Cytoplasm and chloroplasts are not suitable subcellular locations for β-zein accumulation in transgenic plants

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Abstract

Zeins, the main storage proteins of maize that accumulate in the endoplasmic reticulum of the endosperm cells, are particularly interesting because they are rich in the essential sulphur amino acids. Overexpression of certain zein genes in plants such as alfalfa would be expected to improve the nutritional characteristics of this crop. Recently, significant accumulation values have been reached, but still far from those considered useful for nutritional purposes. This study investigates whether targeting to compartments other than the endoplasmic reticulum (cytosol and chloroplasts) could result in increasing β-zein accumulation in transgenic plants. To address β-zein to the cytosol, the fragment which codes for the signal peptide has been removed. β-zein has also been targeted to alfalfa and tobacco chloroplasts by a transit peptide signal. Both tobacco, as a model plant species, and alfalfa have been transformed with the assembled constructs. An alternative route to accumulate β-zein in the chloroplasts is to synthesize β-zein directly in the plastid lumen. Thus, the β-zein gene has also been inserted into tobacco plastid DNA. The β-zein gene in each different type of transformed plant was properly transcribed, as determined by northern blot analysis, but no accumulation of β-zein was detected, either in the cytoplasm or in the chloroplasts of alfalfa and tobacco transformed plants. Therefore, it is concluded that chloroplasts and the cytosol are not favourable subcellular locations for zein protein accumulation.

Key words: β-zein, chloroplast, cytoplasm, green fluorescent protein, improved nutritional quality, plastome transformation, transgenic alfalfa.

Introduction

The nutritional quality of crop plants depends mainly on their content in essential amino acids but plant proteins are often deficient in some of them. Therefore, various strategies have been developed to improve the nutritive value of crops by modifying their protein composition through genetic engineering (Tabe and Higgins, 1998). High-methionine or high-lysine proteins have been expressed in transgenic plants (Galili et al., 2002), either using genes coding for seed storage proteins (SSPs) (Molvig et al., 1997) or genes coding for vegetative storage proteins (VSPs) (Guenoune et al., 1999). Essential amino-acid-rich SSPs were largely used because they are biochemically well characterized. Genes encoding the Brazil nut 2S albumin and its homologue from sunflower were expressed under the control of seed-specific promoters in several species and 2S heterologous proteins accumulated from 5% to 10% of the total seed protein (Muntz et al., 1998). In the transgenic seeds, the 2S albumins were stably deposited in protein bodies (PBs) derived from the vacuolar compartment. Conversely, SSPs did not accumulate to high levels in transgenic vegetative tissues, either when they were directed to the protease-rich vegetative vacuoles (Galili et al., 2002) or when they were directed to the endoplasmic...
reticulum (ER) (Bellucci et al., 2002). Only lysine-rich soybean VSPs accumulate to high levels (between 2% and 6% of the leaf soluble proteins) inside vacuoles of vegetative tobacco tissues, representing a promising protein source to enrich the nutritional quality of lysine-poor forage crops (Guenoune et al., 1999). Furthermore, targeting soybean VSPs by a transit peptide to the chloroplasts results in a significant protein accumulation even in this organelle (Guenoune et al., 2002). A lot of other foreign proteins have been successfully expressed in chloroplasts of higher plants (Daniell and Dhingra, 2002; Nawrath et al., 1994), even if sometimes the accumulation of heterologous proteins in this organelle is limited by protein stability (Birch-Machin et al., 2004). Legume forage crops are usually deficient in sulphur amino acids, but stable expression of methionine- and cysteine-rich maize zein proteins has recently been achieved in alfalfa (Medicago sativa) and birdsfoot trefoil (Lotus corniculatus). Nevertheless, zeins accumulated in small quantities in ER-derived PBs in transgenic cells of vegetative tissues (Bagga et al., 2004; Bellucci et al., 2002). Therefore, the aim of this study was to investigate the cytosol and chloroplasts as target organelles as an alternative to the ER for zein protein accumulation in transgenic vegetative tissues.

Materials and methods

Recombinant DNA techniques for nuclear transformation

The β-zein cDNA (G2) was amplified by polymerase chain reaction (PCR) from the pBS.G2 vector (Bellucci et al., 2002) using the forward primer 5’G2tp (5’-GGCTCCTCTAGAATGCAGATGCCCTGCCCCTG-3’) and the backward primer G2SacI.
(5’-GGCTCGAAGCCTCGATGGTAGGGCCGAATGG-3’), to insert a XbaI and a SacI restriction site (underlined) at the 5’ and 3’ end of the G2 gene. Primer 5’G2p anneals 60 bp downstream of the start codon, in order to remove the signal peptide sequence from the coding region of β-zein. The PCR product was cleaved with XbaI and SacI and inserted first in the same sites of pSP73 (Promega), obtaining in this way plasmid pSP73.NSP.G2, and then into the XbaI/SacI sites of p121.1 binary plasmid (Jefferson et al., 1987), which contained the nptII marker gene coding for kanamycin resistance, to form p121.NSP.G2. In order to obtain chloroplast targeting of the green fluorescent protein (GFP), β-zein, and a fusion protein between β-zein and GFP, by the transit peptide (tp) of the tobacco ribulose bisphosphate carboxylase small subunit (rbcS), plasmids pJT117.tpGfp, pJT117.tpG2, and pJT117.tpG2:tpG2 were constructed (Bellucci et al., 2003). These three genes tpGfp, tpG2, and tpG2:tpG2 were excised from the corresponding pJT117 vector and then cloned into the binary vector p121.1. In detail, each pJT117 vector was digested first with the restriction enzyme HindIII and then with the Klenow fragment of DNA polymerase I to convert the HindIII ends into blunt ends. Each gene was then excised digesting the corresponding pJT117 vector again with the restriction enzyme Smal. The excised genes were inserted first into the Smal site of plasmid pSP73, thus obtaining plasmids pSP73.tpGfp, pSP73.tpG2, and pSP73.tpG2:tpG2, and then into the XbaI/SacI sites of plasmid p121.1, to form p121.tpGfp, p121.tpG2, and p121.tpG2:tpG2 (Fig. 1A). Nicotiana tabacum cv. Petit Havana SR1 plants were transformed with plasmids p121.NSP.G2, p121.tpGfp, p121.tpG2, and p121.tpG2:tpG2 harboured by Agrobacterium tumefaciens strain LBA4404 following the leaf disc method (Horsch et al., 1985). Alfalfa (Medicago sativa cv. Regen SY) transformation, using plasmids p121.NSP.G2 and p121.tpG2, was essentially carried out according to the procedure of Austin et al. (1995). Ten independent T0 transgenic plants were regenerated for each species/gene construct combination on kanamycin and transferred to the greenhouse.

Chloroplast transformation vector and E. coli protein extraction

The G2 CDNA was amplified from pBS.G2 using the EcorVG2 (5’-GGCTCGAATCAGCTATGGGAGGAGGATCGCGATGGCCTGCCCTG-3’) and the HindIII G2 (5’-GGCTCAAGCCTCTCACTAGGGCGGAGATGGCGGAGTGGAGTGGG-3’) oligonucleotides to introduce at the 5’ the gene both an EcorV site (underlined) and a Shine-Dalgarno consensus sequence (in bold) as well as at the 3’ an HindIII site (underlined). This PCR product was cloned into the EcorV and HindIII sites of the pBSazaadA2(2) plasmid (Bellucci, 2003): so the azaad gene was replaced by the β-zein one, to obtain pBSazG2 in which the G2 cDNA is under the control of the Prm promoter and regulated by the 3’ untranslated region of the plastid rbcL gene from Chlamydomonas reinhardtii. Oligonucleotides Prm5’ (5’-GGCTCTGTCAGCCTCTGCTAGGAGAACATGCCGATGGCCTGGAGTGGG-3’) and RbcL3’ (5’-GGATCGAATCAGCTATGGGAGGAGGATCGCGATGGCCTGCCCTG-3’) were then used as the T4 polynucleotide kinase and used to amplify the Prm-G2-rbcL expression cassette from pBSozG2. This PCR product was finally cloned into the Smal site of the pBR94 (Ruf et al., 2001) plasmid to create pBR94.G2. Since the chloroplast protein synthetic machinery is similar to that of E. coli, pBR94.G2 expression was initially tested in E. coli. Forty cultures of two E. coli cultures transformed with pBR94.G2 or pBR94, grown overnight in LB and spectinomycin 500 mg l⁻¹, were centrifuged at 6 000 g for 15 min and 4 °C. Pellets were washed twice with 50 mL TRIS–HCl pH 8.0, resuspended in ice-cold 50 mL TRIS–HCl, 50 mL NaCl, 50 mL EDTA, 5% glycerol, 1 mL DTT, 1 mL PMSF, pH 8.0 (1 ml g⁻¹ fresh weight) and hydrolysed at 4 °C for 4 h adding lysozyme to a final concentration of 300 µg ml⁻¹. After centrifugation at 20 000 g for 30 min and 4 °C the supernatants were conserved and named TE, while the pellets obtained were washed twice with ice-cold 50 mL TRIS–HCl pH 8.0, dissolved in 50 mL TRIS–HCl, 10 mL EDTA, 4 mL urea, 1 mL PMSF, pH 8.0, and placed at 37 °C for 1 h. After centrifugation at 12 000 g for 15 min and 4 °C, the supernatants were conserved and named IBs, while the pellets were dissolved in 70% EtOH, 2% β-MeEtOH, 1 mL PMSF, and 1× protease inhibitor mix (Boehringer), vortexed, incubated at 65 °C for 10 min, and centrifuged as described above. The supernatants obtained were named IBa.

Bombardment and selection of transplastomic plants

Transplastomic plants were obtained by delivering microprojectiles coated with pBR94.G2 by particle bombardment into tobacco leaves using the biolistic device PDS1000/He (Bio-Rad), as described by Daniell (1997). The sequence of the primers used to verify plastome transformation were: G1, 5’-ATGATGGCGCAGAACATGCCG-3’; C1, 5’-GGCTGATCGCAGAATGATG-3’; P11, 5’-AGCGAAAATGGTAGCTGGTT-3’; 3M, 5’-GAGCCGTTGTTCATCAAGGAGTGG-3’.

Isolation and analysis of nucleic acids

RNA was extracted with Nucleo Spin® RNA Plant Kit (Macherey-Nagel, Germany) and analysed according to Bellucci et al. (2002), whereas total DNA was isolated and analysed according to the same authors.

In vitro translation

In vitro translation of pSP73.NSP.G2, pSP73.tpG2, pSP73.tpGfp, and pSP73.tpG2:tpG2 was performed with the TNT Coupled Wheat Germ Extract System (Promega) in 50 µl with 35S-methionine. Plasmids were cleaved at the SacI site located at the 5’ end of the DNA insert. Translation product was directly resuspended in a loading buffer and separated by SDS-PAGE. Following SDS-PAGE, gels were fixed in 10% (v/v) methanol and 30% (v/v) glacial acetic acid for 30 min. Gels were then impregnated with Amplify™ (Amersham Biosciences),
dried, and subjected to fluorography according to the manufacturer’s instructions.

**Protein immunoblot analysis and detection of GFP**

Protein was isolated and analysed as described in Bellucci *et al.* (2003). GFP was visualized using a 1:400 dilution of polyclonal anti-GFP A.v. Peptide Antibody (BD Biosciences, Clontech) and β-zein was detected with a polyclonal antiserum utilized at 1:400 dilution. The protein bands were visualized with peroxidase-linked goat anti-rabbit secondary antibody (1:5000, Pierce) using SuperSignal West Pico chemiluminescent substrate (Pierce). The GFP fluorescence was observed under a laser scanning confocal microscope as described in Bellucci *et al.* (2003). Images were processed with the Imaris software (Bitplane, Zurich, CH) following the depth-analysis module and the shadow projection reconstruction technique. For each of the fluorescence images of the GFP expressing cells a negative control was photographed for comparison (data not shown). Bright-field images of the GFP expressing cells were used to identify the different kinds of cells.

**Results and discussion**

Zeins are naturally synthesized by membrane-bound polypeptides and transported into the lumen of the ER, where they assemble in PBs (Khoo and Wolf, 1970). Nascent proteins acquire their mature structure in the ER assisted by several ER-resident molecular chaperones (Vitale and Denecke, 1999). The induction of chaperones such as BiP and GRP94 is triggered by the presence of non-native structures and/or misassembled proteins in the ER (Gething and Sambrook, 1992). Therefore, the ER is a tightly regulated subcellular environment where, particularly in leaves, it seems difficult for heterologous proteins to accumulate at exceptionally high levels. Thus, to enhance the zein expression level in transgenic plants, this study’s strategy was to redirect the β-zein both to the cytosol and to the chloroplasts. Alfalfa and tobacco nuclear genomes were
transformed with p121.NSP.G2 and p121.tpG2 plasmids which target β-zein to the cytosol and the chloroplast, respectively. The β-zein gene in transformed plants was properly transcribed, as determined by northern blot analysis, yielding the predicted 0.6 and 0.8 kb mRNA (Fig. 1B, lanes 1–2 and 3–4). However, the two constructs failed to produce detectable amounts of β-zein in either alfalfa or tobacco, by western blot using proteins isolated from different tissues in aqueous or in alcoholic solvents. In vitro translation of the two gene constructs revealed two protein bands with apparent molecular mass of 15 kDa and 25 kDa, respectively, as expected (Fig. 2, lanes 1, 2), indicating that, at least in vitro, the RNA of the β-zein transgenes is effectively translatable. Thus, no accumulation of β-zein in the cytoplasm and chloroplasts of alfalfa and tobacco transformed plants can be ascribed to post-transcriptional effects. It was concluded that the cytosol is not a favourable subcellular location for zein proteins, even though the cytosol sometimes increased the levels of alien proteins when they were redirected to it (Avesani et al., 2003).

To elucidate the reasons of β-zein instability in the chloroplasts, the efficiency of the rbcS transit peptide used in this study in targeting heterologous proteins to the chloroplast was verified in tobacco using GFP. Transformed tobacco p121.tpG2 plants accumulate GFP inside the chloroplast. Western analysis on these plants showed an immunologically detectable band of 27 kDa (Fig. 3A), which corresponds to the GFP whose transit peptide has been cleaved after entering into the chloroplast, and a very faint band of 34 kDa which is the tpGFP not cleaved (Fig. 2, lane 3). Moreover, confocal laser-scanning microscope detected GFP fluorescence inside the chloroplasts which are localized around the cell periphery, either in the mesophyll cell or in the petal cell (Fig. 3A). Thus, even tpβ-zein, which uses the same transit peptide of tpGFP, should enter into the chloroplasts, but it does not seem not to be stable in these organelles.

In order to clarify the fate of tpβ-zein in the chloroplasts, tpβ-zein was fluorescence-labelled at the C-terminal with GFP. Tobacco plants transformed with the vector p121.tpG2:gfp were expected to express the fusion protein tpβ-zein:GFP with a molecular mass of 50 kDa (Fig. 2, lane 4). These transformants were tested for tpβ-zein:GFP production by western blot analysis. Proteins were first extracted in an aqueous solvent and the pellet was then resuspended in an alcoholic solvent. Anti-GFP antibody recognized a peptide of 27 kDa using proteins isolated in aqueous solvent (Fig. 3B). Anti-β-zein did not detect any specific peptide (data not shown). Under confocal laser-scanning microscope tpβ-zein:GFP fluorescence was not diffused in the chloroplasts as it was for p121.tpG2 transformants, but it was detected as small green fluorescent spots both in the cytoplasm and in the chloroplasts (Fig. 4).

Fig. 4. Expression of the transgene cassette in E. coli and its integration into the tobacco chloroplast genome. (A) Schematic representation of the rps14/trnfM region of the tobacco chloroplast genome. Arrows over the genes indicate the direction of transcription. The annealing positions of primers p11/C1 and G1-3M are shown. (B) Twenty μg of bacterial proteins from E. coli transformed with pRB94 or pRB94.G2 was separated by SDS-PAGE and subjected to western analysis. Proteins were soluble in saline extraction buffer (TE), or were obtained from inclusion bodies (IB) and solubilized in saline extraction buffer with urea (IBs) or in alcohol (IBE). C+, a 0.5 μg aliquot of alcoholic protein extract from maize endosperm was used as standard. (C, D) PCR analyses of pRB94.G2 transformed plants with primer pairs p11/C1 and G1-3M. Prrn, 16S rRNA promoter; rbcL, 3'-untranslated region of the gene coding for the large subunit of the ribulose 1,5-bisphosphate carboxylase; aadA, aminoglycoside 3'-adenylyl transferase gene; wt, wild type; C–, PCR reaction with no DNA; C+, PCR reaction with pRB94.G2 DNA.
fraction of the terminal part is relatively stable in the cytoplasm. Another until full degradation, whereas the remaining GFP-C-fusion protein is rapidly removed by cytosolic proteases entering into the chloroplasts. The N-terminal part of the pRB94.G2 linearized with Ndel. The transformed plants have a band of 7.2 kb (lanes 2–5), which is the result of the 4.7 kb band of the wild type (lane 1) plus the 2.5 kb fragment of the transgene cassette (Fig. 4A). Plasmid pRB94.G2 linearized with Ndel produced the expected 8.6 kb band (lane 7).

3B). GFP localization in the chloroplasts is clearly visible in the stomatal guard cells (white arrows), but there are also several fluorescent spots in the cytoplasm. In the petal cells, vacuoles are surrounded by green fluorescence that indicates the presence of GFP in the cytoplasm. This study’s hypothesis is that, immediately after translation, a part of the tpβ-zein:GFP is degraded in the cytoplasm just before entering into the chloroplasts. The N-terminal part of the fusion protein is rapidly removed by cytosolic proteases until full degradation, whereas the remaining GFP-C-terminal part is relatively stable in the cytoplasm. Another fraction of the tpβ-zein:GFP molecules enters into the chloroplast, as demonstrated already by transient assays (Bellucci et al., 2003), but these polypeptides are rapidly degraded at the N-terminal part, probably with the same mechanism described above.

These data indicate that tpβ-zein:GFP has real stability problems before its import into the chloroplasts and even after its import. To overcome the difficulties related to import into the chloroplasts and to understand if β-zein can really accumulate in the chloroplasts, the β-zein gene was inserted into the tobacco chloroplast DNA, so that β-zein mRNA could be directly translated in the stroma. The β-zein gene was cloned in a plastid expression cassette which was then introduced into the plastid transformation vector pRB94, upstream of, and in the same orientation as, the aadA selectable marker gene (Fig. 4A). The functionality of the β-zein expression cassette was initially investigated in E. coli. Either total protein extract or protein extract from inclusion bodies solubilized in aqueous solvent or in ethanol was examined by immunoblot analysis with an anti-β-zein antibody. Zein expression was detected in inclusion bodies as two polypeptides soluble in ethanol with a molecular mass between 15 kDa and 20 kDa, slightly higher than expected (Fig. 4B, lane 6). Other non-specific polypeptides were recognized by the anti-β-zein antibody. Thus overexpression of β-zein in bacteria leads to the production of insoluble aggregates, probably due to its tendency, as in the plant cell, to form protein bodies of polypeptides linked by sulphur bonds (Bagga et al., 1995). Ten chloroplast-transformed tobacco plants were generated by leaf bombardment with pRB94.G2, followed by four rounds of shoot regeneration on 500 mg l⁻¹ spectinomycin. Total DNA samples were examined by PCR using the primer pairs p11/C1 and G1/3M to confirm that both the β-zein and aadA genes were correctly inserted into the plastome (Fig. 4C, D). The primers amplified the predicted DNA fragments in the resistant transformants, except the one in which the 2.1 kb product was not amplified (Fig. 4C, lane 3). Southern blot analysis was carried out on four of these plants to determine whether they were homoplasmic. Total leaf DNA cut with Ndel, and probed with a rps14/trnfM probe (Fig. 4A), produced a band of 7.2 kb, as expected for transplastomic DNA, while the 4.7 kb band expected for wild-type DNA was absent in the transformed plants (Fig. 5). β-zein mRNA accumulation was studied by northern blot analysis of total RNA extracted from leaves of chloroplast-transformed plants. Probing of the gel blot with the β-zein gene showed a complex hybridization pattern (Fig. 6). The β-zein-encoding mRNA species included two main messages of 0.6 kb (monocistronic β-zein transcript), and 2.2 kb (dicistronic β-zein-aadA transcript), due to the lack of efficient transcription-termination, as already reported (Staub and Maliga, 1994). The two minor bands of 0.4 kb and 2.9 kb could derive from the transcription activity of another gene upstream of the β-zein gene like trnfM, for example. A 0.6 kb band was also obtained in the nuclear-transformed p121.NSP.G2 plant, but the northern blot had to be overexposed to observe the nuclear transcript. Another northern blot using the aadA gene as a probe confirmed the previous results (data not shown).

Despite the fact that a significant level of β-zein transcript was detected in transplastomic pRB94.G2 plants, and that the β-zein expression cassette in pRB94.G2 was functional in E. coli, it was not possible to detect the β-zein
protein in pRB94.G2 plants. Very similar results have recently been obtained by Magee et al. (2004), who introduced a dicistronic expression cassette encoding the α and β-subunits of human haemoglobin into the tobacco plastid DNA. These authors explained the lack of haemoglobin expression in the chloroplasts with the inefficient initiation of haemoglobin mRNA translation or with proteolysis of recombinant haemoglobin. Considering the fact that β-zein was not detected in the cytoplasm or in the chloroplasts using both nuclear and plastome transformation, it is reasonable to assume that β-zein accumulation in these two subcellular compartments has been prevented by a common mechanism which could be the activity of endogenous proteases. β-zein degradation could be triggered by the lack of very specific molecular chaperones such as those (BiP?) which could help β-zein to fold in the ER, but which are not found outside this compartment.

Fig. 6. Transcription pattern of transgenic plants. Northern blot analysis of β-zein transcript accumulation in pRB94.G2 plants (lanes 1–4) and in a nuclear-transformed p121.NSP.G2 plant (lane 5). The filter, containing approximately equal quantities (10 μg) of total RNA, was hybridized with a PCR fragment corresponding to the G2 coding region. Ethidium bromide-stained RNA was used to assess loading. A scheme of the transcription pattern expected for the β-zein gene integrated in the chloroplast genome (two transcripts of 0.6 and 2.2 kb showed by horizontal arrows) is shown at the bottom of the figure.
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