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IMMUNOLOCALIZATION OF LIPOXYGENASE IN THE ANTHER OF *GAGEA LUTEA* (L.) KER.-GAW.

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Immunogold labelling revealed the presence of lipoxygenase (LOX) in different parts and types of anther cells of *Gagea lutea*. LOX was found in the cytoplasm and close to ER elements in epidermal and endothecial cells, and close to the cell walls of the latter. The positive immunoreaction to LOX was less intense in the middle layers and the loculus of the anther, where single immunogold particles were concentrated at the cell walls of these layers and in the protoplast masses, in vacuoles, close to mitochondria, inside plastids, and in the liquid of the anther cavity. LOX occurred in the cytoplasm and around ER elements of pollen grains as well as in the exine layer, particularly in contact regions between the outer and inner exine layers. The correlations between LOX localization in different anther cells and the functioning of particular anther parts are discussed.

Key words: *Gagea lutea,* Lipoxygenase, anther, antibodies, pollen, pollen coat.

INTRODUCTION

Lipoxygenase (LOX, EC 1.13.11.12) catalyzes oxygenation of the long chain of fatty acids containing *cis*, *cis*–1,4-pentadiene structure to hydroperoxides. The enzyme is widespread in both the plant and the animal kingdoms. It is involved in a number of important processes in plant cells, but its physiological function is still not fully understood. Lipoxygenase is known to play a role in the membrane degradation observed during senescence, wounding, and hypersensitive response to a pathogen attack (Hildebrand et al., 1988; Siedow, 1991). Increased LOX activity was observed in the defense response to infection of plants by pathogens (Koch et al., 1992) and under treatment of plant and cell cultures with elicitors (Peever and Higgins, 1989; Rickauer et al., 1990).

According to literature data, high activity of LOX is strictly related to early stages of plant growth (Holtman et al., 1996; Matsui et al., 1988; Schmitt and van Mechelen, 1997). Moreover, in *Arabidopsis thaliana* the LOX gene is differentially regulated in plant organs. It is expressed in Arabidopsis leaves, roots, inflorescences and young seedlings. In mature plants, LOX mRNA increased upon treatment with ABA and methyl jasmonate (Melan et al., 1993). Mechanical wounding increases local systemic expression of LOX in *A. thaliana*, and it is induced by methyl ester of jasmonate (León and Sánchez-Serrano, 1999). cDNA sequence analysis showed that LOX has a transit peptide sequence for chloroplast targeting (Bell and Mullet, 1993); this peptide is located in chloroplasts (Bell et al., 1995).

Recent decades of research have yielded much genetic evidence about the expression of LOX during germination and in the early growth stages of plants (Holtman et al., 1996; Melan et al., 1994). Increased lipoxygenase activity is suggested to be caused mostly by an increase of the amount of enzymatic protein (Matsui et al., 1992; Maccarrone et al., 2000). Lipoxygenase was isolated from lupin seedlings 48 h old; its activity changed during germination (Beneytout et al., 1988). In barley, lipoxygenase appears during the development of grain (Schmitt

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Abbreviations: LOX – lipoxygenase; LOX PAb – anti-lipoxygenase polyclonal antibody.

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and Van Mechelen, 1997). Plant organs and tissues have shown some differences in LOX activity. In *Brassica* sp., LOX activity is higher in flowers than in leaves and fruits (Galliard and Chan, 1980). Kato et al. (1992) reported the appearance of new LOX isozymes in soybean cotyledons after germination. Individual isoforms of LOX were found in different cellular compartments, the bulk of them in cytosol (Siedow, 1991), with some reports indicating the presence of the enzyme in chloroplasts (Bowsher et al., 1992), in microsomal membranes (Todd et al., 1990), bound with the plasmalemma (Droillard et al., 1993; Vianello et al., 1995) and in vacuoles in cultured embryo cotyledons (Wang et al., 1999). LOX is also used as a storage protein during vegetative growth (Fischer et al., 1999). The activity of some LOX metabolites in plants is known or has been suggested (Porta and Rocha-Sosa, 2002).

In view of the many functions of individual compounds of the LOX pathway, we decided to examine the localization of LOX on the cytological level in the anther of *Gagea lutea* by electron microscopy and immunogold labelling technique.

MATERIALS AND METHODS

PLANT MATERIAL

Plants of *Gagea lutea* (L.) Ker.-Gaw. (Liliaceae) growing in natural habitats in the vicinity of Gdańsk (northern Poland) were used in this study. Bulbs with buds of this early-spring plant species were collected in February and March.

IMMUNOCYTOCHEMISTRY

For immunogold labelling, small segments (2–3 mm) from anthers of *Gagea lutea* were fixed in 2% formaldehyde (freshly prepared from paraformaldehyde) and 1% glutaraldehyde dissolved in PBS (0.1 M phosphate buffer, pH 7.4) for 24 h at 4°C. The samples were rinsed several times in PBS and 0.5 M NH_4Cl in PBS, dehydrated in ethanol, embedded in LR White resin (Sigma), and polymerized at 60°C overnight. Thin sections were collected on nickel grids, treated with aqueous 0.56 M sodium periodate for 30 min, thoroughly washed with distilled water, and treated with 0.1 M HCl for 10 min followed by a 5 min water wash. Sections were incubated first in 1% BSA in PBS for 30 min at room temperature, then with pre-immune rabbit serum (Agrisera, Sweden) diluted 1/1000 in PBS-BSA for 1 h at room temperature. After triple washing with PBS-BSA (10 min each wash) the sections were incubated with PBS-BSA containing rabbit anti-LOX antiserum diluted 1/1000 for 1 h and repeatedly washed with PBS-BSA. Goat anti-rabbit immunoglobulins conjugated to 10 nm gold particles (GAR-gold) (Sigma) were diluted 1/50 in PBS-BSA and then applied for 40 min at room temperature. Next, the sections were washed several times with PBS and redistilled water. As an additional control, samples were incubated with pre-serum and GAR-gold or with GAR-gold only, omitting the primary antiserum. The sections were stained with 2% uranyl acetate for 5 min and Reynolds reagent (lead nitrate and sodium citrate) for 1 min. All sections were examined with a LEO 912 AB electron microscope.

RESULTS

IMMUNOGOLD LOX LOCALIZATION

Immunogold labelling revealed the subcellular localization of epitopes binding LOX PAb in different parts and types of anther cells (Figs. 1–14). At the investigated stage of anther development (pollen grains in the anther loculus), several concentric cell layers form the outer anther wall, consisting of epidermis, endothecium and middle layers.

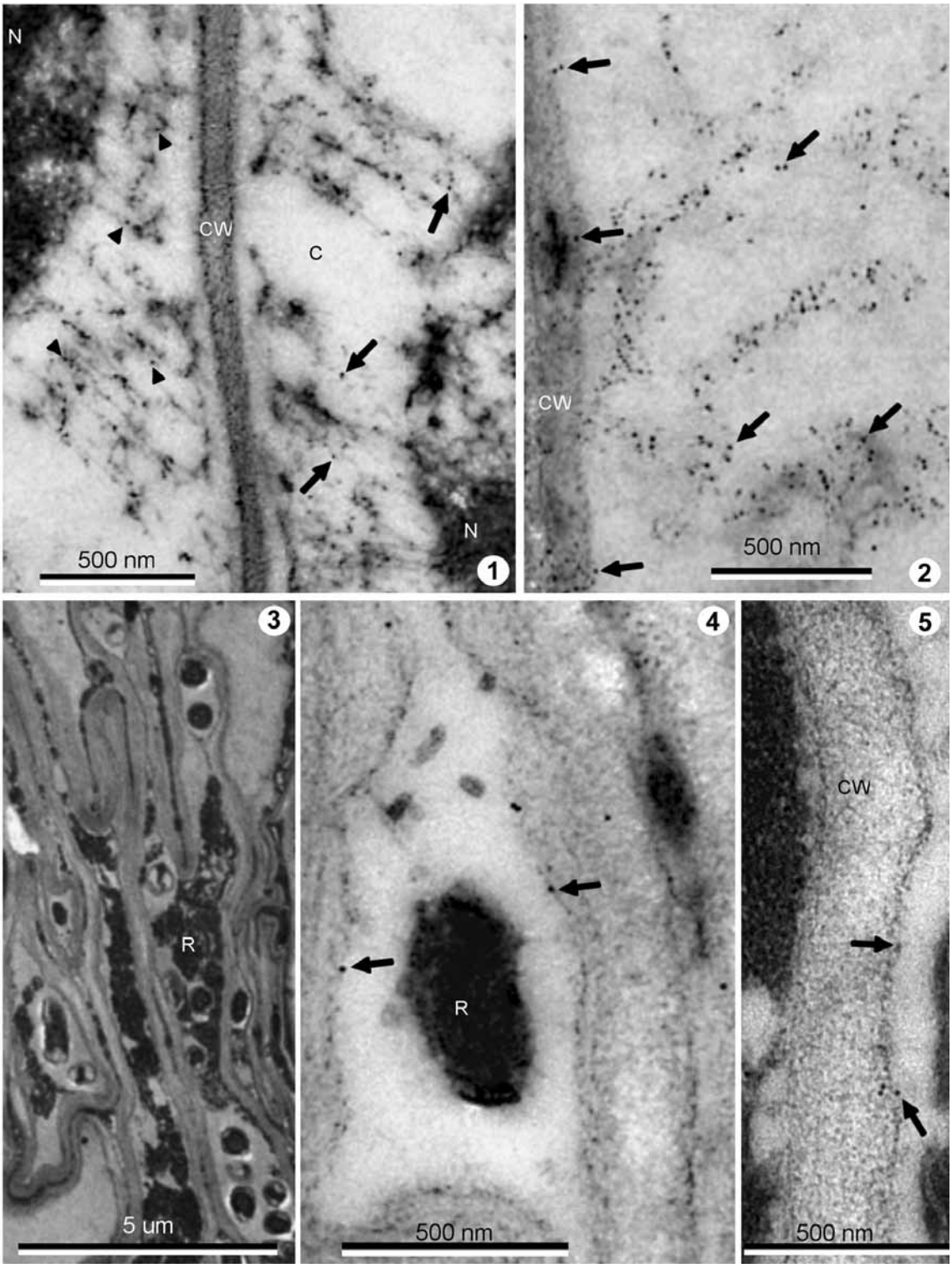
The epidermal cells are slightly stretched and flattened. In these cells, a positive immunoreaction to LOX was observed in the cytoplasm and close to ER elements (Fig. 1). No immunogold particles were observed in the area of the nucleus, nor in the numerous vacuoles occurring in the epidermal cells.

Immunolabelling to LOX in the endothecial cells was more intense than in the epidermal cells (Fig. 2). Immunogold particles were observed in the cytoplasm of cells of this subepidermal layer. Particles in different concentrations formed large spread-out groups gathered mainly in the peripheral cytoplasm around long ER elements. Smaller groups close to the cell walls were distributed in strands in denser cytoplasm adjacent to the thick radial cell walls.

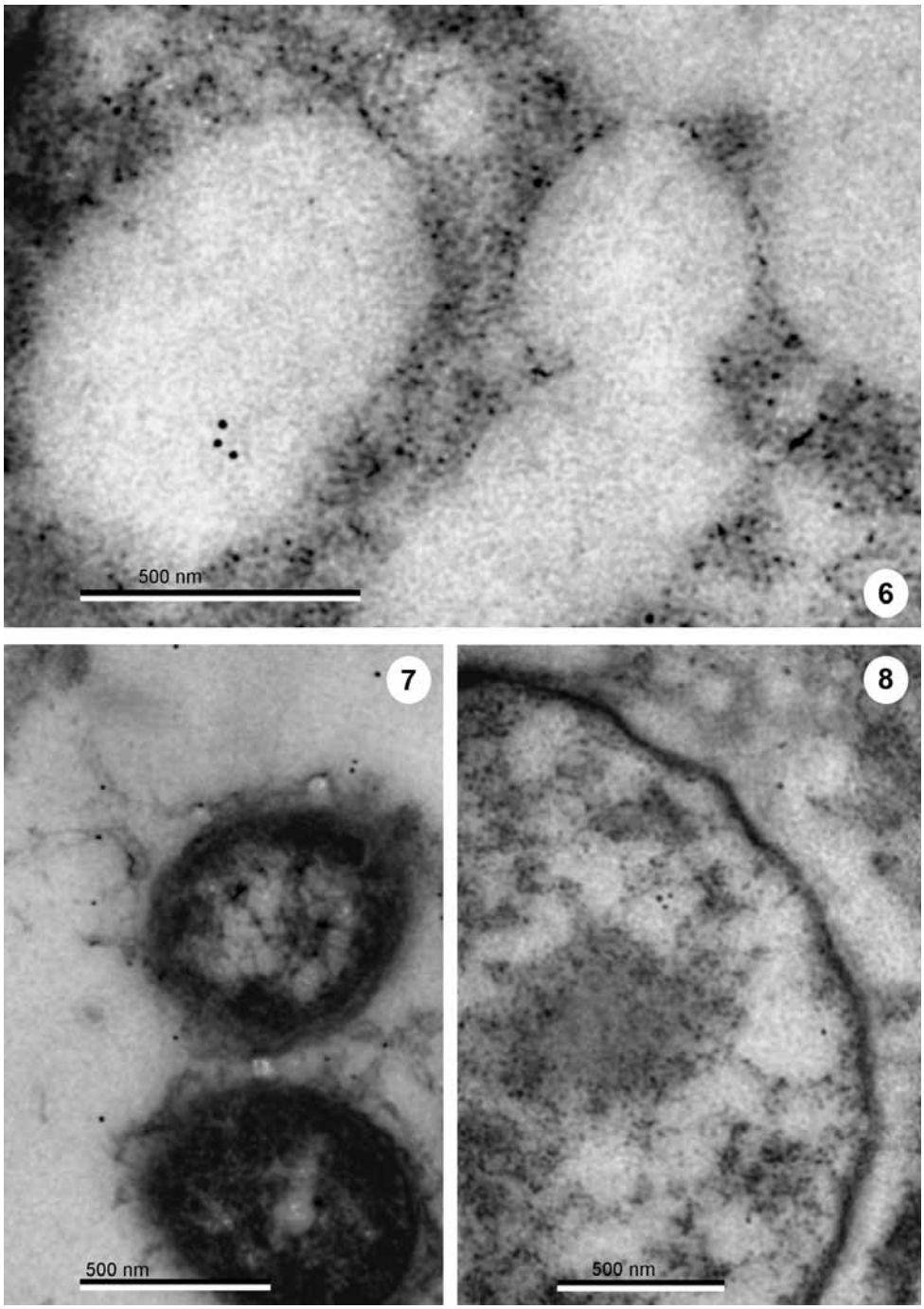
The flattened and crushed cells of the middle layers contained remnants of disintegrated cytoplasm with starch grains (Fig. 3). The positive immunoreaction to LOX was less intense than in the cells of both external layers. Single immunogold particles were concentrated at the edges of cell walls (Figs. 4, 5).

At the investigated stage of development, the innermost layer of the anther wall and tapetum were totally disintegrated. The content of the tapetal cells was distributed in the loculus and surrounded the developing pollen grains. Protoplast masses contained clearly visible mitochondria, plastids, lipid bodies and vacuoles. Single immunogold particles indicating sites of LOX PAb-binding epitopes were found in protoplast masses, in small vacuoles (Fig. 6), close to mitochondria, in the liquid of the anther cavity (Fig. 7), and inside plastids (Fig. 8).

In the pollen grain, positive immunoreaction to LOX was observed in the cytoplasm and around

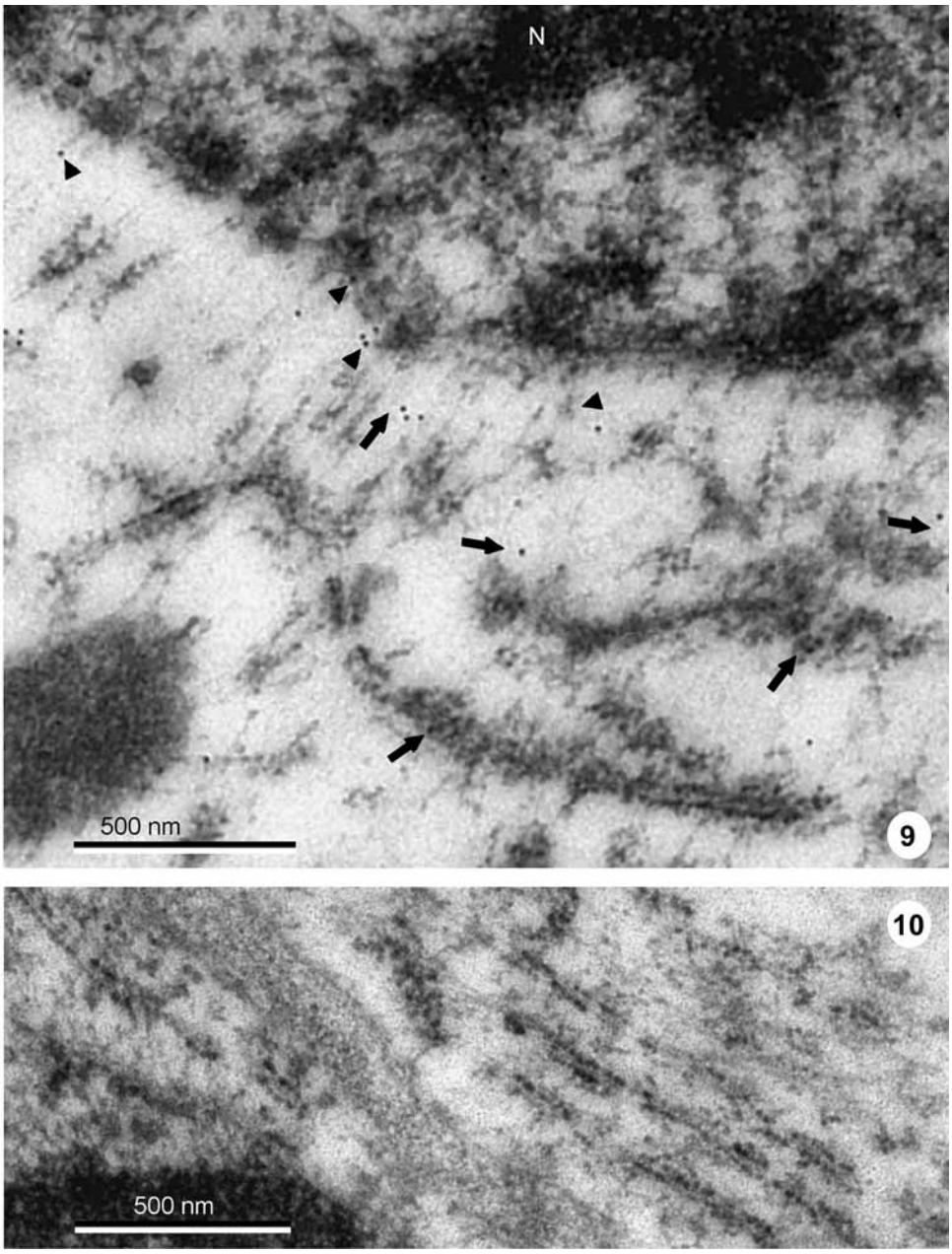


Figs. 1–5. Immunolabelling of lipoxygenase in anther wall cells of *Gagea lutea*. **Fig. 1.** Immunogold LOX PAb localization in epidermal cells. Immunogold particles in the cytoplasm (arrows) and close to ER elements (arrowheads). C – cytoplasm; N – nucleus; CW – cell wall. **Fig. 2.** Portion of endothecial cell. Immunogold particles in the peripheral cytoplasm, some of them grouped close to cell walls (arrows). CW – cell wall. **Fig. 3.** Middle layers of anther wall, with flattened and crushed cells filled with remnants of cell content. **Figs. 4, 5.** Cell walls of degenerated cells of anther wall, with single gold particles and clusters of them around the internal surfaces (arrows). R – remnants of cell content; CW – cell wall.



Figs. 6–8. Immunogold particles in protoplast masses of anther loculus. **Fig. 6.** In the protoplast and in small vacuoles. **Fig. 7.** Close to mitochondria and in the liquid of the anther cavity. **Fig. 8**. Inside plastid.

short and longer ER elements (Fig. 9). The distribution of immunogold particles around ER elements was intense and rather uniform in the cytoplasm. Labelling was not observed in the area of the nucleus, although immunogold particles were found close to the nuclear envelope. Groups of immunogold particles were visible at the exine layer wrapping the pollen grain, particularly at the exine surface in the space between neighboring pollen grains (Figs. 11, 12). Similar groups of immunogold particles were also observed at the interstices between the outer and inner exine (Fig. 13).

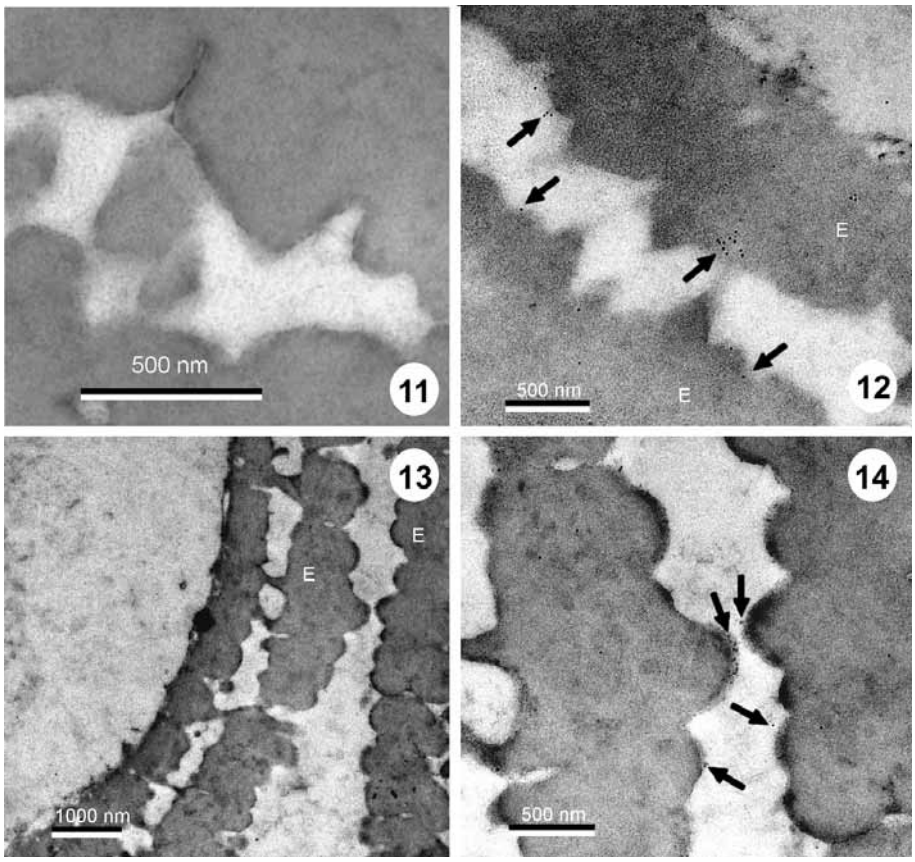


Figs. 9, 10. Portions of pollen grain with immunogold LOX PAb localization. **Fig. 9.** Immunogold particles in cytoplasm, around short and longer ER elements (arrows), and at the nuclear envelope (arrowheads). N – nucleus. **Fig. 10.** Control micrograph of cytoplasm with ER elements and a fragment of the nucleus.

To determine the degree of specificity of the immunogold reaction, a control reaction for the whole procedure was run. The control reaction omitted incubation with the primary antibody but not incubation with or without pre-serum. The reactions showed no gold particles (Figs. 10, 11). In the illustrations we show the control reactions only in pollen grain cytoplasm and in a portion of the exine layer. In all control reactions not shown here the results were negative.

DISCUSSION

In *Gagea lutea*, lipoxygenase was found in the cells of all investigated anther wall layers: epidermal, endothecial and middle. It was also localized in the anther cavity liquid and pollen grains. LOX was observed in the cytoplasm of all types of cells. This was expected, in view of the variety of reactions in which the enzyme is engaged. LOX is known to prefer free fatty acids as substrates for reactions in the



Figs. 11–14. Portions of pollen grain with immunogold LOX PAb localization. **Fig. 11.** Control micrograph of a portion of exine layer of the pollen grain. **Fig. 12.** Immunogold particles (arrows) located at the interstice between the inner and outer exine. E – exine. **Fig. 13.** Portion of the exine layers of neighboring pollen grains. E – exine. **Fig. 14.** Immunogold particles at the exine surface in the space between interstice between neighboring pollen grains (arrows).

cytoplasm; LOX activity has been demonstrated with polyunsaturated fatty acids (PUFAs) esterified to phospholipids, and with PUFAs esterified to neutral lipids such as triglycerides (Feussner and Wasternack, 2002). The long-chain fatty acids most abundant in plants, linolenic and linoleic acids, have cis, cis 1,4-pentadiene structure; thus they are good substrates for LOX. Earlier, LOX was shown to be randomly distributed throughout the cytoplasm only in storage parenchyma cells of germinating soybean seeds (Vernooy-Gerritsen et al., 1984).

In the epidermal and endothecial cells of the anther, the enzyme was observed mainly in the cytoplasm and close to ER elements, and occurred close to the cell walls of the latter. In the anther cavity, LOX was found in protoplast masses, in vacuoles, close to mitochondria, and inside plastids. Lipoxygenase also occurred in the cytoplasm and around ER elements of pollen grains. These observations find partial support in the work of other authors. Using immunocytochemical analysis, Feussner et al. (1995) localized this enzyme in chloroplasts. Thylakoid membrane-associated lipoxygenase was found in tomato fruits; LOXs were localized in the stroma, but substantial LOX activity was detected in the envelope fraction (Bowsher et al., 1992). In cotyledons, besides soluble LOXs, particulate LOXs were also found in microsome membranes, plasma membranes and lipid bodies (Feussner and Wasternack, 2002). These results on LOX localization were not the focus of those authors' interest, however. We should also emphasize that LOX localization in cells is problematic, in part because soluble lipoxygenases tend to adhere nonspecifically to membranes (Siedow and Girvin, 1980).

The immunoreaction to lipoxygenase differed in intensity between cell types. The positive immunoreaction to lipoxygenase was less intense in the middle layers and the loculus of the anther, where only single immunogold particles were found. The most intense immunoreaction (immunolabelling to LOX) was in endothecial cells. Such a result is connected with the function of this layer. The cells of the

endothecium attain their maximum development when the anther is ready to dehisce for the discharge of pollen grains. In the degenerated cells of the middle layers, single immunogold particles were concentrated mainly at the cell wall edges. In many species, cells of the middle layers are storage centers for starch anther reserves, which are mobilized during later development of pollen. After pollen grains mature, the middle layers disappear.

Both the tapetum and pollen are responsible for synthesis of the four main lipidic structures of pollen grains. The tapetum synthesizes precursors of two extracellular lipidic structures – the sporopollenin and pollen coat – the biosynthesis of which is temporally separated, but some compounds (such as fatty acids) are common to both processes. Sporopollenin is extremely resistant to non-oxidative physical, biological and chemical degradation processes (Domingez et al., 1999). Sporopollenin is made up of a series of related polymers derived from long-chain fatty acids, plus more modest amounts of oxygenated aromatic rings and phenylpropanoids (Piffanelli et al., 1998). The occurrence of LOX at the exine layer of pollen grains and in the space between neighboring pollen grains seems very important. Some compounds of the LOX pathway can induce programmed cell death, or show antifungal activity. Some products of the hydroperoxide lyase pathway (branch of the LOX pathway) such as C6-volatiles (aldehydes and alcohols) are attractants, odors, show antimicrobial activity, or play a role in plant signaling (Porta and Rocha-Sosa, 2002). Many pollen grains produce volatile derivatives in their pollen coats, which act as either attractants or deterrents to insects and may also play a role in defense against microbiological attack. Some volatiles like xylene, limonene and hectadecanone are known to be released by various types of pollen.

The pollen coat is a lipoidal substance filling the spaces between the baculae of the highly sculpted exine surface. The acyl lipid composition of the pollen coat is completely different from that in the pollen grain. In the pollen coat of *Brassica napus*, for example, is a non-polar neutral ester fraction containing medium- and long-chain saturated fatty acids. The intracellular lipids of the pollen are triacylglycerols and phospholipids, enriched in C18 polyunsaturated fatty acids, particularly in linolenic acid (Piffanelli et al., 1997). In the Liliaceae family, besides the elaioplast, another type of lipid body has been shown. The tapetal cytoplasmic lipid bodies probably are derived from the ER (for references see: Piffanelli et al., 1998).

The vegetative cell of the pollen grain also synthesizes oil bodies and intracellular membranes (Piffanelli et al., 1998). Thus, LOX activity was expected to occur in the pollen coat and cytoplasm, ER elements and close to the nuclear envelope of the pollen grain in *Gagea lutea*. In another branch of the LOX pathway a precursor of jasmonic acid and some ketol products are formed. Jasmonic acid and its methyl ester are cited as factors responsible for growth inhibition, abscission, senescence and induction of certain leaf proteins (Gardner, 1991). The observed effects on senescence have been suggested to be the result of high, nonphysiological concentrations of jasmonate (León and Sánchez-Serrano, 1999). A high level of jasmonate was detected in growing tissues (Creelman and Mullet, 1997). The requirement of JA for male fertility in *Arabidopsis thaliana* was demonstrated in its mutant deficient in linolenic acid (McConn and Browse, 1996). Recently, Mandaokar et al. (2003) showed that JA was essential for anther development and pollen fertility in *Arabidopsis thaliana* plants.

When tapetal cells reach the end of their maturation, they undergo lysis. The lipidic cell content is released into the loculus, eventually accumulating in the baculae of the exine. LOX was found in the loculus in the anther of *Gagea lutea*. It was localized as single immunogold particles in the protoplast masses and close to cell organelles.

To our knowledge this is the first report on LOX immunolocalization in different cells of the anther. The occurrence of LOX in the anther of *Gagea lutea* may be related to the presence of some lipid compounds in the different layers of the anther. The enzyme may be responsible for production of some volatile derivatives acting as attractants or deterrents to insects, or may play a role in defense against pathogen attack.

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