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Immunogold localization of trans-zeatin riboside in embryo and endosperm during early fruit drop of *Malus domestica*

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Abstract

The specificity of a monoclonal antibody IgG_1, raised against trans-zeatin riboside-keyhole limpet hemocyanin conjugate, was investigated by means of inhibition experiments with soluble competing antigens. A competitive enzyme immunoassay was set up, with immobilized antigen. The analysis of the cross reaction profile enabled a study of the specificity of the antigen-antibody interaction. The antibody was able to distinguish the trans form of zeatin riboside from the cis form (cross reaction index = 1 %); cross reactions with ribose, adenine, adenosine and other related heterologous antigens were not detectable over the range of concetration tested. The recognition centres for the antibody seem to be the purine ring and the R substituent, especially in its hydroxymethyl group. Employment of this monoclonal antibody to localize cytokinins in control and shedding affected fruits of *Malus domestica* Borkh. evidenced high content of trans-zeatin riboside in developing seeds, differences in its content in embryo and endosperm, and a strong reduction of its content in the tissues of drop fruits. This decrease may be an important component responsible for early fruit abscission.

Additional key words: cytokinins, fruit abscission, immunohistochemistry, monoclonal antibodies, seed development.

Introduction

Production of *Malus domestica* is affected by an early fruit drop, starting about two weeks after anthesis and lasting several weeks. As a rule, these fruits show a reduction in size, detectable approximately 1 - 2 weeks before abscission (Zucconi *et al.* 1978). Moreover, Vernieri *et al.* (1992), analyzing the seeds of abscinding fruits, describe delayed development, precocious cytological differentiation, chromatin pyknosis and frequent cell degeneration. Ruffini Castiglione (1995) shows

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that chromatin changes observed in the seeds of abscinding fruits of *M. domestica* may be influenced by altered chromatin compactness, depending on DNA-associated proteins and DNA methylation. Moreover she concludes that differential genome repatterning might influence the fate of a developing fruit without excluding other concomitant causes.

In fact reproductive structures, such as developing seeds, are normally characterized by a high sink strength and an ability to attract nutrients in competition with the other developing structures (Wolswinkel 1992). A combination of exogenous and endogenous factors, including phytohormones, is probably responsible for regulation of the sink strength and for the control of fruit set.

To enable a better characterization of the variations in hormonal content occurring during early fruit drop, this paper considers trans-zeatin riboside (tZR), one of the prevalent cytokinin that plays diverse roles in plant growth and development. A monoclonal antibody with high specificity for tZR has been produced to localize it in embryo and endosperm tissue, and the overall topography of the antigen binding regions has been described.

Materials and methods

Standards and reagents: Analytical grade reagents, trans-zeatin riboside (tZR) and other purified cross-reactants, as well as keyhole limpet hemocyanin (KLH) type VIII, bovine serum albumin (BSA) fraction V, complete and incomplete Freund's adjuvants, were supplied by *Sigma Chemical Co.*, St Louis, USA; polyethylene glycol (PEG) 1550 by *Serva Feinbiochemica*, Heidelberg, Germany. *Linbro* polystyrene microtitration plates 96 flat bottom wells, 24-well culture plates and the culture media were obtained from *ICN Biomedicals* Inc., Costa Mesa, USA. *Tween 20*, EIA grade affinity purified goat anti-mouse IgG (H+L) horseradish peroxidase conjugate, peroxidase substrate, 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) were supplied by *Bio-Rad*, Richmond, USA. Immunobed Tm resin were supplied by *Polysciences*, Inc., Warrington, U.S.A. Immunogold conjugate LM goat anti-mouse IgG (5nm Gold), Normal Goat Serum and Silver Enhancing Kit - Light Microscopy were supplied *by British BioCell International*, Cardiff, UK.

Preparation of the hapten-carrier conjugates and production of monoclonal antibodies (Mabs): tZR was coupled to either the keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) using periodate-borohydride procedure, according to the Erlanger and Beiser (1964). Hapten to protein substitution molar ratios were estimated by means of UV absorption (*LKB* 2238 *Uvicord S II*) with the results of 64 and 18, respectively, for the KLH or BSA conjugate.

Eight-week-old female Balb/c mice were immunized by i.p. injection of 200 μg of tZR-KLH conjugate, solubilized in 100 mm^3 of 0.1 M phosphate buffered saline, pH 7.4, 0.08 % NaCl (PBS), emulsified with 100 mm^3 of Freund's incomplete adjuvant. Booster injections and subsequent procedure were given according to Mertens *et al.* (1983) and to Eberle *et al.* (1987).

Two of the positive hybridoma were cloned twice by limiting dilution. The antigen specificity of the antibodies from different clones was tested by ELISA. The subclass of the Mab was determined by using the *Mouse Typer Sub-isotyping Kit* from *Bio-Rad*, Richmond, USA.

ELISA: Positiveness of hybridoma culture supernatants was screened by using an ELISA non-competitive assay design, performed in 96-well microtitration plates, coated with 100 mm^3 of an 8 $\mu g\ cm^{-3}$ tZR-BSA solution in 0.1M PBS, pH 7.4. Before use, the coated plates were saturated with 2 % BSA in PBS/Tween 20 (0.05 %) and rinsed three times in the same buffer. To each well 100 mm^3 of the supernatants was added. Following a 2-h incubation at 37 °C and after washing four times, 100 mm^3 IgG goat anti mouse horseradish peroxidase conjugate (diluted 1:2 500) were added. After 1 h standing at 37 °C and washing as above, the peroxidase activity was developed with 100 mm^3 of an ABTS-H_2O_2 solution. After 30 min the reaction was stopped with 2 % oxalic acid and absorbance of the samples was measured (λ = 415 nm; *Labsystem Multiscan Plus MK II,* Stockholm, Sweden).

A competitive enzyme immunoassay was set up to investigate the specificity of the purified Mab. Optimum conditions were found for antigen coating and antibody dilution such as to give rise to a sigmoid dose-response curve over the range of 4×10^{-3} - 2.5×10^{-8} M free homologous antigen in solution. Specificity was tested by incubating individual heterologous antigens in the same assay conditions. In accordance with Abraham's criteria (Abraham 1969), the cross-reactivity was expressed as the percentage ratio between the mass of the homologous antigen and that of the heterologous ligand required to displace 50 % of the antibody bound to the immobilized antigen.

Plant material: Fruits of *Malus domestica* Borkh. cv. Supergolden were sampled 22 and 32 d after anthesis from plants grown at the Experimental Agricultural Farm of the University of Pisa (Italy). Two types of fruits were picked: *A*) from control apples (collected 22 d after anthesis), and *B*) from apples affected by fruit-drop, but not yet detached from the trees (32 d after anthesis). Even if the embryo developmental level was not always homogeneous, the embryo generally appeared to be at a heart stage in the seeds of fruits A and B (Fig. 1*a,b*).

Immunohistochemical procedure: Isolated seeds were treated to maintain the cytokinin ribosides *in situ*: oxidation reaction of the ribosides producing Schiff bases and stabilization by reduction with sodium borohydride was performed following the procedure described by Sossountzov *et al.* (1988). The samples were then fixed with 4 % paraformaldehyde + 1 % glutaraldehyde mixture in 0.15 M phosphate buffer, pH 7.4, for 18 h and embedded in *Immunobed Tm* resin. Semithin sections (3 μm thick) were obtained by means of an ultracut (*Ultratome Nova, LKB,* Stockholm, Sweden) using a glass knife. For monoclonal antibody binding, slides of samples A and B were rehydrated using 0.1 M PBS (pH 7.4) + 1 % BSA + 0.1 % Tween 20 (solution A). Following this, all steps described below were performed at room temperature in a moist chamber. The slides were covered for 15 min with 1.5 %

normal goat serum in solution A to block non-specific sites. Incubation with the monoclonal antibody against tZR was carried out for two hours using 1:200 diluted ascites fluid in solution A. After three washes in solution A, the slides were exposed to immunogold conjugate LM goat antimouse IgG (5 nm Gold) diluted 1:100 in solution A for 1 h, and then rinsed three times in the same solution and once in distilled water. For light microscopy, the gold signal was enhanced with silver (Silver enhancing Kit - Light Microscopy); three drops of the initiator and three drops of the enhancer (*British BioCell International*, Cardiff, UK) were mixed together and placed on the slides in the dark. After 7 min, the reaction was stopped in tap water. The slides were then stained with acid fuchsin, air dried and mounted in DPX mountant for microscopy (*BDH Laboratory*, Poole, UK).

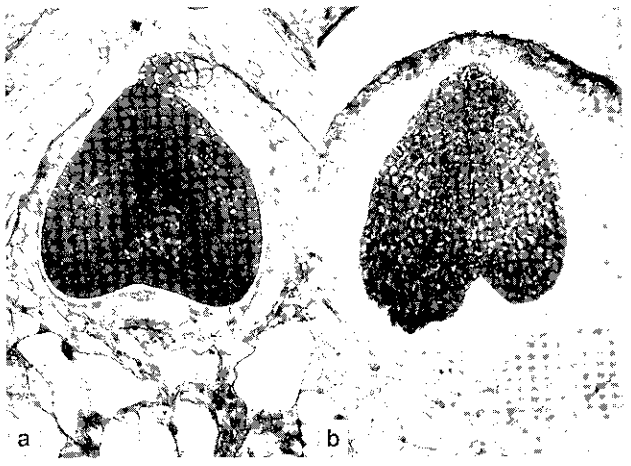

Fig. 1. Longitudinal sections of control seed (*a*) and abscinding fruit seed (*b*); haematoxylin staining (× 100).

Observations were carried out using a *Leitz Dialux 22EB* microscope (Wetzlar, Germany). Quantification of labelling, calculated with the *NIH Image analysis programme* (1.4 version, *MacIntosh*) was expressed as the absorbance of gold particles μm^{-2}. A calibration curve was performed by recording standard gray values. The analysis was performed for 200 cells randomly choosen of 5 seeds for each sample and for each system.

Immunohistochemical controls: Conventional controls of the method were performed in every experiment and the specificity of immunolabelling was tested by means of: *1*) treatment with a non-immune mouse IgG instead of the primary antibody; *2*) further dilution and omission of the primary antibody; *3*) omission of the immunogold conjugate LM goat anti-mouse IgG; *4*) inhibition of the immunoresponse by preincubating the antibodies with an excess of homologous antigens before adding to the slides; *5*) omission of the coupling step used to maintain *in situ* the cytokinin ribosides.

Results and discussion

The selected monoclonal antibody was isotypized as an IgG_1 and applied to a competitive enzyme immunoassay, with the antigen (ag) immobilized on the solid phase. The addition of 2.5×10^{-8} - 4×10^{-3} M tZR to aliquots of Mab at optimum dilution titre, produced binding inhibition of the antibody to the insoluble ag. The degree of inhibition increased with the amount of free ag in solution with lower and upper detection limits of 2.5×10^{-8} - 4×10^{-3} M (defined, respectively, as the lowest [ag] exceeding the zero-dose precision, and the higher [ag] giving an absorbance value different from that measured omitting the primary antibody, at a 99.73 % confidence limit) (Fig. 2). The assay allowed the assessment of Mab specificity

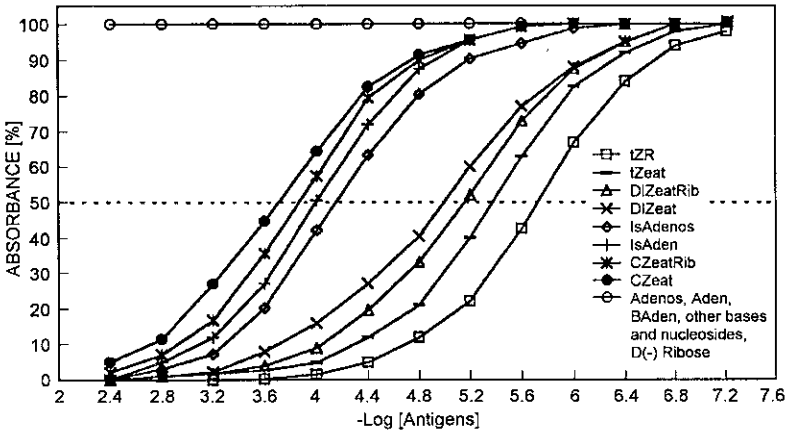

Fig. 2. Displacement curves of monoclonal antibody binding to immobilized trans-zeatin riboside by the primary antigen and heterologous antigens in solution (see Table 1 for the abbreviations).

against heterologous ags (Table 1). The monoclonal antibody against tZR was able to distinguish between the cis and the trans forms of zeatin riboside. However, the presence of a ribosyl moiety did not seem to influence the antibody binding to a great degree, given the fact that: *1*) trans-zeatin strongly competed with tZR for the binding site of the Mab; *2*) no relevant difference was detected in the cross-reaction profile between the glycosylated and nonglycosylated heterologous antigens; *3*) D(-)ribose did not show any inhibitory effect within the range considered (Fig. 2; Table 1). As a consequence, in the ag molecule, the recognition centres for the Mab seems to be *1*) the purine ring, although its importance is probably limited, as it is not possible to detect a cross reaction with adenine over the range of concentration tested; *2*) the R substituent of the purine ring. In stabilizing the antigen-antibody interaction it is to underline the major relevance of the hydroxymethyl group in comparison to the methyl group on the R purine substituent. Indeed, from the differences in the antigen displacement curves (Fig. 2) obtained with dihydrozeatin riboside and isopentenyl-adenosine, it is clearly shown a highest level of cross reaction with dihydrozeatin (CR = 28 %), containing a hydroxymethyl group, in comparison to isopentenyl-

adenosine (CR = 2 %), in which is present a methyl group. This overall topography of the antigen binding regions confirmed the high specificity of the Mab and also its analytical power in histological preparations.

Table 1. Cross-reaction profile of the anti trans-zeatin riboside monoclonal antibody. The inhibition constant (Ki) equals the concentration (mol dm^{-3}) of the competing antigen causing a 50 % inhibition of the antibody binding to the solid-phase immobilized primary antigen. The percent cross-reaction index (C.R.) is defined as 100 × Ki(tZR)/Ki(heterologous antigen). The 50 % inhibition of antibody binding was not attained at any concentration with benzyladenosine, benzyladenine, adenosine, adenine, other bases and nucleosides and D(-)ribose.

Antigen	Ki [M]	C.R. [%]
Trans-zeatin riboside (tZR)	1.91×10^{-6}	100
Trans-zeatin (tZeat)	4.15×10^{-6}	46
Dihydrozeatin riboside (DIZeatRib)	6.82×10^{-6}	28
Dihydrozeatin (DIZeat)	1.01×10^{-5}	19
Isopentenyladenosine (IsAdenos)	9.55×10^{-5}	2
Isopentenyladenine (IsAden)	9.54×10^{-5}	2
Cis-zeatin riboside (CZeatRib)	1.91×10^{-4}	1
Cis-zeatin (CZeat)	1.90×10^{-4}	1

The validity and the specificity of the immunogold technique to detect tZR distribution used in both embryo and endosperm was confirmed analyzing the sections belonging to the immunohistochemical controls (data not shown). Further dilutions of the primary antibody gave a parallel reduction in the silver staining; the substitution of the primary antibody with a non-immune mouse IgG and the omission of the immunogold conjugate LM goat anti-mouse IgG did not produce any labelling signal; preincubating the antibodies with an excess of homologous antigens caused the inhibition of the immunoresponse; the omission of the coupling step used to maintain *in situ* the cytokinin ribosides revealed a drastic and almost total reduction in the immunoreactivity.

As regards density and distribution of silver particles, in sample A a high immunoreactivity was clearly evident for both nuclear and cytoplasmic compartments (Figs. 4*a*, 5*a*); while embryo showed a more homogeneous labelling distribution (Fig. 4*a*), endosperm appeared especially stained in the nucleus and cytoplasmatic labelling regarded the peripheral portion, adjacent the plasmalemma (Figs. 3, 5*a*). Sample B was very poorly immunoreactive in comparison to the control (Figs. 4*b*, 5*b*); cytoplasmic immunolabelling was reduced about three and two times for embryo and endosperm, respectively. Moreover nuclear labelling was practically absent (Fig. 3).

The large amount of data concerning plant hormones have been obtained by biochemical and physicochemical determinations while very few papers describe histological and/or intracellular localization of growth regulators (Sotta *et al.* 1985, Eberle *et al.* 1987, Sossountzov *et al.* 1988, Bertrand and Benhamou 1992).

Since cytokinins are key hormones involved in the regulation and co-ordination of plant metabolism, growth and morphogenesis (for review see Binns 1994), *in situ* localization of trans-zeatin riboside could further increase the understanding of its physiological role and of its mechanisms of action.

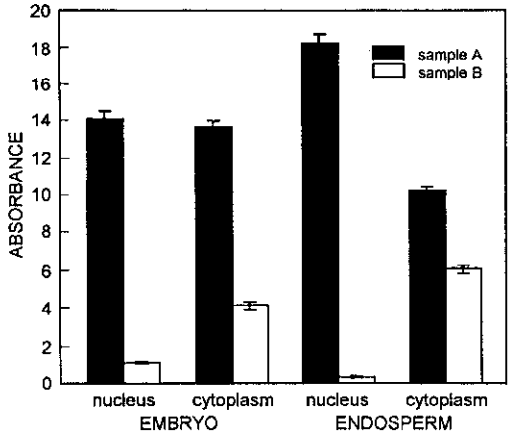

Fig. 3. Density of labelling obtained with the anti trans-zeatin riboside antibody - goat anti mouse gold conjugated in the embryo and endosperm cells of *Malus domestica*. Density is given as the absorbance (arbitrary units) of gold particles per μm^2 (*bars* indicate SE).

Specific cytological details emerge from our data: tZR is localized either in the cytoplasmic or in the nuclear compartment (Figs. 4*a*, 5*a*). A similar cytological distribution was obtained on terminal buds of *Craigella tomato* (Sossountzov *et al.* 1988) by means of polyclonal antibodies for the same antigen. Moreover, it was shown that not only the embryo of *Malus domestica*, but the endosperm is also a very immunoreactive system, especially in the nuclear compartment. A large amount of literature confirms that endosperm is characterized by a high content of gibberellins (Ceccarelli and Lorenzi 1983), abscisic acid (Vernieri *et al.* 1992) and cytokinins (Lorenzi *et al.* 1988) and, as a consequence, seems to be extremely important in metabolism during seed and fruit development.

The reduced content in tZR in embryo and endosperm of sample B, in comparison to sample A, may partially explain its cyto-histological characteristics: precocious differentiation and/or degeneration (Vernieri *et al.* 1992, Ruffini Castiglione 1995). From the 1960's it is well documented that cytokinin content in apple fruit is high and considerably greater than in other organs with a crucial role during fruit set (Letham 1969, Letham and Williams 1969). Cytokinins are a class of growth regulators that promote cell division (Quatrano 1987, Zhang *et al.* 1996) and influence fruit growth (Skoog and Armstrong 1970), including apples (Letham 1963). This generally occurs with a first peak during rapid cell divisions soon after anthesis, followed by a second massive accumulation stage as the fruit reaches maturity (Lewis *et al.* 1996). A reduced synthesis and/or accumulation of cytokinins in our abscinding

system may be one of the concomitant causes influencing the different fate of seeds and as a consequence, of fruit development.

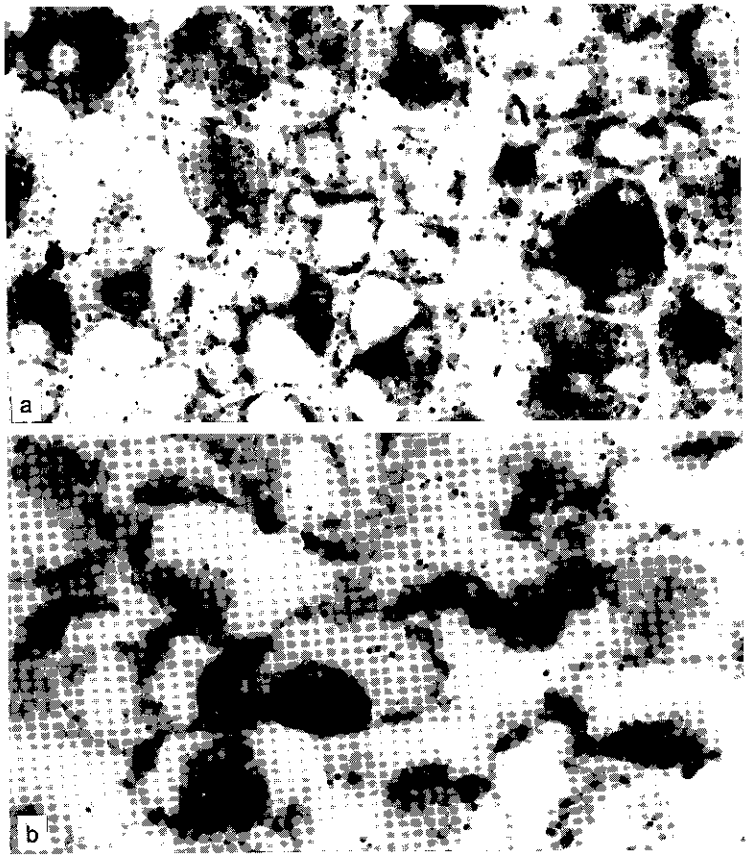

Fig. 4. Silver labelled embryo sections after binding with an anti-trans zeatin riboside antibody. Detail of embryo of sample A (*a*) and sample B (*b*). Acid fuchsin staining (× 1 500).

Regulation of assimilate partitioning and competitive ability of a sink is the result of a balanced content of molecular signals in response to genetic and environmental conditions. While gibberellins may be involved only in a limited part of fruit abscission (Avanzi *et al.* 1988), endogenous content of abscisic acid was clearly higher in the seeds belonging to abscinding apple fruit in comparison to the control (Vernieri *et al.* 1992). Probably a strong tZR reduction, together with an increase of ABA content may influence morphological and structural changes observed in the seeds of abscinding fruits and may determine the precocious differentiation of the developing seed by modulation of a different morphogenetic programme.

Considering that fruit shedding is a heterogeneous phenomenon, it is difficult to decide if this irregular development and an unbalanced hormonal content are the

cause or the effect of failed competition for nutrient substances and, as a consequence, of abscission. Even if additional studies are needed for a better understanding of fruit abscission, we can conclude that tZR, together with other growth substances, plays a fundamental role for the correct growth and morphogenesis in *Malus domestica* seeds.

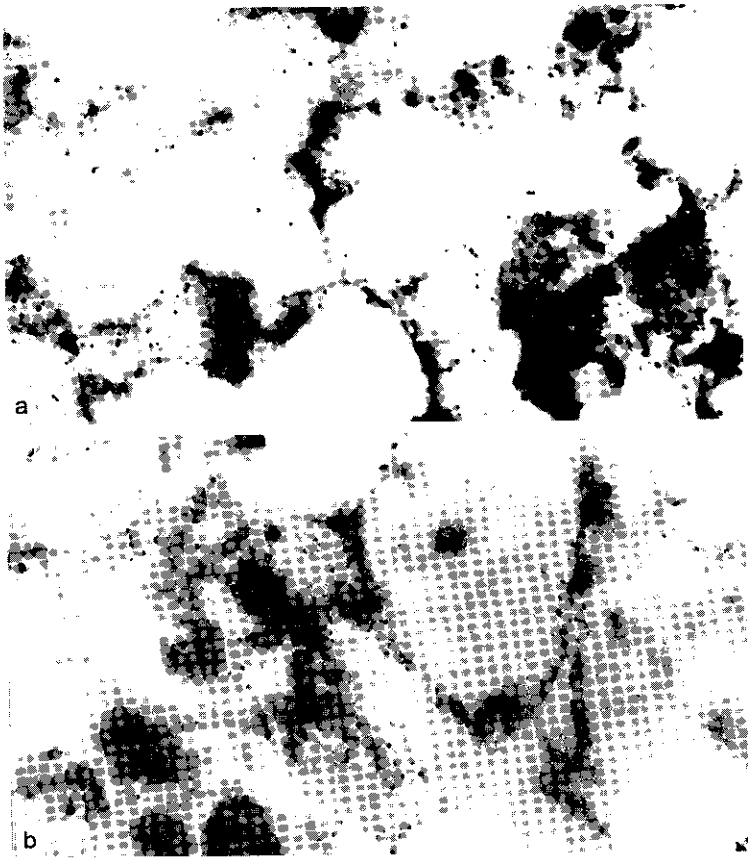

Fig. 5. Silver labelled endosperm sections after binding with an anti-trans zeatin riboside antibody. Detail of endosperm of sample A (*a*) and sample B (*b*). Acid fuchsin staining (× 1 500).

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