**Communication**

**Ethylene Effect on Extensin and Peroxidase Distribution in the Subapical Region of Pea Epicotyls**

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**ABSTRACT**

In dark grown pea (*Pisum sativum*) seedlings ethylene causes the triple response in which elongation growth is inhibited, radial growth is promoted, and orientation of shoots to gravity is altered. The distribution of extensin and peroxidase activity in pea epicotyls upon ethylene treatment was studied by tissue printing on nitrocellulose paper. It was found that the localization of extensin and peroxidase activity changes after 72 and 96 hours of ethylene treatment. In untreated plants, peroxidase activity is detected only in the vascular bundles. Nonetheless, after 72 and 96 hours of ethylene treatment peroxidase activity is hardly detected in the vascular system but present in the epidermal and cortical cells. Extensin increases in the epidermal and cortical cells upon ethylene treatment but it also appears in the vascular system when peroxidase activity is no longer detected.

Extensins constitute a class of cell wall hydroxyproline-rich glycoproteins present in a wide variety of plants (11). It has been recently shown that extensin is primarily localized in the sclerenchyma tissue of soybean seed coats, and in the vascular tissue of cotyledons (4). Plant peroxidases play a major role in the biosynthesis of secondary cell wall and in wound healing, and might be involved in auxin catabolism and defense against pathogen attack (5–7, 10). Plants contain several peroxidase isoforms whose pattern of expression is tissue specific, developmentally regulated and controlled by environmental stimuli (9, 10). In tobacco there are 12 isoforms classified in three subgroups; the anionic, the moderately anionic, and the cationic (10). The cationic peroxidase isoforms have been localized in the central vacuole (12), and both the moderately anionic and anionic isoforms have been found in the cell wall compartment (10).

Ridge and Osborne (14) reported that in etiolated pea seedlings ethylene increases both peroxidase activity and hydroxyproline levels in the cell walls of the immature region including the apical hook. It was suggested that these changes in ionically bound peroxidase and hydroxyproline are involved in the ethylene regulation of cell growth. Ethylene causes the triple response in dark grown pea seedlings (13) in which elongation growth is inhibited, radial growth is promoted and orientation of shoots with respect to gravity is altered. Cross sections through the subapical zone of pea seedlings treated with ethylene for 48, 72, and 96 h showed a progressive increase in size of the epidermal and cortical cells as well as a thickening of their cell walls (1). To test whether the increased hydroxyproline observed in pea epicotyls was due to the accumulation of extensin, we have used an antibody to extensin in order to visualize its localization by means of tissue printing on nitrocellulose paper (4). At the same time, peroxidase activity in pea epicotyls with or without ethylene treatment was also examined by a tissue printing method.

Here, we report that in etiolated pea epicotyls the activity of peroxidase and the extensin distribution coincide in the epidermal and cortical cells in the subapical region after 72 and 96 h of ethylene treatment. In control plants, peroxidase activity is detected only in the vascular bundles; however, after 72 and 96 h of ethylene treatment, peroxidase activity is barely detected in the vascular system, including cortical fibers. The disappearance of peroxidase activity seems to correlate with the inhibition of lignification of the vascular system already reported (2).

**MATERIALS AND METHODS**

Seeds of *Pisum sativum* cv Alaska were surface-sterilized in 10% commercial bleach (5% NaOCl) for 10 min, and soaked for 7 h in H2O. The seeds were sown in vermiculite and grown in darkness for 7 d at 25°C. Two pots of seedlings were then placed in each bell jar and treated with ethylene (Fisher Scientific) by injecting the gas through a vaccine cap to a final concentration of 50 ppm. Control seedlings were placed in bell jars with no ethylene added. Jars were maintained in the dark for 4 d. All manipulations were carried out under dim green light.

Plants were harvested every 24 h and 15 mm of the subapical region was collected for: (a) free-hand sections for phenolglucosinol staining in 20% HCl (8); (b) tissue-prints on nitrocellulose paper (4) either for peroxidase activity using 2 mm o-phenylenediamine (BRL, Bethesda, MD) and 0.012% H2O2 in 60 mm citrate buffer pH 4.5 as a substrate, or for extensin distribution using a specific antibody to soybean seed coat extensin as described by Cassab and Varner (4). Two replicate plants were used for each treatment. The detection of alkaline phosphatase-conjugated second antibody on tissue prints was performed as reported by Blake et al. (3). Primary antibody against soybean seed coat extensin was diluted 1:15,000 and anti-rabbit IgG (Fc) alkaline phosphatase conjugate was diluted 1:20,000; and 5-bromo-4-chloroindoxyl phosphate (BCIP) and nitroblue tetrazolium (NBT; Promega Biotec, Madison, WI) were used to detect the precipitated indoxyl group. The localization of peroxidase by tissue printing on nitrocellulose paper has been recently reported (15), but the activity was detected with 0.2% guaiacol and 0.2% H2O2, or diamino-benzidine and H2O2.

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EXTENSIN AND PEROXIDASE DISTRIBUTION IN PEA EPicotyls

RESULTS AND DISCUSSION

Figure 1 (a, c, g, k, o) shows that peroxidase activity in dark grown epicotyls without added ethylene is mostly found in the vascular bundles. On the other hand, extensin is detected in the epidermal and cortical cell walls (b, f, j, n, r). However, when the pea seedlings are treated with ethylene for 72 and 96 h, peroxidase activity is lower in the vascular bundles (l, p) and peroxidase activity can be detected in the epidermis and cortex. After 48 h of ethylene treatment, extensin increases greatly in the epidermis and some extensin is detected in the vascular bundles. Extensin is absent from these tissues in the untreated seedlings. After 72 and 96 h of ethylene treatment, distribution of peroxidase activity and extensin coincide in the epidermal and cortical cells (l, m, p, q). The extensin visualized by the tissue printing technique will be transferred to the paper only from those walls which contain extensin not yet insolubilized. Therefore, any one print cannot reflect the total accumulated insolubilized extensin. Nonetheless, when series of prints are made at different developmental stages it seems likely that a correct sense of extensin deposition can be derived from an integration of the information obtained from the ordered series of prints.

Phloroglucinol stain of the subapical region of 7-d-old dark grown pea seedlings treated with ethylene for 24, 48, 72, and 96 h was in agreement with the results reported by Apelbaum et al. (2). Lignification of the vascular bundles was inhibited in ethyl-

![Peroxidase and Extensin](image)

**Fig. 1.** Effect of ethylene on distribution of extensin and peroxidase in the subapical region of pea epicotyls. Cross-sections of pea epicotyls were printed onto nitrocellulose paper. The prints were assayed for peroxidase activity using o-phenylenediamine as a substrate (a, c, d, g, h, k, l, o, p); or reacted with extensin antibody diluted 1:15,000, and detected with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (b, e, f, i, j, m, n, q, r). Seven-d-old pea seedlings (a, b); ethylene-treated pea seedlings after 24 h (d, e), 48 h (h, i), 72 h (l, m), and 96 h (p, q); untreated pea seedlings 8-d-old (c, f), 9-d-old (g, j), 10-d-old (k, n), and 11-d-old (o, r) as controls. vb, vascular bundles; e, epidermis. All photographs at the same magnification.
ene treated plants, but lignification of these elements in control plants occurred rapidly (Fig. 2). The distribution of peroxidase activity changed from the vascular bundles in untreated plants to the epidermis and cortex in ethylene treated plants (Fig. 1). Thus, it seems that when lignification is prevented in the vascular tissue by ethylene, the peroxidase activity in these elements is no longer present.

The triple response in pea is a set of morphological and physiological effects on the seedling, and one effect is on cell shape. There is a progressive increase in size after ethylene treatment of the epidermal and cortical cells as well as a thickening of their cell walls in the subapical zone of pea seedlings (1). Extensin and peroxidase activity coincide and accumulate in the epidermal and cortical cells of the pea subapical region after 72 and 96 h of ethylene treatment. It is not known if these changes in ionically bound peroxidase and extensin are involved in the ethylene effect on cell allometry, or they are just a consequence of ethylene addition. On the other hand, it is not known whether the peroxidase activity detected by tissue printing is anionic, moderately anionic, or cationic. It has been reported, however, that the ionically bound peroxidase removed from cell walls of pea epicotyls contains two anionic isozymes (14). Interestingly, the anionic peroxidases are involved in the formation of lignin (7).

Extensin was found to be distributed in the vascular bundles of the subapical region of pea epicotyls upon ethylene treatment when peroxidase activity and lignification were inhibited. This extensin may play a role in maintaining the structure of vascular elements once lignification is stopped. Tissue printing on nitrocellulose paper is a simple technique that can be used for studying several physiological and developmental problems. In soybean hypocotyls, tissue printing was used to show that the pattern of extensin distribution changes according to the developmental stage of the tissue, and tissue type (GI Cassab, JE Varner, unpublished results). As we show here, the effect of ethylene in dark grown pea seedlings was readily analyzed at the cellular level by tissue printing.

**LITERATURE CITED**

4. **Cassab GI, JE Varner** 1987 Immunolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. J Cell Biol 105: 2581-2588

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**FIG. 2.** Transverse section of the subapical region of control dark grown 7-d-old pea seedlings, or treated with 50 ppm ethylene after 24, 48, 72, and 96 h. Lignified elements positive for phloroglucinol stain are shown. Redrawn from Apelbaum *et al.* (2).