression, dans le cytoplasme de ladite cellule, d'un précurseur chimérique de ladite protéine ou dudit polypeptide étrangers; ledit précurseur étant codé par une séquence d'ADN chimérique dans le génome de ladite cellule; ladite séquence d'ADN chimérique comprenant: i) une première séquence d'acide nucléique codant pour un peptide de transit d'un précurseur cytoplasmique d'une protéine ou d'un polypeptide de chloroplaste d'une espèce végétale et ii) une deuxième séquence d'acide nucléique codant pour ladite protéine ou ledit polypeptide étrangers, qui est hétérologue à ladite première séquence d'acide nucléique et qui est en aval de, et dans la même unité transcriptionnelle que, ladite première séquence d'acide nucléique; et puis
   B. de transport de ladite protéine ou dudit polypeptide étrangers à partir du cytoplasme de ladite cellule dans ledit chloroplaste, avec élimination dudit peptide de transit de ladite protéine ou dudit polypeptide étrangers.

2. Procédé selon la revendication 1, caractérisé en ce que ladite protéine ou ledit polypeptide étrangers, lorsqu'ils sont présents dans les chloroplastes des cellules dudit végétal, confèrent audit végétal une résistance à un herbicide.

3. Procédé selon la revendication 1 ou 2, caractérisé en ce que ladite séquence d'ADN chimérique comprend également une troisième séquence d'acide nucléique codant pour une région N-terminal de ladite protéine ou dudit polypeptide de chloroplaste de ladite espèce végétale et qui est en aval de ladite première séquence d'acide nucléique; l'extrémité 5' de ladite troisième séquence d'acide nucléique étant sensiblement contiguë à l'extrémité 3' de ladite première séquence d'acide nucléique.

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4. Procédé selon la revendication 3, caractérisé en ce que ladite troisième séquence d'acide nucléique code pour au moins une partie d'une sous-unité cytoplasmique de ladite protéine ou dudit polypeptide de chloroplaste de ladite espèce végétale.

5. Procédé selon la revendication 4, caractérisé en ce que ladite troisième séquence d'acide nucléique ne code que pour ladite sous-unité cytoplasmique de ladite protéine ou dudit polypeptide de chloroplaste de ladite espèce végétale.

6. Procédé selon l'une quelconque des revendications 3-5, caractérisé en ce que lesdites première et troisième séquences d'acide nucléique comprennent un exon.

7. Procédé selon la revendication 6, caractérisé en ce que ladite troisième séquence d'acide nucléique comprend un intron.

8. Procédé selon l'une quelconque des revendications 3-7, caractérisé en ce que ladite troisième séquence d'acide nucléique est constituée essentiellement d'une séquence d'acide nucléique présentant une homologie de séquence avec les séquences d'acides nucléiques codant pour les parties N-terminales des sous-unités cytoplasmiques de protéines ou de polypeptides de chloroplaste qui sont communs au soja, communs au pois, à la lentille d'eau et au blé.

9. Procédé selon la revendication 8, caractérisé en ce que ladite troisième séquence d'acide nucléique code pour le pentapeptide M-Q-V-W-P.

10. Procédé selon la revendication 1 ou 2, caractérisé en ce que l'extrémité 3' de ladite première séquence d'acide nucléique est essentiellement contiguë à l'extrémité 5' de ladite deuxième séquence d'acide nucléique.

11. Procédé selon la revendication 10, caractérisé en ce qu'un linker de nucléotides est présent entre lesdites première et deuxième séquences d'acide nucléique.

12. Procédé selon la revendication 10 ou 11, caractérisé en ce que ladite deuxième séquence d'acide nucléique ne code pas pour une région N-terminale d'une protéine ou d'un polypeptide de chloroplaste.

13. Procédé selon la revendication 12, caractérisé en ce que ladite séquence d'ADN chimérique est dépourvue d'intron entre lesdites première et deuxième séquences d'acide nucléique.

14. Procédé selon l'une quelconque des revendications 10-13, caractérisé en ce que le premier codon de ladite deuxième séquence d'acide nucléique code pour la méthionine et est adjacent au dernier codon de ladite première séquence d'acide nucléique.

15. Procédé selon l'une quelconque des revendications 1-14, caractérisé en ce que ladite séquence d'ADN chimérique comprend également un promoteur qui est en amont de ladite première séquence d'acide nucléique de telle sorte qu'à la fois lesdites première et deuxième séquences d'acide nucléique sont sous le contrôle transcriptionnel dudit promoteur; et en ce que ledit promoteur contient une séquence d'acide nucléique qui est reconnue par les polymérases de ladite cellule.

16. Procédé selon la revendication 15, caractérisé en ce que ledit promoteur et ladite première séquence d'acide nucléique sont hétérologues.

17. Procédé selon la revendication 15, caractérisé en ce que ledit promoteur est obtenu à partir d'un gène de la plastocyanine, d'un gène de la ferredoxine-$NADP^+$ oxydoréductase ou d'un gène de la nopaline synthase ou est normalement associé avec ladite première séquence d'acide nucléique.

18. Procédé selon l'une quelconque des revendications 1-17, caractérisé en ce que la première séquence d'acide nucléique code pour un peptide de transit d'un précurseur cytoplasmique d'une petite sous-unité d'une ribulose-1,5-bisphospbate carboxylase-oxygénase.

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19. Procédé selon l'une quelconque des revendications 1-17, caractérisé en ce que ladite deuxième séquence d'acide nucléique code pour une protéine ou un polypeptide bactériens ou végétaux.

20. Procédé selon la revendication 19, caractérisé en ce que ladite deuxième séquence d'acide nucléique code pour une protéine ou un polypeptide de chloroplaste ou une protéine ou un polypeptide de chloroplaste mutés.

21. Cellule susceptible d'être obtenue par le procédé selon l'une quelconque des revendications 1-20 ou un végétal ou une culture de cellules végétales contenant de telles cellules; chacune de telles cellules contenant i) ladite séquence d'ADN chimérique dans son génome, de préférence son génome nucléaire, et ii) un chloroplaste contenant ladite protéine ou ledit polypeptide étrangers, dépourvu dudit peptide de transit.

22. Semence du végétal de la revendication 21.

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