Correct Targeting of the Bean Storage Protein Phaseolin in the Seeds of Transformed Tobacco

JOHN S. GREENWOOD AND MAARTEN J. CHRISPEELS*
Department of Biology, C-016, University of California, San Diego, La Jolla, California 92093

ABSTRACT

The storage protein phaseolin accumulates during seed development in protein bodies in cotyledons of the common bean Phaseolus vulgaris. Hall et al. (In L. Van Vloten-Doting, TC Hall, eds, Molecular Form and Function of the Plant Genome, 1985 Plenum Press, In press) recently reported the expression of a gene coding for phaseolin and the accumulation of phaseolin protein in developing seeds of tobacco plants regenerated from transformed callus cells. The protein did not accumulate in other organs of the plants. Mature seeds from normal and transformed tobacco plants were obtained and the subcellular distribution of phaseolin in the seeds was examined using both light and electron microscopic immunocytochemical methods. Phaseolin was found in six of seven transformed tobacco embryos examined, but was present in only one endosperm of five. When present, phaseolin was located exclusively in the protein bodies of the embryonic and endospermic cells. Furthermore, phaseolin was restricted solely to the amorphous matrix of the protein bodies and was excluded from the globoid and proteinaceous crystalloid components of these organelles. The subcellular location of phaseolin in seeds from transformed tobacco plants is similar to that seen in mature seeds of the common bean indicating that in the transformed cells the protein is targeted to the right subcellular compartment.

Research in our laboratory is concerned with the intracellular transport of proteins to the protein bodies of developing seeds, and with the protein targeting mechanisms which exist in plant cells. Transformation of cells with genes from a different organism is a powerful aid in the study of protein targeting, because the genes can be modified in vitro, and the transport of altered gene products studied. Before attempting any gene modification it is necessary to establish that the gene product is targeted to the correct cellular compartment in the transformed system.

Recently, Sengupta-Gopalan et al. (28) and Hall et al. (20) reported the successful transformation of tobacco (Nicotiana tabacum var Xanthi) callus culture cells using Agrobacterium tumefaciens containing a T, plasmid into which a gene for the bean (Phaseolus vulgaris) seed protein, phaseolin, had been inserted. Plants were regenerated from the callus cultures and various tissues were analyzed for phaseolin content. Levels of the protein in the seeds from transformed plants were up to 1000-fold higher than in other plant tissues and made up approximately 1% of the total seed proteins on average. Similarly, phaseolin mRNA levels were several orders of magnitude higher in developing seeds than in the leaves from the transformed plants (28). The protein was found primarily in the embryos of the tobacco seeds, relatively little being found in the seed endosperm tissue. The authors concluded that the flanking sequences of the inserted gene contained regions which allowed correct temporal and tissue-specific expression of the gene.

Phaseolin accounts for approximately 50% of the total protein content in mature bean seeds (4). It is synthesized during seed development and sequestered within protein bodies during cotyledon development. A similar accumulation of phaseolin in the protein bodies of transformed tobacco seeds would indicate that the gene product is targeted to the correct compartment. Using postembedding immunofluorescence and immunogold localization methods on plastic-embedded tissues of mature seeds from transformed tobacco plants we have determined the subcellular localization of phaseolin in the seeds. We present evidence that phaseolin is restricted to the protein bodies in the cells of embryonic and endospermic tissues of tobacco seeds.

MATERIALS AND METHODS

Plant Material. Mature seeds from normal and transformed plants of Nicotiana tabacum var Xanthi were kindly supplied by T. C. Hall and J. D. Kemp, Agrigenetics Advanced Research Division, Madison, WI. Transformed plants contain the gene for the bean seed protein phaseolin. The gene is maximally expressed in the developing tobacco seeds 15 to 31 d postanthesis, phaseolin accumulating in the seeds during this time (20, 28). The mature seeds from both transformed and normal tobacco plants were allowed to imbibe for 16 h on moistened filter paper prior to fixation.

Purification of Phaseolin and Preparation of Antiserum. Phaseolin was extracted from mature seeds of Phaseolus vulgaris L. cv greensleeves and purified by column chromatography and velocity sedimentation as described previously (4). Phaseolin-specific antiserum was produced in New Zealand white rabbits (5); the specificity of the antiserum has been described (2).

IgG2 specific for the oligosaccharide side chains of phaseolin were removed from the antiserum by affinity chromatography using ovulbin-linked Sepharose 4-B (Pharmacia, Uppsala, Sweden) prepared according to the manufacturer’s specifications. Crude phaseolin-specific antiserum (2 ml + 0.02% NaN3) was added to 4 ml of the ovulbin affinity matrix preswollen in phosphate-buffered saline (PBS; 0.15 M NaCl in 10 mM K-phosphate [pH 7.2]) and was rotated overnight at 4°C. The column was washed with cold PBS until A280 was minimal (15 ml), the washes combined and concentrated to the original volume (2 ml) using an Amicon pressure cell (Amicon Corp., Lexington, MA). After centrifugation at 12,000g for 10 min, 0.1 ml aliquots of the supernatant were stored, with 0.02% NaN3

1 Supported by grants from the National Science Foundation (Metabolic Biology) and the United States Department of Agriculture (Genetic Mechanisms for Crop Improvement) to M. J. C.; J. S. G. received a fellowship from the National Sciences and Engineering Research Council of Canada.

2 Abbreviation: IgG, immunoglobulin G.
Preparation of Rhodamine and Colloidal Gold-Conjugated Goat Antibodies against Rabbit IgG. Affinity purified goat-anti-rabbit IgG was conjugated to lissamine rhodamine B (Polysciences Inc., Warrington, PA) according to the method of Brandtzaeg (7). Colloidal gold with an average particle diameter of 3 to 4 nm was prepared according to Keller et al. (23), and affinity purified goat-anti-rabbit IgG adsorbed to the gold particles as described (15, 16).

Fixation and Embedding. Imbied seeds from normal and transformed tobacco plants were dissected under the fixative appropriate for either light or electron microscopic immunocytochemistry. Seed coats were removed prior to fixation. Endosperm tissue and whole embryos were fixed separately.

For light microscope immunocytochemistry, endosperm tissue and embryos were fixed for 2 h at 22°C and 14 h at 4°C in 4% paraformaldehyde in 25 mM K-phosphate (pH 7.2), 0.5 mM sucrose with gentle agitation. Following five hourly changes using the same buffer, the tissues were dehydrated through 10, 30, 50, and 90% ethanol, 30 min per step, and infiltrated overnight with 1:1 (v/v) 90% ethanol/glycol/methacrylate. The glycol methacrylate mixture contained 95% (v/v) hydroxyethyl methacrylate, 5% (v/v) PEG 400, and 0.3% (w/v) 2,2'-azobis-[2-methylpropionitrile (Ladd Research Industries, Inc., Burlington, VT). Hydroxethyl methacrylate was purified prior to use (17). After three further 2-h incubations with 100% glycol methacrylate the endosperm and embryo tissue were placed in flat embedding molds with glycol methacrylate, the molds sealed to exclude air, and the plastic polymerized for 18 h at 50°C under nitrogen. Sections, 0.5 to 1 μm thick, were cut using dry glass knives and mounted on glass slides then processed for the immunocytochemical localization of phaseolin.

Tissues for electron microscopic immunocytochemistry were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 25 mM K-phosphate (pH 7.2), 0.5 mM sucrose for 2 h at 22°C then 14 h at 4°C. After five 1-h washes in the same buffer, the material was postfixed in 1% OsO₄ in 0.1 M K-phosphate (pH 7.2), 0.5 mM sucrose for 4 h at 4°C, washed overnight then twice further for 1 h in the same buffer and dehydrated through 10, 30, 50, and 70% ethanol, 30 min each step. The tissues were infiltrated with 5:2 (v/v) L. R. White acrylic resin (Fullam Inc., Schenectady, NY): 80% ethanol for 4 h followed by four changes of 100% L. R. White resin over the next 16 h (13). Further processing was as described above with a 24-h polymerization. Ultrathin sections (~50 nm thick) were placed on formvar, carbon-coated nickel grids and processed for the immunocytochemical localization of phaseolin.

Immunofluorescence Localization of Phaseolin. Phaseolin localization on 0.5 to 1 μm thick sections of glycol methacrylate embedded tissue was done using an indirect immunofluorescence procedure adapted from Tokuyasu and Singer (29). Sections were air-dried onto glass slides. All pretreatments and washes were performed in moistened chambers using small drops of the reagents placed directly on the sections. Immunoreagents were centrifuged at 10,000g for 4 min in a Beckman (Beckman Industries Inc.) microfuge immediately prior to use. The following treatments were given: four washes of 2.5 min each in PBS, 15 mM glycine; one 10-min incubation in PBS glycine, 2% gelatin; four 2.5-min washes with PBS glycine; a 7-min incubation in either crude or ovalbumin-adsorbed, phaseolin-specific rabbit antiserum with 2% gelatin added; four washes of 2.5 min each with PBS glycine; a 7-min incubation in rhodamine-conjugated goat-anti-rabbit IgG diluted to 20 μg IgG/ml with PBS, 2% gelatin; four 2.5-min washes in PBS. The slides were rinsed briefly with distilled H₂O and coverslips mounted using 90% glycerol. Control treatments included: substitution of phaseolin-specific antiserum with preimmune rabbit serum, omission of phaseolin-specific antiserum, and incubation of sections in buffer only to determine if the cells were autofluorescent. Sections of endosperm and embryo tissues of seeds from normal tobacco plants were also examined.

Sections were observed using a Zeiss Photomicroscope III equipped with Nomarski optic and epifluorescence capabilities. Rhodamine excitation was elicited using an Osram 50-w mercury lamp and a Zeiss rhodamine interference filter. Observations of the sections prior to photography was kept to a minimum.

Electron Microscopic Immunocytochemical Localization of Phaseolin. Phaseolin localization on ultrathin sections of glutaraldehyde and OsO₄-fixed, L. R. White embedded tissues was done using an indirect immunogold procedure (3, 11), using a pretreatment of the sections with NaIO₄ and HCl to unmask antigenic determinants. Grids were floated section-side down on drops of solution and transferred between solutions with a wire loop. The following treatments were given: 10 min on saturated aqueous NaIO₄, five washes of 1 min on glass-distilled H₂O; 10 min on 0.1 n HCl; six washes of 1 min on 0.45 M NaCl, 30 mM K-phosphate (pH 7.2), 0.2% Tween 20, 15 mM glycine (3 × PBST-glycine); 10 min on 3 × PBST-glycine, 2% gelatin; six washes of 1 min on 3 × PBST-glycine; 10 min on adsorbed phaseolin-specific rabbit antiserum containing 0.2% Tween 20 and 2% gelatin; seven 1-min washes on 3 × PBST-glycine; 30 min on colloidal gold-conjugated goat-anti-rabbit IgG Diluted 1:30 (v/v) with 3 × PBST, 2% gelatin (A₅₃₀ = 0.175); ten 1-min washes on 3 × PBS. Grids were rinsed briefly with glass distilled H₂O, stained for 10 min using 3% uranyl acetate in 0.05 M veronal acetate adjusted to pH 6.4 with HCl, rinsed and blotted dry.

RESULTS

Mature tobacco seeds are ovoid in shape, 0.5 to 0.8 mm long and contain a torpedo-shaped embryo that is completely surrounded by a 3 to 4 cell layer thick endosperm. Storage parenchyma cells of the mature endosperm and embryo are characterized by a large central nucleus, many protein bodies with globoid and proteinaceous crystalloid inclusions embedded in an amorphous protein matrix, and numerous lipid bodies (Fig. 1). Mitochondria, proplastids, ER, and Golgi complexes are rarely observed due to the dense packing of the lipid bodies and to a lack of reorganization of membranous systems at this early stage of imbibition. There are no readily apparent differences in either the morphology of mature seeds or the ultrastructure of equivalent endosperm and embryo cell types between seeds from normal and transformed tobacco plants.

Initially, immunofluorescent localizations of phaseolin were performed using crude phaseolin-specific rabbit antiserum. Using this crude antisera we observed a high degree of cell wall-associated fluorescence. The fluorescence was not phaseolin-specific as cell-wall fluorescence was also obtained using embryo and endosperm tissue from normal tobacco plants. Control sections demonstrated that the cell walls were not autofluorescent except for cuticle-like regions which surrounded the endosperm and embryo. Cell walls did not bind rhodamine-conjugated goat-antibody rabbit IgG when crude phaseolin-specific antiserum was either omitted or replaced with preimmune rabbit serum during the immunofluorescent staining procedure. We suspected that antibodies specific for the oligosaccharide side chains of phaseolin were reacting with similar determinants located in the cell walls. The oligosaccharide side chains of phaseolin are of the high-mannose type, containing two residues of N-acetyl glucosamine and eight to nine mannose residues (8, 14). They are similar in composition and, presumably, similar in structure to the high-mannose oligosaccharide side chains of animal glycoproteins (8). Ovalbumin is known to contain a variety of oligosaccharide side chains, all of the high-mannose type (see Kornfeld
and Kornfeld [24]), and was selected as an affinity substrate for removing oligosaccharide-specific antibodies from the anti-phaseolin serum. When this purified antiserum was used for immunofluorescent localization of phaseolin cell wall-associated fluorescence was minimized. (Fig. 2, B, D, and F). Cuticle-like areas of endosperms and embryos remained autofluorescent (Fig. 2, D, F).

Observations of immunofluorescent-labeled sections revealed that phaseolin was indeed present within some, but not all, of the mature embryos and endosperms from transformed tobacco plants (Fig. 2, A, B, E, and F). When present, phaseolin was found to be restricted to areas within the embryonic and endospermic cells tentatively identified as protein bodies (Fig. 2, A, B, E, and F). Thin sections of embryos and endosperms from untransformed tobacco plants, when stained for phaseolin, gave no fluorescence (Fig. 2, C and D). Similarly, no fluorescence was observed in cells of embryos and endosperms from transformed or normal plants when antiphaseolin serum was omitted or replaced with preimmune rabbit serum during the staining procedure (not shown).

Confirmation that phaseolin was restricted to the matrix component of the protein bodies was obtained using ultrathin sections of embryonic tissue from transformed plants labeled by the immunogold method. Pretreatment of the sections with sodium metaperiodate and HCl greatly enhanced the phaseolin-specific labeling when compared to nonpretreated sections. Gold particles, indicating the presence of phaseolin, were concentrated over the matrix component of the protein bodies. Few gold particles were observed over either the crystalloid (Fig. 3, A and B) or globoid cavity (Fig. 3B) components of the protein bodies. It should be noted that the periodate and HCl pretreatment of the section prior to immunolabeling extracted the electron dense material, presumably phytin (25), from the protein body globoids. Lipid bodies and the cytoplasm between lipid bodies and protein bodies remained unlabeled except for a few gold particles attributed to background contamination from observation of control sections. Thus, phaseolin is located solely in the matrix fraction of the protein bodies when present in mature embryos from transformed plants. Additional observations at the ultrastructural level revealed that the sequestering of phaseolin is not tissue-specific in the embryos, all cells of the protoderm, provascular tissue, and storage parenchyma were found to contain phaseolin.

Only one of five observed endosperms from transformed tobacco plants was found to contain phaseolin. However, six of seven embryos contained phaseolin based on combined results from immunofluorescent and immunogold labeling studies. Qualitatively, the immunofluorescence studies suggested that the
Fig. 2. Immunofluorescent localization of phaseolin in mature seed tissues from transformed tobacco. All sections were incubated with antibodies against phaseolin followed by rhodamine-conjugated goat-anti-rabbit IgG. Pairs of micrographs (i.e. A and B) illustrate identical areas from each tissue as examined using Nomarski optics (A, C, E) and epifluorescence microscopy (B, D, F). A and B, Cotyledon storage parenchyma from transformed tobacco seeds (× 1190); E and F, endosperm tissue from transformed tobacco seeds (× 1340). When present, phaseolin is restricted solely to the protein bodies of the embryo (A, B) or endosperm (E, F) of transformed tobacco. The specificity of the reaction is demonstrated by the lack of fluorescence in identically treated sections of normal tobacco seeds (C, D); cuticular areas in these tissues were autofluorescent. N, Nucleus; CW, cell wall; PB, protein body; Cu, cuticle. Micrograph pairs are labeled identically; bars = 10 μm.
Phaseolin is restricted to the matrix component of protein bodies in embryonic tissues of transformed tobacco seeds. Sections were incubated with phaseolin-specific antiserum followed by 5 nm colloidal gold conjugated goat-anti-rabbit IgG. Gold particles are concentrated over the matrix of the protein bodies (A, B). Few gold particles are found over the crystalloid (A, B) or globoid cavity (B) of the protein bodies or over the lipid bodies or strands of cytoplasm. L, lipid body; c, protein body crystalloid; gc, globoid cavity of protein body; m, protein body matrix. A, × 32,700; B, × 36,800; bars = 1 μm.
embryos contained differing amounts of phaseolin. Under identical immunolabeling conditions phaseolin-specific fluorescence was found to be high in one embryo of the five observed, moderate in three of the five, and nonexistent in the remaining. Section thickness cannot account for the variations in the intensity of phaseolin-specific fluorescence since the use of plastic sections only allows detection of antigenic sites at the section surface. Thus variations in phaseolin-specific fluorescence must reflect the relative quantities of available antigen on the section surfaces which, in turn, reflects the relative quantity of phaseolin within the observed tissues.

DISCUSSION

Immunocytochemistry on Sections of Plastic-Embedded Plant Tissues. The selection of methods used for the subcellular immunolocalization of proteins generally involves a compromise between the need to retain the antigenicity of the target molecules and the need to retain cellular and subcellular integrity. A third criterion, the properties of the tissue being investigated, must also be taken into consideration. Although hand sections of fresh tissue have been previously used for immunofluorescence studies on seeds (9, 26), they could not be used here due to the small size of the tobacco seeds and the requirement of relatively high resolution of localization. Thin and ultrathin frozen sections of fixed tissues have been used successfully for high resolution localization of plant proteins at both the light (1) and electron microscopic (2, 18, 19) levels. However, the high lipid content (~40% dry weight, see Tso [30]) of tobacco seeds imparted very poor sectioning properties to the frozen tissues. Thus, cryosectioning techniques were not applicable in this study. Recent advances in postembedding immunolabeling using sections of plastic embedded tissues (3, 11-13) prompted the use of more conventional fixation, dehydration, and embedding methods for the study of the subcellular localization of phaseolin in mature seed tissues from transformed tobacco. The results of this investigation illustrate that high resolution localization of antigens at both the light and electron microscopic levels can be successfully performed using sections of conventionally prepared plant tissues. This confirms and extends the observations of Craig and co-workers (11-13).

Fixation, chemical dehydration, and infiltration with plastic resins during preparation of tissues for light microscopic analyses all have adverse effects on the antigenicity of plant proteins (10). Some plant proteins retain sufficient antigenicity following formicdehyde fixations and polyethyleneimine embedding to allow their detection using postembedding immunofluorescence techniques (12; this study). Similarly, the preparation of plant tissues for routine TEM generally requires fixation using glutaraldehyde and OsO₄ prior to embedding in plastic resins in order to preserve ultrastructural detail. Both of these fixatives, and especially OsO₄, greatly reduce or abolish the antigenicity of plant proteins (10, 32). Thus, glutaraldehyde concentrations are often kept low and OsO₄ fixation is omitted during the preparation of tissue for postembedding immunolocalization at the ultrastructural level (10, 21, 22, 31, 32). Although relatively high levels of antibody labeling are obtained, ultrastructural detail is often quite poor as a result of the omission of OsO₄ fixation (see Zur Nieden) [31, 32]). Recent observations by Bendayan and Zollinger (3) and Craig and Goodchild (11) indicated that sodium metaperiodate and HCl pretreatment of sections of OsO₄ fixed, exoxy resin embedded tissues resulted in a 10- to 20-fold increase in specific antibody labeling. In this investigation, pretreatment of sections of L. R. White-embedded tissue dramatically increased phaseolin-specific immunogold labeling. Thus, the technique is applicable to sections of both epoxy resin and acrylic resin embedded tissues (see also Craig and Miller [13]). The presumed action of sodium meta-periodate and HCl in unmasking antigens on the surface of the sections has been discussed previously (3, 11). It should be noted, however, that phaseolin (this study) and pea seed legumin and vicilin (11-13) are storage proteins which are highly concentrated in the protein bodies of mature seed tissues. The procedures used here may not be appropriate for the immunofluorescence or immunogold localization of less abundant or less stable plant proteins.

Subcellular Localization of Phaseolin in Mature Embryo and Endosperm Tissues from Transformed Tobacco Plants. Immunocytochemical localization of phaseolin in the mature embryo and endosperm tissues of a single seed lot from transformed plants supports many of the results of Sengupta-Gopalan et al. (28). The occurrence of phaseolin in six of seven mature embryos as compared to only one of five endosperm examined indicates that the gene product accumulates primarily in the embryonic tissues of the seed. Qualitative results from the immunofluorescence studies indicated that one embryo of five contained a high level of phaseolin, three of five contained moderate levels, and one had no detectable phaseolin. Although statistical analyses on this low sample number would be meaningless, the results do agree closely with the 1:2:1 ratio of embryos containing high/moderate:nondetectable levels of phaseolin determined in a more extensive statistical study using 77 embryos (28).

When present in the embryos of transformed tobacco, phaseolin was restricted to the matrix component of the protein bodies in all tissue types (Figs. 2, A, B, and 3, A, B). The restriction of phaseolin to the matrix component may be due either to its exclusion from the crystalloid during protein crystallization or due to a temporal difference in sequestering of crystallloid versus phaseolin storage proteins or both. Phaseolin was found to accumulate in the seeds between 15 and 28 d after pollination (28) whereas native tobacco storage proteins accumulate between 9 and 18 d following pollination (27). Thus there is a temporal difference in the accumulation of phaseolin and that of native tobacco proteins.

Protein bodies are the sites of phaseolin accumulation in the developing cotyledon cells of Phaseolus vulgaris (2, 18), as well as in the embryonic cells of mature seeds of transformed tobacco. Thus, the gene product accumulates in the same organelles in these genetically distinct organisms. In beans, phaseolin is synthesized by ER-associated polyribosomes and sequestered within the lumen of the ER due to the presence of a signal peptide on the nascent polypeptide chain (5, 8). Phaseolin is cotranslationally glycosylated (6, 8) and subsequently transported to the Golgi apparatus, transportation to the protein bodies occurring via Golgi-derivied vesicles (6, 8, 18). Phaseolin in seeds of transformed tobacco probably undergoes cleavage of the signal peptide and is also glycosylated (20, 28) implicating the ER in the sequesterion process.

In beans phaseolin accumulates as a family of polypeptides with an Mᵦ ≈ 48,000, whereas in tobacco embryos smaller polypeptides (Mᵦ ≈ 25,000) were also found (20, 28). These smaller polypeptides may be breakdown/processing products of the larger ones. In endosperm, intact phaseolin polypeptides were absent and only smaller polypeptides were present. The authors suggested that the cytosol was the site of accumulation of these breakdown products. The results reported here show that phaseolin or immunologically cross-reacting polypeptides (e.g. breakdown products) were present in the protein bodies of endosperm cells. In agreement with the biochemical findings (20, 28) we observed phaseolin in only one out of five endosperms. The results show that the expression of the phaseolin gene in tobacco is not restricted to embryonic tissues. It should be pointed out, however, that it is not known if phaseolin genes are expressed in bean endosperm as this tissues is transiently present during seed development. We suggest that the small polypeptides derived from phaseolin that are present in both the embryonic and
endosperm tissues of transformed tobacco are the result of a premature partial digestion of the protein within the protein bodies. The polypeptides have electrophoretic mobilities comparable to those derived from specific cleavages of phaseolin following germination of bean seeds (4), and digestion of storage proteins during germination is a protein body-associated event (1). The relatively rare occurrence of phaseolin-derived polypeptides in the mature tobacco endosperm (this study) and the lack of authentic phaseolin in this tissue (28) may be due to an accelerated premature digestion of the protein compared with that occurring in the embryo cells.

**LITERATURE CITED**

30. Tso TC 1972 Physiology and Biochemistry of Tobacco Plants. Dowden, Hutchinson and Ross, Inc., Stroudsburg, PA