Structural Requirements of Oleosin Domains for Subcellular Targeting to the Oil Body

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We have investigated the protein domains responsible for the correct subcellular targeting of plant seed oleosins. We have attempted to study this targeting in vivo using "tagged" oleosins in transgenic plants. Different constructs were prepared lacking gene sequences encoding one of three structural domains of natural oleosins. Each was fused in frame to the Escherichia coli uid A gene encoding β-glucuronidase (GUS). These constructs were introduced into Brassica napus using Agrobacterium-mediated transformation. GUS activity was measured in washed oil bodies and in the soluble protein fraction of the transgenic seeds. It was found that complete Arabidopsis oleosin-GUS fusions undergo correct subcellular targeting in transgenic Brassica seeds. Removal of the C-terminal domain of the Arabidopsis oleosin comprising the last 48 amino acids had no effect on overall subcellular targeting. In contrast, loss of the first 47 amino acids (N terminus) or amino acids 48 to 113 (which make up a lipophilic core) resulted in impaired targeting of the fusion protein to the oil bodies and greatly reduced accumulation of the fusion protein. Northern blotting revealed that this reduction is not due to differences in mRNA accumulation. Results from these measurements indicated that both the N-terminal and central oleosin domain are important for targeting to the oil body and show that there is a direct correlation between the inability to target to the oil body and protein stability.

During seed and pollen development triacylglycerols accumulate in spherical structures called oil bodies (Huang, 1992; Roberts et al., 1993). Seed oil bodies from a number of plant sources appear to contain proteins in addition to triacylglycerol and phospholipids (Yatsu and Jacks, 1972). The amount of protein associated with these oil bodies seems to vary among species. Estimates vary from 0.2% by weight for oil bodies in peanut, equivalent to approximately 0.3% of total seed protein (Yatsu and Jacks, 1972), to 20% of the total seed proteins in rapeseed (Murphy et al., 1989a, 1989b). Some oil bodies, notably those in tissues or organs that do not undergo desiccation (e.g. olive or avocado mesocarp) are devoid of proteins (Ross et al., 1993).

The most abundant oil-body-associated proteins are called oleosins (Huang, 1992). Oleosins are unique to oil bodies, as indicated by subcellular fractionation experiments (Herman, 1987; Murphy et al., 1989a) and immunocytochemistry (Herman, 1987; Murphy et al., 1989b). The function of oleosins, however, is not fully understood. Clearly, oleosins play a structural role in maintaining stability of discrete oil bodies during compression caused by desiccation of seed cells (Murphy, 1990; Huang, 1992). It has also been suggested that (part of the) oleosin may function as a lipase attachment site (Vance and Huang, 1987). These suggestions are based mainly on predicted amino acid sequences of oleosins and structural predictions derived therefrom.

All oleosins may be resolved into three structural domains. These include an N-terminal amphipathic domain, a central hydrophobic core, and an amphipathic C-terminal domain (Huang, 1992). A comparison of deduced amino acid oleosin sequences of different plant species, including angiosperms (monocots and dicots) and gymnosperms, revealed that the central domain has been highly conserved (Lee et al., 1994). The N- and C-terminal domains of the different oleosins diverge in both size and amino acid composition. It seems likely therefore that the central domain is essential for oleosin function and/or final localization of the oleosin in the half-unit membrane of the oil body. The interspecies variability permitted in the N and C termini of oleosins (Huang, 1992) suggests that these regions are unimportant or that primary structure is not the primary determinant.

There have been two reports of in vitro systems that have elucidated part of the targeting process. However, in these systems delivery of oleosins to oil bodies is difficult to achieve (Hills et al., 1993; Loer and Herman, 1993). In the work reported here we have assembled an in vivo targeting system that is capable of yielding information concerning essential targeting motifs in oleosins. Initially we planned to extend an oleosin gene with additional in-frame codons that might specify an immunological "tag." In the course of these experiments, we discovered that quite large "tags" including functional proteins such as GUS could be produced as in-frame oleosin fusions that can accumulate on oil bodies in vivo (van Rooijen and Moloney, 1995). Therefore, we have exploited the properties of different oleosin-GUS fusions to determine the relative importance of the major oleosin structural domains in the correct processing and targeting of seed oleosins.

MATERIALS AND METHODS

Generation of Arabidopsis Oleosin Deletion Constructs

In these experiments, all of the constructs were made using the Arabidopsis 18-kD oleosin genomic clone (van...
Rooijen et al., 1992). This has previously been shown to be highly homologous to the native Brassica oleosin genes (Huang, 1992; van Rooijen et al., 1992) and to be correctly processed and targeted in transgenic Brassica (van Rooijen and Moloney, 1995).

Three deletions corresponding to the three main structural domains of the oleosin were made in the coding region of the Arabidopsis oleosin gene in such a way that the gene products would lack one of these domains (Fig. 1). The deletions were designed in such a way that (a) the coding sequence remained in frame, (b) no foreign amino acids were introduced, and (c) at the position of the deletion a restriction site, unique for the Arabidopsis (not the native Brassica) oleosins, was present. The deletions were made by PCR and conventional cloning techniques. The PCR amplification mixture contained 16 µL of deoxynucleoside triphosphates (1.25 mM), 10 µL of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% [w/v] gelatin), 5 µL of 5' primer (20 µM), 5 µL of 3' primer (20 µM), 1 µL of Tag DNA polymerase (1 unit/µL), 1 µL (1 ng/µL) of DNA template, and 64 µL of H₂O. The reactions were carried out for 30 cycles. Each cycle consisted of 1 min of denaturation at 92°C, 1 min of annealing at 50°C, and 1 min (X = 1 for every kb of amplified extension at 72°C). Unless otherwise stated, h2.1, the nucleotide of the GUS sequence had to be changed from T to G, changing the TTA codon (Leu) into GTA (Val). GVR20 5'-GAGGATCCATGGTACGGTCACGGTC-3', was synthesized. This oligonucleotide contained sequences complementary to the GUS gene (Jefferson et al., 1986) (indicated in bold) and the sequence for the restriction sites BamHI and NcoI (underlined) to facilitate cloning. To create these restriction sites the fourth nucleotide of the GUS sequence had to be changed from T to G, changing the TTA codon (Leu) into GTA (Val). GVR20 and the universal sequencing primer (5'-GTAAACGACGGCCAGCT-3') and 1 ng of pBI121 (Clontech) were used in a PCR to amplify the BamHI NcoI GUSNOS intervening region. This fragment was cloned, cut at the appropriate restriction sites, and together with the GVR01/GVR10 fragment (described above) ligated into pCGN1559. The resulting plasmid was called pCGYOBPGUSE (full-length oleosin-GUS). After construction, the in-frame ligation of the oleosin and GUS was confirmed by dideoxy sequencing across the ligation sites.

As a control, a construct was made in which the GUS gene (Jefferson et al., 1986) was fused upstream of 834 bp of the Arabidopsis oleosin gene promoter. This construct is referred to as pCGY800GUS and was described previously by Plant et al. (1994), who referred to it as d0.8 (= deletion 0.8).

Transformation of Recombinant pCGN1559 Plasmids into Agrobacterium tumefaciens Strain EHA101

A single colony of Agrobacterium tumefaciens strain EHA101 (Hood et al., 1986) was used to inoculate 5 mL of Luria broth plus 100 µg/mL kanamycin. This culture was grown for 48 h at 28°C. The 5-mL culture was used to inoculate 500 mL of Luria broth plus 100 µg/mL kanamycin. This culture was grown at 28°C until it reached A₆₀₀ = 0.5 (approximately 4 h). The cells were spun down (10 min, 5000g) and resuspended in 500 mL of sterile H₂O (repeated twice). The cells were spun again and resuspended in 3 mL of sterile H₂O containing 10% glycerol. Forty microliters of the cells were dispersed into Eppendorf tubes and either used directly for electroporation or stored at −80°C for future use. Electroporation was carried out according to the method of Dower et al. (1988). The pulse generator (Bio-Rad) was set to the 25 µF capacitance, 2.5 kV discharge, and 200 Ω resistance in parallel with the sample chamber. After the pulse, the cells were dispersed into 1 mL of sterilized Luria broth plus 100 µg/mL kanamycin. This culture was grown for 16 h at 28°C. The resulting culture was used to inoculate 5 mL of Luria broth plus 100 µg/mL kanamycin. This culture was grown for 24 h at 28°C. The resulting culture was used to inoculate 500 mL of Luria broth plus 100 µg/mL kanamycin. This culture was grown at 28°C until it reached A₆₀₀ = 1.0 (approximately 4 h). The cells were spun down (10 min, 5000g) and resuspended in 500 mL of sterile H₂O (repeated twice). The cells were spun again and resuspended in 3 mL of sterile H₂O containing 10% glycerol. Forty microliters of the cells were dispersed into Eppendorf tubes and either used directly for electroporation or stored at −80°C for future use. Electroporation was carried out according to the method of Dower et al. (1988). The pulse generator (Bio-Rad) was set to the 25 µF capacitance, 2.5 kV discharge, and 200 Ω resistance in parallel with the sample chamber. After

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2 In these experiments the thrombin cleavage site was not used. For details of use of this proteolytic cleavage motif, please see van Rooijen and Moloney (1995).
Figure 1. Oleosin-GUS deletion constructs used to transform B. napus. I. The deduced amino acid sequence of the Arabidopsis oleosin gene used in this study (van Rooijen et al., 1992). II, top, Hydrophobicity plot (Kyte-Doolittle) of the deduced amino acid sequence of the different Arabidopsis oleosin gene deletions. N, N terminus; C, C terminus; L, lipophilic core. Bottom, The complete oleosin gene (A); missing the gene sequence encoding A^4 to A^107 (B); missing T^106 to F^113 (C); and missing E^123 to H^173 (D). The different deletion constructs were ligated in frame to GUS from which the putative N-glycosylation site was removed (Farrell and Beachy, 1990) and moved into pCGN1559 (McBride and Summerfelt, 1990) to create the constructs pCGYOBPGUSA, pCGYOBPGUSB, pCGYOBPGUSC, and pCGYOBPCUSD, respectively. A construct, pCGYOBPGUSE, was also made that is identical with pCGYOBPGUSA, except for the GUS gene, which still contained a putative N-glycosylation site (Iturriaga et al., 1990); nos, Nopaline synthase terminator; RB, right T-DNA border; ori ColE1, origin of replication for high maintenance in E. coli; Gm, gentamycin resistance gene; 3'tml-npt-35S, tumor morphology large locus 3'-neomycin phosphotransferase gene-cauliflower mosaic virus 35S promoter; LB, left T-DNA border; ori pRI, origin of replication for high stability in A. tumefaciens.
electroporation, the EHA101 cells were grown for 2 h at 28°C before plating on Luria broth plates containing kanamycin (100 μg/mL) and gentamycin (100 μg/mL).

Transformation of Brassica napus cv Westar

The procedure used is an adaptation of the procedure developed by Moloney et al. (1989). The details are described below. Unless otherwise stated, the tissues were grown and maintained in a culture room (24°C, 16-h light/8-h dark photoperiod, light intensity 60 to 80 μE m⁻² s⁻¹). Brassica napus cv Westar seeds were sterilized in 20% commercial bleach (Javex, Colgate-Palmolive, Toronto, Canada) for 30 min with shaking. The seeds were washed five times with sterile double-distilled H₂O and were placed on Murashige-Skoog medium (Murashige and Skoog, 1962) containing the recombinant plasmid was grown in minimal medium, including 100 μg/mL kanamycin and 100 μg/mL gentamycin, in a 28°C shaker for 2 d. The Agrobacterium cells were spun down (10 min, 5000 rpm) and resuspended in 5 mL of minimal or Murashige-Skoog medium.

For transformation, cotyledonary petioles of 4- to 5-d-old plants were used. The cut end of cotyledonary petioles were dipped into the Brassica cotyledons were placed onto Murashige-Skoog medium. The sequence and position identical with the Arabidopsis oleosin gene as reported by van Rooijen et al. (1992), and the GUS gene is as reported by Jefferson et al. (1986). Restriction sites are underlined.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Template</th>
<th>sequence 5'→3'</th>
<th>restriction site(s)</th>
<th>position relative to translational start</th>
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<tr>
<td>GVR01</td>
<td>Oleosin</td>
<td>5'-AACCTCCGAAACCTCCTGGAACGAGTAGTGTCTGCTGACCACGTCCAGTGC-3'</td>
<td>NcoI/BamHI</td>
<td>759 to 725</td>
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<tr>
<td>GVR10</td>
<td>Oleosin</td>
<td>5'-CACTGGAGAAGACCTCTGGTGGAAAC-3'</td>
<td>PstI</td>
<td>833 to 815</td>
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<tr>
<td>GVR14</td>
<td>Oleosin</td>
<td>5'-TGGCGGCTACAATTTTTTGTTCGGCAGACTGGTTGCGC-3'</td>
<td>SacII</td>
<td>15 to 3</td>
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<tr>
<td>GVR15</td>
<td>Oleosin</td>
<td>5'-AACCTGGTCAACAGCTGGTTGCGC-3'</td>
<td>SacII</td>
<td>146 to 168</td>
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<tr>
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<td>605 to 585</td>
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<td>GVR19B</td>
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<td>5'-CAAGTGCACCTCTGGTTCACGAGGAG-3'</td>
<td>KpnI</td>
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<td>GUS</td>
<td>5'-GGAGATCATTGATGACGTCGCTGTGAGAAAAC-3'</td>
<td>NcoI/BamHI</td>
<td>1 to 21</td>
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<td>SmaI</td>
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<tr>
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<td>GUS</td>
<td>5'-CTAGGGTTTGGGTGGTTTAC-3'</td>
<td>SmaI</td>
<td>32 to 13</td>
</tr>
</tbody>
</table>

Suc, 500 mg/L carbenicillin, 20 mg/L kanamycin, pH 5.8. Roots emerged after 2 to 3 weeks and the plantlets were transferred to potting mix (Redi Earth, W.R. Grace and Co., Ajax, Ontario, Canada) and put in a misting chamber (75% RH). At this stage leaf samples were taken for neomycin phosphotransferase assays (Moloney et al., 1989). After about 3 weeks the plants were transferred to the greenhouse and allowed to flower, self-fertilize, and set seed. Plants transformed with pCGYOBPGUSA were called "A" plants, plants transformed with pCGYOBPGUSB were called "B" plants, etc. Plants transformed with pCGY800GUS were called "GB" plants (described by Plant et al., 1994).

GUS Activity Measurements and Determination of Oleosin Targeting

Twenty seeds of wild-type or transgenic B. napus cv Westar were ground in 1.0 mL of extraction buffer (0.1 M Mes, pH 5.0) for exactly 1 min with a mortar and pestle. This extract was spun at 14,000g for 15 min at room temperature. The pellet was discarded, and the "fat pad" was removed and resuspended in the same volume (typically 800 μL of extraction buffer A) as the "unternatant" (liquid fraction between the pellet and the fat pad). GUS activity associated with the oil bodies and the unternatant was determined according to the method of Jefferson (1987) and set to be the total GUS activity. The fat pad was washed five times with sterile double-distilled H₂O and was placed on Murashige-Skoog medium containing 0.7% phytagar and 3% Suc, pH 5.8. The GUS activity associated with the oil bodies was determined according to the method of Jefferson (1987) and set to be the total GUS activity.
pendent extractions were performed on a single transgenic plant, and of the plants transformed with pCGYOBPGUSB and pCGYOBPGUSC, three independent extractions were performed on three independently transformed plants.

For the GUS specific activity measurements, 10 seeds were ground in 500 μL of GUS extraction buffer and activity was measured as described by Jefferson (1987). Protein content in the seed extract was measured according to the method of Bradford (1976).

Northern Blotting, Hybridization, and Densitometry Scanning

Total RNA was extracted according to the procedure of Verwoerd et al. (1989). Thirty micrograms of total RNA was loaded onto a 1% agarose formaldehyde gel and transferred to Hybond nylon membrane according to the manufacturer’s protocol (Amersham).

An Arabidopsis oleosin cDNA clone, YAP230, identical to the coding region of the oleosin gene used to prepare the fusions, was obtained from the Arabidopsis Biological Resource Center at The Ohio State University (kindly donated by Dr. Raynal, Université de Perpignan, France). The oleosin cDNA insert was released from this clone after digestion with EcoRI. This insert was labeled by a random oligonucleotide-priming method. Prehybridization and hybridization were performed according to the Hybond protocol (Amersham). The filter was washed successively in 50 mL of 5× SSPE for 15 min at 42°C, 50 mL of 1× SSPE for 15 min at 42°C, 50 mL of 0.1× SSPE, 0.1% SDS for 15 min at 42°C, and 50 mL of 0.1× SSPE, 0.1% SDS for 45 min at 65°C. For Figure 5, the filter was exposed to Kodak XAR film at -70°C for 88 h. For the scanning densitometry measurements, a Hewlett-Packard ScanJet IIC instrument was used. The data were analyzed using Macintosh Deskscan II and Image 1.33f software. To get a value in the linear range of the scanner/film, the oleosin transcript signals were scanned after 9 h and the oleosin-GUS transcript signals after 88 h of exposure at -70°C. The oleosin-GUS/oleosin ratio was determined using the formula: ([oleosin-GUS densitometry scanning value after 88 h]/[88/9 × oleosin densitometry scanning value after 9 h]) × 100%.

RESULTS

Stable Integration of Oleosin-GUS Gene Fusions in B. napus

Putative, transformed plants were tested for expression of the neomycin phosphotransferase gene (Moloney et al., 1989; results not shown). This served as a selectable marker in transformed tissue. Confirmation of stable integration of the different oleosin-GUS gene fusions was also obtained using PCR (Fig. 3). Oligonucleotide GVR25 (5'-AACACTC-CTACCTCTTCC-3', position -92 to -74 relative to the translational start of the GUS gene [Jefferson et al., 1986]) were used in a PCR to amplify the enclosed regions using 50 ng of genomic DNA from plants A1, B7, C12, D10, E1, F1, and control (wild-type B. napus cv Westar) as a template and oligonucleotides GVR25 and GUSSEQ to amplify the intervening regions. The total GUS activity in several plants transformed with different constructs as template. Given the different sizes of the deletions in the constructs, the PCR products are predicted to have different sizes. As expected, plants transformed with pCGYOBPGUSA, pCGYOBPGUSB, pCGYOBPGUSC, pCGYOBPGUSD, and pCGYOBPGUSE yielded amplification products of 902, 764, 702, 755, and 902 bp, respectively. B. napus genomic DNA from a wild-type plant did not yield an amplification product with these oligonucleotides.

Relative GUS Activity of Transgenic Plants Transformed with Different Oleosin Deletion Constructs

The total GUS activity in several plants transformed with the different oleosin deletion constructs was measured (Fig. 4). When the GUS activity in plants transformed with pCGY800GUS was compared with the GUS activity in plants transformed with pCGYOBPGUSA and pCGYOBPGUSE, it was clear that the fusion of the oleosin to GUS has a limited effect on its activity. The activity in plants transformed with pCGYOBPGUSE was only slightly higher than pCGYOBPGUSA. The GUS in pCGYOBPGUSA has been modified to destroy a putative N-glycosylation site and maintained approximately 25% of its activity in E. coli (Farrell and Beachy, 1990). As shown in Figure 4, this difference in GUS activity was not as pronounced in vivo. However, the N-terminal and central domain deletions had a dramatic effect on the GUS activity in the transgenic seeds. The N-terminal oleosin deletion resulted in a 10-fold reduction and the central domain deletion resulted in a 20-fold reduction in the GUS activity compared to the full-length oleosin. When the C-terminal oleosin domain was deleted, the GUS activity was reduced by approxi-
oleosins reported so far in the GenBank and EMBL data bases. In this experiment we used the B. napus oleosins as an internal standard to correct for any differences in loading and/or stage of seed development of the samples used. To get a more quantitative estimate of the mRNA levels we used densitometry scanning on different exposures (see "Materials and Methods"). When this method was used the ratio of oleosin-GUS: B. napus oleosin transcripts in plants A1, B7, C12, D10, and E1 was 4.0, 5.7, 4.4, 3.6, and 4.3%, respectively.

Determination of Percentage of GUS Activity Associated with the Oil Bodies of Different Transgenic Plants

As is clear from the results described above, the transcript levels in the different transgenic plants are very similar and cannot account for the difference in GUS activity. We therefore sought to investigate whether there is a relationship between the (in)ability of the GUS to be targeted to the oil body and its total activity.

Transgenic seeds were extracted and the percentage of the GUS activity associated with washed oil bodies was compared to the total GUS activity in the extract. As shown in Figure 6, plants transformed with pCGY800GUS (transcriptional GUS fusion) had less than 10% of the GUS activity associated with the oil bodies. This is to be expected, since there is no signal/targeting signal preceding the GUS, and this protein will therefore be synthesized on the free ribosomes and accumulate in the cytosol. Plants transformed with pCGYOBPGUSA and pCGYOBPGUSE contain the complete oleosin-GUS fusion, and about 80% of the GUS activity was found to be associated with the oil bodies.

Oleosin-GUS Transcript Accumulation in Mature Green Seeds

To exclude the possibility that this may be due to differences in oleosin-GUS mRNA accumulation, a northern blotting experiment on RNA extracted from mature green seeds of plants A1, B7, C12, D10, and E1 (highest GUS activity) was performed. The filter was hybridized to a radiolabeled oleosin cDNA identical with the coding region of the Arabidopsis gene used in these experiments (see "Materials and Methods").

As shown in Figure 5, the probe hybridized to both the Arabidopsis oleosin-GUS and the B. napus oleosins present in the sample. This is not surprising, since the Arabidopsis oleosin cDNA used is >90% identical with several B. napus oleosins reported so far in the GenBank and EMBL data bases.
Oleosin Domains Important for Targeting to the Oil Body

The C-terminal domain of the oleosin does not seem to affect the targeting of GUS to the oil body; plants transformed with construct pCGYOBPGUSD also showed 80% of its GUS activity associated with the oil bodies. The N-terminal and central domain of the oleosin both seem to be important for targeting because plants transformed with pCGYOBPGUSB (lacking the N-terminal domain) and plants transformed with pCGYOBPGUSC (lacking the central domain) had only 58 and 39%, respectively, of the GUS activity associated with the oil bodies.

Immunoblotting of the Oleosin-GUS Fusions

In addition to (mis)targeting, the different GUS activities corresponding to the various truncations, as reported in Figure 4, might have resulted from inactivation of GUS in the particular oleosin-GUS fusion or instability of the mistargeted fusion. We therefore performed immunoblotting to determine whether a quantitative relationship between GUS activity and protein accumulation could be detected.

Proteins were extracted from seeds from plants A1, B7, C12, D10, E1, and GB5. These were run on SDS-denaturing 7.5% polyacrylamide gels and blotted onto polyvinylidene difluoride membranes and visualized immunologically using GUS antiserum (Clontech). All lanes were loaded with equal amounts of protein (as can be judged from the amount of a 66-kD seed protein that cross-reacts with the primary and/or secondary antibody), except for the GB5 lane, which is underloaded. Based on the predicted amino acid sequence of the oleosin-GUS fusions, A1, B7, C12, D10, and E1 should accumulate proteins of 87.6, 82.5, 81.2, 82.1, and 87.7 kD, respectively. The GUS in GB5 (68.2 kD), which is not expressed as a fusion, was used as a marker control. As shown in Figure 7, the oleosin-GUS fusions in plants A1, B7, D10, and E1 are clearly visible, albeit at different steady-state levels, and appear to have the predicted sizes. In contrast, C12 did not show a detectable GUS fusion protein. In fact, several plants transformed with pCGYOBPGUSC were tested, and in all cases no GUS fusion proteins could be detected by immunoblotting.

DISCUSSION

The GUS gene has been widely used as a reporter gene to investigate the regulation of plant promoter sequences. In these experiments the GUS protein stably accumulates in the cytosol (e.g. GB5, this report). It has also been used in targeting studies to different organelles such as the nucleus (Carrington et al., 1991), chloroplasts (Kavanagh et al., 1988), and mitochondria (Schmitz and Lonsdale, 1989). However, when GUS is used in secretory or vacuolar targeting studies, GUS activity is found to be blocked and/or the GUS protein is degraded (Iturriaga et al., 1990; Pang et al., 1992). In both cases, N-glycosylation of the GUS was believed to be responsible for the lack of activity and protein accumulation, respectively.

The fact that plants transformed with pCGYOBPGUSE show GUS activity is important in the light of a possible involvement of the ER in posttranslational processing of the oleosins. It has been shown (Qu et al., 1986) that oleosins are synthesized on the ER. In addition, two research groups (Hills et al., 1993; Loer and Herman, 1993) independently demonstrated the interaction of a newly synthesized oleosin with an ER preparation in vitro. They found that oleosins are co-translationally integrated into canine microsomal membranes. When oil bodies were added to this microsomal preparation, no oleosins were integrated into the oil-body monolayer membrane. This suggests that oleosins are synthesized and processed on the ER before they are transferred to the oil body. In addition, proteinase K protection experiments showed that in these in vitro experiments about 15 kD of the oleosins are protected from degradation (Hills et al., 1993). This might suggest that one domain (N or C terminus) or portions of both domains are exposed to the cytosol during ER association. The observation that plants transformed with pCGYOBPGUSE show GUS activity seems to indicate that the C-terminal part of the oleosin (including the GUS) has not been exposed to the luminal side of the ER during its synthesis. If this had happened, GUS would likely have undergone inactivation through glycosylation. When furnished with an endomembrane signal sequence for the ER, GUS is N-glycosylated, resulting in near loss of enzymatic activity (Iturriaga et al., 1990; Pang et al., 1992).

When the first oleosin cDNA sequence (Vance and Huang, 1987) was reported, it was suggested that based on the deduced amino acid sequence the hydrophobic nature of the central domain most likely played a role in the localization of the oleosin in the oil-body half-unit membrane. Since that time, more than a dozen oleosin sequences have been reported, and in all cases the deduced amino acid sequence indicates the conserved nature of the central hydrophobic domain. The work presented in this paper provides experimental evidence for the importance of the central domain and reveals the possible involvement of the N-terminal domain in the final localization and targeting of oleosin to the oil body.
From Figure 4 it is clear that the different oleosin deletions have a dramatic effect on GUS activity. As demonstrated in Figure 5, this is not due to differential transcript accumulation or a reduction of the specific activity of GUS when preceded by a particular oleosin fusion, as shown in Figure 7. Rather, it seems that especially the oleosin central domain and to a lesser extent the N-terminal oleosin deletion have an effect on their ability to target and stably accumulate the fusion protein.

In all of the oleosin sequences reported so far the region immediately preceding the central hydrophobic domain is positively charged. It has been suggested (Hills et al., 1993) that this positive charge might be important for determining the orientation in the ER membrane as is the case for several mammalian proteins. In the N-terminal deletion all of the positively charged residues were deleted and thus might have had an effect on the orientation of the oleosin in the ER membrane and possibly an effect on protein stability of the "inverted oleosin."

To our knowledge, oleosins are the only plant proteins with a single membrane-spanning domain and uncleavable signal peptide. It has been suggested (Vance and Huang, 1987; Lee et al., 1991) that the hydrophobic domain of the oleosins could both contain a cryptic signal sequence and targeting and stability.

This manuscript confirms, with evidence obtained in vivo, the widely held belief that the central hydrophobic domain is essential for correct targeting. It further shows that oleosin protein stability is dependent on unimpeded targeting to the oil body. It appears that the N-terminal domain might have an important role in this process as well. We are currently using site-directed mutagenesis and truncation experiments to determine which parts of these domains are responsible for the observations described in this manuscript.

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