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Histochemical Demonstration and Localization of H_2O_2 in Organs of Higher Plants by Tissue Printing on Nitrocellulose Paper[1]

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A sensitive tissue-print assay for the detection and histological localization of H_2O_2 in freshly cut organ sections was developed by impregnating nitrocellulose paper with a mixture of KI and soluble starch. H_2O_2 transferred from the cut surface of the section to the dried paper forms I_2, which can be visualized by the intensely colored I_2-starch complex. The detection limit of the assay is in the range of 0.1 to 0.2 mmol L^{-1} H_2O_2. Due to the rapid immobilization of H_2O_2 in the paper, very clear prints of the tissue distribution of H_2O_2 can be obtained with a spatial resolution on the level of single cells. The application of this rapid and simple assay is explored in five experimental examples demonstrating that the in vivo level of H_2O_2 varies strikingly in different tissues and can be regulated by developmental factors such as hormones, light, and wounding. The results show that: (a) In the hypocotyl of soybean (*Glycine max* L.) seedlings the apoplastic H_2O_2 level increases strongly from top to base, accompanied by characteristic changes in its histological distribution. (b) In the epicotyl of pea (*Pisum sativum* L.) seedlings the induction of lateral expansion by ethylene is correlated with a depletion of H_2O_2 in the cell walls of the expanding tissues. (c) In the hypocotyl of bean (*Phaseolus vulgaris* L.) seedlings H_2O_2 is primarily localized in a ring of parenchymatic tissue between xylem and cortex next to lignifying cells but not in the lignifying cells themselves. (d) In the hypocotyl of sunflower (*Helianthus annuus* L.) and cucumber (*Cucumis sativus* L.) seedlings the light-mediated inhibition of elongation growth is correlated with a strong increase in H_2O_2 in the epidermis and in the vascular bundles. (e) Potato (*Solanum tuberosum* L.) tubers show high levels of H_2O_2 only in the outer cell layers but are able to accumulate H_2O_2 in the inner tissue upon wounding.

H_2O_2 is a constituent of oxidative plant metabolism. It is a product of peroxisomal and chloroplastic oxidative reactions (Elstner, 1987, 1991) and it is probably a substrate for apoplastic cross-linking of phenolic compounds by peroxidase in the formation of cell-wall polymers such as lignin, suberin, or insoluble Hyp-rich glycoproteins (Fry, 1986; Lewis and Yamamoto, 1990; Olson and Varner, 1993; O'Malley et al., 1993). The induced synthesis and secretion of H_2O_2 ("oxidative burst") upon pathogen attack or application of related elicitors is regarded as an early defense reaction against microbial infection (Apostol et al., 1989; Peng and Kuc, 1992). Despite these essential physiological functions there is only sporadic information on the formation of H_2O_2 by living plant cells (e.g. Goldberg et al., 1987; Angelini and Federico, 1989; Apostol et al., 1989; Schwacke and Hager, 1992; Svalheim and Robertsen, 1993), mainly because of difficulties in the reliable determination of H_2O_2 levels in plant tissues. Due to its extremely positive redox potential, H_2O_2 is an unstable molecule that reacts with many reduced compounds liberated during homogenization and extraction of plant materials. Moreover, the ubiquitous presence of catalase, which decomposes H_2O_2 to H_2O and O_2, complicates the determination of H_2O_2 in tissue extracts. There are several useful spectrophotometric or luminescence-based assay procedures for the chemical determination of H_2O_2 in stabilized solutions (Warm and Laties, 1982; Frew et al., 1983). A procedure based on the oxidation of KI to I_2 by H_2O_2, visualized by the formation of the blue-black I_2-starch complex (Smith, 1970), has recently been used for a histochemical assay of H_2O_2 in freshly cut plant tissue sections (Olson and Varner, 1993; Varner, 1993). Using this reaction I have developed a sensitive tissue-printing assay for the rapid detection and localization of H_2O_2 in higher plant organs. The present communication describes the application of this assay to several problems related to the physiological function of H_2O_2 in plant growth and development.

MATERIALS AND METHODS

Seedlings of soybean (*Glycine max* L. cv Kalmit), sunflower (*Helianthus annuus* L.), garden bean (*Phaseolus vulgaris* L. cv Saxa), pea (*Pisum sativum* L. cv Alaska), and cucumber (*Cucumis sativus* L. cv Bidretta) were grown in closed plastic boxes on wet vermiculite at 25°C in darkness or continuous white fluorescent light (9 W m^{-2}). Other plants were purchased at local markets.

H_2O_2 printing paper was prepared by soaking strips of nitrocellulose membrane (BA 83, 0.2 μm; Schleicher & Schuell, Dassel, Germany) with a solution containing 100 g L^{-1} soluble starch (Merck, Darmstadt, Germany) and 0.5 mol L^{-1} KI in distilled water. The starch was solubilized by boiling before KI was added to the cooled solution. The strips were dried in a stream of warm air (30°C) and stored in the dark before use. To minimize background staining by autoxidation of KI, the reagent solution was used no longer than 5 h and the printing paper no longer than 2 h after preparation.

Tissue printing was performed at room temperature (18–20°C) essentially as described by Cassab and Varner (1989). Small pieces of H_2O_2 printing paper were placed on a pad of

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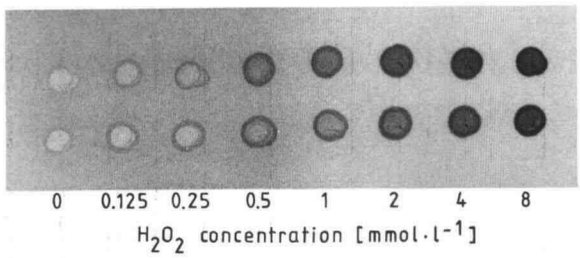

Figure 1. Calibration of the H_2O_2 printing assay. Drops of 2.5 μL of H_2O_2 standard solutions (in distilled water) were applied to KI-starch-impregnated nitrocellulose paper. The photograph was taken after 5 min of air drying. H_2O_2 concentrations of $\geq$0.25 mmol L^{-1} produce a staining reaction that can be clearly discriminated from the background response of the water control.

five layers of chromatographic paper. Hand-cut tissue cross-sections (0.5–1.0 mm thick) were prepared with a new razor blade and immediately transferred to the printing paper; displacement of the sections after contacting the paper was carefully avoided. Usually, three to six sequential sections were placed on a piece of printing paper simultaneously. It took about 15 s to prepare a sequence of six sections and to transfer them to the printing paper. The group of sections was covered with a pad of soft cleaning tissue (16 layers) and gently pressed with the forefinger for 60 s (the effective load on the sections was about 200–300 g). Then the sections were carefully removed with a forceps and the prints examined under a dissection microscope at 16-fold magnification after 5 to 10 min of drying. H_2O_2 staining develops within 0 to 5 min depending on the amount of H_2O_2 transferred. Because the prints intensify with time, it is necessary to examine them after a defined period, e.g. after 5 min. Prints can be stored with little background staining for at least 2 h in darkness. Photographs were taken with a camera mounted on a microscope using Agfa Pan 25 or Kodak Ektachrome 64 T film.

Experiments were repeated at least three times on different occasions with very similar results. The pictures shown in the figures are typical examples from these experiments.

RESULTS AND DISCUSSION

Tissue prints on nitrocellulose paper that can be developed with specific reagents for visualizing certain RNAs or proteins (enzymes) provide a simple, ingenious tool for the detection and histological localization of these macromolecules (McClure and Guilfoyle, 1989; Bailey et al., 1991; Lagrimini, 1991; Taylor et al., 1993). This technique, which was introduced simultaneously by Cassab and Varner (1987) and Spruce et al. (1987), has an inherent potential for a large variety of additional applications (Reid et al., 1992). For example, the basic principle of tissue printing can be utilized for the demonstration of H_2O_2 by impregnating nitrocellulose paper with a mixture of KI and starch, a reagent for the histochemical assay of H_2O_2 (Olson and Varner, 1993; Varner, 1993). Application of small drops of H_2O_2 solution to the dried paper results in the appearance of blue-black dots (or halos), which turn brown upon drying of the paper. A test with a series of freshly prepared H_2O_2 solutions in distilled water (Fig. 1) shows that the detection limit of this assay is in the range of 0.1 to 0.2 mmol L^{-1} H_2O_2 (0.25–0.5 nmol/dot). Similar tests performed by mixing equal volumes of H_2O_2 solution and reagent solution demonstrated visible coloration at $\geq$2 mmol L^{-1} H_2O_2. Thus, due to the absence of dilution during the assay and to the optical amplification by the diffusely reflecting membrane support, the sensitivity of the printing assay for H_2O_2 is increased by a factor of about 10. Moreover, thanks to the rapid immobilization of H_2O_2 on the surface of the impregnated nitrocellulose paper, this material provides an excellent substrate for the histochemical localization of H_2O_2 in tissue slices.

As an example of the high fidelity and resolution of this method, Figure 2 shows typical prints prepared from sections through the basal region of a soybean seedling root with an emergent side root. The discrete appearance of the cell-wall pattern indicates that H_2O_2 is localized primarily in the apoplastic space, from where it is sucked up by the paper and captured there by the immobilized reagent. Optimal sensitivity and spatial resolution were obtained at high concentrations of KI and starch; however, more than 0.5 mol L^{-1} KI and 100 g L^{-1} starch proved to be supraoptimal, since these

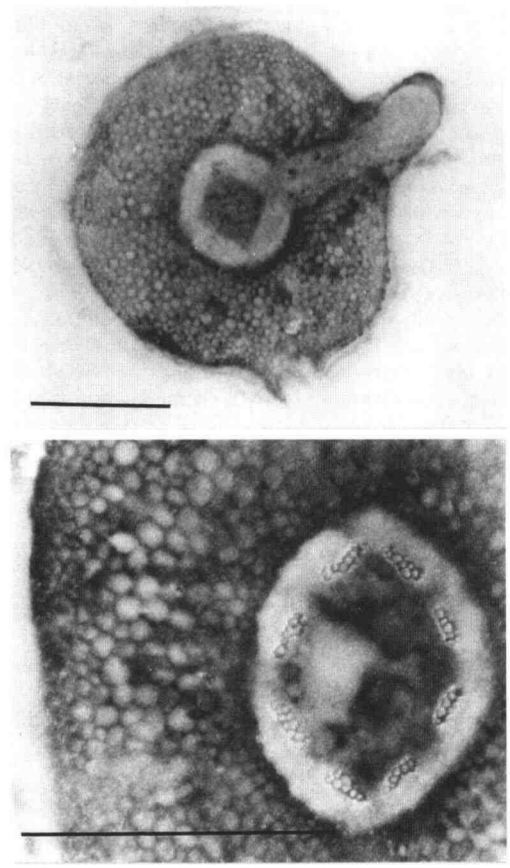

Figure 2. Demonstration of the spatial resolution of the H_2O_2 printing assay. Handcut sections from the root base of a 5-d-old light-grown soybean seedling were blotted on KI-starch-impregnated nitrocellulose paper. Photographs were taken after 5 min of air drying. Note the physical imprints of xylem strands in the lower figure. Bars = 1 mm.

amounts caused the reagent solution to become too viscous and background staining to increase markedly.

The specificity of the test reaction for H_2O_2 was checked by pretreating the sections with catalase immediately before transferring them to the nitrocellulose paper. Figure 3 shows a typical result obtained for a soybean hypocotyl section. Although the pretreatment per se decreased the sharpness of the prints considerably (and obliterated the prints at pretreatment times longer than 1 min), the effect of catalase in decreasing the amount of H_2O_2 transferred to the paper was clearly detectable. The sensitivity to catalase eliminates the interference by KI-oxidizing substances other than H_2O_2 (false-positive results) in the assay. However, as pointed out by Olson and Varner (1993), false-negative results due to the interference by endogenous reductants such as ascorbate released from injured cells cannot be strictly ruled out. This problem may apply particularly to photosynthetic tissues containing high levels of ascorbate.

The H_2O_2 detected by the tissue-print method was not due to wounding. Fully developed prints were obtained after only 15 s of contact with the paper, i.e. 30 s after cutting the sections, and staining did not intensify when the time between cutting and printing was increased up to 5 min. Performing the printing on ice with precooled plant material (5°C) led to results similar to those obtained at room temperature conditions (about 20°C) except for a delay in the development of the prints. This indicates that the method demonstrates steady-state levels of H_2O_2 in the intact tissues.

The H_2O_2-printing assay was tested in this laboratory with a variety of plant tissues demonstrating large differences in H_2O_2 content. Whereas seedlings of soybean and pea showed rather high H_2O_2 contents, which were easily detected both with the direct histochemical assay of Varner (1993) and the H_2O_2-printing assay, seedlings of garden bean, sunflower, cucumber, radish, and maize displayed much lower levels of H_2O_2 that could be demonstrated only with the latter method. Significant amounts of H_2O_2 were also found in pear fruits (in a pattern representing the pattern of stone cells) and potato tubers (tissue close to the periderm), whereas the fruits of apple, tomato, radish, zucchini, cucumber, and kiwi were negative. As a rule, high levels of H_2O_2 were detected in adult (no longer growing) tissues and much lower levels were detected in actively growing or meristematic tissues of stems and roots. However, characteristic species-specific patterns of H_2O_2 distribution were found in the organs of different plants. In general, H_2O_2 was preferentially associated with the epidermis of growing organ regions (with the notable exception of the pea epicotyl; see below). In adult organ regions parenchymatic tissues (cortex, pith parenchyma) also demonstrated comparable H_2O_2 levels. Cotyledons of soybean, pea, and bean seedlings demonstrated moderate levels of H_2O_2, which was homogenously distributed in the organ. In the following sections, five examples are described in which the H_2O_2-printing technique was applied to problems concerning the putative function of H_2O_2 in higher plants.

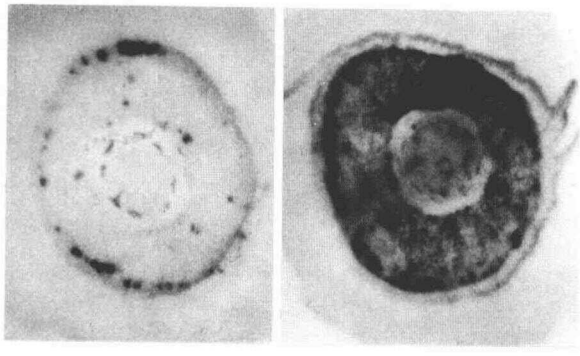

Figure 3. Effect of catalase on the H_2O_2 printing assay. Left, The surface of a freshly cut section from the hypocotyl base of a 5-d-old dark-grown soybean seedling was treated with 0.25 μL of catalase solution (1600 units μL^{-1} in 50 mmol L^{-1} K-phosphate buffer, pH 7.0) for 1 min and then blotted as in Figure 2. Right, Control print from a similar section treated for 1 min with 0.25 μL of buffer.

Example 1: Distribution of H_2O_2 along Hypocotyl and Root of Soybean Seedlings

The gradient of the distribution of H_2O_2 in the cross-section of the axis organs of a dark-grown soybean seedling is illustrated in Figure 4. There is a striking increase of the overall level of H_2O_2 from the hook region toward the lower parts of the hypocotyl and the root. In the elongation zone below the hook, only the epidermis produced distinct and strong staining, with much weaker staining in the cortex and almost no staining in the inner tissues. This pattern changes dramatically toward the lower part of the hypocotyl, where the cortex and the pith of the central cylinder show strong staining. The ring of small-celled parenchyma between cortex and pith containing the xylem and phloem strands does not elicit a detectable H_2O_2 reaction on the print.

A somewhat different H_2O_2 pattern is displayed in the root, which shows very high H_2O_2 levels in the cell walls of the cortex and the xylem but no positive reaction in the phloem region of the central cylinder. Considering the small size of the molecule, it is amazing to see how strictly H_2O_2 is confined to the apoplast of particular tissues within the organ.

These results show that in the younger, growing parts of the soybean hypocotyl H_2O_2 is restricted mainly to the epidermis. In the adult parts of the organ the cortex and the pith also contain high levels of H_2O_2, whereas the vascular bundles and associated tissues appear to be devoid of H_2O_2. Although the presence of H_2O_2 in the symplast cannot be excluded by this technique, the major fraction of H_2O_2 detectable on the prints is clearly derived from the apoplastic space of the various tissues. The availability of H_2O_2 in the cell-wall fluid is a prerequisite for its proposed role in cross-linking phenolic groups of wall polymers that leads to cell-wall stiffening and resistance against pathogen attack. Moreover, the demonstration of millimolar concentrations of H_2O_2 in the solution surrounding the symplast implies that the plasma membrane is capable of tolerating rather high levels of H_2O_2 at its outer surface without serious damage.

Example 2: Changes in the H_2O_2 Pattern during Ethylene-Induced Radial Expansion of the Pea Epicotyl

Ethylene, applied in the form of the ethylene-releasing growth-regulator substance ethephon, leads to characteristic

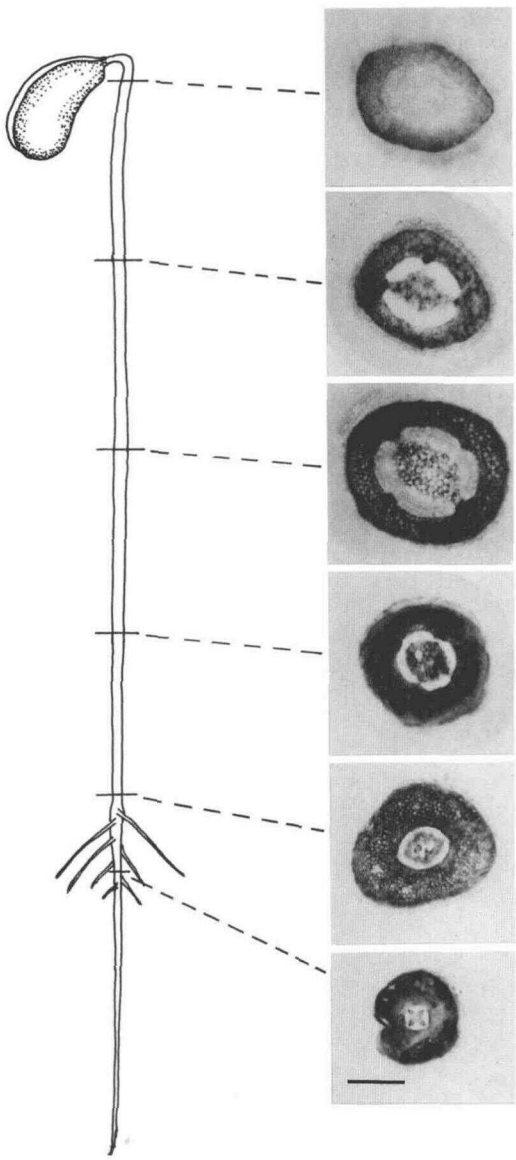

Figure 4. Distribution of H_2O_2 in the axis tissues of a 5-d-old dark-grown soybean seedling. Sections were prepared from the hypocotyl (5, 25, 50, 75, and 105 mm below the cotyledonary node) and from the root (10 mm below the root base) and blotted as in Figure 2. Bar = 1 mm.

changes in the morphology of the pea seedling, most conspicuously to an inhibition of elongation and an increase in radial expansion (swelling) of the growing zone below the epicotyl hook (Eisinger, 1983). Cassab et al. (1988) have shown that this reorientation of the growth direction is accompanied by marked changes in the tissue distribution of peroxidase activity and soluble extensin. Figure 5 shows that ethylene also has significant effects on the distribution of H_2O_2 in this system. Whereas untreated control seedlings, as well as the nonswollen lower epicotyl region of treated seedlings, demonstrated a strong and homogenous staining of the cell walls in the cortex, there was a striking radial gradient of decreased staining from the center to the periphery of the epicotyl region that is laterally expanded in the presence of ethylene. Closer inspection of Figure 5, a and b, reveals that staining is restricted to the inner, narrow cells of the cortex, which obviously do not participate in the swelling response, whereas the outer, expanded cells demonstrate little or no H_2O_2 staining. Therefore, ethylene-induced cell expansion is obviously correlated with a drastically decreased level of H_2O_2 in the expanding cell walls.

In contrast to the situation in most other plants investigated, the epidermal region of the pea epicotyl did not also stain for H_2O_2 in seedlings not treated with ethylene (Fig. 5c). Interestingly, a comparison with peroxidase tissue prints reported by Cassab et al. (1988) indicates that H_2O_2 and peroxidase activity demonstrate complementary patterns in the cross-section of the pea epicotyl, peroxidase being most strongly expressed in the vascular bundles, the sclerenchyma fibers, and the epidermis, i.e. in those tissues with little or no H_2O_2 content, both in the presence and absence of ethylene. Moreover, there appears to be a mutual exclusion between the presence of H_2O_2 and lignification in this material.

Figure 5 shows a comparison between the traditional histochemical staining directly on the sections (left) and tissue prints produced from similar sections (right). Although both methods provide basically the same information, the spatial resolution of the former is superior to that of the latter method. This has been a general experience with plant materials containing high levels of H_2O_2, which tend to produce strongly stained but more or less blurred prints.

Example 3: Localization of H_2O_2 and Lignin in the Hypocotyl of Bean Seedlings

H_2O_2 is regarded as an essential reaction partner in the oxidative formation of lignin in certain cell walls; therefore, one would expect that lignifying cells produce relatively high amounts of this substrate. This question was investigated in the hypocotyl of bean seedlings, which form a cylindrical sheath of lignified tissue containing the xylem elements around the pith (Fig. 6, left). Because of the rigid structure of the xylem strands, tissue printing of the cross-section produces a pronounced physical imprint of the xylem cells on the nitrocellulose paper, which can be easily visualized by oblique illumination (Fig. 6, middle). Figure 6, right, shows the same print after full development of the H_2O_2-staining reaction. It is evident that H_2O_2 is not associated with the lignifying xylem proper but with a ring of small-celled parenchymatic tissue between the xylem and cortex containing sclerenchyma and phloem tissues. Thus, a precise separation of lignified and H_2O_2-producing tissues is possible by exploiting the structural profile produced by tissues of differing rigidity on the nitrocellulose paper (Cassab and Varner, 1989; Varner and Taylor, 1989).

Using the direct histochemical H_2O_2 assay, Olson and Varner (1993) have recently provided evidence for a co-localization of lignin and H_2O_2 in the phloem fibers and xylem of *Zinnia* stems. Our results with etiolated soybean hypocotyls (Fig. 4), pea epicotyls (Fig. 5), and bean hypocotyls (Fig. 6) indicate that this correlation may not be generalized. The possibility that the polymerization of lignin monomers

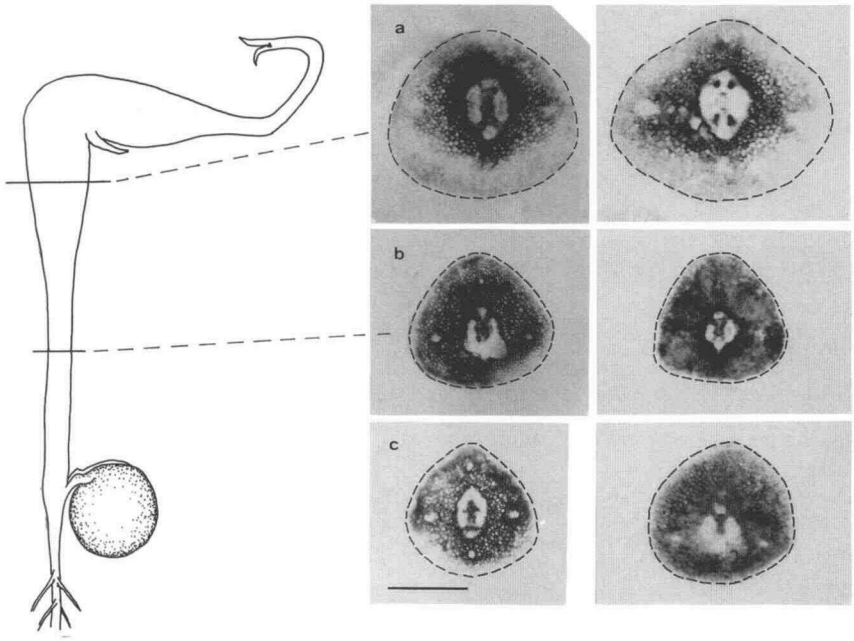

Figure 5. Distribution of H_2O_2 in the growing zone of the first internode of pea stems treated with ethylene for induction of radial expansion. Pea seedlings were transferred, after 3 d of germination in darkness, to a root medium containing water with 100 mg L^{-1} ethephon for inducing the ethylene-specific radical expansion growth in the upper part of the first internode. After a further 3 d of growth in darkness, sections were prepared from the indicated regions of the epicotyl (a and b) and either incubated in a drop of KI-starch reagent (left) or blotted as in Figure 2 (right). For comparison, sections from the same region of the first internode of nontreated control seedlings are also shown (c). Bar = 1 mm.

can be catalyzed in the absence of H_2O_2 by laccase was recently emphasized by O'Malley et al. (1993).

Example 4: Change of Epidermal H_2O_2 Levels during Light-Induced Growth Inhibition of Sunflower and Cucumber Hypocotyls

The light-mediated inhibition of hypocotyl elongation is a central feature of photomorphogenesis in dicotyledonous seedlings. The hypothesis has been proposed that this response is mediated by a stiffening of the cell wall in the elongation zone of the hypocotyl by peroxidase-catalyzed cross-linking of load-bearing wall polymers (Goldberg et al., 1987; Angelini et al., 1990; Zheng and Van Huystee, 1992). In this context it was of interest to check whether light has a promoting effect on the level of H_2O_2 in the elongation zone of the hypocotyl. Figure 7 shows that there are in fact significant differences in the intensity of H_2O_2 staining in tissue prints produced by hypocotyl sections from illuminated and dark-grown sunflower and cucumber seedlings. In both plants staining was strongest in the epidermis and in the vascular bundles, with less intense staining in the cortex. Illumination with white light produced a marked increase in staining in all tissues, especially in the epidermis. Similar results were obtained with soybean seedlings (data not shown). Interestingly, sunflower and cucumber produced a dotted staining pattern instead of a meshwork in the H_2O_2-positive regions of the prints, suggesting that H_2O_2 is transferred to the paper from the cell lumens rather than from the cell walls. Taken together, these results indicate that light-grown hypocotyls contain higher levels of H_2O_2 in their elongation zone than dark-grown hypocotyls. Moreover,

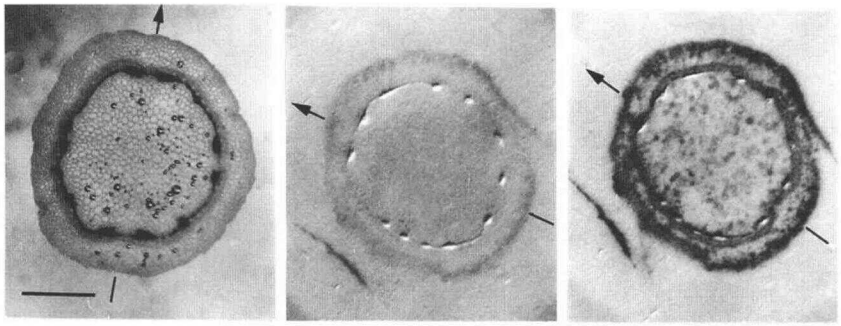

Figure 6. Distribution of lignin and H_2O_2 in the hypocotyl of a bean seedling. Sections were prepared from the growing region of the hypocotyl (20 mm below the cotyledonary node) of a bean seedling grown for 6 d in darkness. Sequential sections were either stained for lignin with phloroglucinol-HCl (left, dark regions) or blotted as in Figure 2. Middle, Blot photographed immediately after removal of the section showing the mechanical imprints of the xylem bundles. Right, The same blot photographed 20 min later showing the pattern of H_2O_2-mediated staining. The orientation of the sections is indicated by arrows. Bar = 1 mm.

H_2O_2 seems to be located primarily in the epidermis, the putatively growth-controlling tissue of the organ (Hohl and Schopfer, 1992). Whether this effect can be causally related to the light-mediated inhibition of growth remains uncertain. At any rate, these experiments do demonstrate that the H_2O_2 level of plant tissues can be regulated by environmental factors such as light and that tissue printing is an effective method to document this phenomenon.

Example 5: Induction of H_2O_2 Formation in Wounded Potato Tuber Tissue

Wound healing of injured storage parenchyma of the potato tuber is characterized by the formation of a new suberized periderm from a cork cambium (phellogen) regenerated at the surface of the wound that closely resembles the original periderm of the nonwounded tuber surface (Kahl, 1978). Figure 8 shows that the outer cell layers of tuber, including the periderm, contain high amounts of H_2O_2. Moreover, the air-exposed surface of a freshly cut tuber disc also acquires the ability to accumulate levels of H_2O_2 within 24 h after wounding that are detectable with the H_2O_2-printing method. This response to mechanical injury may be related to the biosynthesis of suberin in the newly formed periderm (Kolattukudy, 1981). In addition, H_2O_2 could serve as a chemical defense barrier against the invasion of fungal and bacterial pathogens. H_2O_2 printing may provide a useful tool for investigating these protective processes in more detail.

GENERAL CONCLUSIONS

These few examples of application of the H_2O_2-printing assay to various plant materials illustrate that H_2O_2 is generally produced by many plant tissues, although in highly variable amounts. This assay should be useful for quickly screening large numbers of samples in vivo for the presence and histological distribution of H_2O_2. The rapidity of the assay minimizes the possibility of unwanted side reactions, although a possibility for false-negative results remains. Moreover, because the results can be obtained within less than 1 min after preparation of tissue sections, wound-induced changes in the H_2O_2 level can be largely excluded. After standardization, the method can be used for semiquantitative estimation of H_2O_2 and comparative studies on changes in H_2O_2 levels mediated by developmental factors such as hormones, light, or wounding. H_2O_2 appears to be a developmentally tightly regulated metabolite in the plant, the study of which may provide a multitude of new and unexpected insights into growth and development.

Finally, it should be noted that this assay can be modified for the histochemical localization of other oxidizing agents. For example, as pointed out by Joe Varner (personal communication), nitrite will oxidize KI to I_2 at pH 4.0. This reaction can possibly be used for the localization of nitrite as well as nitrite reductase.

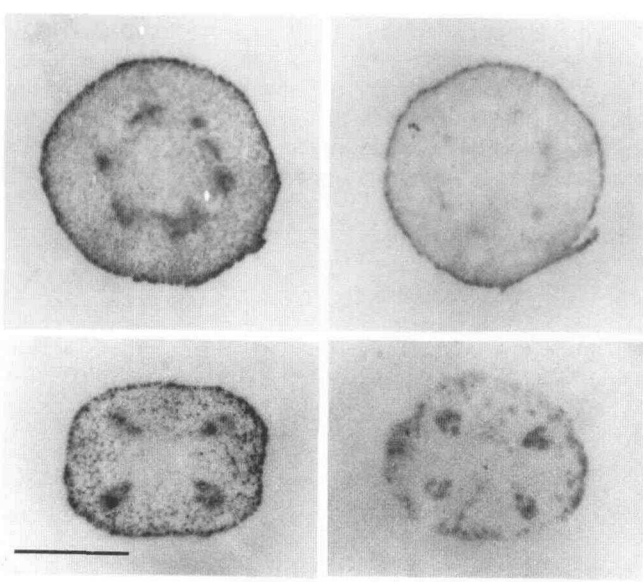

Figure 7. Distribution of H_2O_2 in the growing zone of the hypocotyl of sunflower (top) and cucumber (bottom) seedlings grown in the light (left) or in darkness (right). Seedlings were grown for 6 d in white light or darkness. Sections were prepared from the hypocotyl 10 mm below the cotyledonary node and blotted as in Figure 2. Bar = 1 mm.

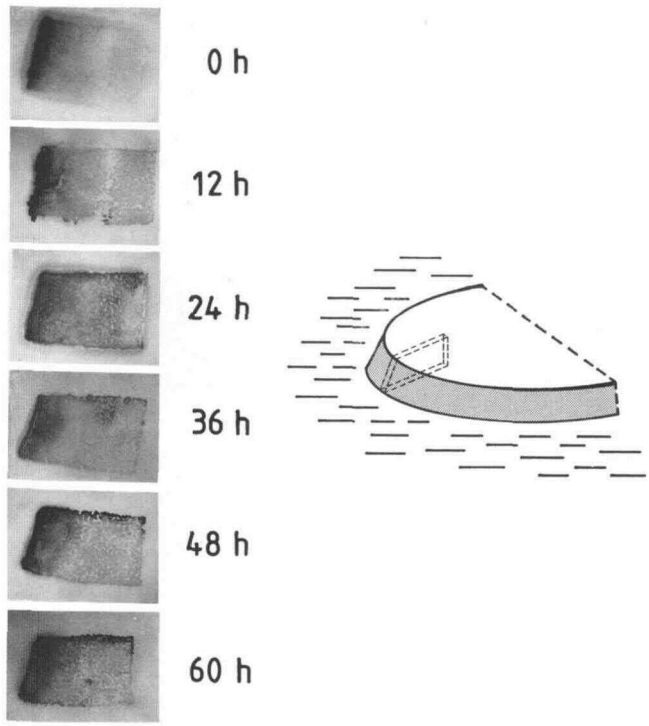

Figure 8. Wound-induced formation of H_2O_2 in potato tuber sections. A 5-mm disc cut from the middle of a potato tuber was stored with its lower surface on wet filter paper in a closed box at 25°C. Left, Various times after wounding, thin sections (0.5 mm) were cut at right angles to the wound surfaces from the periphery of the disc, including the periderm, and blotted as in Figure 2. Note that staining occurs after ≥24 h in a thin layer adjacent to the upper (air-exposed) wound surface but not at the lower surface (facing the filter paper). Also, the inner edges of the sections, which result from the cutting immediately before the assay, show no response.

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