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Simultaneous targeting of pea glutathione reductase and of a bacterial fusion protein to chloroplasts and mitochondria in transgenic tobacco

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Summary

N-terminal presequences from cDNAs encoding mitochondrion- or chloroplast-specific proteins are able, with variable efficiencies, to target preproteins to their respective organelles. In the few cases studied in which a nuclear-encoded protein is found in both these organelles, each compartment-specific isoform is encoded by a separate gene. Glutathione reductase (GR) from peas is encoded by a single nuclear gene and yet GR is distributed between chloroplasts, mitochondria and the cytosol. Previous sequence analysis of a full-length GR cDNA revealed the presence of a putative plastid transit peptide. However, expression of this cDNA in transgenic tobacco resulted in substantially elevated GR activities in both chloroplasts and mitochondria in four independent lines examined. There was no effect on expression of the endogenous tobacco GR genes. Replacement of the GR presequence with presequences from pea *rbcS* (chloroplast) and *Nicotiana plumbaginifolia Mn-SOD* (mitochondrion) resulted in targeting of GR only into the appropriate organelle. Expression of a fusion protein between the amino terminal region of GR and phosphinothricin acetyl transferase resulted in targeting of the foreign protein to chloroplasts and mitochondria. Thus, the pea GR presequence is capable of co-targeting this enzyme or a foreign protein to chloroplasts and mitochondria *in vivo*. This is the first example of co-targeting by a higher plant preprotein.

Introduction

The majority of chloroplast and mitochondrial proteins are encoded by nuclear genes and are synthesized on cytosolic ribosomes as preproteins. Targeting of these preproteins to the correct organelle is generally specified by an amino-terminal presequence or transit peptide which is proteolytically removed after import into the appropriate organelle (Braun *et al.*, 1992; Hawlitschek *et al.*, 1988; Robinson and Ellis, 1984). Preproteins destined for the inner mitochondrial membrane or for the thylakoid lumen are further processed by specific peptidases to generate the mature polypeptide (Hageman *et al.*, 1986; Musgrove *et al.*, 1989; Schneider *et al.*, 1991).

Analysis of mitochondrial and chloroplast presequences reveals that these signals are extremely heterogeneous both in terms of length and sequence composition. There is little structural conservation within either class, although there is evidence that mitochondrial presequences have a tendency to form amphiphilic helices (Endo *et al.*, 1989; von Heijne, 1986; Roise *et al.*, 1986). Comparison of a relatively small number of chloroplast presequences indicated the presence of three 'homology blocks' (Karlin-Neumann and Tobin, 1986), however subsequent examination of a wider range of sequences indicated that there are no regions of highly conserved amino acids in chloroplast presequences, although there is evidence for structurally distinct domains (von Heijne *et al.*, 1989). Both classes of presequence show some similarities in their overall amino acid composition, being low in acidic residues (asp, glu) and high in serine. In other respects they differ markedly, with mitochondrial presequences having higher arginine and leucine content and lower threonine content than chloroplast presequences (von Heijne *et al.*, 1989).

Irrespective of the possible similarities or differences between these two presequence classes there is little evidence for mistargeting of preproteins. Indeed, experiments using chimaeric gene constructs, where a presequence has been fused to a reporter gene sequence, confirmed the fidelity of organelle targeting in transgenic plants (Boutry *et al.*, 1987; Kavanagh *et al.*, 1988). Similarly, *in vitro* studies indicated a high organellar specificity for preproteins (Whelan *et al.*, 1990). Two exceptions to this high specificity were found using heterologous systems. The chloroplast transit peptide from the small subunit of Rubisco from *Chlamydomonas reinhardtii* targeted a mouse dihydrofolate reductase into yeast mitochondria *in vivo* and *in vitro* (Hurt *et al.*, 1986). In the other example, a fusion protein between a yeast mitochondrial presequence and chloramphenicol acetyl transferase was expressed in transgenic tobacco and was found to be targeted to the chloroplasts with an efficiency equal to or greater than targeting to the mitochondria, depending on the growth conditions (Huang *et al.*, 1990).

In this paper we report for the first time the simultaneous

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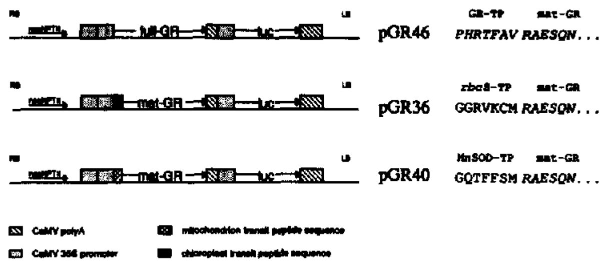

Figure 1. Chimaeric glutathione reductase constructs.
Full-GR refers to the polypeptide(s) encoded by the full-length pea GR cDNA sequence (λGR201: Creissen *et al.*, 1992). The polypeptide encoded by the truncated cDNA, which encodes an active GR polypeptide (defined by its ability to restore GR activity to the *E.coli gor* mutant) is termed mat-GR. The firefly luciferase gene (luc) is towards the left border of the T-DNA. The amino acid sequences of the region around the presumed cleavage site of the mature GR polypeptide are shown on the right. Sequences encoded by the pea GR cDNAs are italicized.

targeting of a higher plant protein, pea glutathione reductase (GR), into chloroplasts and mitochondria of transgenic tobacco plants and we demonstrate that the GR presequence is also capable of targeting a bacterial protein, phosphinothricin acetyl transferase into both organelles.

Results

The binary vector, pGR46, shown in Figure 1, contains the full-length pea glutathione reductase cDNA, including its presequence (Creissen *et al.*, 1992), under the control of the CaMV35S promoter (with duplicated enhancer region) and polyadenylation sequences. On the left-border side of the chimaeric GR gene is a chimaeric CaMV35S-firefly luciferase gene (Mullineaux *et al.*, 1990) which allowed rapid screening for complete T-DNA transfer in kanamycin-resistant shoots. Twelve independent transgenic (kanamycin-resistant, luciferase positive) plants were regenerated (T_1 generation) and allowed to self-pollinate. Seed from these plants (T_2 population) was harvested and progeny were screened for segregation of the T-DNA by assaying for luciferase (luc) activity in seedling leaves (see Experimental procedures). Four independent transgenic lines which exhibited a 3:1 segregation of the luc marker in the T_2 population (i.e. contained a single T-DNA locus) were selected for further analysis. These were GR46.1, GR46.23, GR46.27, and GR46.30.

Subcellular compartmentation of glutathione reductase

Chloroplasts and mitochondria were isolated from luc-positive (i.e. homozygous or heterozygous for both luciferase and GR transgenes) and luc-negative (i.e. recessive for the transgenes) T_2 progeny of the each of the GR46 lines and the GR activity in each of the subcellular fractions was determined. Figure 2 shows the specific activities for GR in total leaf protein extracts, isolated chloroplasts and isolated mitochondria from each of the transgenic lines. The specific activities for the enzyme in total extracts were consistently higher in the luc-positive (transgenic) progeny than in their recessive siblings. Furthermore, the specific GR activities in each of the subcellular fractions were also higher than in equivalent fractions from luc-negative siblings. The high levels of GR activity in either compartment were not caused by contamination with the other organelle: there was little or no detectable mitochondrial marker enzyme (cytochrome c oxidase) activity in the chloroplast fractions and although there was some chloroplast contamination (indicated by GAPDH activity) in three of the lines (Table 1), this level of cross contamination could not account for the high levels of GR activity detected in these fractions from the luc-positive samples. Furthermore, GR46.27, in which no GAPDH activity could be detected in the mitochondrial fraction, had high GR activities in this fraction as well as in the chloroplasts of transgenic progeny. Measurement of the activity of the cytosol marker, pyrophosphate-dependent phosphofructokinase, indicated that there was no significant contamination of the organelles by cytosol proteins. Consequently, it was concluded that the pea glutathione reductase preprotein encoded by pGR46 contains all of the necessary information for simultaneous targeting (co-targeting) of GR to both mitochondria and chloroplasts in the transgenic tobacco plants.

Identification of the functional co-targeting component of pGR46

To investigate whether the co-targeting phenomenon described above was a function of the pea GR transit peptide sequence, two further binary constructs were made. These were based on a truncated pea GR cDNA (λGR27; Creissen *et al.*, 1992) encoding a polypeptide which extended approximately eight residues beyond the presumed N-terminus of the mature GR polypeptide by comparison with the amino terminus of the *Pinus sylvestris* glutathione reductase (Karpinski, 1994). This truncated GR cDNA was able to restore GR activity in the *Escherichia coli* glutathione reductase (*gor*) deletion mutant SG5 (Greer and Perham, 1986), and therefore encodes an active GR polypeptide (data not shown). The λGR27 sequence was fused either to the sequence encoding the chloroplast transit peptide from pea *rbcS* under the control of the CaMV35S promoter and polyadenylation sequences in the expression cassette pJIT117 (Guerineau *et al.*, 1988) or to the Mn-superoxide dismutase presequence from *Nicotiana plumbaginifolia* (Bowler *et al.*, 1989) for targeting of the pea GR to the mitochondrion. These chimaeric genes were ligated into the pBinluc binary vector to create pGR36 and pGR40, respectively (Figure 1). Two independent transgenic tobacco lines, which segregated as single loci for the

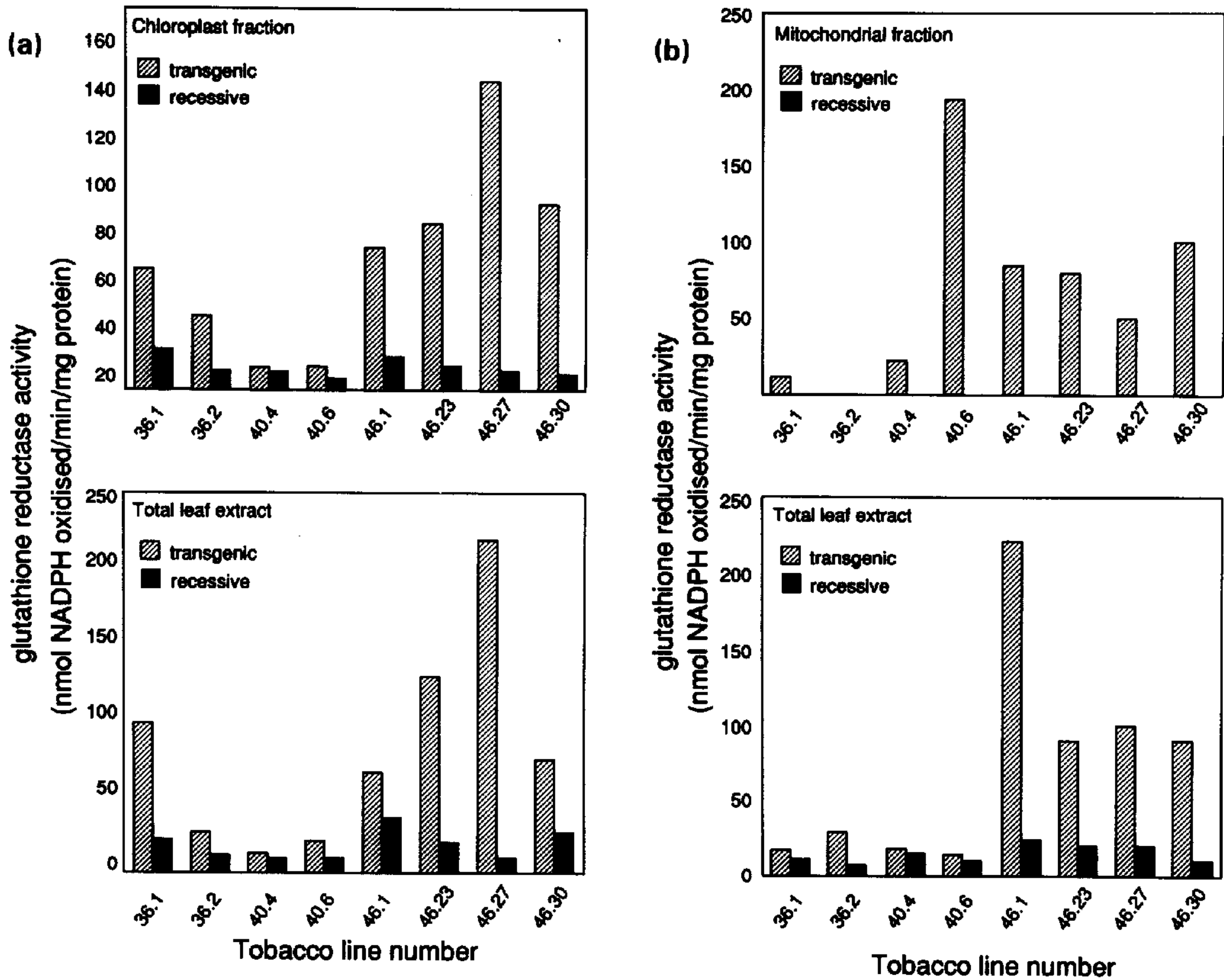

Figure 2. Glutathione reductase activities in (a) total leaf extracts and purified chloroplasts, and (b) total leaf extracts and isolated mitochondria from transgenic (luc-positive) and recessive (luc-negative) T_2 progeny of transgenic tobacco lines containing the chimaeric GR constructs: GR36.1, GR36.2 (chloroplast-targeted); GR40.4, GR40.6 (mitochondrion targeted); GR46.1, GR46.23, GR46.27, GR46.30 (co-targeted).

Table 1. Marker enzyme data for organelle fractions from GR46 lines

	GAPDH		CCO		PPiPFK	
Fraction	luc+	luc−	luc+	luc−	luc+	luc−
Chloroplast						
GR46.1	70	107	ND	ND	ND	ND
GR46.23	30	30	ND	ND	ND	ND
GR46.27	30	20	ND	ND	ND	ND
GR46.30	57	42	0.6	ND	ND	ND
Mitochondrion						
GR46.1	12	2	150	250	ND	ND
GR46.23	20	10	345	400	ND	ND
GR46.27	ND	ND	1900	1900	ND	ND
GR46.30	4	2	380	440	ND	ND

GAPDH (glyceraldehyde phosphate dehydrogenase—chloroplast marker); CCO (cytochrome C oxidase–mitochondrial marker); PPiPFK (pyrophosphate-dependent phosphofructokinase–cytosol marker). Enzyme activities are nmol substrate oxidized (min mg protein)$^{-1}$. ND indicates that the activity was below detectable limits.

transgene in T_2 progeny, were selected for compartmentation analysis for each of the constructs. These were GR36.1 and GR36.2 (chloroplast transit peptide) and GR40.4 and GR40.6 (mitochondrion presequence).

The specific activities for GR in total extracts and in purified chloroplasts and mitochondria from luc-positive and for luc-negative progeny of each of the four lines are shown in Figure 2. In every case, elevated GR activity was only associated with the predicted target organelle. However it was noted that, in general, the GR activities in total extracts from the GR36 and GR40 plants were lower than those obtained with pGR46. Where the mature pea GR sequence was introduced with no presequence there was no elevation of GR protein or activity in either organelle (data not shown). Western blots of protein extracts from isolated chloroplasts and mitochondria confirmed the presence of the GR protein of the predicted molecular weight in the appropriate subcellular compartment (Figure 3). The pea anti-GR antiserum recognized both pea and tobacco GRs with equal efficiency when equivalent activities of partially purified GR were compared by Western blotting (data not shown). However, the low abundance of GR in non-transformed tobacco tissues meant that it was not possible to detect the protein on Western blots of total extracts. Therefore, the GR protein detected in the transgenic samples represents a significant elevation over the wild-type levels.

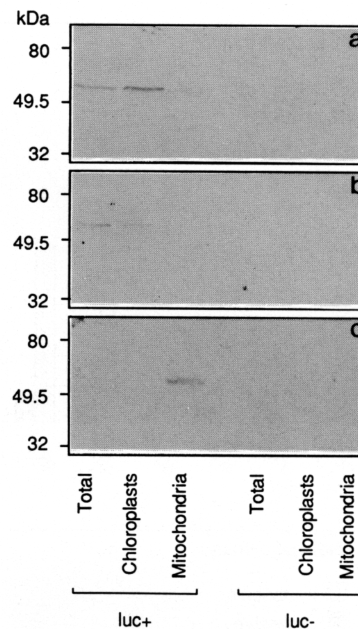

Figure 3. Western blots of proteins from whole leaves (total), isolated chloroplasts and isolated mitochondria from transgenic (luc+) T_2 progeny and their recessive siblings (luc-). One hundred micrograms of protein were loaded per lane: (a) GR46.1; (b) GR36.1; (c) GR40.4. GR was detected using the anti-pea leaf-GR antiserum (Edwards *et al.*, 1990).

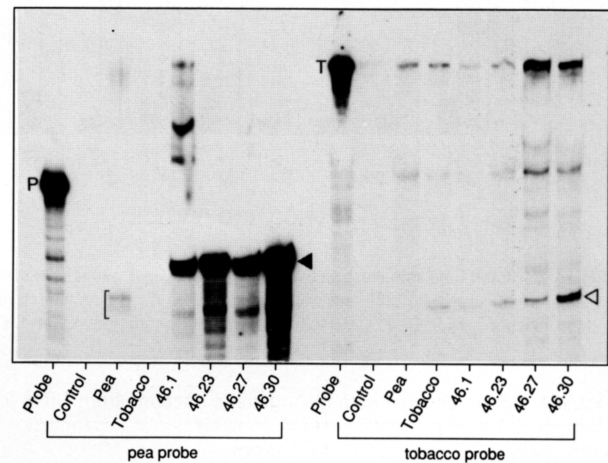

Figure 4. RNaseA/T1 protection analysis of pea and tobacco GR transcripts in control pea and tobacco RNA and in RNA isolated from GR46 lines. Protected fragments corresponding to the pea and tobacco transcripts are indicated by open and closed arrowheads, respectively. The undigested pea and tobacco probes are designated P and T. Lane 1, pea RNA control (predigested with RNaseA); 2, pea; 3, control tobacco; 4 and 11, GR46.1, 5 and 12, GR46.23; 6 and 13, GR46.27; 7 and 14, GR46.30. Samples in lanes 1–7 were hybridized with the pea GR fragment, samples in lanes 11–17 were hybridized with the tobacco GR fragment.

Transcription of the native and pea GRs in the transgenic lines

It was not possible to differentiate between the pea GR and the native tobacco GR in the transgenic plants by immunodetection on Western blots of protein extracts resolved by SDS–PAGE. Furthermore, due to the large numbers of pea and tobacco GR isoforms visualized on non-denaturing gels (Edwards *et al.*, 1994; Foyer *et al.*, 1992) it was not possible to resolve the transgene-encoded GR from the tobacco GR by this method. However, we were able to confirm by RNaseA/T1 protection analysis using *in vitro* synthesized GR probes from the pea cDNA (pGR201 co-ordinates 2030–1788: Creissen *et al.*, 1992) and from the tobacco cDNA clone pGRT4 (co-ordinates 1348–918; EMBL accession number X76533) that, although there was some variation in the levels of transcription of the native and introduced GR sequences in the different GR46 lines, this variability did not correlate with the increase in GR levels. No protected fragments were obtained when the pea probe was used to protect RNA from control tobacco (Figure 4).

Targeting of phosphinothricin acetyl transferase

Having established that pea GR is targeted to chloroplasts and mitochondria in transgenic tobacco, we then attempted to ascertain whether this phenomenon could be repeated with a foreign protein. We constructed a chimaeric gene construct in which the GR presequence and part of the mature protein was fused to the coding sequence for phosphinothricin acetyl transferase (pat), which tolerates fusions to its amino terminus (Botterman *et al.*,1991). This

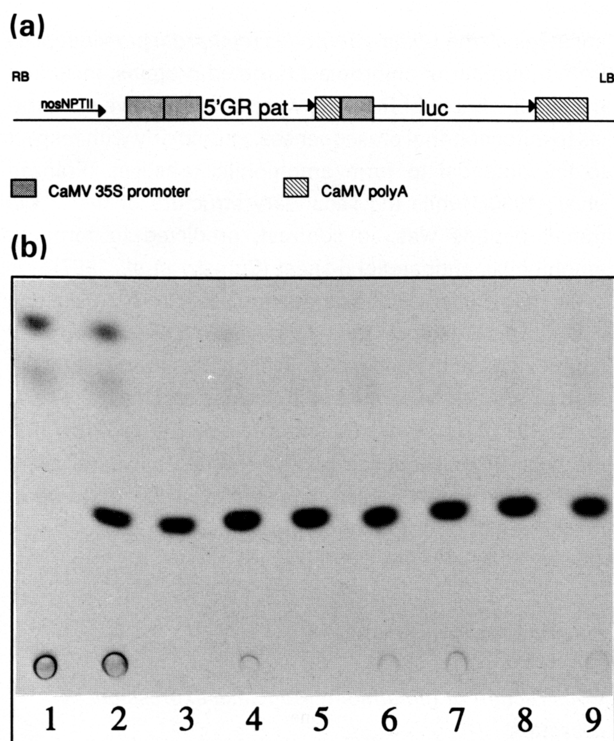

Figure 5. (a). Map of pGR50.
(b) Phosphinothricin acetyl transferase activities in tobacco plants transgenic for the GR-pat fusion (pGR50). Lane1, total leaf extract from control tobacco; 2, control tobacco plus partially purified pat protein; 3, partially purified pat protein; 4, GR50.2, total extract; 5, GR50.2, chloroplast fraction; 6, GR50.2, mitochondrion fraction; 7, GR50.3, total leaf extract; 8, GR50.3, chloroplast fraction; 9, GR50.3, mitochondrion fraction. Assays were performed using 5 μg protein (plant extracts) or 0.14 μg partially purified pat protein (lanes 2 and 3).

construct (pGR50; Figure 5a) was transformed into tobacco by *Agrobacterium* co-cultivation of leaf discs. Regenerants were allowed to self-pollinate and chloroplasts and mitochondria were purified from two lines containing the fusion protein (GR50.2 and GR50.3). Marker enzyme assays confirmed that there was no significant cross-contamination of either organelle fraction (Table 2). Assays for pat activity were carried out on total leaf extracts and on isolated organelles using 5 μg of protein (Figure 5b). No pat activity was detected in total leaf extracts from control tobacco (Figure 5b, lane 1). However, in both GR50.2 and GR50.3, pat activity was detected in both the chloroplast (lanes 5 and 8) and the mitochondrion fractions (lanes 6 and 9). A control plasmid containing the pat coding sequence with no presequence gave pat activity in the total leaf extracts of transgenic plants, but none of this activity was associated with either the chloroplast or the mitochondrion (data not shown).

Discussion

Glutathione reductase is considered to be a key enzyme in protection against oxidative stress in plants and animals.

Table 2. Marker enzyme data for organelle fractions from GR50 lines

	Chloroplast fraction		Mitochondrion fraction	
Fraction	GAPDH	CCO	GAPDH	CCO
GR50.2	160	ND	ND	310
GR50.3	87	ND	ND	260

GAPDH (glyceraldehyde phosphate dehydrogenase—chloroplast marker); CCO (cytochrome C oxidase—mitochondrial marker). Enzyme activities are nmol substrate oxidized (min. mg protein)$^{-1}$. ND indicates that the activity was below detectable limits.

In animal cells most of the GR activity is cytosolic, with a small proportion located in the mitochondrial matrix (Taniguchi *et al.*, 1986). In higher plants the majority of the GR activity is distributed between the chloroplast and cytosol (Bielawski and Joy, 1986; Gillham and Dodge, 1986). However, Edwards *et al.* (1990) showed that in pea leaves 3% of the total GR activity was located in the mitochondria while the remainder was distributed between the chloroplasts (77%) and cytosol (20%). While the mitochondrial GR represents only a small proportion of the total GR in pea leaves, it may be distinguished from other compartment-specific isoforms on the basis of differences in isoelectric point and in K_m for oxidized glutathione (Edwards *et al.*, 1990). We have cloned GR cDNAs from pea leaf (Creissen *et al.*, 1992) and used them to overexpress the enzyme in transgenic tobacco plants as part of our work to understand the role of GR in stressed plants. Surprisingly, although we were unable to detect the GR protein or enzyme activity in mitochondria from control tobacco plants, we found high specific activities of the enzyme in the mitochondria as well as in the chloroplasts of all of the transgenic lines examined that expressed the full-length GR cDNA (GR46 lines). Owing to the different growth requirements and extraction conditions used for isolating uncontaminated mitochondria and chloroplasts from tobacco leaves, it was not possible to determine the percentage distribution of GR between the different compartments, nor was it possible to determine the contribution of the cytosolic GR fraction to the total activity. The data for the compartment-specific marker enzymes and from the detection of the GR polypeptide on Western blots of organelle protein extracts confirmed that the elevated GR activities in each of the organelle fractions could not be the result of cross-contamination of the subcellular fractions.

Transgenic plants that expressed a truncated pea GR cDNA sequence (which was found to encode an active enzyme by restoration of GR activity in the *E. coli* glutathione reductase deletion mutant) did not accumulate the enzyme in the organelles. Furthermore, fusion of this truncated cDNA to either a chloroplast-targeting sequence

(*rbcS* presequence) or a mitochondrion-targeting sequence (Mn-SOD presequence) resulted in elevated GR activity only in the expected subcellular compartment. Furthermore, fusion of the presequence and part of the mature coding sequence for GR to the pat coding sequence resulted in targeting of the pat protein to both chloroplasts and mitochondria in two independent transgenic tobacco lines. Therefore we conclude that all of the information required for targeting GR to either compartment resides entirely within the pea GR presequence. We cannot, however, rule out the possibility that the mature GR coding sequence influences the targeting efficiency, as was the case for the small subunit of Rubisco (Kavanagh *et al.*, 1988).

Current understanding of organelle targeting suggests that where multiple molecular forms of an enzyme are located in different subcellular compartments, each isoform is encoded by a separate nuclear gene. Examples of this include the catalases (Scandalios, 1994) superoxide dismutases (Van Camp *et al.*, 1994) and malate dehydrogenase (Gietl, 1992). In all of these examples the different subcellular isoforms are readily distinguishable biochemically. This is not the case for GR. In pea, GR resolves as a single band of a molecular weight of 55 kDa on SDS–PAGE, although a number of isoforms which differ in their isoelectric points can be identified by isoelectric focusing (Edwards *et al.*, 1990) or by chromatofocusing (Madamanchi *et al.*, 1992). Furthermore, we were unable to identify more than one class of cDNA in pea leaf cDNA libraries (Creissen *et al.*, 1992), and genomic mapping and cloning indicate the presence of a single GR gene in the pea genome (Mullineaux, unpublished data). That the product of a single gene is targeted to both chloroplasts and mitochondria in transgenic tobacco plants provides a possible explanation for the distribution of GR within the pea leaf. It is interesting to note that the cytosolic and mitochondrial GRs from rat liver were essentially indistinguishable biochemically (Taniguchi *et al.*, 1986) and may therefore also be the product of a single gene. No information is available on the subcellular distribution of GR in other animals and although mammalian GR cDNAs have been isolated (Tutic *et al.*, 1990), there are no data concerning the number of GR genes in any other higher eukaryote.

Co-targeting to mitochondria and chloroplasts by a higher plant presequence has not been described before. There are, however, two examples where presequences have been demonstrated to target to the 'wrong' organelle in heterologous systems: the *Chlamydomonas reinhardtii* Rubisco small subunit transit peptide is capable of targeting to yeast mitochondria *in vivo* (Hurt *et al.*, 1986), while the yeast cytochrome oxidase subunit Va (*coxVa*) transit peptide targeted chloramphenicol acetyl transferase to mitochondria and to chloroplasts in transgenic tobacco plants (Huang *et al.*, 1990). Both of these transit peptides are atypical: the *Chlamydomonas reinhardtii* presequences from a number of chloroplast-targeted proteins, including the small subunit of Rubisco, were found to have similarities to mitochondrial presequences, particularly with respect to the potential to form amphiphilic α-helices (Franzén *et al.*, 1990), while the secondary structure of the *coxVa* transit peptide was, in contrast, predicted to form an amphiphilic, antiparallel β-sheet (Cumsky *et al.*, 1987).

We had initially considered the pea cDNA to encode a chloroplast isoform of GR, based on the amino acid composition of the deduced transit peptide, particularly the high proportion of serine and threonine residues (Creissen *et al.*, 1992). However, the data presented here show that the pea GR presequence is bifunctional, that is to say, a single polypeptide which is recognized by the import machinery of both mitochondria and chloroplasts.

Experimental procedures

Construction of glutathione reductase expression cassettes

Three binary constructs were made for expression of pea glutathione reductase.

pGR46. The *Eco*RV–*Bam*HI fragment (co-ordinates 18–2029) from pGR201 containing the full-length pea GR cDNA insert (Creissen *et al.*, 1992) was recovered and inserted into the expression cassette pJIT163–*Bgl*II to create pGR42. pJIT163–*Bgl*II contains 35S promoter and polyadenylation sequences from cauliflower mosaic virus (CaMV) separated by a restriction-site polylinker. pJIT163–*Bgl*II was made from pJIT163 (Guerineau *et al.*, 1992) by insertion of a *Bgl*II linker (5'-GCAGATCTGC-3') into the *Sac*I site of pJIT163 located at the 5' end of the 35S promoter. The CaMV35S-GR-polyA cassette was recovered from pGR42 as a *Bgl*II fragment and ligated into the unique *Bam*HI site of pBinluc to create pGR46 (Figure 1). pBinluc comprises a chimaeric firefly luciferase gene under the control of the CaMV35s promoter and polyadenylation sequences (pJIT53; Mullineaux *et al.*, 1990) ligated into the *Sac*I site of pBIN 19 (Bevan, 1984).

pGR36. The *Nco*I fragment from λGR27 (Creissen *et al.*, 1992) was recovered and treated with bacteriophage T_4 DNA polymerase I and ligated into the T_4 DNA polymerase I-treated *Sph*I site of pJIT117 (Guerineau *et al.*, 1988) which contains a *rbcS* targeting sequence (cpTP) for targeting the resulting fusion peptide to the chloroplast, creating pGR34. The 35S-cpTP-GR-polyA cassette from pGR34 was recovered as a *Bgl*II fragment and ligated into the *Bam*HI site of pBinluc to create pGR36 (Figure 1).

pGR40. The *Nco*I fragment from λGR27 was inserted into the *Nco*I site of pJIT198 to create pGR38. pJIT198 contains CaMV35S promoter and polyadenylation sequences flanking the mitochondrial-targeting (mtTP) sequence from a Mn-superoxide dismutase (Mn-SOD) cDNA recovered from *Nicotiana plumbaginifolia* (pSOD1: Bowler *et al.*, 1989). The chimaeric 35S-MtTP-GR-polyA cassette was recovered as a *Bgl*II fragment and ligated into the *Bam*HI site of pBinluc to generate pGR40 (Figure 1).

Construction of glutathione reductase-pat fusion cassette

The phosphinothricin acetyl transferase (pat) coding sequence from *Streptomyces hygroscopicus* was isolated from pIJ4102 (generous gift of Janet White, John Innes Centre). The coding sequence for pat was isolated by digesting pIJ4102 with *XhoI*, followed by treatment with T_4 DNA polymerase and subsequent digestion with *BglI*. The pat fragment was inserted into pGR42 between the T_4 DNA polymerase-treated *SacI* site (co-ordinate 393 of the GR cDNA) and a *BamHI* site at the 3′ end of the GR coding sequence. This created pGR49, which comprised the first 101 residues of GR fused to the pat coding sequence, under the control of the CaMV35S promoter and polyadenylation sequences. The cassette was then isolated as a *BglII* fragment and ligated into the *BamHI* site of pBinluc to create pGR50.

Transformation of tobacco

The binary GR plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by a triparental mating technique (Ditta *et al.*, 1980). Tobacco (*Nicotiana tabacum* L. cv. Samsun NN) leaf discs were co-cultivated with *A. tumefaciens* containing the different GR plasmids as described by Guerineau *et al.* (1990). Potentially transformed shoots were identified by virtue of their resistance to 100 mg l^{-1} kanamycin sulphate in the growth medium. Putative transformants which had formed roots on 100 mg l^{-1} kanamycin sulphate-containing rooting medium were confirmed by screening the shoots for luciferase activity as described by Mullineaux *et al.*, (1990). Kanamycin-resistant, luciferase-positive (kan^r,luc^+) plantlets were potted in soil and grown to maturity in the glasshouse. Seed was collected from self-pollinated plants (T_2 progeny).

Glutathione reductase assays and immunodetection

Glutathione reductase activities in crude extracts were performed by measuring the GSSG-dependent oxidation of NADPH (Carlberg and Mannervick, 1985). Immunodetection of GR following SDS–PAGE was performed using an antiserum raised against purified pea leaf GR (Edwards *et al.*, 1990)

Phosphinothricin acetyl transferase assay

Assays for phosphinothricin acetyl transferase were performed by thin layer chromatography on 5 µg of protein essentially as described elsewhere (de Block *et al.*, 1987). Partially purified pat was obtained by expression of the protein in *Escherichia coli* containing the plasmid pMV104 (provided by J. Botterman). Cells were grown at 37°C to an OD_{600} of 0.2 and expression of pat was induced by the addition of IPTG to 1 mM. After a further 3 h culture, the cells were harvested by centrifugation (12 000 *g*; 15 min). The pellet was resuspended in phosphate-buffered saline and the cells were disrupted in a French press and centrifuged at 12 000 *g* to remove cell debris. A partially purified pat preparation was obtained by adding polyethylene glycol 6000 to the cleared lysate to a final concentation of 40% (w/v). The extract was chilled on ice and centrifuged at 12 000 *g* for 15 min. Most of the pat activity was retained in the supernatant. Excess PEG was removed by extraction with an equal volume of chloroform, and the extract was subseqently used in the TLC assay.

RNA isolation and RNaseA/T1 protection

Leaf material was frozen in liquid nitrogen and ground to a powder. Nucleic acids were extracted by allowing the powder to thaw in 2 volumes TLES (50 mM Tris–Cl, pH 9.0, 150 mM LiCl, 5 mM EDTA, 5% (w/v) SDS), followed by at least three extractions with phenol:chloroform:isoamyl alcohol (25:24:1). RNA was precipitated by the addition of an equal volume of 4 M LiCl.

GR transcripts were detected in 20 µg of total leaf RNA using an RNaseA/T1 protection assay (Mullineaux *et al.*, 1993). The protected fragments were separated on a 6% (w/v) polyacrylamide 8 M urea sequencing gel and detected by autoradiography (Edwards *et al.*, 1994)

Organelle isolation

Chloroplasts and mitochondria were fractionated using the method of Boutry *et al.*, (1987) with the following modifications. Chloroplasts: plants were shaded for 3 days prior to isolation, the plant material was homogenized with a polytron, EDTA (1 mM) was added to the grinding medium, Percoll was diluted in the grinding medium, rather than second suspension medium, after recovery of the purified chloroplast fraction from Percoll gradients, the chloroplasts were washed in 10 volumes of Hepes-Sorbitol medium (50 mM Hepes, 330 mM Sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM KH_2PO_4, pH 7.6) and resuspended in the same medium.

Mitochondria: mitochondria were isolated as described for chloroplasts with the following modifications. The plants were not shaded, ascorbic acid was omitted from the grinding medium, the mitochondria were layered on to a single concentration of percoll (50% v/v) in grinding medium. Chloroplasts and mitochondria were lysed for enzyme assays by the addition of an equal volume of lysis buffer (50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EDTA, 0.2% Triton X-100).

To confirm the purity of the organelle fractions, enzymes were assayed that are specific to each of the organelles (Edwards *et al.*, 1990). These were glyceraldehyde 3-phosphate dehydrogenase (GAPDH:chloroplast; Wu and Racker, 1959); cytochrome C oxidase (CCO:mitochondria; Tolbert, 1974) and pyrophosphate-dependent phosphofructokinase (PPi-PFK:cytosol; Journet and Douce, 1985).

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