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Sunita Shah
San Jose State University

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**ISOLATION AND IMMUNOLOCALIZATION
OF UNIQUE EPIDERMAL PROTEINS IN LEAVES OF
PISUM SATIVUM VAR. *ARGENTEUM***

**A Thesis
Presented to
The Faculty of the Department of Biological Sciences
San Jose State University**

**In Partial Fulfillment
of the Requirements for the Degree
Master of Science**

**by
Sunita Shah
May 1995**

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APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. David K. Bruck, Professor

Dr. John Boothby, Professor

Dr. Chris Brinegar, Professor

APPROVED FOR THE UNIVERSITY

ABSTRACT

ISOLATION AND IMMUNOLOCALIZATION OF UNIQUE EPIDERMAL PROTEINS IN LEAVES OF *PISUM SATIVUM* VAR. *ARGENTEUM*

By Sunita Shah

In this study, our goal was to isolate and localize unique proteins expressed in the mature epidermis of *Pisum sativum* var. *argenteum*. Three potential epidermal-specific proteins (ESPs) were identified by analytical SDS-PAGE. One of these ESps was isolated from preparative SDS-PAGE gels and used for polyclonal antibody production. Contradictory results were obtained regarding antibody specificity in immunoblots versus immunohistological sections. The anti-ESP antibodies reacted nonspecifically with all the major proteins of the epidermis on an immunoblot. Localization of fluorescently (AMCA)-tagged anti-ESP antibodies on sections of mature leaves occurred almost exclusively in the epidermis, lending support to the unique, tissue-specific expression of this protein.

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INTRODUCTION

Cell differentiation presumably results from repression and derepression of specific genes, where the stages of differentiation are controlled by the appearance of specific gene products. It is, therefore, of interest to determine the timing of formation and identity of these gene products to understand the mechanism of cell differentiation. To isolate individual gene products, one must gain access to a purified cell type or tissue. The superficial location of the epidermis in plants makes it a suitable and accessible tissue for these manipulations. The epidermis of *Pisum sativum* var. *argenteum* (*arg*) was used in this investigation of epidermal-specific proteins, because this variety bears a single gene mutation (Marx 1978; Hoch *et al* 1980) causing the epidermis to be loosely attached to underlying mesophyll tissue and therefore easy to peel and isolate from other plant tissues. Thus pure preparations of epidermal tissue could be readily obtained.

Evidence has been accumulated that the epidermis is the fundamental cell type in plants (Bruck and Walker 1985a, 1985b). It is the first morphologically recognizable tissue (as protoderm) formed in the embryo. Epidermal tissue has many unique structural and functional characteristics (Bruck and Walker 1985a, b; 1986; Bruck *et al* 1989; Esau 1977; Walker and Bruck 1985) that are useful for identifying it at maturity and during development. The cells are structurally distinctive from other cell types by the presence of a cuticle (Bruck and Walker 1985a). Functionally, epidermal cells,

unlike other parenchymatous cells, have a limited ability to dedifferentiate and redifferentiate (Walker 1983; Moore 1984; Walker and Bruck 1985), as indicated by their failure to participate in graft unions (Bruck and Walker 1986; Bruck *et al* 1989; Walker and Bruck 1985) or to regenerate after wounding (Bruck and Walker 1985b). At an early point in their differentiation, they become restricted to an anticlinal plane of cell division (Stewart and Burk 1970; Bruck and Walker 1985a), unless part of a multiple epidermis. These properties suggest the presence of some unique proteins responsible for these characteristics. Hence the *arg* epidermis was used to investigate the presence of unique tissue-specific proteins.

Similar to the procedure followed in the present investigation, tissue-specific proteins have been identified in *Washingtonia filifera* (Chandra Sekhar and DeMason 1988) and *Cocos nucifera* (DeMason and Chandra Sekhar 1990) by SDS-PAGE. Endosperm-storage proteins extracted from coconuts were separated by SDS-PAGE, isoelectric focusing, and two-dimensional gel electrophoresis. Individual bands were electroeluted and injected into hens to raise polyclonal antibodies. Antibody specificity was confirmed by an immunoblot analysis, and immunolocalization was performed by a silver-enhanced, immunogold technique. Two unique (tissue-specific) proteins were found in the crystalloids and a third in the protein matrix. In our investigation, tissue-specific proteins were isolated from epidermal protein extracts by SDS-PAGE and used for polyclonal antibody production. The antibodies, tagged with fluorescent markers

(AMCA-labeled secondary antibodies), were used to confirm the uniqueness of these antigenic proteins.

Epidermal-specific proteins have been identified in other plants, such as maize (Sossountzov *et al* 1991) and *Pachyphytum* (Clark *et al* 1992). In maize, expression of lipid transfer protein (LTP) gene has been characterized. LTP gene expression was observed to be high in embryos and endosperm during seed maturation. The cotyledons, epicotyl, and root-hypocotyl axis of the embryo, and the embryonic and seedling coleoptile had high LTP mRNA levels, as observed by *in situ* hybridization. The outer epidermis of coleoptiles and leaves had the highest concentrations of immunogold-labeled LTP. In *Pachyphytum*, pure epidermal tissue was collected due to an underlying layer of thick-walled sclerenchyma cells from which the epidermis readily separated during peeling. Epidermal-specific transcripts were obtained by differential screening of a cDNA library and were found to be highly expressed in epidermal cells of developing stems and leaves. They were also expressed in the outer tunica layer of the shoot apical meristem, supporting the contention of Bruck and Walker (1985a) that the superficial cells of the meristem are epidermal in nature. The nucleotide sequence of these epidermal-specific transcripts (Clark and Bohnert 1993) has also been determined.

Another epidermal-specific protein, extracellular protein 2 (EP2), has been identified in carrot (Sterk *et al* 1991) by *in situ* hybridization of mRNA with antisense RNA. EP2 was isolated by means of antibodies

raised against the proteins secreted into the medium surrounding an embryo. The EP2 gene is expressed exclusively in the protodermal cells of globular and heart-shaped somatic embryos. A gradual decrease in EP2 gene expression was observed in the protoderm as embryogenesis proceeded to the torpedo stage. It ceased in mature embryogenic tissues, but continued in the outer tunica layer of the shoot apical meristem and its recent derivatives in the seedling. The EP2 gene is transiently expressed and thus is stage specific as well as tissue specific.

In the present research, screening of epidermal-specific proteins was performed at the protein level rather than the mRNA level as in the investigations of Sossountzov *et al* (1991), Clark *et al* (1992), and Sterk *et al* (1991). Our goal was to identify unique proteins expressed in the mature epidermis for later use in studies of epidermal development during embryogenesis. Cell differentiation and anatomical pattern formation are significant events in plant embryo and adult development. Little is known about the mechanisms leading to cell differentiation in plants at the molecular level. Until recently, only genes for abundant seed proteins had been analyzed for tissue-specific and stage-specific gene expression (Perez-Grau and Goldberg 1989). Now, about 100 embryo-specific proteins have been identified (Goldberg *et al* 1989).

Most of our knowledge about differentiation in plants is at the morphological level. The cells of the youngest (early globular stage) proembryo appear morphologically identical (Bruck and Walker

1985a). The onset of uniquely epidermal morphological features, beyond the secretion of cutin, of the exterior cells of the embryo occurs at different stages in different species. Yet the exterior cells of the youngest embryo, and the zygote itself, secrete a cuticle (Bruck and Walker 1985a), a feature generally considered to be distinctive to the epidermal cell type. Further evidence is required to verify whether these external cells, including the zygote, have become committed to development as, and so determined as, epidermal cells.

We hope to use unique epidermal proteins, identified to some degree in this project, to detect epidermal differentiation at stages prior to the appearance of distinctive morphological differences between embryonic cells. Antibodies raised against these unique proteins will be used as probes to determine the point at which embryonic cells initiate the expression of the proteins. The proteins, therefore, constitute molecular markers of epidermal identity and finer indicators than morphological criteria of a commitment to an epidermal cell type. By this method, the concept of the cell type may be more strictly and precisely defined. This kind of study is fundamental to the ultimate goal of understanding how all cells differentiate and how plants develop at the molecular level.

MATERIALS AND METHODS

Plant Growing Conditions. Seeds of *arg* variety of the garden pea (*Pisum sativum* var. *argenteum*) were sown in 5-inch plastic pots containing Supersoil (Rod McLellan Co., S. San Francisco, CA) plus Nutricote Total fertilizer (N-P-K 13-13-13; McCalif Growers' Supply, Ceres, CA). Seedlings were grown in a growth chamber with incandescent and fluorescent illumination (ca. 2000 lux at plant level) provided over a 16 h photoperiod. Temperature was maintained at 21°C during the day and 13.5°C at night.

Extract Preparation. Epidermal and mesophyll tissues were collected from 2-to-4-week-old seedling leaves removed from the stem. The epidermis was peeled from both leaf surfaces with jeweler's forceps. The remaining internal tissue is referred to herein as mesophyll tissue despite also containing vascular tissues. The peeled epidermal and mesophyll tissues were frozen separately in liquid nitrogen and stored at -20°C. Whole epidermal extracts (WEEx) and whole mesophyll extracts (WMEEx) were prepared by homogenizing the tissues in a 1.5 ml microcentrifuge tube with a plastic pestle in extraction buffer (ca. 0.5 ml, pH 7.0, per 1 mg frozen tissue) on ice. The extraction buffer consisted of 50 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, and 100 mM NaCl, plus a protease inhibitor cocktail of 5 mM ϵ -amino-*n*-caproic acid, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were

centrifuged at 10,000 x *g* for 15 min at 22°C, and the supernatant was concentrated in Centricon 10 (Amicon, Danvers, MA) microconcentrators in a Sorvall superspeed RC2-B centrifuge at 5000 x *g* for 6 h at 4°C. The concentrated protein extracts were stored at -80°C. *n*-Butanol was added to the protein extract in a 1:1 (vol/vol) ratio to eliminate lipids that might interfere during electrophoresis; the top butanol solution was pipetted off the remaining protein solution. The total content of soluble protein for WEEx and WMEEx was determined by a Bradford protein assay using bovine serum albumin (BSA) as a standard (Bradford 1976).

Gel Electrophoresis. WEEx and WMEEx (0.2 mg each) were separated by SDS-PAGE (Laemmli 1970). Analytical SDS-PAGE was performed using a 10% acrylamide (30% acrylamide/0.8% bisacrylamide) resolving gel and a 5% stacking gel. Gels measuring 15 x 20 x 0.15 cm were run at 30 mA for 4 h. Gels were stained first with Coomassie Blue R-250 in 50% methanol for 1 h, destained with 50% methanol and 10% acetic acid overnight and then with 5% methanol and 7% acetic acid for 1-2 h. After Coomassie staining, gels were silver stained (Merril *et al* 1981) to detect the presence of minute amounts of protein on the gel. The protein bands from WEEx were compared with the bands from WMEEx to detect the presence of unique proteins in the epidermal extract. The molecular weights of these bands were estimated by reference to molecular weight markers (Sigma, St. Louis, MO) at 205, 116, 97, 66, 45, and 29 kD.

Preparation of Epidermal-Specific Proteins. Epidermal-specific proteins (ESP = ESPs) were prepared by two methods.

(1) Immunoaffinity Adsorption. Rabbit antibodies (0.5 ml) against WEEEx and WMEx (see below) were purified on a 5 ml Protein A-cyanogen bromide-activated column (Sigma, St. Louis, MO) and concentrated by ammonium sulfate (50%) precipitation. The precipitate was analyzed for the presence and purity of antibodies on a 10% acrylamide, 8 x 5 x 0.15 cm, SDS-PAGE gel. The gel was run for 2 h at 20 mA. Molecular weight markers (Sigma, St. Louis, MO) used were 66, 45, 36, 29, 24, 20 and 14 kD. The purified anti-WMEx and anti-WEEEx antibodies were run through a PD-10 sephadex column (Bio-Rad, Hercules, CA) to remove the ammonium ions from the precipitate. An activated immunoaffinity support of 1 ml Affi-Gel 10 gel (Bio-Rad, Hercules, CA) was coupled with 3 ml purified anti-WMEx antibodies (0.5 mg/ml). Additional sites on the gel were blocked with 1 M ethanolamine-HCl, pH 8.0, for 1 h and then washed with distilled water. Three times at 3 h intervals, 1 ml WEEEx was added to 300 μ l of the gel adsorbed with anti-WMEx antibodies in a batch process in microfuge tubes at 4°C, with agitation every half hour. WEEEx was treated in this manner to remove all the proteins that were present in both epidermis and the mesophyll (common proteins). This batch process made possible the isolation of proteins present only in the epidermis (unique proteins). The WEEEx components, unbound and bound to the anti-WMEx antibodies that were coupled to the Affi-Gel

10 and WEEx were centrifuged at 10,000 x *g* for 30 s at 22°C. The supernatant containing the unique epidermal proteins, referred to as unbound proteins, was collected. Common proteins, which were adsorbed onto the anti-WMEx antibodies and referred to as bound proteins, were eluted off of the gel with 100 mM glycine, pH 3.0. The bound and unbound proteins were analyzed on two 10% acrylamide, 15 x 20 x 0.15 cm, SDS-PAGE gels. The gels were run at 30 mA for 4 h. Another gel was run as described above, except that a 12% acrylamide gel was used to analyze the lower molecular weight proteins.

(2) Preparative SDS-PAGE. WEEx (0.5 mg) was run on two preparative, 12% acrylamide, 15 x 20 x 0.3 cm, SDS-PAGE gels. From both gels, a narrow vertical slice containing separated WEEx proteins was excised, silver stained, and compared with the WMEx protein profile for the presence of unique bands. The bands on the unstained part of the gels corresponding in position to the unique band in the stained part of the gels was cut out and the slice stored at -20°C.

Preparation of Rabbit Antisera. Preimmune blood samples were collected from the ear vein of four 7-week-old female rabbits. The gel slice (see above), containing an ESP, was homogenized in an equal volume of 0.01 M phosphate buffered saline (PBS), pH 7.2. Two rabbits were each subcutaneously injected with 1 ml of the homogenate per shoulder. WEEx and WMEx (1 ml each of 0.5 mg/ml with Freund's complete adjuvant) were injected subcutaneously into

each of two other rabbits. To all rabbits, a second booster dose was given after 4 weeks and a third booster dose after another 2 weeks. All the rabbits were bled 1 week after the third injection, and the blood was allowed to clot at room temperature for 1 h. The supernatant was then centrifuged at 1000 x *g* for 15 min, and the sera were collected and stored at -20°C.

Immunoblot. WEE_x and WME_x were run on 12% acrylamide, 8 x 5 x 0.15 cm, SDS-PAGE gels, and the bands were transferred onto S & S NYTRAN (Schleicher and Schuell, Keene, NH), a nitrocellulose membrane with 0.45 µm diameter pores, in a Trans-Blot system (Bio-Rad, Hercules, CA). Immunoblots (Harlow and Lane 1988) were prepared using preimmune serum, anti-WEE_x, and anti-ESP (all at 1:400 dilution) under identical conditions. An ELISA (Envall and Perlmann 1972) was also performed separately on preimmune serum, anti-WEE_x, anti-WME_x, and anti-ESP using a goat anti-rabbit IgG/HRPO conjugate (Antibodies, Inc., Davis, CA) with 40 mM ABTS as the substrate. All the above mentioned sera were tested against WEE_x (0.25 mg/ml) and WME_x (0.25 mg/ml) individually at 405 nm with a 450 nm baseline correction.

Microtechnique. Mature leaves (the fourth, fifth, and sixth leaves produced by the seedling) of *arg* were cut into 1 mm² segments and fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.8, for 2 h at room temperature. The

segments were rinsed 3 times in the same buffer. The tissue segments were then partially dehydrated in a graded series of acetone solutions (25%-95%) for 5 min each. All the segments were infiltrated in the dark in the hydrophilic acrylic resin, LR White (Ted Pella, Inc., Redding, CA), for 1 h. A second change of LR White was made for 12 h and a final change for 1 h. This resin was used because it does not require total dehydration or excessive heating for polymerization and so maintains antigenicity better than most other resins. The resin was cured at 60°C for 16 h. The tissue blocks were sectioned with a Sorvall JB-4 rotary microtome to obtain 1 µm thick sections, which were affixed to glass slides on a hot plate at 42°C overnight. Tissue sections were stained with 1% crystal violet and photographed with Kodak TP 2415 film, or stained for immunocytochemistry (see below).

Immunocytochemistry. Tissue sections were treated with 1% non-fat dry milk in 10 mM potassium phosphate buffer, pH 7.6, for 2 h at 4°C to block non-specific sites. Dilutions at 1:10 of pre-immune, anti-WEEEx, anti-WMEx, and anti-ESP were used as primary antisera on different slides for 2 h at room temperature. The slides were first rinsed with 0.1% Tween-80 in 10 mM potassium phosphate buffer, pH 7.6, for 10 min (4 changes) and then with indirect stream of running water for 15 min. The slides were incubated with goat anti-rabbit IgG (H+L) conjugated to an AMCA fluorophore (ICN Biomedicals, Costa Mesa, CA) at a 1:75 dilution for 2 h at room temperature. After rinsing

the slides, as described above, sections were viewed under epifluorescence optics on a Zeiss research microscope and photographed on Kodak Royal Gold 100 film.

RESULTS AND DISCUSSION

Protein Extraction. The *arg* mutant of garden pea (Fig. 1) was used because the pectinaceous middle lamella that cements the epidermis to the underlying mesophyll is poorly formed. Thus it is easy to peel the epidermis from the mesophyll to obtain pure preparations of the tissues (Fig. 2). Epidermal cells are relatively thick-walled parenchyma cells, highly vacuolate with limited peripheral cytoplasm. Hence it was possible to extract only a limited quantity of protein from the cells. The average soluble protein content of several different extracts of epidermal and mesophyll tissues was low, less than 0.01 mg/ml and about 0.1 mg/ml, respectively. Consequently, the protein extracts were concentrated with microconcentrators to obtain more of the proteins, including the ones present in minute amounts. The average protein content after concentration was *ca.* 0.15 mg/ml (total 1.5 mg) in WEEx and 0.50 mg/ml (total 5 mg) in WMEEx.

Protein Separation. A comparison of silver-stained protein bands (Fig. 3) of SDS-PAGE-separated WEEx and WMEEx indicated a number of potential epidermal-specific protein bands. A higher number of unique proteins occurred in mesophyll extracts than in epidermal extracts. The majority of the bands were common to both tissues. Of the potential epidermal-specific bands, five were suitable candidates for isolation due to higher band intensity and greater separation from adjacent bands.

Immunoaffinity Adsorption. The antibodies raised against WEEx and WMEx were purified on a Protein-A column and analyzed by SDS-PAGE. The purification of an IgG fraction containing rabbit anti-WMEx antibodies by Protein-A column chromatography is shown in Fig. 4. Average sample concentration before chromatography was 600 μ g. The concentration of bound antibodies was 125 μ g after elution at pH 3.0.

Purified anti-WMEx was coupled to Affi-Gel 10 gel for use in the elimination of proteins common to both epidermis and mesophyll from WEEx. Elimination of shared bands from the crowded electrophoretic profiles would make the unique proteins more accessible. Unique bands would then be eluted or excised and antibodies raised against them. However, only limited removal of shared proteins from WEEx was accomplished (Table 1), probably due to inefficient coupling of the antibodies to the Affi-Gel. The poor efficiency of coupling could be due to residual sites or to competition from contaminating proteins. In addition, our estimates of binding efficiency did not account for antibodies free in solution that were associated with but not bound to the beads. This omission would lead to erroneously high estimates of binding efficiency. Other reasons could be low levels of antibodies against high concentration proteins or absence of antibodies against some shared proteins. Nevertheless, three epidermal-specific bands, at molecular weights 40, 21, and 13 kD, were visible on gels loaded with the eluent from this procedure (Fig. 5 and 6).

Preparative Gels. Preparative gels (3 mm) were run with a wide lane (10 mm) and concentrated sample application to maximize the yield of unique proteins. In all three preparative 10%, 10%, and 12% acrylamide SDS-PAGE gels, few bands were visible. Of the three bands identified as epidermal-specific proteins in Fig. 5 and 6, the 13 kD band was faint but visible, having a concentration of only about 5 ng/ml, an approximation based on band intensity. The 12% acrylamide SDS-PAGE was successful in retarding migration of the 13 kD band, but the 40 and 21 kD bands were absent in all of the preparative gels. The gels were stained for a longer period of time in an attempt to visualize the other two bands, but overstained gels resulted in poor resolution of any bands. The reason for the absence of these two bands on the gel, even after increasing the concentration of the epidermal extract, is unknown. The 13 kD band (called hereafter the ESP) was faint but detectable in all of the preparative gels.

Antibody Reactivity and Specificity. An ELISA was conducted on all of the rabbit antisera collected to test for their reactivity at 405/450 nm against WEEx and WMEEx (Table 2). The preimmune sera were more reactive with WEEx than with WMEEx, undoubtedly as a result of non-specific binding. Anti-WEEx reacted with both WEEx and WMEEx to about the same extent, an anticipated result given the commonality between the two tissues of the majority of the proteins. WMEEx probably had a higher concentration of common proteins than WEEx,

as the overall protein concentration in mesophyll was much higher than that in epidermal tissue and hence was equally reactive as WEEEx. As expected, the rabbit anti-WMEx was more reactive to WMEx than to WEEEx.

Low but significant reactivity of anti-ESP was expected with WEEEx because of the low proportion of ESPs in the whole epidermal extract. This pattern was in evidence with the anti-ESP collected from the blood sera of one rabbit (Rabbit No. 1). Surprisingly, however, the reactivity of anti-ESP from Rabbit No. 1 was less to WEEEx than to WMEx. Negligible reactivity of anti-ESP with WMEx was expected, so that the relatively substantial degree of reactivity was probably a result of non-specific binding. Non-specific binding of the secondary antibody or the conjugate to the ELISA plates might have been prevented by blocking plates with BSA. The reactivity of anti-ESP from a second rabbit (Rabbit No. 2) was almost negligible with both WEEEx and WMEx. The amount of ESP injected into each rabbit was very low, as it was electrophoretically obtained by excision from a preparative gel in a purified state. Therefore, only 10-15 ng was recovered per gel, perhaps an inadequate amount to elicit a significant immune response.

The reactivity of anti-WEEEx antibodies toward both epidermis and mesophyll was confirmed by an immunoblot (Fig. 7). An immunoblot was also conducted on the preimmune and anti-ESP antibodies (Fig. 8). Like the ELISA absorbance values for reactivity, preimmune sera reacted with the molecular weight markers, WEEEx, and WMEx non-

specifically. The anti-ESP did not react with any molecular weight markers or WME_x bands. They were expected to react only with the unique 13 kD protein band but instead reacted non-specifically with all WEE_x bands. It is conceivable, although highly improbable, that the ESPs bore an epitope common to all WEE_x proteins so that antibodies against it bound to a number of bands. More likely, the 13 kD band consisted of a number of small subunits fragmented from the higher molecular weight proteins, as it was at the dye front in an extremely low molecular weight position. If so, when those fragments were injected into the rabbits as antigens, antibodies were produced against a great number of small subunits of the larger proteins. Thus these antibodies bound to many bands.

Immunocytochemistry. Transverse sections of *arg* leaves revealed a poorly defined, single-layered palisade mesophyll and an extensive spongy mesophyll interrupted by closely spaced veins (Fig. 9). Because the epidermal cells bear large central vacuoles, AMCA-specific (blue) fluorescence was observed only on the thin cytoplasmic periphery of the cells. As expected, little AMCA-specific fluorescence occurred on sections treated with preimmune antisera (Fig. 10a). The few spots observed lacked tissue and cell type specificity or occurred in the resin surrounding the sections, suggesting that this low-level binding of the secondary antibodies was random and non-specific. Similar results were obtained when control sections were treated with AMCA-conjugated secondary antibodies only, without the primary antibodies.

Chlorophyll (yellow) autofluorescence occurred throughout the mesophyll in all sections treated with either preimmune or immune sera. Anti-WEEEx antibodies bound in about equal amount to the epidermis and spongy mesophyll (Fig. 10b), consistent with the abundance of protein shared by the two tissues. Anti-WMEEx antibodies bound preferentially to internal tissues of the leaf, including spongy mesophyll, palisade mesophyll, and xylem parenchyma (Fig. 10c). Limited localization occurred in the epidermis as a result of the lower concentration of protein present in that tissue in comparison to internal tissues. Binding of anti-ESP antibodies was restricted to the epidermis (Fig. 10d). The label was nearly absent in internal tissues, although occasional non-specific binding was evident in the spongy mesophyll. These results are curious in light of the lack of specificity of the anti-ESP antibodies in the immunoblots discussed above.

The immunocytochemical investigation described above suggested the successful localization of a unique epidermal protein and, therefore, the isolation of antibodies specific to this determinant. However, this possibility was contradicted by the immunoblots, in which the anti-ESP antibodies recognized virtually all epidermal proteins, whether unique or shared. Further contradiction occurred with the reactivity of preimmune serum with proteins of both tissue extracts and molecular weight standards. Additional work is clearly necessary to confirm the localization of epidermal-specific proteins. This investigation has, nonetheless, identified several useful procedures for this purpose. Modifications of the present procedure

might include greater concentration of the epidermal extract in the rabbit injections to elicit a stronger immune response. A more concentrated extract would allow an Affi-Gel column removal of common proteins, in which washings are more efficient, rather than reliance on the batch immunoadsorption process. Many of the details of the procedure for localizing tissue-specific proteins have been worked out in this research, providing a headstart for future studies.

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Figure 1. Photograph of 4-week-old leaves of the wildtype (left) and *argenteum* (right) varieties of *Pisum sativum*. Extensive intercellular air spaces occur between the epidermis and mesophyll or cortical tissue, resulting in a silver cast to the leaves and stems of the *arg* mutant.

Figure 2. Peeling of the epidermis from the *arg* leaf mesophyll with jeweler's forceps. Pure preparations of epidermis and mesophyll are easily obtained due to the loose attachment of the epidermis and underlying mesophyll.

Figure 3. SDS-PAGE protein profiles of epidermal and mesophyll extracts of leaves of *P. sativum* var. *argenteum* resolved on a 10% acrylamide gel and silver stained. Lane 1: epidermal protein extract. Lane 2: mesophyll protein extract. Lane 3: two-fold diluted epidermal protein extract. Lane 4: two-fold diluted mesophyll protein extract. Lane 5: molecular weight markers (in kD). The arrows point to potential epidermal-specific protein bands.

Figure 4. Protein content (Bradford assay), as measured by absorbance at 595 nm, of fractions of rabbit anti-WME_x eluted from a Protein-A column. Arrow indicates point of addition of elution buffer (100 mM glycine, pH 3.0).

Table 1. Concentration of the WEEx adsorptions onto Affi-Gel 10 gel coupled with anti-WEEx antibodies. The percent efficiency refers to the degree of anti-WEEx bound to the Affi-Gel.

Affinity Run	Percent Efficiency	WEEx (mg)	Unbound Protein (mg)	Bound Protein (mg)
1	30	0.16	0.12	0.004
2	52	0.32	0.28	0.035
3	52	0.20	0.16	0.020

Figure 5. SDS-PAGE of silver-stained proteins from epidermis and mesophyll of leaves of *P. sativum* var. *argenteum* prepared from Affinity Run No. 1 and resolved on a 10% acrylamide gel. Lane 1: molecular weight markers (in kD). Lane 2: WEEEx. Lane 3: mixture of WEEEx and WMEEx. Lane 4: WMEEx. Lane 5: epidermal proteins adsorbed onto anti-mesophyll antibodies (referred to as "bound proteins I"). Lane 6: epidermal proteins that failed to adsorb onto anti-mesophyll antibodies (referred to as "unbound proteins I"). Lane 7: bound proteins II. Lane 8: unbound proteins II. Proteins designated by I and II are products of sequential adsorbances on Affi-Gel. The arrows point to potential epidermal-specific protein bands.

Figure 6. SDS-PAGE of silver-stained proteins from epidermis and mesophyll of leaves of *P. sativum* var. *argenteum* prepared from Affinity Run No. 2 and resolved on a 10% acrylamide gel. Lanes 1 and 10: molecular weight markers (in kD). Lane 2: WEE_x. Lane 3: WME_x. Lane 4: unbound proteins I. Lane 5: bound proteins I. Lane 6: unbound proteins II. Lane 7: bound proteins II. Lane 8: unbound proteins III. Lane 9: bound proteins III. Proteins designated by I, II and III are products of sequential adsorbances on Affi-Gel. The arrows point to epidermal-specific protein bands.

Table 2. Absorbance values of different rabbit antisera at different dilutions against whole mesophyll extract (WME_x) and whole epidermal extract (WEE_x), as measured by ELISA at 405 nm with a 450 nm baseline correction.

Antiserum	Antigen	Antiserum Dilutions					
		1:100	1:200	1:400	1:800	1:1600	1:3200
Preimmune	WEE _x	0.040	0.036	0.021	0.013	0.006	0.002
	WME _x	0.027	0.016	0.014	0.008	0.005	0.004
Anti-WEE _x	WEE _x	0.444	0.221	0.193	0.085	0.018	0.001
	WME _x	0.460	0.367	0.256	0.176	0.106	0.069
Anti-WME _x	WEE _x	0.552	0.343	0.250	0.118	0.064	0.026
	WME _x	0.995	0.791	0.554	0.305	0.144	0.064
Anti-ESP No. 1	WEE _x	0.056	0.025	0.020	0.016	0.008	0.004
	WME _x	0.064	0.041	0.033	0.018	0.009	0.003
Anti-ESP No. 2	WEE _x	0.044	0.034	0.021	0.011	0.009	0.004
	WME _x	0.020	0.011	0.008	0.004	0.002	0.001

Figure 7. Immunoblot of anti-WEEEx antibodies against different dilutions of WEEEx and WMEEx. Lane 1: WEEEx. Lane 2: 2-fold diluted WEEEx. Lane 3: 4-fold diluted WEEEx. Lane 4: 8-fold diluted WEEEx. Lane 5: WMEEx. Lane 6: 2-fold diluted WMEEx. Lane 7: 4-fold diluted WMEEx. Lane 8: 8-fold diluted WMEEx. Lane 9: prestained molecular weight markers (in kD).

Figure 8. Immunoblots of rabbit sera against ESP. (a) Pre-immune and (b) immune (anti-ESP) sera. Both sera were diluted 1:400 in 1% BSA in 50 mM TBS, pH 7.2. Lane 1: molecular weight markers (in kD). Lane 2: epidermal protein extract. Lane 3: mesophyll protein extract.

Figure 9. Transverse section of mature leaf of *P. sativum* var. *argenteum*. Palisade mesophyll is poorly defined, whereas spongy mesophyll (*M*) is extensive. A vein is visible within the spongy mesophyll. Large chloroplasts (*C*) are numerous in the mesophyll cells. The epidermal cells (*E*) are highly vacuolate, and their walls are relatively thick, especially the outer tangential walls.

Figure 10. Immunofluorescence micrographs of transverse sections of leaves of *P. sativum* var. *argenteum*. (a) Application of preimmune rabbit antisera followed by goat anti-rabbit IgG-AMCA (blue fluorescing) conjugate. (b) Application of rabbit antibodies prepared against WEEx followed by goat anti-rabbit IgG-AMCA (blue fluorescing) conjugate. (c) Application of rabbit antibodies prepared against WMEx followed by goat anti-rabbit IgG-AMCA (blue fluorescing) conjugate. (d) Application of rabbit antibodies prepared against ESP followed by goat anti-rabbit IgG-AMCA (blue fluorescing) conjugate. Chlorophyll (yellow fluorescing) autofluorescence visible in chloroplasts of mesophyll of all sections.