Localisation of Barley α-Amylase/Subtilisin Inhibitor, α-Amylase and Starch Granules in Developing and Germinating Barley Kernels

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Plant Science
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Winnipeg, Manitoba

ISBN 0-315-81806-9

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LOCALISATION OF BARLEY a-AMYLASE/SUBTILISIN, INHIBITOR, a-AMYLASE AND STARCH GRANULES IN DEVELOPING AND GERMINATING BARLEY KERNELS

BY

SHARON MARGARET GUBBELS

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Acknowledgments

Special thanks to my committee members: Dr. R.D. Hill, Dr. A.W. MacGregor, Dr. M.J. Sumner, and Dr. B. Fristensky, who were consulted extensively throughout this project and offered excellent advice and encouragement.

Len Dushnicky of the Canadian Grain Commission, Garry Burgess of Botany, and Bert Luit of Plant Science all spent many hours teaching me the basics of light and electron microscopy and photography. Thank you.

Many thanks to the members of Dr. Hill's lab, particularly Doug Durnin, Doug Monroe, James Astwood, Andre Silvanovich, Dave Audette and Dr. Ma Luo, for day to day advice and encouragement.

I would like to thank a number of people from the University of Guelph who taught me the basics of molecular biology. They include: Kathy Walker, Bill Wong, Don VanRyk, Doreen Culham, Carla Taylor, Janice Schmidt, Stacey Charleton, John Kim, Juan Pablo Martinez, Greg Boland, Annette Nassuth, Lloyd Berger, Brian Dixon, Lloyd Hutchinson, and Shin Woo Lee.

My family has given me tremendous support over the last few years as I struggled to complete this thesis. Special thanks to Elisabeth's grandparents, aunts & uncles for the babysitting services which enabled me to finish the thesis revisions.

Jim Astwood has been the most influencial person in my life during the past 8 years. He has given me support and encouragement as my husband and sound technical advice as a co-worker. He was 100% responsible for formatting this document on the computer.

Also, thanks to my daughter, Elisabeth, for making me happy.

ABSTRACT

The biological role of a proteinaceous α-amylase inhibitor, which inhibits both barley α-amylase and bacterial subtilisin *in vitro*, was investigated in developing and germinating barley (*Hordeum vulgare* cv. Bonanza) kernels by comparing the location of the inhibitor to that of both α-amylase and starch granules, using immunocytochemistry and polarizing microscopy. Barley α-amylase/subtilisin inhibitor (BASI) and starch granules, but not α-amylase, were found in endosperm, scutellum, and coleoptile of developing kernels. BASI was dispersed throughout the cells of the scutellar parenchyma of mature, unimbibed kernels but appeared to become increasingly associated with starch granules after kernels were imbibed for 3 days (3 DI). BASI was also associated with starch granules in the cells of both coleoptile and root at 1 - 4 DI. α-Amylase was detected in the scutellar parenchyma after 2 DI and in the coleoptile parenchyma after 4 DI. At 1 DI, α-amylase and BASI were abundant in the endosperm adjacent to the scutellar epithelium and the adjoining aleurone. Both proteins moved towards the central and distal portions of the grain as imbibition continued. Using transmission electron microscopy, BASI was determined to be located in protein bodies of developing endosperm and 1 DI scutellar cells. These results indicate that BASI is closely associated with α-amylase during germination and is generally found in kernel tissue containing starch granules. This suggests that, *in vivo*, the function of BASI is to attenuate starch granule degradation by binding α-amylase.

List of Abbreviations

ABA - Abscisic Acid
BASI - Barley α-Amylase/Subtilisin Inhibitor
BGA - Barley Germ Agglutinin
cDNA - Complementary DNA
DI - Days of Imbibition
DIC - Differential Interference Contrast
ICC - Immunocytochemistry
IgG - Immunoglobulin G
LEA - Late Embryogenesis Abundant
LLP - Lectin-Like Protein
mRNA - Messenger RNA
PAS - Periodic Acid/Schiff's
PHA - Phytohaemagglutinin
SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STI - Soybean Trypsin Inhibitor
WGA - Wheat Germ Agglutinin
WPA - Weeks Post-Anthesis

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INTRODUCTION

The barley α-amylase/subtilisin inhibitor (**BASI**) is a member of a large group of proteins that are produced primarily during seed development in a wide variety of plant species and are able to inhibit various types of α-amylases and/or serine proteinases. It is possible that the majority of these inhibitors provide seeds with protection from invaders since, in most cases, they inhibit proteinases and amylases of other organisms (Garcia-Olmedo *et al.*, 1987). In addition to strongly inhibiting a bacterial subtilisin, **BASI** is one of the few inhibitors that is able to inhibit an endogenous α-amylase *in vitro* (Mundy *et al.*, 1983).

One of the most promising suggestions for the *in vivo* role of **BASI** is that it attenuates α-amylase activity in barley kernels upon germination (Hill *et al.*, 1987) . α-Amylase II, which is specifically inhibited by **BASI**, is produced in large amounts at the time of germination. It is proposed that **BASI** binds the newly synthesised α-amylase II. As increasing amounts of α-amylase pour into the system the inhibitor is saturated with α-amylase. The unbound α-amylase then proceeds to degrade the starch granules. Thus, **BASI** may act to attenuate the release of sugars early in germination until the embryo is able to metabolise them.

The research documented in this thesis was undertaken to strengthen the mounting evidence that **BASI** in fact plays a role in attenuating starch granule degradation by inhibiting endogenous α-amylase. This hypothesis was tested by comparing the locations of **BASI**, α-amylase, and starch granules during development and germination of barley grains. If BASI and α-amylase are co-located near starch granules during germination then it is physically possible for BASI to inhibit α-amylase *in vivo*.

LITERATURE REVIEW

Barley Kernels

The barley kernel consists of the caryopsis, husk (lemma and palea), and rachilla - a bristle that lies in the ventral crease of the kernel (Reid, 1985). The caryopsis includes the embryo, endosperm, aleurone, testa, and pericarp (See figure **1A**). The embryo, which arises from an egg cell that has been fertilized with one of the sperm cells (Bewley and Black, 1978), contains developing meristems and scutellar tissue. The plumule, and the radicle and seminal roots are surrounded by coleoptile and coleorhiza, respectively. Long, finger-like scutellar epithelial cells develop at the interface of the scutellum and endosperm (See figure **2A**). Endosperm, which contains the majority of starch and protein reserves, develops from the fusion of two polar nuclei in the embryo sac central cell with the other sperm cell (Bewley and Black, 1978). The peripheral endosperm cells differentiate into aleurone cells (See figures **2C** and **2D**) which are still considered part of the endosperm (Briggs, 1978). The testa or seed coat develops from the inner integument that surrounds the ovule. The pericarp, which is derived of ovary wall, provides the developing caryopsis with nutrients via vascular connection with the parent plant (Duffus and Cochrane, review in press). Kernel development is often measured in days or weeks post anthesis (DPA/WPA) since the ovule is fertilized soon after anthesis.

Upon imbibition of mature kernels, gibberellic acid, a plant growth regulator, stimulates aleurone and scutellar epithelium cells to synthesize and secrete hydrolytic enzymes into the endosperm (Mundy and Munck, 1985). The scutellum releases its own stored nutrient reserves and absorbs nutrients provided by the endosperm for the growing

seedling. Since imbibition begins the cascade of events leading up to germination, the germination process can be measured in hours or days after imbibition (HI/DI).

Endosperm Reserves

Starch

The deposition of starch in the endosperm of barley kernels begins soon after fertilization. Starch is visible within amyloplasts from 2 to 3 days after anthesis (Baxter and Duffus, 1971). By 2 WPA, starch is rapidly filling endosperm cells (Jennings *et al.*, 1963). Amyloplast division ceases before the beginning of cell division in the endosperm (Briarty *et al.*, 1979).

During early development, one starch granule is formed per amyloplast. After two WPA these large type A granules (up to 35 μm in diameter in mature kernels) are no longer initiated and the smaller type B granules (up to 10 μm) begin to be observed within amyloplasts. Plastids containing type B granules bud off from parent amyloplasts (Buttrose, 1960). Although, depending on barley variety, there may be 5.5 to 30 times the number of type B granules relative to type A granules (Williams and Duffus, 1977), the type A granules may occupy as much as 90% of the total volume of starch in the endosperm (May and Buttrose, 1959). At kernel maturity, the majority of starch is in the form of amylopectin in both type A and type B granules (Williams and Duffus, 1977). Starch represents 65 - 70 % of kernel dry weight at maturity (Duffus, 1978).

Protein

Synthesis of protein reserves begins early in grain development, but lags behind starch synthesis until 3 WPA when protein synthesis in the endosperm is very rapid (Jennings *et al.*, 1963). Most protein is deposited in protein bodies which are specialized vacuoles consisting of protein enclosed loosely within a single lipoprotein membrane (Buttrose, 1963). Protein bodies range in diameter from 1 to 15 μm in the endosperm of mature grains (Jennings *et al.*, 1963). Protein bodies are difficult to see on thick sections (50 μm) using optical microscopy since the large and predominant type A starch granules tend to obscure them (Jennings *et al.*, 1963). At maturity, protein accounts for about 15 % of the endosperm tissue (Duffus and Cochrane, review in press).

Most proteins that are to be transported, intra- or extra-cellularly, are synthesised as higher molecular weight precursors (Keegstra, 1990). The extra peptide, which is cleaved from the protein at the time of translocation, is called a signal peptide if the protein is destined to go through the secretory pathway, or is called a transit peptide if the protein is destined for a plastid. Proteins routed through the secretory pathway may be targeted to protein bodies if additional information is included in a separate peptide or within the mature protein itself (Sebastiani *et al.*, 1991).

Essential functional regions of identified signal and transit peptides have not been successfully defined. However, characteristic amino acid compositions are apparent for both types of peptides. Signal peptides generally have one or two positively charged amino acid residues followed by a continuous string of hydrophobic amino acid residues and often include many leucine residues (Darnell *et al.*, 1990). Transit peptides are rich

in the hydroxylated amino acids serine and threonine (about 20 -35 % of total amino acids) and small hydrophobic amino acids such as alanine and valine. Transit peptides are generally deficient in acidic amino acids and are usually positive in net charge (Keegstra et.al., 1989 and Keegstra, 1990). The majority of transit peptides have a loosely conserved cleavage-site motif : (Val/Ile)-X-(Ala/Cys)↓Ala (Gavel and von Heijne, 1990). If such a motif is present there is a 90% probability that it is the correct cleavage site. It is possible to speculate on the cellular location of a protein by analyzing its prepeptide.

Scutellum Reserves

Starch

Starch is synthesized in amyloplasts within the scutellum during the first few weeks of barley kernel development. Starch granules are concentrated in the basal third of the scutellum (Smart and O'Brien, 1979). Upon germination, both the size and number of amyloplasts increase (Gram, 1982). The amyloplasts contain one or more starch granules, which may be up to several micrometers in diameter (Gram, 1982). After 3 days of germination the starch granules are the dominating feature of the basal two thirds of the scutellum (Smart and O'Brien, 1979).

Starch granules are absent from all scutellar tissue of wheat caryopses at maturity (Swift and O'Brien, 1972a&b). A few small starch granules may be observed in the scutellar parenchyma and epithelium after 6 hours of imbibition and, as germination proceeds, the size and number of granules increase (Swift and O'Brien, 1972b). Many

small starch granules are observed in the scutellar epithelium but the starch granules in the scutellar parenchyma are much larger and more abundant. The increase in starch reserves is followed by a decrease in starch content, first in the epithelial cells and then in the parenchyma cells beginning about 4 days after imbibition (Swift and O'Brien, 1972b).

Protein

By the time barley caryopses reach maturity, protein bodies have become the major reserve component in the scutellum (Smart and O'Brien, 1979). Protein bodies range from 2 to 4 μm in size and contain a granular protein matrix and one or more inclusions of phytin (Gram, 1982). All protein bodies in the scutellum are completely degraded by 7 days after imbibition (Smart and O'Brien, 1979).

In the scutellum of wheat caryopses, protein degradation may be observed within protein bodies as early as 12 hours after imbibition (Swift and O'Brien, 1972b). Breakdown of protein bodies is evident throughout the scutellum after 24 hours of imbibition. Partially hydrolysed protein may be observed in the protein bodies. The membranes of these vacuolated protein bodies fuse together so that later in germination only a few very large vacuoles remain of the protein bodies (Swift and O'Brien, 1972b).

Localisation of α-amylase

There have been numerous studies involving α-amylase immunolocalisation. The majority of these experiments focus on the presence of α-amylase in the aleurone tissue

and its mode of secretion into the adjacent endosperm (Gubler *et al.*, 1986 and Zingen-Sell *et al.*, 1990). Ranki (1990) observed a significant level of α-amylase in the scutellar epithelium after 1 day of imbibition but much less after 2 days. Similarly, Pogson *et al.* (1989) reported that α-amylase was not present in the scutellar epithelium in mature kernels but that it was detected in 24, 48, and 72 hour-imbibed kernels. Both Ranki and Pogson *et al.* used fluorescent antibodies to detect the anti-amylase antibody. This method of detection is difficult to interpret because of autofluorescence in scutellar tissue. Pogson *et al.* speculated that α-amylase signal was present in the scutellar parenchyma but concluded that a more sensitive assay was necessary to confirm this observation. MacGregor *et al.* (1984) found marginal amounts of α-amylase I and II in extracted scutellar protein samples during the first 2 days of imbibition and marked increases in both isozymes in 3 and 4 DI scutellum.

Immunolocalisation of α-amylase in the endosperm of germinating barley has been explored by Gibbons (1979) and Gibbons and Nielsen (1983). α-Amylase signal appears along the scutellum after 1 day of imbibition, becomes darker after 2 days of imbibition, and then spreads through the endosperm towards the distal end of the kernel over the next few days of imbibition. The location of α-amylase over time corresponds very closely to the breakdown of cell walls in the endosperm.

Seed-Specific Proteins

Introduction

At any one stage in seed development, roughly 20,000 different genes are expressed (Goldberg *et al.*, 1989). The most abundant species of mRNA transcripts may be divided into distinct groups based on the times that they are present in the seed (Dure III *et al.*, 1983). The majority of seed proteins are produced by two gene groups (Goldberg *et al.*, 1989). The group of genes expressed during early to late embryogenesis produces storage proteins, lectins, and α-amylase/serine protease inhibitors. The second group are late embryogenesis abundant (LEA) genes. Many of the genes from both groups are sensitive to exogenous ABA in culture. *In situ*, the expression of the first group of genes is well under way by the time significant levels of ABA appear in the seed. (see Table 2)

Many seed-specific genes are members of multigene families. Smith (1990) has proposed that differential regulation of these genes may represent the molecular basis of phenotypic plasticity, which is the expression of variability in the phenotype of individuals of identical genotype (Bradshaw, 1965). Smith considers the sophistication of controls over the synthesis and action of protein to be a major factor in the ability of an individual to adapt to environmental conditions. The genes expressed in plant seeds must be especially sensitive to environmental signals in order to correctly express the responses that will ensure the survival of a new generation of plants.

Inhibitors of α-Amylases and Serine Proteinases

Numerous proteinaceous inhibitors of α-amylases and serine proteinases have

evolutionary and structural relationships and are found in a wide variety of plant seeds (Garcia-Olmedo *et al.*, 1987). α-Amylase/proteinase inhibitors may be divided into several families based on the degree of sequence similarity between members. Three of the best characterized families include the soybean (*Glycine max*) trypsin inhibitor (Kunitz) family, the cereal trypsin/α-amylase inhibitor family, and the potato (*Solanum tuberosum*) inhibitor I family.

An evolutionary link between seed storage proteins and α-amylase/proteinase inhibitors has been proposed by Kreis *et al.*(1985). The non-repetitive domain of the sulphur-rich prolamins of cereals contains regions of similarity with cereal inhibitors of trypsin and α-amylase, and with 2S storage proteins (also known as napins, members of the cereal trypsin/α-amylase inhibitor family) of rapeseed (*Brassica napus*) and castor bean (*Ricinus communis*). Thus, it is not unexpected to find that the gene regulation of these proteins is similar. All of these proteins are synthesised at the same time in seed development and are generally targeted for protein bodies in the cells.

There appears to be a standard mechanism of inhibition for all of the α-amylase/serine proteinase inhibitors (Laskowski and Kato, 1980). Although free enzyme and inhibitor appear to be in simple equilibrium with the enzyme/inhibitor complex, the inhibitors are actually highly specific substrates for target enzymes. The inhibitors undergo a limited and extremely slow proteolysis when bound to the enzyme. Each inhibitor has at least one reactive bond (P1-P'1) which interacts with the active site of the enzyme. The amino acid residue in the P1 position determines the specificity of the enzyme.

Soybean Trypsin Inhibitor (Kunitz) Family The members of the soybean trypsin inhibitor (STI) family have molecular weights of about 20,000 and contain two disulphide bridges (Garcia-Olmedo *et al.*, 1987). In addition to the soybean multigene family, many proteins from different plant species have been characterized including the α-amylase/subtilisin inhibitors of barley, wheat (*Triticum aestivum*), and rye (*Secale cereale*) which will be discussed in greater detail under the section entitled "BASI". The majority of identified inhibitors are specific for trypsin and/or chymotrypsin. They may or may not be double headed.

The STI multigene family consists of at least 10 members which are differentially regulated (Jofuku and Goldberg, 1989). The KTi3 gene encodes the prominent STI (Ti^a) of soybean seeds which has a molecular weight of 21.5 kDa (Vodkin, 1981). KTi3 is most highly expressed during embryogenesis but is also found among leaf mRNA transcripts. Transcripts for both KTi3 protein and ß-conglycinin storage protein accumulate in a wave-like pattern progressively from the outer edge to the centre of the cotyledon during embryogenesis (Perez-Grau and Goldberg, 1989). According to Horisberger and Tacchini-Vonlanthen (1983), who used transmission electron microscopy to observe immunogold labelled anti-STI antibodies, STI is associated with most protein bodies, cell walls, the chromatin deposits and nucleolus of nuclei, and to a lesser extent the cytoplasm between the lipid-containing spherosomes. A function in germination has been suggested because a precise proteolytic cleavage involving five amino acid residues at the carboxyl terminus occurs during germination (Hartyl *et al.*, 1986).

The major albumin of cocoa (*Theobroma cacao*) seeds has homology with STI

proteins (Spencer and Hodge, 1991). Cocoa albumin is 221 residues in length at maturity and has a 26 residue leader peptide. The cocoa protein has been located in membrane-enclosed organelles within cocoa embryos (Spencer and Hodge, 1991).

Cereal Trypsin/α-Amylase Inhibitor Family Members of the cereal trypsin/α-amylase inhibitor family have monomers of about 12,000 MW and are active in the monomeric, dimeric and tetrameric forms (Garcia-Olmedo *et al.*, 1987). Some of these proteins are only active against heterologous α-amylases, while others inhibit only trypsin/chymotrypsin. Only one inhibitor, isolated from ragi (*Eleusine coracana*), has been reported to be bifunctional (Shivaraj and Pattabiranam, 1981). This protein is able to inhibit both heterologous α-amylases and trypsin.

CM-proteins (extracted by **C**hloroform/**M**ethanol mixtures) make up the main components of the A-hordein fraction in the endosperm of barley. There are at least six different members in this multi-gene family (Paz-Ares *et al.*, 1986). CMa is an α-amylase (heterologous) inhibitor. CMc and CMe are trypsin inhibitors. No inhibitory activity has been found for CMb and CMd (Barber *et al.*, 1986). CM genes are expressed between 10 and 30 days post anthesis (DPA). Peak expression is between 15 and 20 DPA, which is slightly earlier than other storage proteins (Paz-Ares *et al.*,1983). The deduced amino acid sequence of a CM cDNA clone, pUP-13, indicates the presence of a signal peptide (Paz-Ares *et al.*, 1986).

In addition to cereals, members of this family are found in a broad spectrum of plant species. The napin (*Brassica napus* storage protein) multigene family has sequence

similarities with these inhibitors, but no inhibitory properties have been reported to date (Crouch *et al.*, 1983). Similarity is also found with the Kazal secretory trypsin inhibitor from bovine pancreas (Odani *et al.*, 1983). Therefore, it is possible that an ancestral gene for this family may have arisen before the divergence of plants and animals.

Potato Inhibitor I Family Members of the potato inhibitor I family are multimeric proteins of about MW 41,000 that are made up of monomers of MW 8,100 (Garcia-Olmedo *et al.*, 1987). They are active against trypsin, chymotrypsin and subtilisin. The best studied examples of this family are found in potato, tomato, broad bean (*Vicia faba*) and barley. Sequence similarity is also found in an invertebrate, the leech (*Hirudo medicinalis*). Also, the conformation of the residues near the reactive bond in a barley chymotrypsin inhibitor, CI-2, is similar to that of analogous residues in a domain of the turkey ovomucoid inhibitor of the Kazal family (McPhalen *et al.*, 1985).

One member of the potato inhibitor 1 family comprises 50% of the soluble protein in unripe fruit of a wild species of tomato (*Lycopersicon peruvianum*) (Wingate *et al.*, 1989). RNA transcripts for the protein are present in fruit from 22 to 66 days after pollination. Thus, the protein is probably synthesized throughout development (Wingate *et al.*, 1991). The *in vitro* translation product of a cDNA clone, lambda clone 1, inhibits both chymotrypsin elastase and subtilisin (Wingate and Ryan, 1991). The immature polypeptide includes a 23 residue signal peptide and a 19 residue propeptide. The mature polypeptide is 69 residues in size. Potato inhibitor 1 protein is present throughout fruit tissue, as shown by tissue blot analysis, except in seeds (Wingate *et al.*, 1991). Early

embryonic tissues are devoid of inhibitor 1. The protein is located primarily in protein aggregates within vacuoles in fruit parenchyma cells (Wingate *et al.*, 1991).

Wheat and Barley Germ Agglutinin

Wheat and barley germ agglutinin (WGA/BGA) are chitin-binding lectins. Ninety-five percent of the amino acid residues of these proteins are identical (Lerner and Raikhel, 1989). WGA and BGA are synthesised during embryo development and slowly decrease in abundance over the first four days of germination (Morris *et al.*, 1985). WGA transcripts increase between 10 - 40 days post anthesis and then decline (Raikhel *et al.*, 1988).

WGA is located in cells that are in direct contact with soil during germination and growth of the seedling (Mishkind *et al.*, 1982). These cells are found in the surface layer of the radicle, seminal roots, coleoptile, and scutellum. WGA is found in the same tissues in barley and rye with the exceptions that it is absent from the coleoptile of developing barley embryos and is present in both the inner and outer surface cells of rye coleoptiles (Mishkind *et al.*, 1983). WGA mRNA and protein are also found in adventitious root caps and tips of mature plants. Mishkind *et al.* (1982) have proposed that WGA has a role in the defense against fungal pathogens because it is found at potential infection sites and is known to possess fungicidal properties.

Within the cell, most WGA is found adjacent to the inner surfaces of protein bodies (Mishkind *et al.*, 1982). Localisation within protein bodies was expected because the leader sequence of a BGA clone has characteristics of a signal peptide (Lerner and

Raikhel, 1989). Also, some WGA is located within electron-translucent regions of the cytoplasm and at the periphery of the cell adjacent to the cell wall (Mishkind *et al.*, 1982).

Phytohemagglutinin Family

Phytohemagglutinin (PHA), a lectin of the common bean (*Phaseolus vulgaris*), is a tetrameric glycoprotein composed of two different subunits called PHA-E (erythroagglutinating) and PHA-L (leucoagglutinating) (Leavitt *et al.*, 1977). The DNA sequences of two genes, *dlec1* and *dlec2* which encode, respectively, PHA-E and PHA-L, indicate that signal peptides are initially present on both subunits (Hoffman and Donaldson, 1985). PHA-E has a 21 amino acid signal peptide and is 254 amino acids long at maturity. PHA-L has a signal peptide of 20-residues and is 252 amino acids long at maturity. PHA genes are expressed primarily in cotyledons but are also detected at 10 times lower levels of expression in the axis (Chrispeels *et al.*, 1984). PHA is located within the protein bodies of cotyledons (Chrispeels, 1983) and is thought to have a protective function since it is able to kill the larvae of bruchid beetles (*Callosobruchus maculatus*) when added to the normal diet of the larvae (Janzen *et al.*, 1976).

LLP (lectin-like protein) is a PHA-like protein in *Phaseolus vulgaris* that has also been well characterized. LLP has a signal sequence of 20 residues and a mature peptide of 223 amino acids (Hoffman *et al.*, 1982). The LLP gene has about 65% sequence identity with the PHA-E and PHA-L genes (Vitale *et al.*, 1989). LLP, which is also called α-AI (α-amylase inhibitor), is synthesized during early to mid stages of seed

development (Moreno *et al.*, 1990). LLP is located in the cotyledons and, at a reduced level, in the embryonic axis and is present in protein bodies. LLP inhibits the activity of mammalian and insect α-amylases, but not plant α-amylases (Moreno *et al.*, 1990).

Late Embryogenesis Abundant Proteins

Late Embryogenesis Abundant (LEA) proteins are encoded by small multi-gene families expressed only during late embryogenesis (Baker *et al.*, 1988, Dure III *et al.*, 1989). However, they may be induced by abscisic acid or dehydration in other plant tissues such as leaves. All of the LEA proteins are very hydrophilic. Although LEA proteins share the above characteristics they can be subdivided into 3 subgroups on the basis of sequence similarity (Dure III *et al.*, 1989).

Em (early-methionine-labelled) protein of wheat, a Group 3 LEA and one of the best characterized LEAs, is the product of a small multi-gene family (Futers *et al.*, 1990). The 93 residue polypeptide does not contain a leader peptide (Litts *et al.*, 1987). Em protein is located in the cytosol of wheat embryo but not endosperm (Marcotte *et al.*, 1989). The protein is synthesized following the production of ABA during late embryogenesis (Williamson et al, 1985). An ABA responsive element is present in the 5' regulatory region of Em genes (Guiltinan *et al.*, 1990).

It has been proposed that the LEA proteins function in desiccation survival of seeds (Baker *et al.*, 1988). In addition to the expression of LEA genes just prior to the desiccation phase of seed development and their responsiveness to ABA and desiccation stress, this hypothesis is supported by the amino acid composition and structure of the

proteins. Group 2 LEA proteins are high in glycine and hydroxylated amino acids. It is thought that these LEA proteins promote structural integrity of other proteins and lipid membranes during desiccation stress by using the hydroxylated groups to solvate structural surfaces. Group 2 LEA proteins are better than sugars at alleviating desiccation stress because they are less likely to crystallize and can cover greater surface area. Group 3 LEA proteins may reduce the effects of increased ionic strength of the cytosol during osmotic stress. Group 3 LEA proteins contain a conserved domain of 11 amino acid residues repeated numerous times. These domains can exist as amphiphilic helices which have a high concentration of charged residues on the surface for the binding of ions. LEA proteins may be routinely used during the desiccation phase of seed development and may be on call for other times of desiccation stress.

Barley α-Amylase Inhibitor

Introduction

The barley α-amylase/subtilisin inhibitor (BASI) is a double headed inhibitor that contains separate active sites directed against α-amylase and subtilisin. It was first isolated and characterized in 1983 by two independent research groups (Weselake *et al.*, 1983a & 1983b, and Mundy *et al.*, 1983) and found to be identical to a previously isolated subtilisin inhibitor from barley (Yoshikawa *et al.*, 1976). Homologues of BASI are found in wheat, rye, and triticale (*Triticosecale wittmack*) but not in sorghum (*Sorghum bicolor*), oats (*Avena sativa*), millet (*Pennisetum americanum*), rice (*Oryza* spp.), and maize (*Zea mays*)(Weselake *et al.*, 1985b).

Inhibitory Effects

BASI is unusual in that it inhibits endogenous α-amylase; most α-amylase inhibitors in plants inhibit exogenous α-amylases. BASI inhibits hydrolysis of soluble starch by the high pI isozyme of α-amylase (α-amylase II) but does not affect the low pI isozyme (α-amylase I) (Weselake *et al.*, 1983a). It is inactive towards α-amylases from sorghum malt, hog pancreas, *Aspergillus oryzae*, and *Bacillus subtilis* (Mundy *et al.*, 1983).

The inhibitor binds α-amylase in a 2:1 (inhibitor:enzyme) molar ratio (Halayko *et al.*, 1986). It optimally inhibits α-amylase II at about pH 7, which is close to BASI's pI of 7.2-7.3. The inhibitor-enzyme dissociation constants are 1 X 10^{-6}M at pH 7.0 and 7 X 10^{-6}M at pH 5.5. There is a 7 fold increase in binding affinity when the pH is changed from pH 5.5, the pH optimum for α-amylase activity, to pH 7.0. Inhibition is decreased when the salt concentration is raised from 0 to 0.2M NaCl. This suggests that the complex is stabilized by ionic bonding (Halayko *et al.*, 1986). Inhibition of starch granule hydrolysis was independent of starch concentration and temperatures ranging from 15 to 35°C (Weselake *et al.*, 1985a).

BASI has been tested for inhibitory effects on the proteinases: trypsin, chymotrypsin, papain, *Aspergillus sulphureus* proteinase, *A. sydowi* proteinase, *Streptomyces griseus* protease (pronase), subtilisin BPN', subtilisin amylosaccharitics, neutral subtilopeptidase, and thermolysin (Yoshikawa *et al.*, 1976). Only the two subtilisins were strongly inhibited. BASI inhibited these proteinases most strongly above pH 8.0. Other researchers have tested wheat, rye and triticale homologues against a number of the above mentioned proteinases and also subtilisin Carlsberg and pancreatic elastase (Mosolov and

Shul'gin, 1986). The homologues strongly inhibit subtilisins but are unreactive or only slightly reactive against other proteinases. The inhibitors form complexes with subtilisin in a 1:1 molar ratio. Methionine residues are essential for inhibitor activity. They are located in the P1 position of reactive sites that are similar to those of other inhibitors active against microbial serine proteinases.

Sequence Similarities with other Proteins

Of the many families of proteinaceous inhibitors of proteinases and α-amylases that are found in plant seeds, BASI has the most sequence similarity with the soybean trypsin inhibitor (kunitz) family (Hejgaard *et al.*, 1983). Between BASI and soybean trypsin inhibitor, 26% of the residues are identical (Svendsen *et al.*,1986). The similarity extends throughout these proteins. Wheat α-amylase/subtilisin inhibitor, which shows 96% identity in the first 45 residues with BASI (Mundy *et al.*, 1984), has a high content (58%) of ß-sheet structure (Maeda, 1986). Soybean trypsin inhibitor has ß-sheet in 60% of its secondary structure. The ß-sheet regions for the two proteins generally correspond to each other. Although the members of the soybean trypsin inhibitor family have now diverged in inhibitory action, it is probable that they evolved from a common ancestor.

Molecular Characteristics of RNA and protein

The mature BASI protein consists of a single peptide chain of 181 residues with a molecular weight of 19,865 (Svendsen *et al.*, 1986). It has two intramolecular disulfide bridges which closely correspond to those found in the soybean trypsin inhibitor (Maeda,

1986). The RNA transcript of BASI is about 800 nucleotides in length (Leah and Mundy, 1989). Its coding region is high in guanine and cytosine (69%) which is not unusual for a cereal gene.

The immature protein has a 22 amino acid residue prepeptide (Leah and Mundy, 1989) that has characteristics of both signal and transit peptides. It is like a signal peptide in that it has two positive amino acid residues at the beginning of the sequence followed by a string of hydrophobic amino acid residues. However, there are four serine residues between the positive amino acids and the hydrophobic amino acids which are unusual for signal peptides but common in chloroplast transit peptides (Keegstra *et al.*, 1989). The positive amino acid at the end of the peptide and the high content of hydroxylated (serine and threonine) amino acids (27%) is also characteristic of transit peptides. The residues at the peptide cleavage site, Ser-Arg-Ala↓Ala, are similar but not identical to the conserved cleavage-site motif, (Val/Ile)-X-(Ala/Cys)↓Ala, found in many transit peptides (Gavel and von Heijne, 1990). The sequence would be unusually short for a transit peptide. Thus, this leader sequence may act as a signal peptide. Subtle changes in the ancestral signal peptide may have converted it to a functional transit peptide, or it may act as a bifunctional peptide. The leader peptide of BASI has similar characteristics to the leader peptide of the major albumin of cocoa seeds (Spencer and Hodge, 1991). Immunolocalisation is desirable to determine the cellular location of BASI.

Protein and RNA Synthesis

BASI protein is first detected in extracts of whole kernels at about 2 WPA (Robertson and Hill, 1989, and Munck *et al.*, 1985). The level of BASI increases to a maximum level at 4 - 5 WPA and then remains constant through to seed maturity. The level of BASI detected in the kernel remains constant during early germination (Chisholm, 1989, and Lecommandeur *et al.*, 1987). Lecommandeur *et al.* (1987) found that between 2 DI and 5 DI the amount of BASI in whole kernels (cv. Menuet) decreased by one third. According to Robertson *et al.* (1989) the accumulation of BASI in the embryo is different than for the whole kernel. These workers reported that rapid synthesis of BASI in the embryo does not occur until 4 WPA. Embryonic BASI increases until about 7 WPA and again remains in the mature kernel. The increase in embryonic BASI follows the increase of ABA in the embryo. The majority of BASI is already synthesised in the endosperm before ABA is produced in the kernel.

BASI mRNA, in immature kernels, is first detected about 4 days post anthesis, peaks at about 2 WPA and is absent by 5 WPA (Robertson, 1989). This fits well with the profile of protein accumulation since RNA accumulation peaks just before the major accumulation of protein and declines at the point where the amount of BASI remains constant through to kernel maturity.

Location of BASI Protein

Extracted RNA and proteins from dissected tissues have given some clues to the location of inhibitor protein in the kernel and young seedling. By examining pearling

fractions of barley, Weselake *et al.* (1985b) were able to determine that BASI was distributed throughout the endosperm of mature kernels. Although it is known that there is BASI in the embryo during development (Robertson *et al.*, 1989), it is not known if BASI is evenly distributed among different embryonic tissues. In experiments examining protein, which was translated *in vitro* from isolated mRNA of barley (cv.Bonus) kernels, Mundy *et al.* (1986) were able to detect BASI in cultured mature aleurone but not in developing aleurone. Lecommandeur *et al.*(1987) have also shown that BASI is located in the aleurone (5%) and endosperm (95%) of germinating barley (cv.Menuet) kernels. Only trace amounts of inhibitor protein were found in the scutellum of 3 and 5 day old seedlings. Lecommandeur *et al.* (1987) also performed immunofluorescent studies on resting and germinating kernels which confirmed that the inhibitor was present throughout the endosperm and showed that it was found between the starch granules. More immunocytochemical work is required to determine the exact location of BASI in specific tissues and cells.

Possible Functions

Deposition of some α-amylase/serine proteinase inhibitors in the endosperm is thought to compensate for the low lysine content of hordein, the major storage protein of barley kernels (Rasmussen *et al.*, 1988). Although BASI is not particularly lysine-rich (4.5 mol.% lysine), production of its RNA transcripts is regulated by single recessive genes, *lys* 3A and *lys* 1, found in high lysine barley cultivars (Rasmussen *et al.*, 1988, and Leah and Mundy, 1989). The *lys* 3a allele of cultivars, 'Riso No.1508' and 'Piggy', exerts

a positive effect on BASI expression, while the *lys* 1 allele of cultivar 'Hiproly', exerts a negative effect. The transcripts of other high-lysine containing inhibitors (eg. CI-1, CI-2, and protein Z) are significantly increased in 'Hiproly' compared to 'CI4362', its low-lysine sister line. This gene regulation may be a consequence of the evolutionary ties of the inhibitory proteins with storage proteins.

Given that BASI strongly inhibits bacterial subtilisins and that it is part of a family of genes that inhibit heterologous enzymes, it is possible that in the past and perhaps today it acts by protecting the kernel from pathogens. However, the fact that it has evolved to inhibit a major hydrolytic enzyme, α-amylase, that is endogenous to barley suggests that it has another important function(s).

The possibility of a role for the inhibitor in preventing preharvest sprouting has been investigated by a number of research groups (Munck *et al.*, 1985, Abdul-Hussain and Paulsen, 1989, Audette, 1990). A heritable relationship between the level of inhibitor production and sprouting susceptibility has not been found. However, environmental conditions affect the levels of BASI within a particular cultivar and this may, in turn, affect sprouting resistance. Robertson *et al.* (1989) have shown that dehydration stress and abscisic acid greatly increase the amount of inhibitor found in barley seedlings. Field data for various wheat cultivars over two years, one with drought conditions, indicated that in the drought year the levels of inhibitor were significantly higher than in the non-drought year (Audette, 1990). Thus BASI may have a role in stress resistance.

One of the most promising suggestions for the *in vivo* role of the inhibitor is that it attenuates the degradation of starch granules by α-amylase upon germination (Hill *et al.*,

1987) . α-Amylase II, which is specifically inhibited by BASI, is produced in large amounts at the time of germination. It is proposed that the inhibitor binds the newly synthesised α-amylase II, but as amylase pours into the system it saturates the inhibitor and proceeds to rapidly degrade the starch granule. The inhibitor may act to attenuate the release of sugars early in germination until the embryo is ready to metabolise them. The fact that BASI is detected throughout the germination process supports a role in germination.

MATERIALS AND METHODS

Plant Material

Barley, *Hordeum vulgare* cv. Bonanza, was grown under a 16 hour day with 18/14°C day/night temperature in a growth cabinet. Heads were tagged when the middle kernels were at mid-anthesis and were harvested at the required time intervals (2, 3, 4, 5, and 6 WPA) for developmental studies. Samples for microscopy were taken from the middle four rows of each head.

Barley grains to be imbibed for germination studies were surface sterilized by rinsing with distilled water twice, washing in 0.2% silver nitrate for 20 minutes, rinsing several times with 0.5M NaCl until the cloudy precipitate disappeared, and rinsing with sterile water twice (McFadden *et al.*, 1988). They were placed in sterile petri dishes containing filter paper soaked with sterile water and incubated at room temperature in the dark. Each day the average coleoptile length was determined (Day 1 : 2.5-3mm measured from root base, Day 2 : 4-6mm, Day 3 : 1.3-1.5cm, Day 4 : 3.2-3.5cm) and a number of samples with coleoptiles of this length were removed for fixation/embedding. Samples of mature grains were sterilized and imbibed for 3 hours.

Antibody Stock

Antibody Serums

Lab stocks of polyclonal anti-α-amylase (Doug's Rabbit A), anti-inhibitor (Rabbit H), and non-immune (Rabbit T) serums were used for this research. The protein specificity of the antibody serums, tested by western analysis, has been previously documented

(Robertson and Hill, 1989). The anti-inhibitor serum is specific for a single protein of identical size to the α-amylase inhibitor protein. The anti-α-amylase serum is specific for α-amylase but does not distinguish between the high and low pI isozymes.

The concentration of antibodies in the crude serums was found, in preliminary studies, to be too low to adequately show ICC signal above the background level of silver-enhanced immunogold. Thus, further purification of the antibody was necessary. Immunoglobulins were first purified by Protein A-sepharose affinity chromatography. Anti-α-amylase immunoglobulin was further purified by affinity chromatography on purified α-amylase to obtain an enriched preparation of anti-α-amylase antibody.

Protein A-Sepharose Purification

Immunoglobulins (mainly IgG) were purified on a Protein A-sepharose 4B (Pharmacia) column according to Goding (1976). The elution of proteins from the column was monitored using a Hewlett-Packard diode array spectrophotometer (model 8452A). A wavelength of 280 nm was used to detect the protein. After allowing 1 ml crude serum to run into the gel, the column was washed with PBS (0.02M sodium phosphate, 0.13M NaCl, 0.005% merthiolate, pH 7.4) until the peak of unbound proteins returned to the baseline. The immunoglobulins were eluted with elution buffer (0.15M NaCl, 0.58% glacial acetic acid, about pH 3). The peak was collected in a tube containing 0.5M Tris pH 8 to rapidly neutralize the pH of eluted solution. The antibody was dialysed over night in 20mM Tris pH 7.5.

Affinity Purification

An α-amylase-sepharose 4B column was prepared according to the manufacturer's instructions (Pharmacia). Six milligrams of α-amylase (courtesy of Dr. A.W. MacGregor, Canadian Grain Commission) were coupled to 2 mls of swollen sepharose 4B gel. Protein A purified anti-α-amylase antibody was run into the affinity column in 1 ml aliquots. The column was stored at 4°C for at least a half hour between aliquots. The column was washed with 0.02M Tris-HCl, pH 6.5/0.5M NaCl and anti-α-amylase antibody was eluted with alkaline dioxane buffer (0.1M phosphate buffer, pH 12 and 10% dioxane)(Higgins *et al.*, 1982). The eluent was kept on ice and quickly titrated to pH 7 with HCl. The antibody was immediately dialysed against 20mM Tris-HCl, pH 7.5, over night.

Quality and Quantification

After each purification step a quick protein assay (Bradford, 1976) was done to determine the concentration of antibody. Samples of antibody from before and after purification were run on a 15% polyacrylamide stacking gel (Laemmli, 1970). Part of the gel was silver stained (Merril *et al.*, 1981) to ensure that the proteins were intact and that the protein-A sepharose column had removed most of the non-immunoglobulin protein. Proteins in the remaining gel were electroblotted onto nitrocellulose and horseradish peroxidase linked goat anti-rabbit IgG (BioRad) was used to detect the antibody. This confirmed that the purified samples contained a much higher concentration of immunoglobulin than did crude serum.

Immunocytochemistry Controls

Non-immune antibody (i.e. from an uninoculated rabbit) was the negative control in all experiments. Immunoglobulins were purified from non-immune serum in the same manner as the anti-α-amylase and anti-inhibitor antibodies by passing the serum over a Protein-A sepharose column. Sections from each sample of seed tissue were incubated with anti-α-amylase, anti-BASI, and non-immune antibody.

Initially, some slides were incubated with secondary (2°) antibody only to ensure that the 2° antibody was not causing background. Since this was never a problem, this control was not used regularly.

An immuno-adsorbed control (Gubler *et al.*, 1986), which required pre-adsorbing anti-inhibitor antibody with inhibitor before using the antibody for ICC experiments, reduced the inhibitor signal. However, the same control strategy for α-amylase did not reduce α-amylase signal. This resulted from an inability to determine the appropriate amount of antigen needed to completely block all of the antibody, because the affinity purified anti-α-amylase stock, compared to the anti-BASI immunoglobulin stock, is enriched for anti-α-amylase.

Hutson *et al.* (1979) have argued that preadsorption of antibody with antigen is not a definitive control for detecting cross reactive tissues. Theoretically, saturation of all antigen binding sites with the primary antigen before use in ICC will also reduce the binding of the antibody to cross reactive tissue. Therefore, false positives may result from this control experiment. Hutson *et al.* (1979) suggest that physiological controls (ie. similar tissues that do not contain the protein of interest for ICC) are necessary to

detect cross reactive tissues. Since there are no homologues of BASI in maize, signal should not appear when anti-inhibitor antibody is used in ICC of maize kernels. This was indeed the result that was obtained when the experiment was done with cryosections of 2 DI maize kernels.

Immunocytochemistry and Optical Microscopy

Embedding and Sectioning Tissues

Fresh grain samples were quickly cut in half longitudinally along the ventral crease, placed cut side down in a plastic mould, covered with an embedding media for frozen tissue (Tissue Tech OCT compound, Miles Inc, Elkhart, Indianna) , and frozen in liquid freon. Sample blocks could be stored at -70°C for weeks. A Cryocut E cryostat (Reichert-Jung) was used to cut 8 μm frozen sections. The sections were thaw mounted onto clean uncoated slides and immediately placed on a 30 - 45°C hot plate to quickly dehydrate tissue. The slides were then placed directly into fixative (2% paraformaldehyde, 0.5% glutaraldehyde, 30mM PIPES/pH 7.5, 10mM $CaCl_2$ (Pogson *et al.*, 1989)) and fixed for 7 minutes at room temperature. The sections were dehydrated in a series of 50%, 70%, and 95% EtOH washes (5 minutes each at room temperature), and heated over night in a 37° oven. The dehydrated cryosections remained in good condition for weeks provided they were kept dry. For each sample time, 6 OCT blocks of grain halves were embedded and about a dozen slides per block with at least 3 good sections per slide were cut.

Immunocytochemistry

The slides were blocked in PBS (10mM sodium phosphate buffer/pH7.2, 0.15M NaCl (Gubler *et al.*, 1986) and 0.2% triton-X-100) and 5% PM (powdered milk, Carnation) for 40 minutes at RT. About 50 μl of primary antibody solution (40 μg/ml antibody,PBS,1% PM) was placed on each slide and covered with a coverslip to prevent evaporation. Affinity purified anti-α-amylase stock and anti-inhibitor immunoglobulin stock were used. The slides were placed in a humid chamber at room temperature and incubated for 2-4 hours. The sections were washed 4-6 times in PBS/1%PM for a total of 1 hour. The sections were incubated with secondary antibody (1/100 dilution of 5nm gold conjugated goat anti-rabbit IgG (Dimension labs.), PBS, 1% PM) as outlined for the primary antibody. The slides were again washed 4-6 times in PBS/1%PM for a total of 1 hour. The last 2 washes were without PM. The tissues were dehydrated through a short EtOH series (50%,70%,90%,95% ethanol, 5 minutes each).

Silver Enhancement

SilvEnhance-LM kits (Dimension Laboratories Inc.) were used to make the sites of immunogold binding visible under the light microscope. A 1:1 ratio of initiating solution and enhancing solution was mixed immediately before use. About 100μl of this mixture was placed directly on each dehydrated slide. The slides were carefully watched to ensure that the sections were covered with solution at all times. Numerous test slides were necessary to determine the maximum incubation time allowed before background began to appear. A new kit allowed between 12 - 15 minutes of incubation but this time

decreased to about 8 minutes after two weeks and continued to shorten as the kit aged. After the appropriate time of incubation the slides were washed in a few changes of distilled water to halt the reaction and mounted in 70% sucrose.

Photomicroscopy

An average of 25 sections of each sample date (ie. 2 WPA - 4 DI) were screened per antibody (ie.anti-amylase, anti-inhibitor, or non-immune). Representative photographs of each seed tissue were taken. After printing, each of the photos was rechecked with the sample sections to ensure that it was indeed representative of the tissue for that particular sample date.

The ICC slides were not stained, except for silver-enhancement of the immunogold signal, to prevent confusion as to what was true signal in the photomicrographs. Instead, the sections were photographed using different types of microscopy. Prints of the identical tissue, photographed with different optics, were perfectly aligned in figures 2 - 24 to illustrate different features of the tissue. Bright field microscopy was used to photograph α-amylase and inhibitor signal. Differential interference contrast was used to show tissue morphology. Polarizing microscopy was used to show the location and size of the starch granules.

Two different light microscopes were used to analyze the sections and prepare the micrographs: a Leitz Orthoplan microscope illuminated with a 100w tungsten lamp (figures **3-12**) and a Zeiss Photomicroscope III with a 100w halogen lamp (figures **13-25**).

Technical pan film was used to photograph the sections in both bright field and polarizing microscopy. A green filter was used in the bright field microscopy to enhance the brown colour of the silver-enhanced immunogold. A polarizing filter and analyzer were used for the polarizing microscopy. The film was developed in Kodak D19 for 4 minutes at 20°C and fixed in Kodak Rapid fix for 2-3 minutes.

Plus-X-pan film was used to photograph in differential interference microscopy (DIC). The Zeiss microscope had a Nomarski system of DIC while the Leitz microscope used Jamin/Lebedeff optics. The film was developed in a 1:1 dilution of Kodak D76 : water for 7 minutes at 20°C and fixed in Kodak Rapid fix for 2-3 minutes.

All black and white negatives were printed on Kodak Polycontrast III resin coated paper. The paper was quickly developed in an automatic film developer and, within one day, was permanemtly fixed by washing in water for 1 minute, Kodak rapid fix for 2 minutes, in water for another minute, and then air drying.

A slide micrometer was photographed for every objective/ocular combination used on each microscope. The appropriate micrometer negatives were printed along side the tissue negatives and used to determine the magnification bars on the photographs.

Immunocytochemistry and Electron Microscopy

Embedding and Sectioning Tissue

Fresh grain samples were cut in half longitudinally in fixative and groups of 10 grain halves were placed in 10 ml vials containing fixative (2% paraformaldehyde, 0.5% glutaraldehyde, 30mM PIPES buffer/pH 7.5, 10 mM $CaCl_2$ (Pogson *et al.*,1989)). Vials

were degassed for an hour. Fixative was replaced and the vials were degassed for another hour. Samples were fixed O/N at 4°C. Dehydration and embedding of the samples were essentially as described in Lending and Larkins (1989). Fixed tissues were dehydrated by washing as follows : 3 times for 5 minutes in phosphate buffer, twice for 5 minutes in sterile water, twice for 10 minutes in each of 10%, 30%, 50% EtOH, twice for 20 minutes in 70% EtOH, and twice for 10 minutes in 100% EtOH. Tissues were then infiltrated with LR white resin, medium grade (London Resin Co.), as follows : 2 hours in 30% LR white (in 95% EtOH), 2 hours in 60% LR white, and three days in 100% LR white at 4°C. Vials were on a rotator for the last 2 steps of the dehydration and all of the infiltrating steps. The half grains were arranged face down in the bottom of a small tin plate and a second tin was carefully layered on top of the first to exclude oxygen. Plates were incubated at 55°C over night to harden the resin.

Individual grains were cut from the resin blocks with a jig saw and mounted , using epoxy glue, on top of resin blocks that had been cast in beam capsules. Excess plastic was removed using a hand held grinder (Dremel). A small trapezoid block face was cut using a TM60 block trimmer (Reichert). Initially, 2μm sections were cut on a Porter-Blum JB-4 microtome (Sorvall) and viewed under a light microscope to pinpoint the desired tissue. The block face was then cut even smaller to about 1mm squared. Ultrathin sections (silver-gold, 80 - 120nm thick) were cut on the ultramicrotome (Reichert ultracut) and mounted on nickel grids.

Immunocytochemistry

The protocol is a modified version of Rasmussen et.al. (1990). The grids were placed in the wells (2 ml) of a culture plate and blocked with 1ml PBS (10mM sodium phosphate buffer/pH7.2, 0.15M NaCl, and 0.2% triton-X-100)/ 5% PM (powdered milk) for 10 minutes. They were incubated with 100μl of 1°antibody (40 μg/ml 1° antibody, PBS, 1%PM) for 3 hours and washed with PBS/1%PM 4 times for 5 minutes. Immunoglobulin stocks of both anti-α-amylase and anti-BASI were used. The grids were then incubated with 100 μl of 2° antibody [1/50 dilution of 15 nm goat anti-rabbit colloidal gold (Dimension labs.), PBS, 1% PM] for 1 hour and washed for 5 minutes in PBS/ 1% PM, 3 times 5 minutes in PBS, and twice in distilled water. The grids were then air dried. The lid for the culture plate was fitted with a moist filter paper and used to cover the plate during the incubations with both 1° and 2° antibodies.

Heavy Metal Staining

Grids were stained with uranyl acetate and lead citrate after immunocytochemistry was completed (Hotz, 1991). The grids were floated sample side down on uranyl acetate stain (5% uranyl acetate in 50% methanol) for 30 minutes. They were then rinsed in 50% methanol, washed in 50% methanol for 30 minutes, rinsed in distilled water, and washed in distilled water for 30 minutes. The grids were stained in lead citrate solution (Renold, 1963) for 10 minutes in a CO_2 free environment (petri dish with KOH pellets). They were then rinsed, washed in boiled CO_2 free distilled water for 30 minutes and air dried.

Photomicrography

A Hitachi H-7000 transmission electron microscope, courtesy of the Botany/Microbiology/Zoology departments of the University of Manitoba, was used to view and photograph the sections. Kodak electron microscope film (ESTAR thick base) was developed in D19 diluted 1:2 with water for 4 minutes at 20°C. Negatives were printed as outlined for the light microscopy negatives.

Cytological Staining

Toluidine Blue-O

Toluidine blue O was used to stain test sections of plastic embedded tissues prior to trimming blocks for the ultramicrotome. LR White sections (2μm) were stained in a filtered solution of 0.05% toluidine blue O in benzoate buffer pH 4.4 (0.125% benzoic acid, 0.145% sodium benzoate) at room temperature (O'Brien and McCulley, 1981) for about 1 minute followed by a brief destaining in water. The slides were breathed on twice (water needed for metachromasia) before mounting in 70% sucrose.

Periodic Acid/Schiff's

The periodic acid/Schiff's (PAS) reaction was used to stain sections for starch. A modified method from 0'Brien and McCulley (1981) was used. Free aldehydes in the sections (2μm LR White embedded or 8μm cryosections) were blocked over night at room temperature in a saturated solution (2.5 g in 500 mls, heat then filter) of dimedone. The slides were rinsed in running water for 10 minutes, were placed in 1% periodic acid

solution for 10 minutes to oxidize the sections and were rinsed in running water for 5 minutes. They were then placed in Schiff's reagent (Sigma) for 5 minutes and immediately transferred through a solution of 0.5% sodium metabisulfite/1%conc.HCl 3 times for 2 minutes each. The slides were rinsed in running water for 5 - 10 minutes and dehydrated through an alcohol series.

Aniline Blue Black

The PAS slides were counter-stained with 1% aniline blue black (C.I.20470 MCB)/7% acetic acid at 55°C (Fisher,1968). LR white sections (2μm) required a staining time of 3 minutes. Cryosections (8μm) required only 15 seconds of staining in aniline blue black. The sections were destained briefly in 5% acetic acid and were mounted in glycerol/5% acetic acid.

Photography

Kodak Ektachrome 160 ASA slide film was used for Colour photography. The film was commercially processed. Colour prints were made to match the true colours of the slides. Colour photocopies were made of the original plates.

RESULTS

Protein Bodies and Inclusions

The most prominent storage organelles in scutellar cells at kernel maturity were protein bodies. At 1 DI (figure **2A**), the majority of protein bodies were still densely packed but some protein degradation was apparent. By 3 DI (figure **2B**), most of the protein was degraded and the individual protein bodies had merged together to form large vacuoles. (note: Table 1 - Summary of Results, page 95)

The aleurone of 1 DI kernels (figure **2C**) was packed with protein bodies and inclusions. Although Maltese crosses appeared when the aleurone was viewed using polarizing microscopy (figure **2D**), it was probably the crystalline structure of the type I inclusions (Morrison *et al.*, 1975) that rotated the polarized light, not starch granules. The majority of inclusions in **2C** coincide with the Maltese crosses in **2D**. Starch granules were not detected in the aleurone of 1 DI kernels (figure **2C**).

Cellular Location of BASI, α-Amylase, and Starch Granules

Developing Embryo

General Location α-Amylase inhibitor was found to be present in all tissues of the developing embryo except the radicle, seminal roots and the plumule. The location of inhibitor signal in the scutellum, coleorhiza, and coleoptile is illustrated in Figure **3A** - a representative section of an embryo at 3 weeks post anthesis (WPA). Figures **3B-3D** show the morphology of the embryo presented in Figure **3A**.

Polarizing microscopy was used to detect the location of starch granules in a

cryosection of a 3 WPA barley kernel (figure **4A**). The large starch granules of the endosperm appeared as bright circles that are dissected by dark crosses. The small starch granules of the embryo appeared as faint white speckles. Vascular tissue is observed in the radicle. Silver-enhanced gold particles may also be identified with the use of polarizing microscopy. Figure **4B** illustrates the location of both starch granules and BASI signal in 3 WPA cryosections (same section as shown in Figure **3A**). BASI signal was observed in exactly the same embryonic tissues as those in which starch granules were observed.

α-Amylase signal was not detected in embryonic tissues during kernel development.

Specific Changes in the Scutellum Starch granules (less than 1 μm) began to appear in the basal scutellum at 2 WPA (figure **5**:1b) and increased in size (to 3 - 4 μm) until 4 WPA (figures **5**:2b and **6**:1b). The granules appear as raised 'hills' or depressed 'valleys' on the relief-like presentation of DIC photomicrography. Starch granules (2 μm or less at 5 WPA) were broken down during the later part of development (figure **6**:2b). At maturity, the granules were not detected in the basal scutellum (data not shown).

BASI was first detected at 2 WPA (figure **5**:1a). The quantity of inhibitor increased until 4-5 WPA (figures **5**:2a and **6**:1a&2a) and then remained constant through to maturity. BASI appeared to be dispersed throughout the scutellar parenchyma cells during development.

In the embryo, α-amylase inhibitor signal was most intense and starch granules became the largest in the basal scutellum both during development and germination. The

increase in both BASI and starch over the 2 - 4 WPA period of kernel development is illustrated by polarizing microscopy (figures **5**:1c&2c and **6**:1c). The decrease in starch granule size in 5 WPA kernels is reflected by the reduced intensity of figure **6**:2c.

Embryo Upon Germination

Specific Changes in the Scutellum Upon germination, starch granules began to accumulate throughout the scutellum. The starch granules were very small (1 - 2 μm) at 1 DI (figure **7**:1b&2b) but grew to become the major cellular component (3 - 5 μm) by 3 DI (figure **9**:1b&2b). At 4 DI (figure **10**:1b&2b), starch granule (now less than 3 - 4 μm) breakdown was observed in barley scutellum.

BASI was found to be dispersed throughout the scutellum prior to imbibition. However, upon germination the inhibitor became increasingly associated with starch granules. At 1 DI (figure **7**:1a), inhibitor signal in the basal scutellum appeared as it did at maturity (data not shown, identical to 5 WPA, figure **6**:2a). Over the second and third days after imbibition, the location of inhibitor signal changed from being dispersed throughout the cell (figure **7**:1c) to being adjacent to starch granules (figures **8-9**:1c). The overall intensity of the signal appeared to remain constant (figures **7-9**:1a). At 4 DI (figure **10**), the intensity of BASI signal appeared to diminish.

Although α-amylase was not detected in the embryo during development, it appeared during the germination process in all of the same tissues in which the α-amylase inhibitor and the starch granules were found. α-Amylase signal was first observed in the basal scutellum of 2 DI kernels (figure **8**:2a) and increased in intensity as seedling growth

continued (figures **9-10**:2a). α-Amylase signal appeared to be associated with starch granules (figures **9-10**:2c).

BASI was only detected in scutellar epithelium cells at maturity (figure **17**) and at 1 DI (figure **18**). Neither during development nor germination was α-amylase detected in the scutellar epithelium.

Coleoptile Starch granules were evenly distributed throughout the coleoptile. A wide range in the size of starch granules was observed at any one time. During germination, the size of starch granules increased (figure **12**). The largest starch granules ranged from 2 μm (at 1 DI) to 4 μm (at 4 DI).

BASI was first detected in the developing coleoptile at 3 WPA in areas of starch granule accumulation (figures **3A** and **4B**). BASI was present throughout development and germination in coleoptile tissue, in close association with the starch granules. In 2 DI coleoptiles, inhibitor signal intensity increased from coleoptile tip to base (figure **11**:2a&3a). Signal was not detected at the tip of the coleoptile but was observed around starch granules (2 μm or less) in tissues proximal (1mm) to the tip (figure **11**:2b,c). Maximum signal occurred about half way (2mm) to the base of the coleoptile (figure **11**:3b,c). Inhibitor signal intensity at the base of the coleoptile remained constant from 1 DI to 4 DI (figure **12**:1a & 2a).

α-Amylase first appeared in the coleoptile at about 4 DI (figure **12**:3a). α-Amylase, like the α-amylase inhibitor, was directly associated with the starch granules (figure **12**:3b &c).

α-Amylase inhibitor signal was also observed to surround starch granules in roots after 2 DI (data not shown). BASI intensity was much lower in roots than in other starch bearing tissues of the developing seedling.

Developing Endosperm and Aleurone

α-Amylase inhibitor began to accumulate in the endosperm tissue at about 2 WPA. The quantity increased until 4 - 5 WPA, then remained constant until mature kernels were imbibed. At 2 WPA, the inhibitor signal was just visible in the endosperm (figure **13**:1b, 3b & 4b - note: data in original prints but lost in thesis plates during processing). Starch granules were still small, especially near the scutellar-endosperm interface (figure **13**:1a). By 3 WPA, the inhibitor signal was readily distinguishable (figure **14**:1b,3b,and 4b)and the starch granules were larger (figure **14**:1a). From 4 WPA through to maturity, the intensity of the prominent α-amylase inhibitor signal changed little (figures **15-16**:1b,3b,4b). At maturity, the starch granules were tightly packed in the endosperm cells (figure **17**:1a,2a,3a & 4a). BASI was found uniformly throughout the endosperm tissue.

α-Amylase inhibitor was first detected in the aleurone at 2 WPA (figure **13**:3b & 4b). BASI was most predominant on the dorsal side of the kernel near the embryo (figure **13**:4b). The level of inhibitor signal gradually increased in the aleurone from 2 to 5 WPA (figures **13-16**:3b,4b). The aleurone on the dorsal side was always significantly darker than on the ventral side of the kernel. The quantity of inhibitor signal on the ventral side of the kernel continued to increase until the level of signal was roughly equal

on both sides of the kernel at maturity (figure 17:3b). BASI was not detected in the cell wall or in the nuclei of aleurone cells (figure 17:4a & b).

It should be noted that any dark speckling in the bright field photographs of the aleurone was probably artifactual. Tiny air bubbles were occasionally trapped in the aleurone and the silver appeared to bind to them. An example of this artifact is shown in the control photographs of figure **14** (2a,b).

α-Amylase was not detected in the endosperm or aleurone during kernel development.

Endosperm and Aleurone During Germination

Upon germination, there was an increase in immunocytochemical signal for both α-amylase and α-amylase inhibitor in the endosperm located immediately below the scutellum and beside the aleurone which was adjacent to the embryo. The fine line of signal that appears at 1 DI (figures **18** and **19**) spread toward the central and distal portions of the kernel as germination proceeded (figures **20** - **25**).

At 1 DI, the signal in the endosperm tissue of sections incubated with anti-amylase and in sections incubated with anti-inhibitor were remarkably similar. One subtle difference was that the line that marks the front of α-amylase moving from the scutellum into the endosperm (figure **19**:1b) was crisper than the line of inhibitor signal (figure **18**:1b). In both cases there was more signal in the endosperm along the ventral side than in the endosperm on the dorsal side of the kernel (figures **18-19**:3b & 4b). At 2 DI, the intensity of the signal for both α-amylase and α-amylase inhibitor had significantly increased but the front of signal had not proceeded much further into the endosperm

(figures **20-21**:1b,3b & 4b). DIC microscopy of 3 DI tissues showed that starch granules were being broken down in the areas where α-amylase signal was first located upon imbibition (figures **22**:1a & 3a). By 3 and 4 DI, there was movement of the signal towards the distal and central portions of the endosperm (figures **24-25**:1b,2b,3b & 4b).

α-Amylase signal began to appear in aleurone cells during imbibition of the kernels (figure **19**:3b & 4b). Both α-amylase and α-amylase inhibitor signal continued to be present in the aleurone cells throughout germination (figures **20-25**:3b & 4b).

Subcellular Location of BASI

The location of the α-amylase inhibitor within the cells of endosperm tissue at 3 WPA and basal scutellar tissue at 1 DI was observed using ICC treated grids viewed by transmission electron microscopy. The gold particles, which represent antibody attachment to the inhibitor, were predominant in the protein bodies of both tissues (figure **26A**). Although some gold was found in inclusions within the protein bodies, the majority was observed in the granular protein matrix that surrounds inclusion deposits. Some gold particles were observed in the cytoplasm, but a more detailed study will be necessary to determine if this observation is significant.

FIGURE 1 : Cryosections of developing and germinating barley kernels stained with periodic acid/Schiff's (PAS) and aniline blue black (ABB) : (**A**) 4 WPA kernel, (**B**) 2 DI kernel. Areas highlighted in the following figures were labelled: figures **5-10** (□), figures **11-12** (ⓒ), figures **13-23** (○+⬭), and figures **24-25** (○+ⓓ).

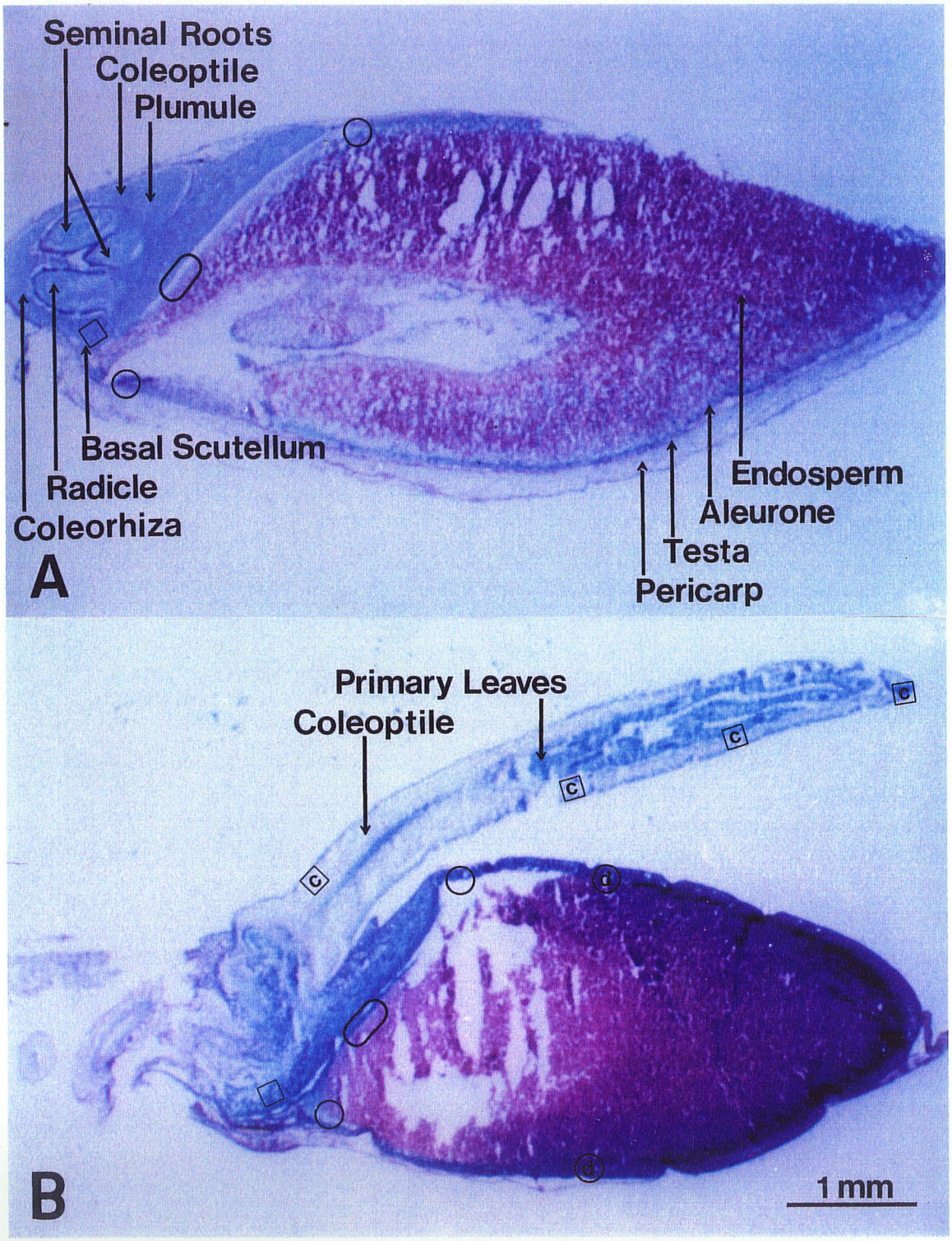

Seminal Roots
Coleoptile
Plumule
Basal Scutellum
Radicle
Coleorhiza
A
Endosperm
Aleurone
Testa
Pericarp
Primary Leaves
Coleoptile
c
c
c
c
d
d
B
1 mm

FIGURE 2 : Scutellum and aleurone cells of germinating barley kernels: (**A**) Scutellum of a 1 DI kernel, (**B**) Scutellar parenchyma cells of a 3 DI kernel, (**C**) Aleurone cells of a 1 DI kernel, (**D**) Aleurone cells of a 1 DI kernel (as in **C**) photographed using polarizing microscopy. Sections of LR-white embedded kernels were stained with periodic acid/Schiff's (PAS) and aniline blue black (ABB). Parenchyma (P) and epithelial (Ep) cells as well as endosperm tissue (E) are labelled in (A). Examples of nuclei (N), starch granules (SG), protein bodies (PB), vacuolated protein bodies (V), inclusions (I) within protein bodies, and cell walls (CW) are labelled.

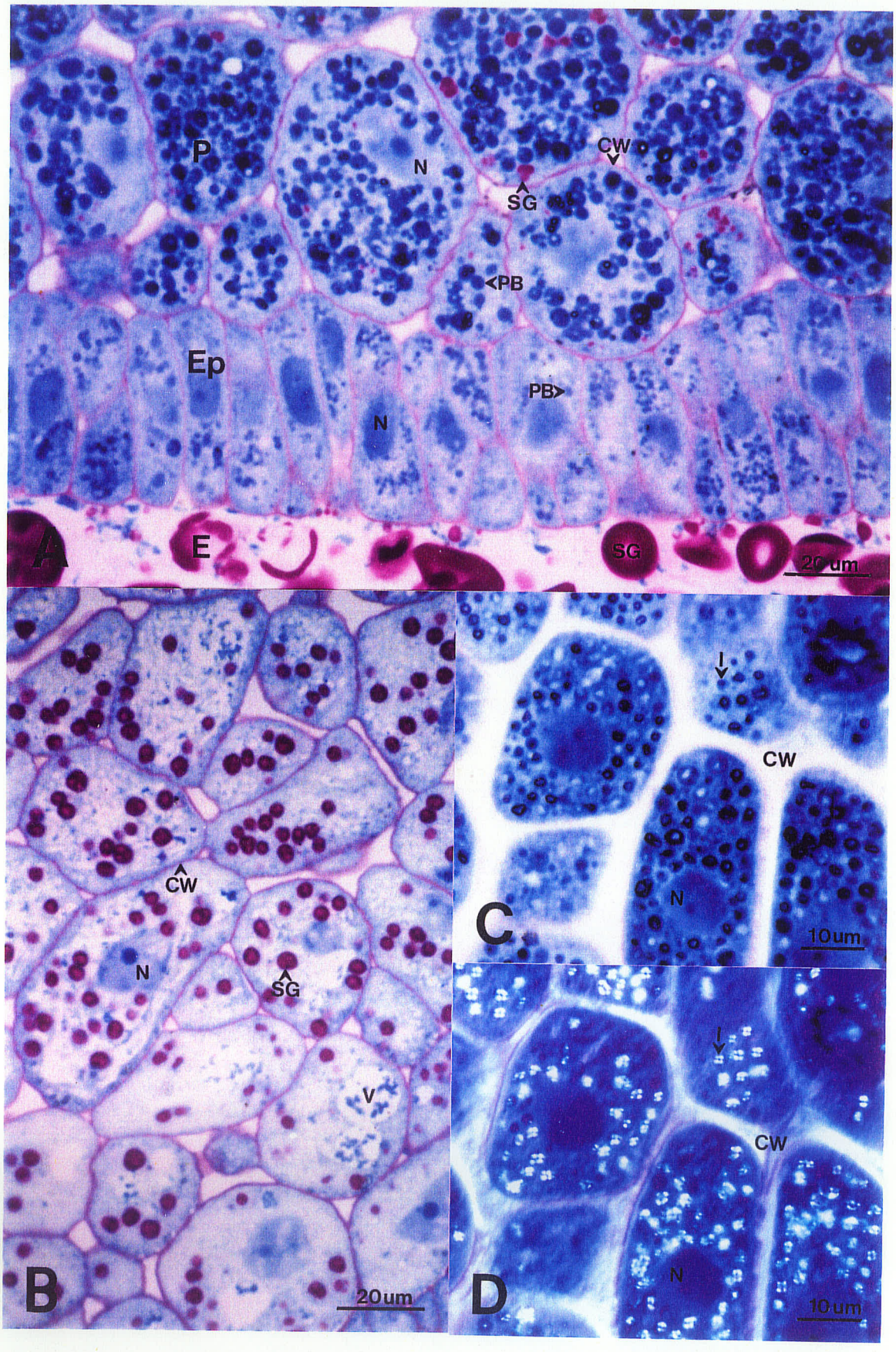

P
N
CW
SG
PB
Ep
PB
N
A
E
SG
20 um
B
CW
N
SG
V
20um
C
I
CW
N
10um
D
I
CW
N
10um

FIGURE 3 : Immunolocalisation of barley α-amylase/subtilisin inhibitor (BASI) in a developing embryo. (**A**) Bright field microscopy of immunolocalised BASI in a 3 WPA embryo. (**B,C,D**) Differential interference contrast microscopy of enlarged areas of the embryo in (**A**) : (**B**) Radicle (R), Seminal root (SR), and coleorhiza (Cr), (**C**) Tip of plumule (P) and coleoptile (Cp), and Scutellum (Sc), (**D**) Apex Scutellum (Sc). The corresponding areas in (**A**) are indicated with large arrows from (**B,C,D**).

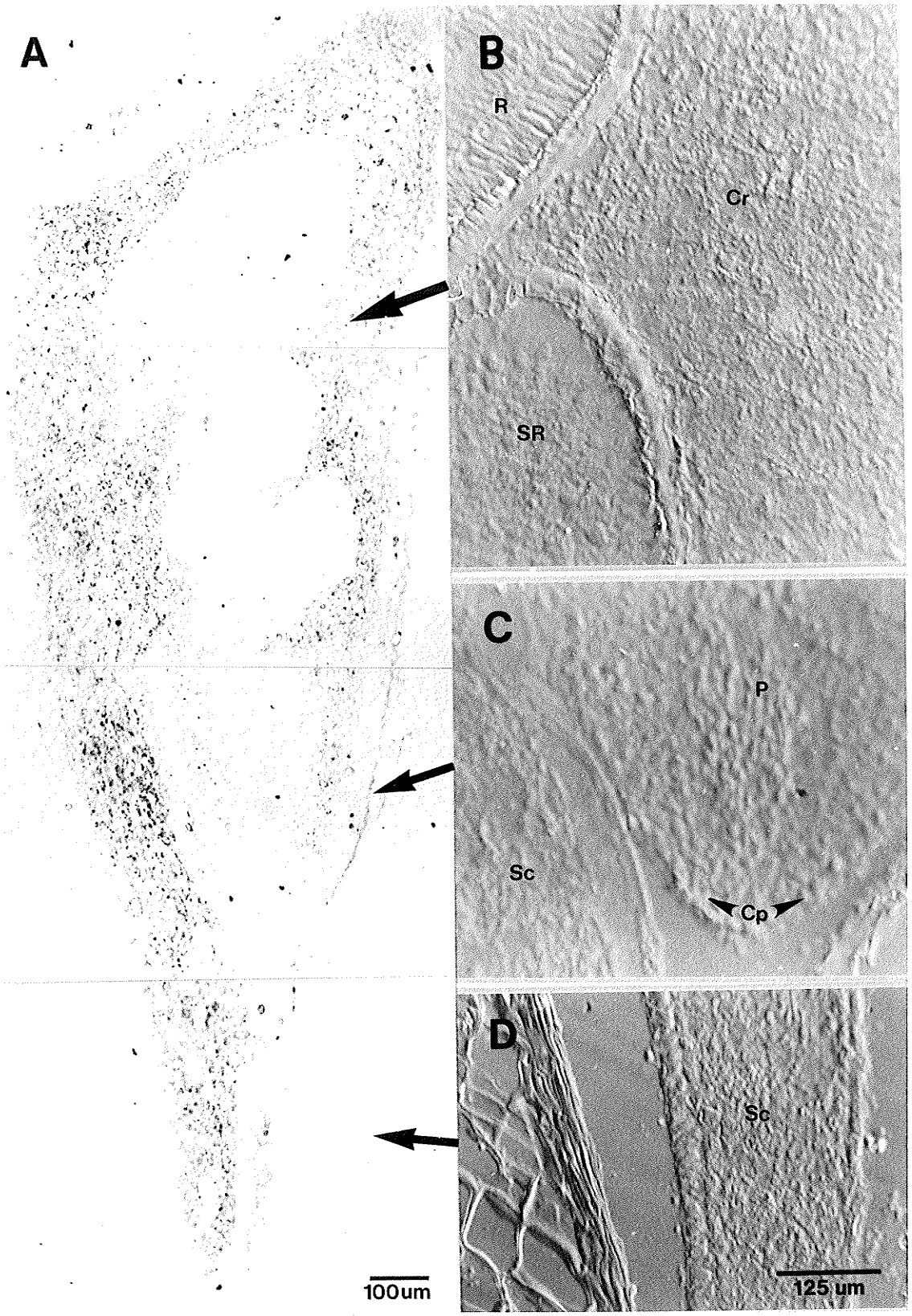

A
B
R
Cr
SR
C
P
Sc
Cp
D
Sc
100um
125 um

FIGURE 4 : Localisation of starch granules and BASI in a developing embryo using polarizing microscopy. Both starch granules and silver (ICC enhancer) are capable of rotating polarized light. (**A**) Starch granule localisation in a 3 WPA embryo treated with non-immune (control) antibody. (**B**) BASI and starch granule localisation in a 3 WPA embryo treated with anti-BASI antibody (identical to embryo in figure 2:A). Examples of starch granules (SG), vascular tissue (V), and BASI signal (S) are labelled. Endosperm (E) is also labelled.

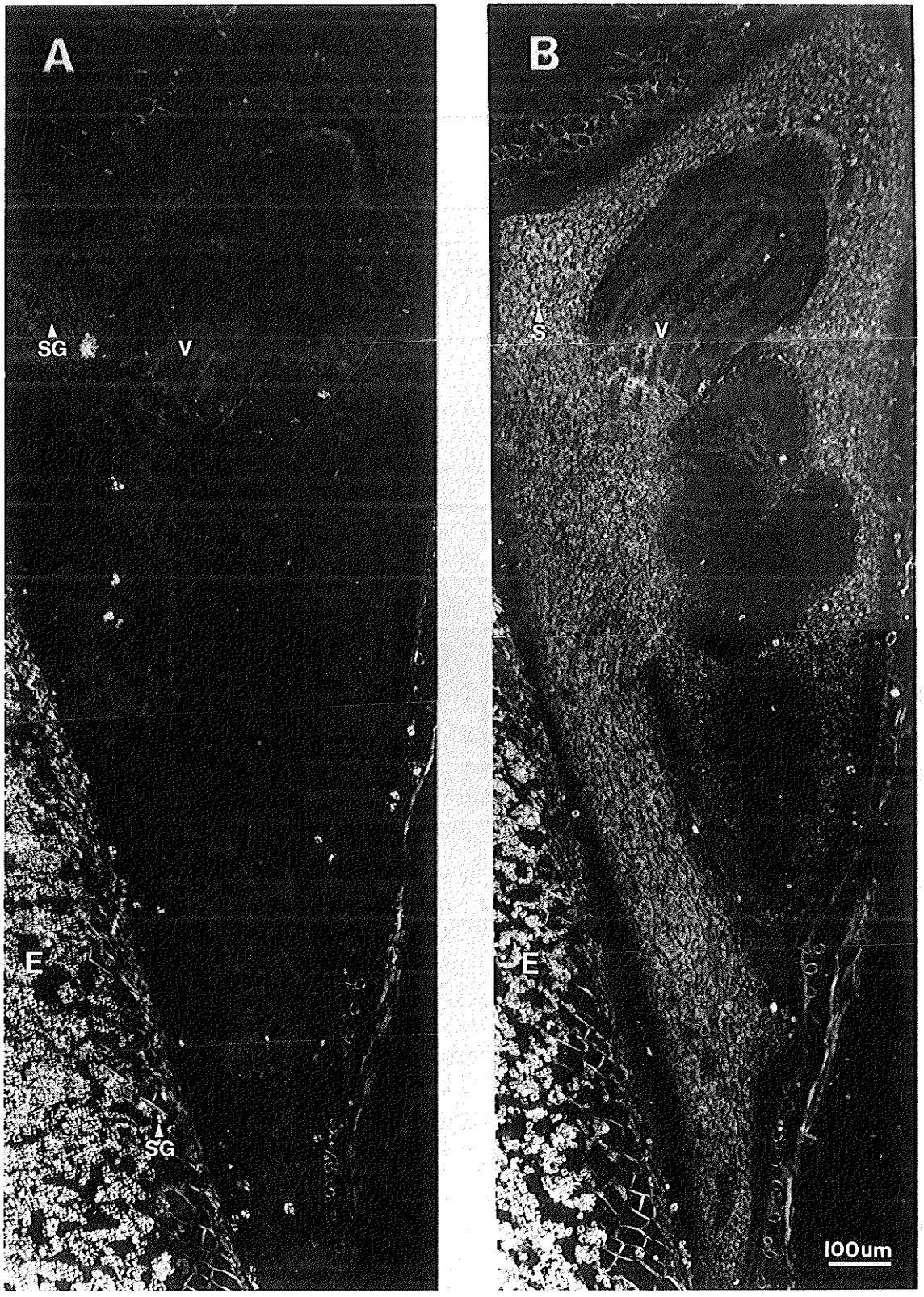

A
SG
V
E
SG
B
S
V
E
100um

FIGURE 5 : Localisation of BASI and starch granules in the basal scutellum of (**1**) 2 WPA and (2) 3 WPA embryos. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. (**2d**) A 3 WPA embryo treated with non-immune antibody is included as a control. Examples of starch granules (SG) are labelled.

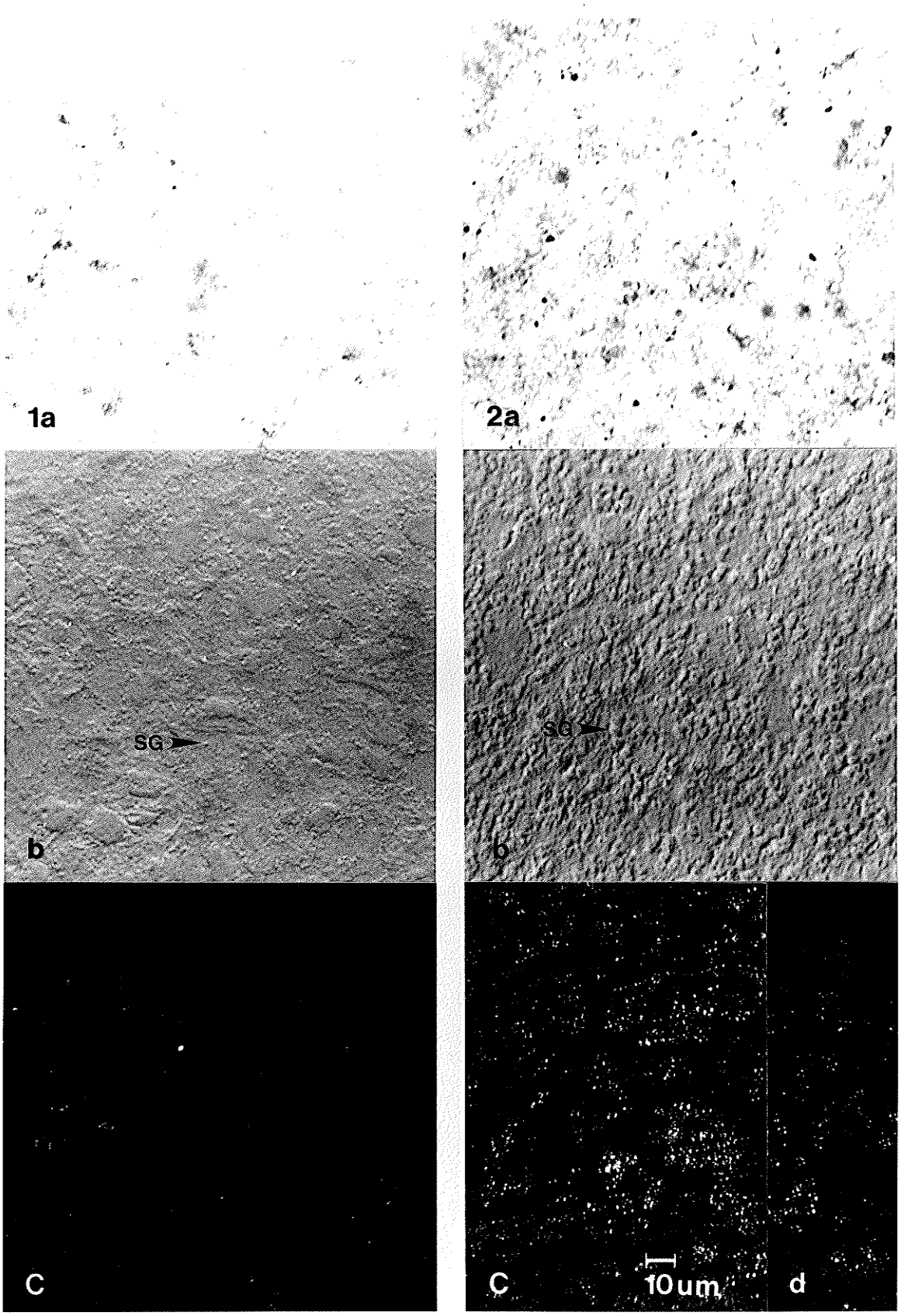

1a
2a
SG
b
SG
b
C
C
10um
d

FIGURE 6 : Localisation of BASI and starch granules in the basal scutellum of (1) 4 WPA and (2) 5 WPA embryos. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. Examples of starch granules (SG) are labelled.

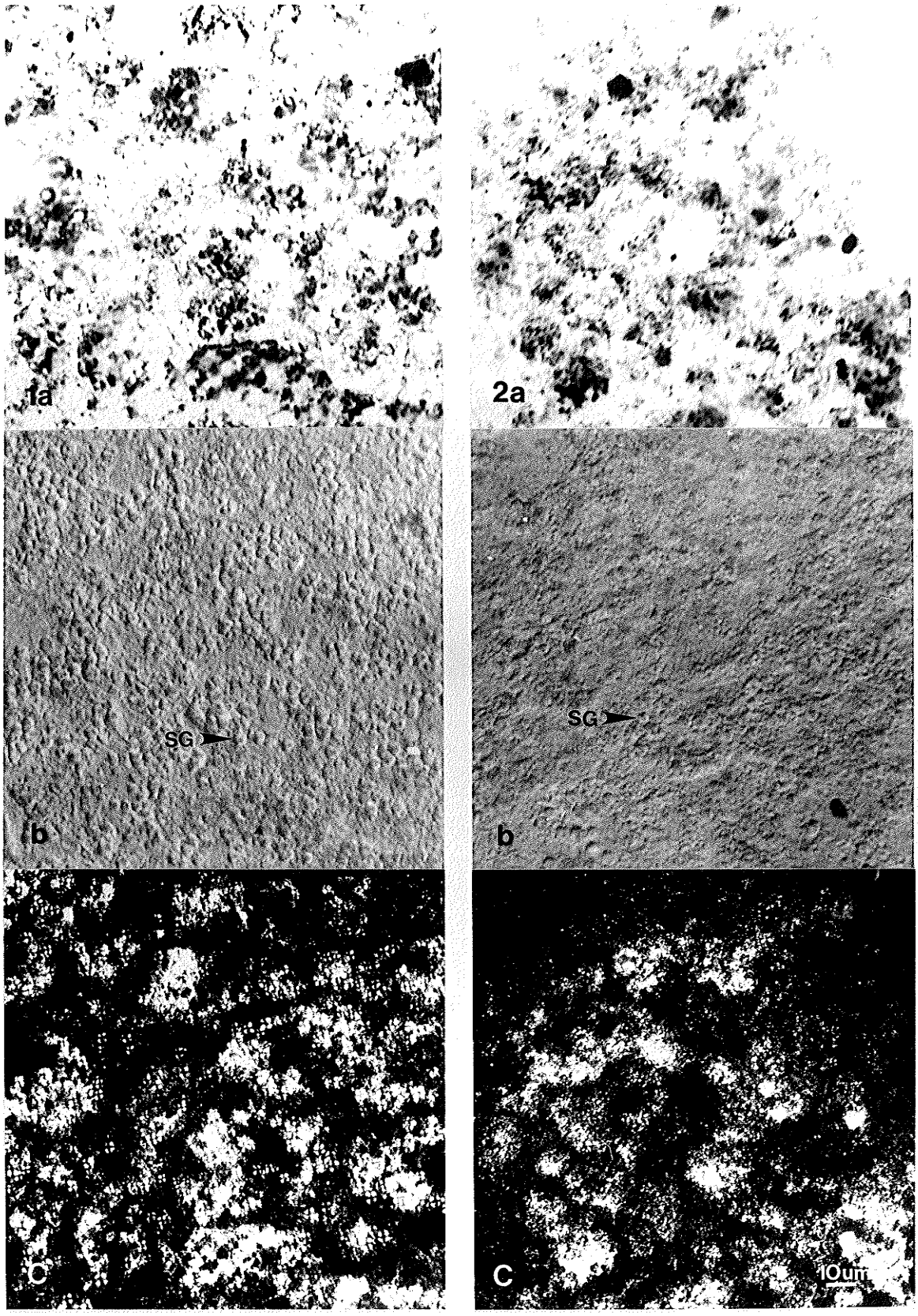

1a
2a
SG
SG
b
b
C
C
10um

FIGURE 7 : Localisation of (1) BASI and starch granules in the basal scutellum of 1 day imbibed (DI) embryos. (2) 1 DI embryos treated with non-immune (control) antibody are completely devoid of signal. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. Arrows point to corresponding areas (**1a,b,c**) containing BASI signal. An example of a starch granule (SG) is labelled.

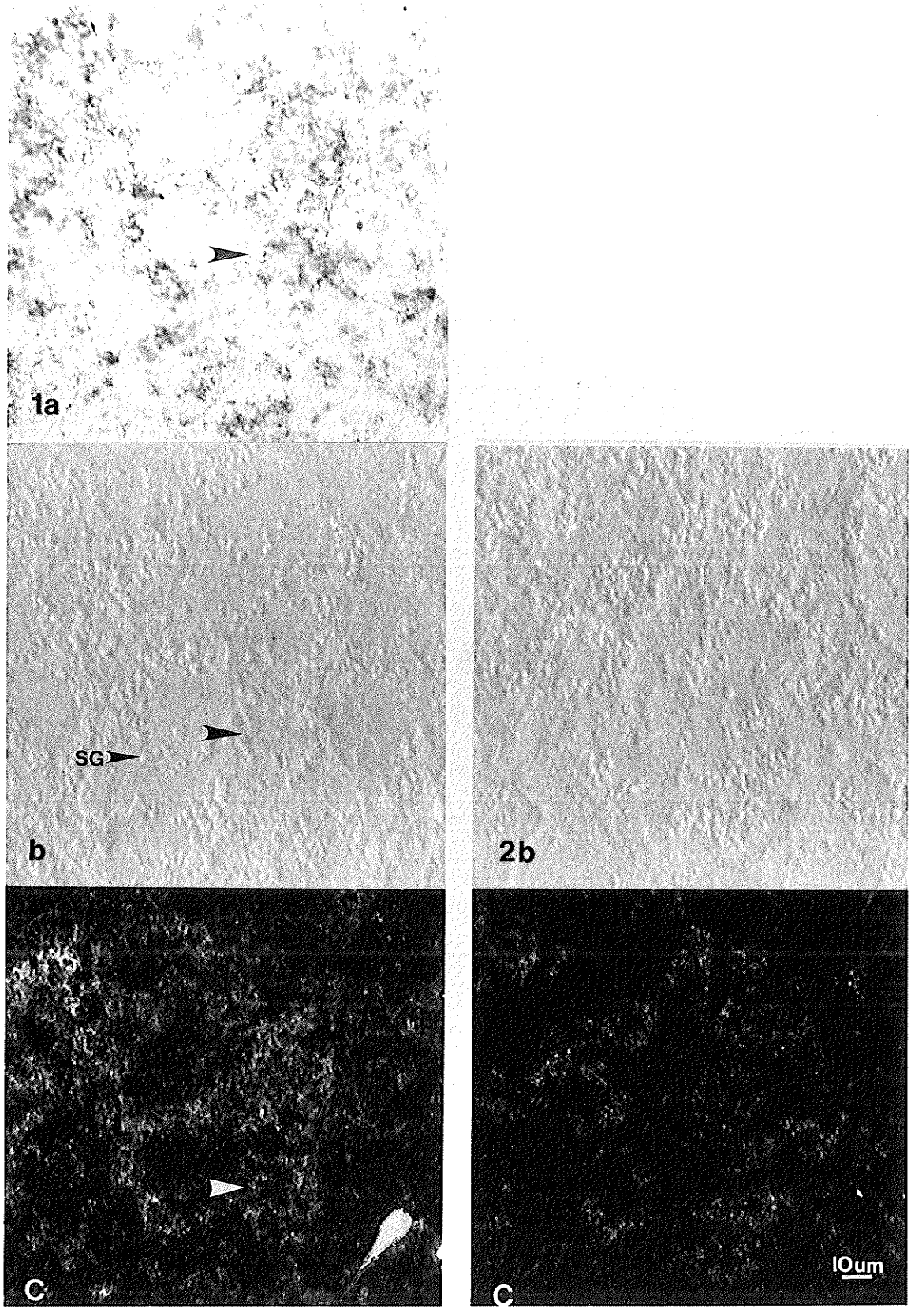

1a
b
SG
2b
C
C
10um

FIGURE 8 : Localisation of starch granules and (1) BASI and (2) α-amylase in the basal scutellum of 2 day imbibed embryos. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. Arrows point to corresponding starch granules in each set of photographs (**1a,b,c** and **2a,b,c**). Artifacts (Art) are distinguished from true signal.

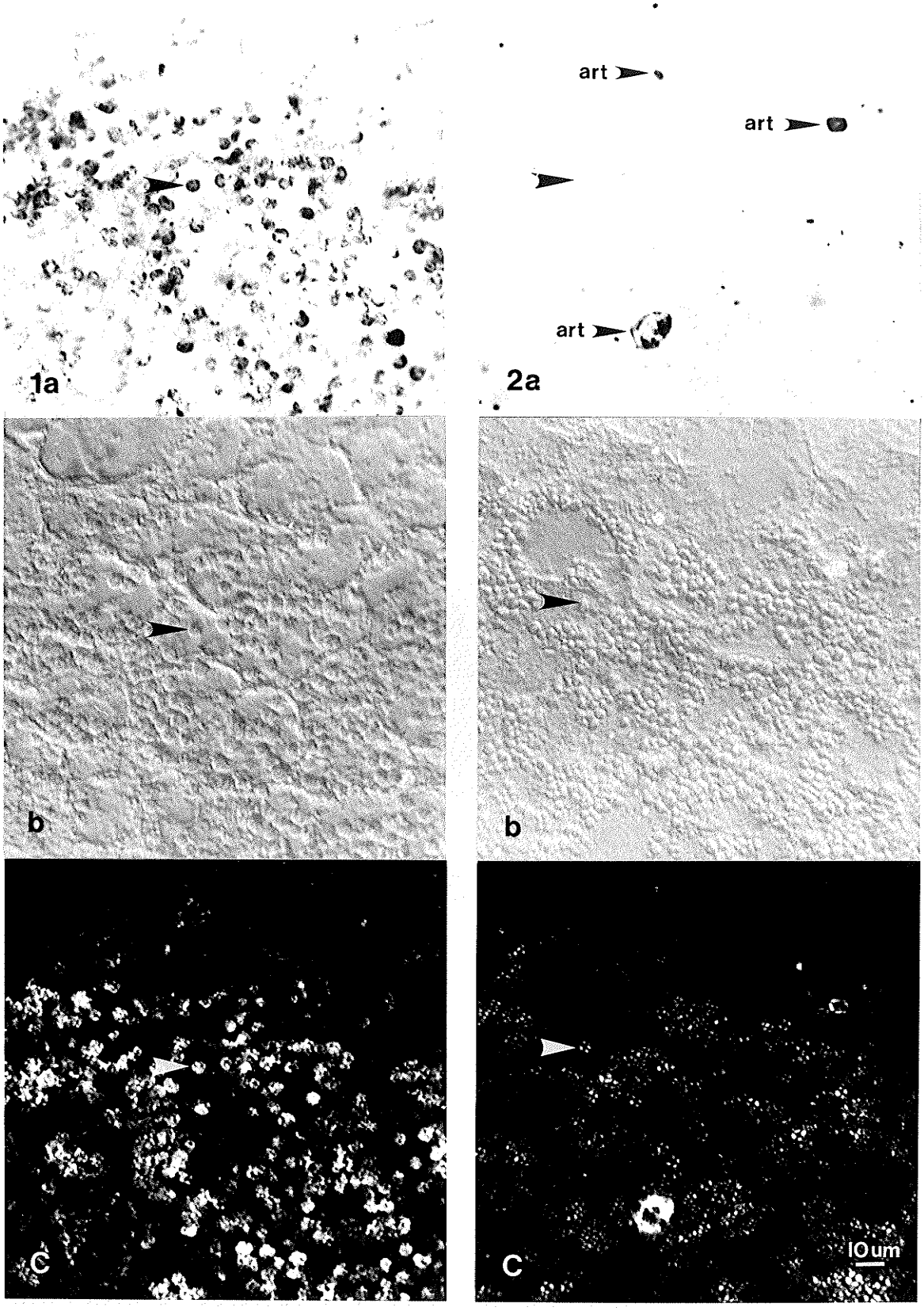

art
art
art
1a
2a
b
b
C
C
10um

FIGURE 9 : Localisation of starch granules and (1) BASI and (2) α-amylase in the basal scutellum of 3 day imbibed embryos. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. Arrows point to the corresponding clusters of starch granules in each set of photographs (**1a,b,c** and **2a,b,c**).

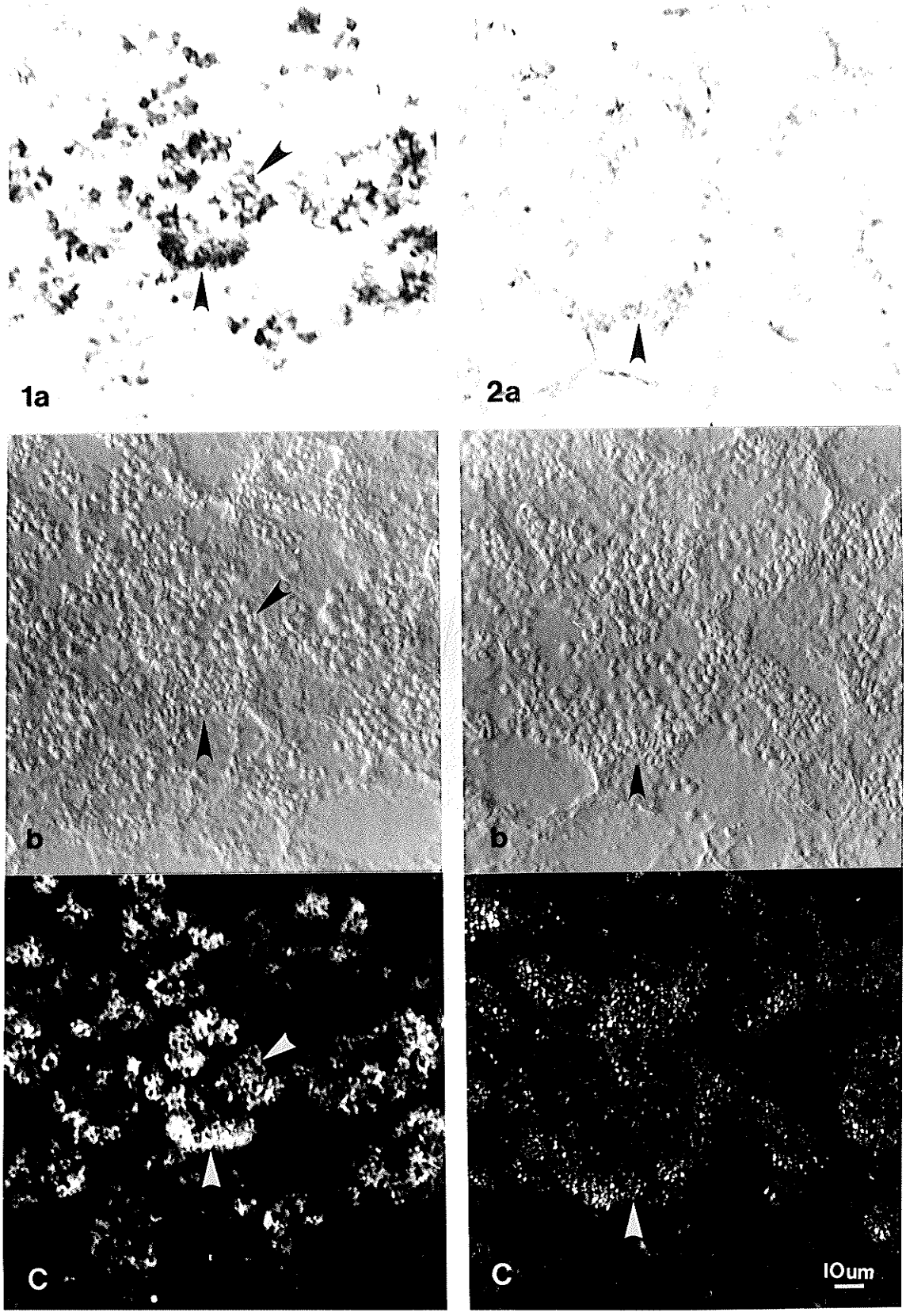

1a
2a
b
b
C
C
10um

FIGURE 10 : Localisation of starch granules and (1) BASI and (2) α-amylase in the basal scutellum of 4 day imbibed embryos. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. Arrows point to the corresponding clusters of starch granules in each set of photographs (**1a,b,c** and **2a,b,c**). Partially degraded starch granules (SG) are labelled (**1b & 2b**).

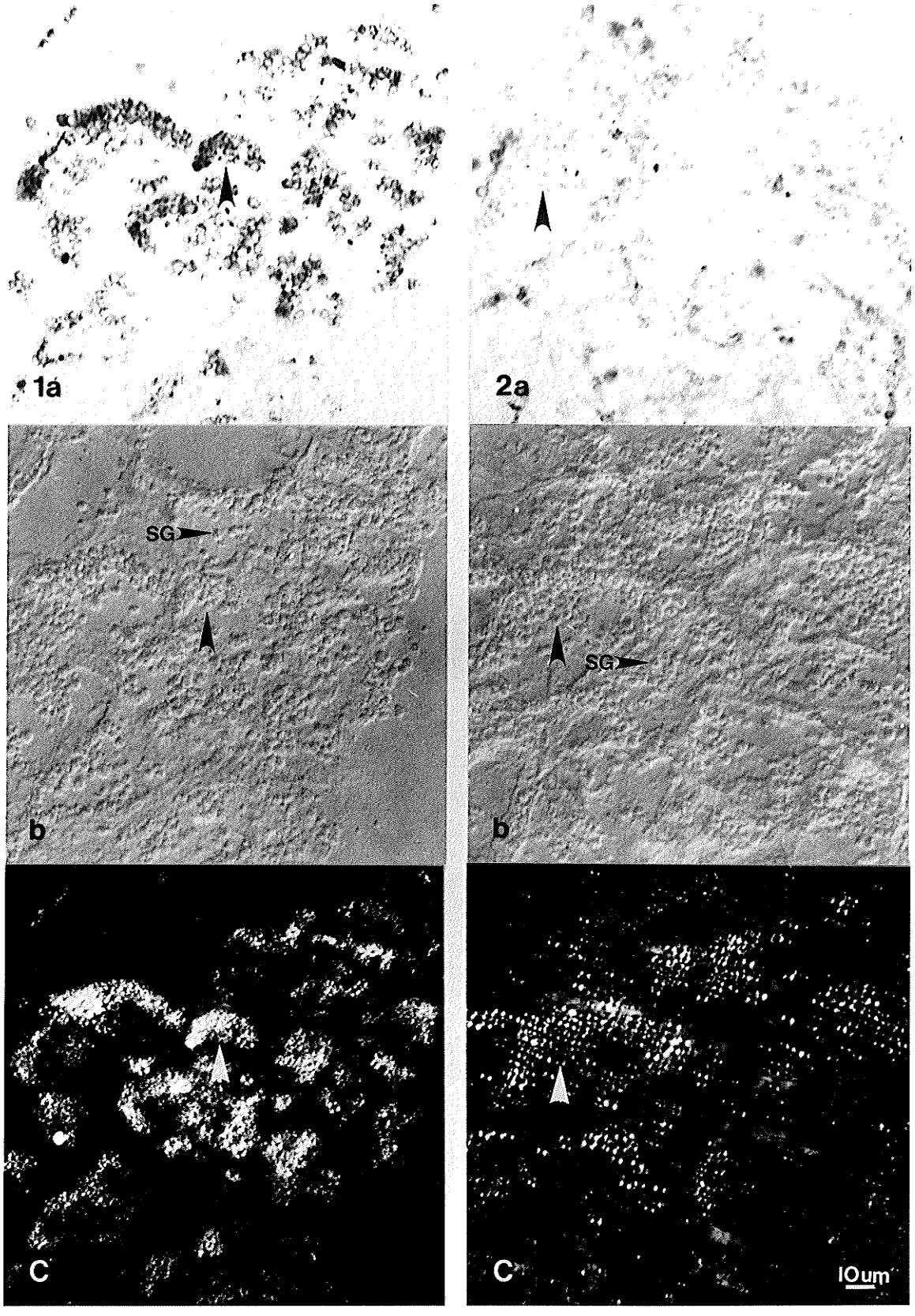

1a
2a
SG
b
SG
b
C
C
10um

FIGURE 11 : Localisation of BASI and starch granules in the coleoptile of 2 day imbibed embryos. Photographs were taken (**1**) at the tip (devoid of signal), and (**2**) at one quarter (1mm) and (**3**) one half the distance (2mm) towards the base of the coleoptile. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. Arrows point to the corresponding single or clustered starch granules in each set of photographs.

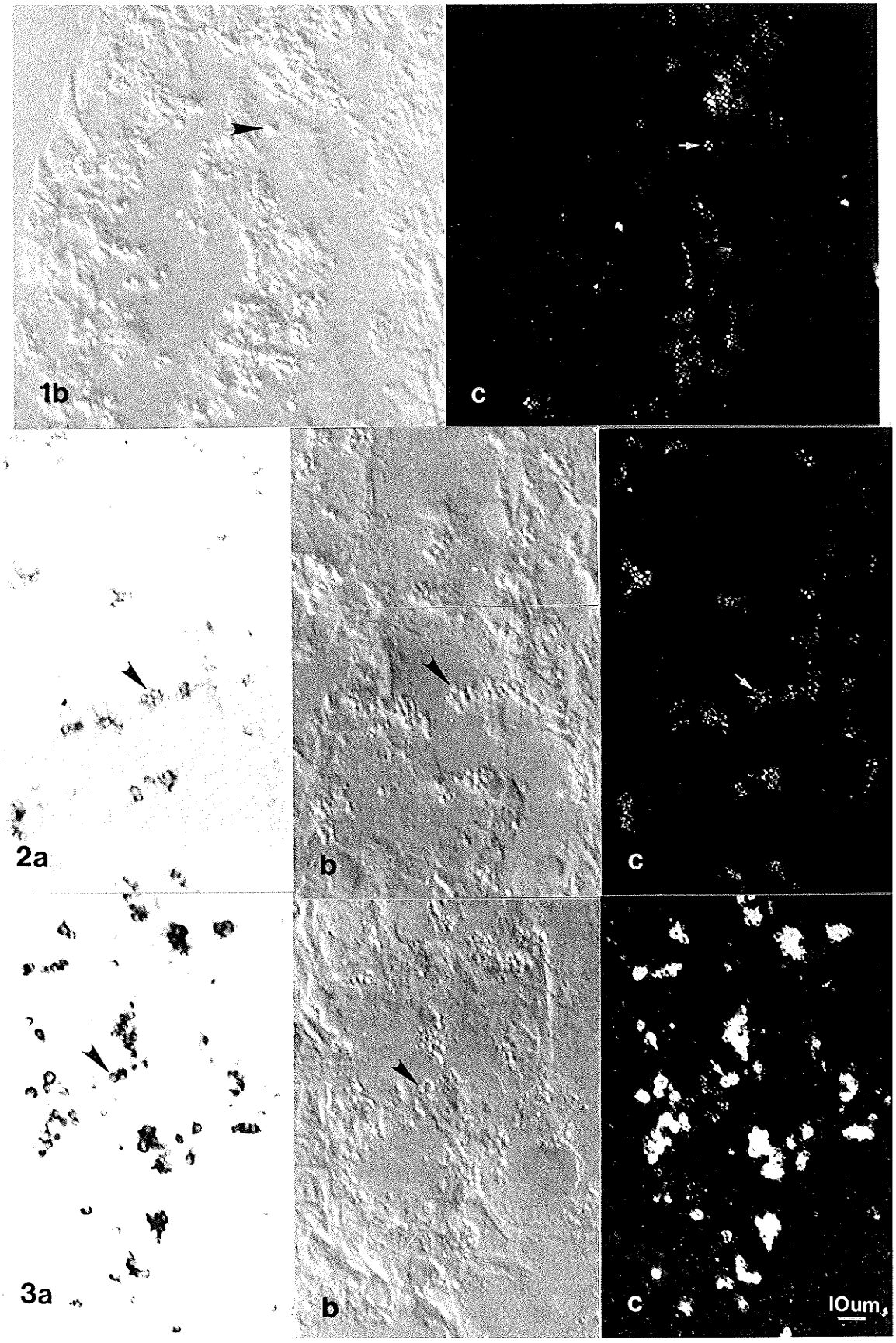

1b
c
2a
b
c
3a
b
c
10um

FIGURE 12 : Localisation of BASI, α-amylase and starch granules in coleoptiles of 1 DI and 4 DI embryos. (1) Location of BASI and starch granules at the base of 1 DI coleoptile treated with anti-BASI antibody. (2) Location of BASI and starch granules at the base of 4 DI coleoptile treated with anti-BASI antibody. (3) Location of α-amylase and starch granules at the base of 4 DI coleoptile treated with anti-α-amylase antibody. (4) Starch granules at the base of a 4 DI coleoptile treated with non-immune (control) antibody. Microscopy includes (**a**) bright field, (**b**) differential interference contrast and (**c**) polarizing microscopy. Arrows point to the corresponding single or clustered starch granules in each set of photographs.

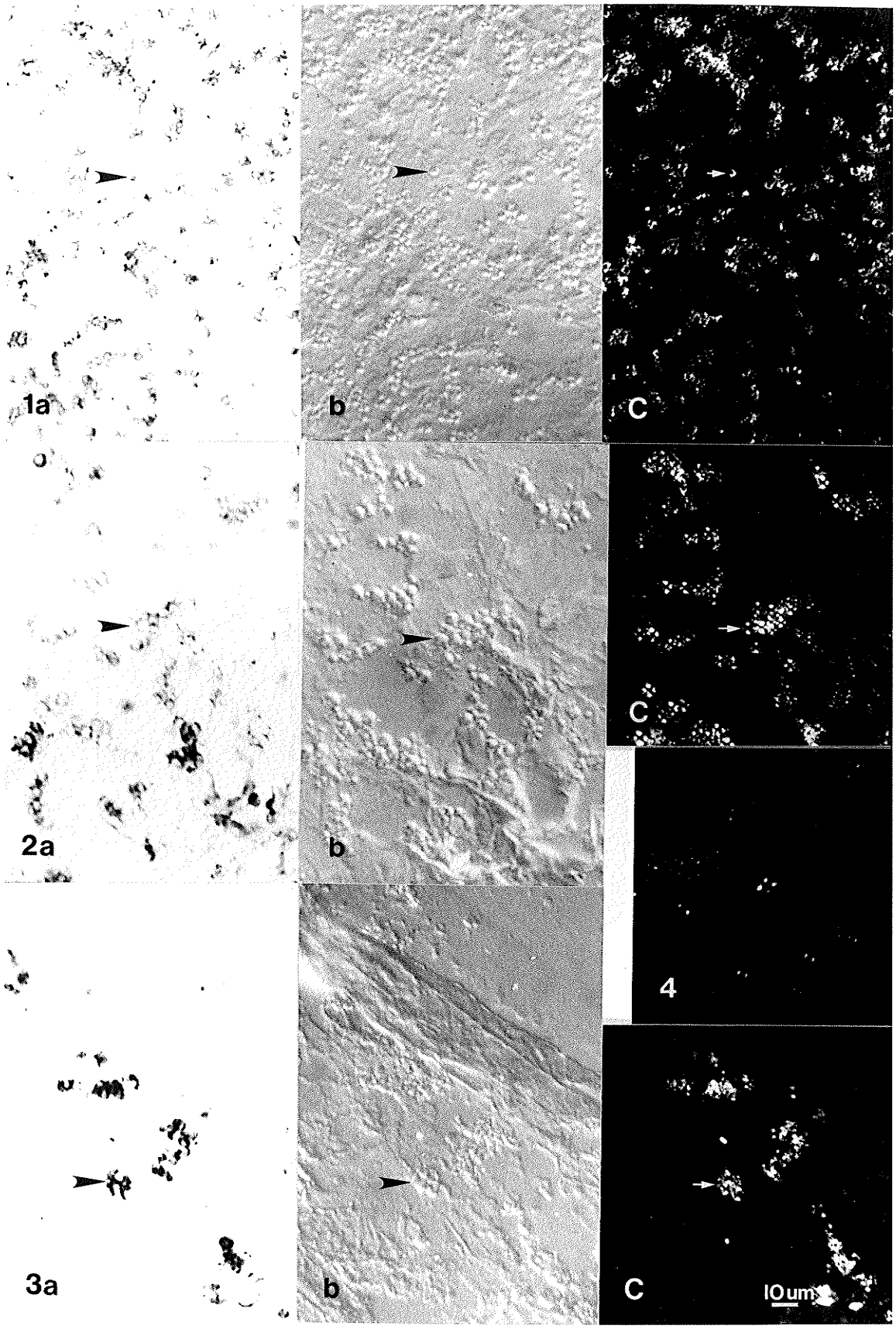
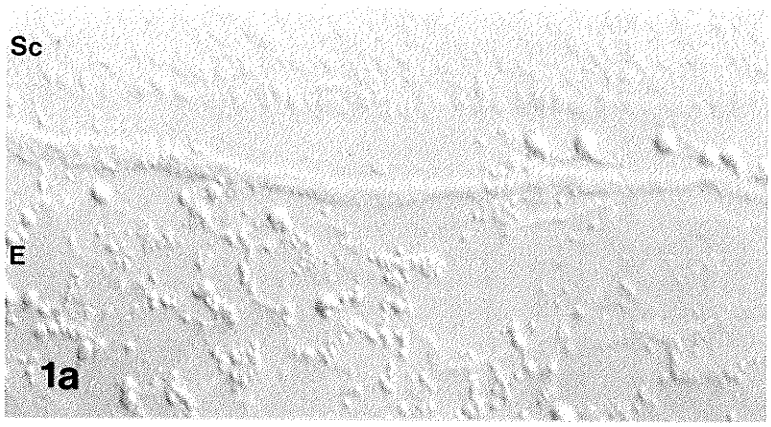

1a
b
C
2a
b
C
4
3a
b
C
10um

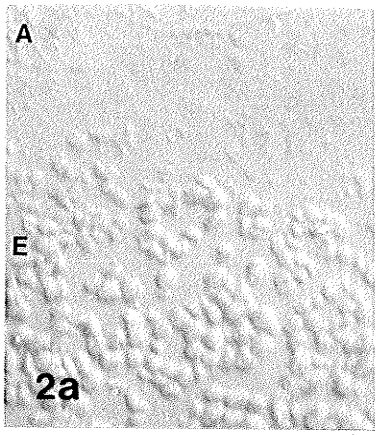
FIGURE 13 : Localisation of BASI and starch granules in the endosperm and aleurone of 2 WPA kernels. Endosperm (E) adjacent to (1) scutellum (Sc) and adjacent to aleurone (A) on the (3) ventral and (4) dorsal sides of a 2 WPA kernel treated with anti-BASI antibody is illustrated. (2) Endosperm and aleurone on the dorsal side of a 2 WPA kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.



Sc

E

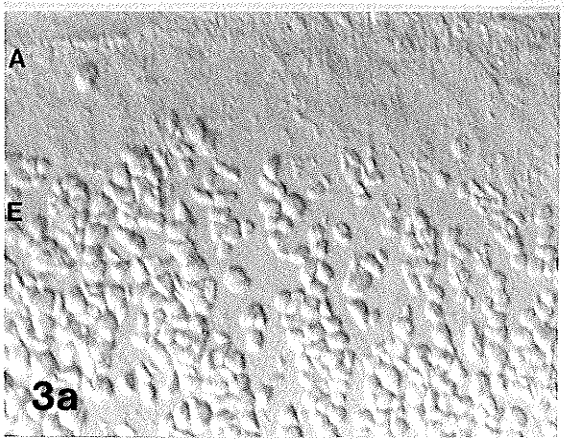
b



A

E

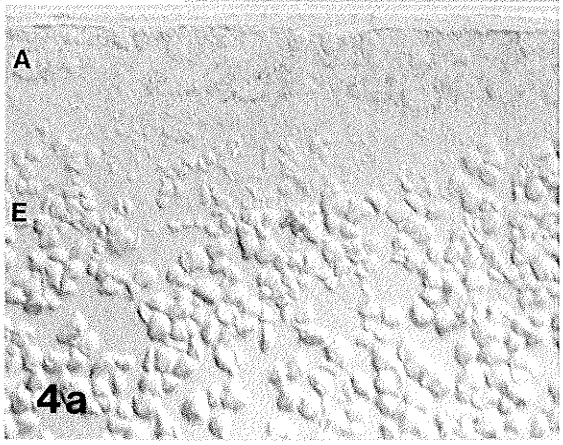
b



A

E

b



A

E

b

50 um

FIGURE 14 : Localisation of BASI and starch granules in the endosperm and aleurone of 3 WPA kernels. Endosperm (E) adjacent to (1) scutellum (Sc) and adjacent to aleurone (A) on the (3) ventral and (4) dorsal sides of a 3 WPA kernel treated with anti-BASI antibody is illustrated. (2) Endosperm and aleurone on the dorsal side of a 3 WPA kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy. An example of artifacts (Art) that occasionally appear due to silver deposition around pockets of air trapped in the aleurone is illustrated in **2b**.

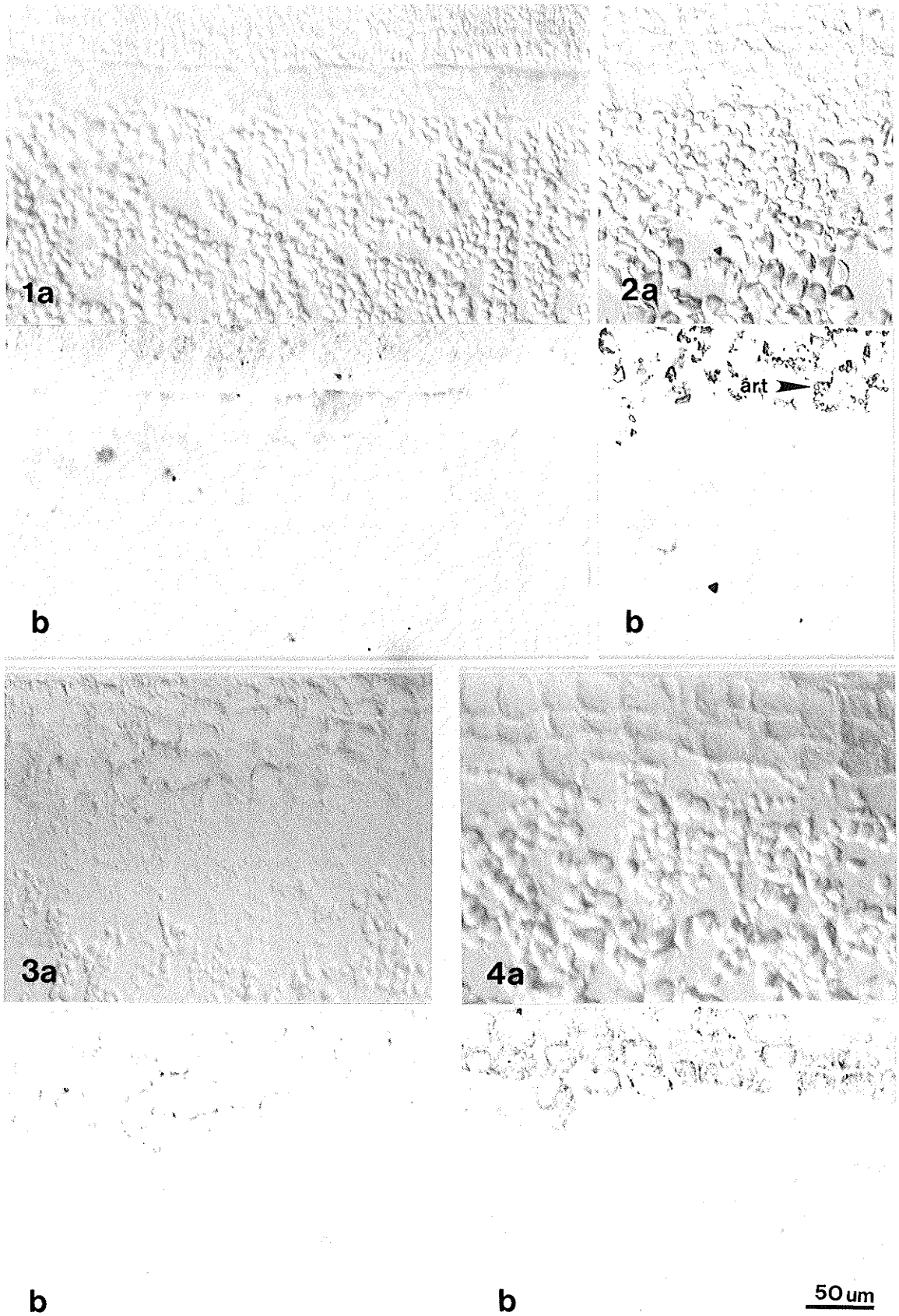

1a
2a
art
b
b
3a
4a
b
b
50um

FIGURE 15 : Localisation of BASI and starch granules in the endosperm and aleurone of 4 WPA kernels. Endosperm (E) adjacent to (**1**) scutellum (Sc) and adjacent to aleurone (A) on the (**3**) ventral and (**4**) dorsal sides of a 4 WPA kernel treated with anti-BASI antibody is illustrated. (**2**) Endosperm and scutellum of a 4 WPA kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.

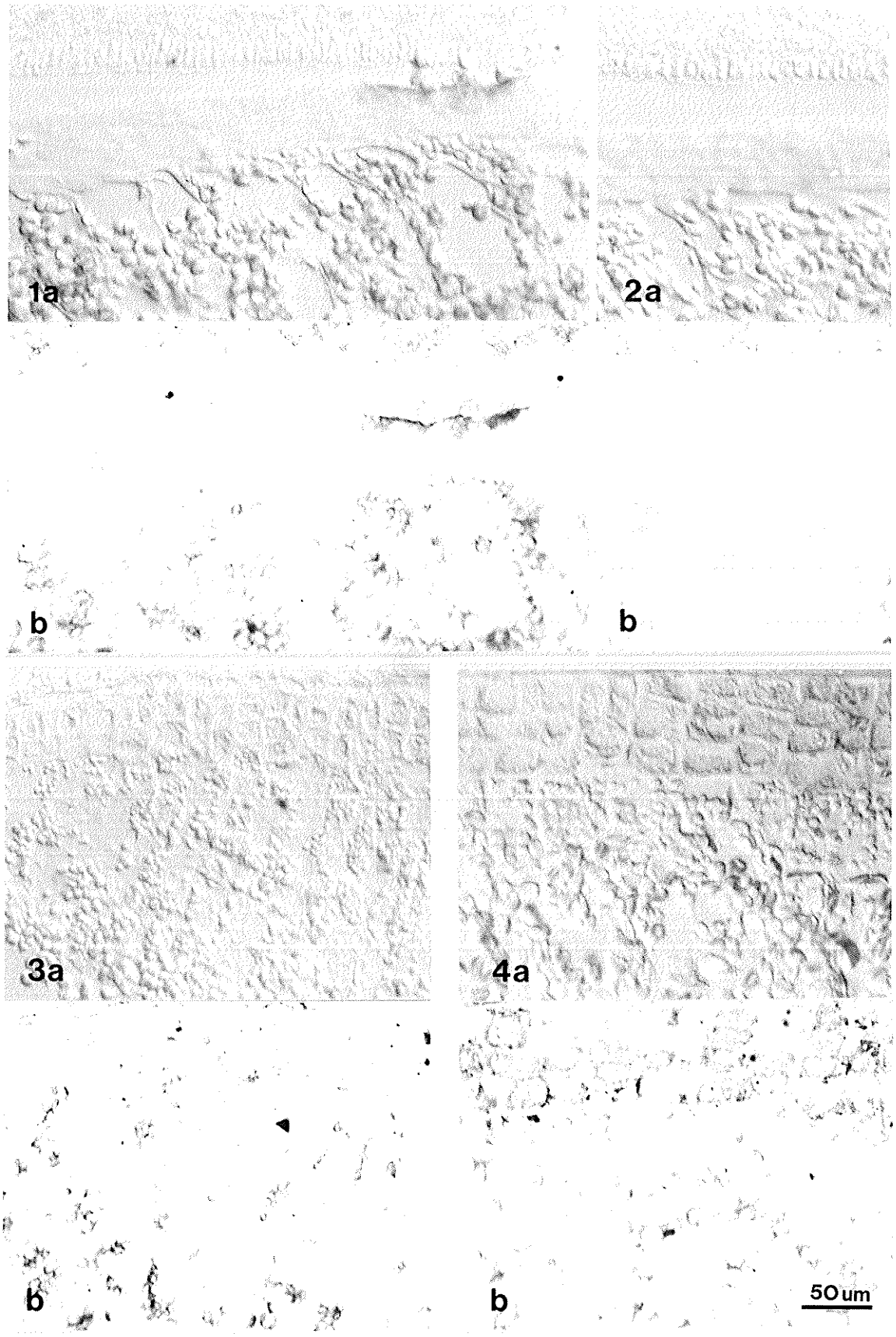

1a
2a
b
b
3a
4a
b
b
50um

FIGURE 16 : Localisation of BASI and starch granules in the endosperm and aleurone of 5 WPA kernels. Endosperm (E) adjacent to (**1**) scutellum (Sc) and adjacent to aleurone (A) on the (**3**) ventral and (**4**) dorsal sides of a 5 WPA kernel treated with anti-BASI antibody is illustrated. (**2**) Endosperm and scutellum of a 5 WPA kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.

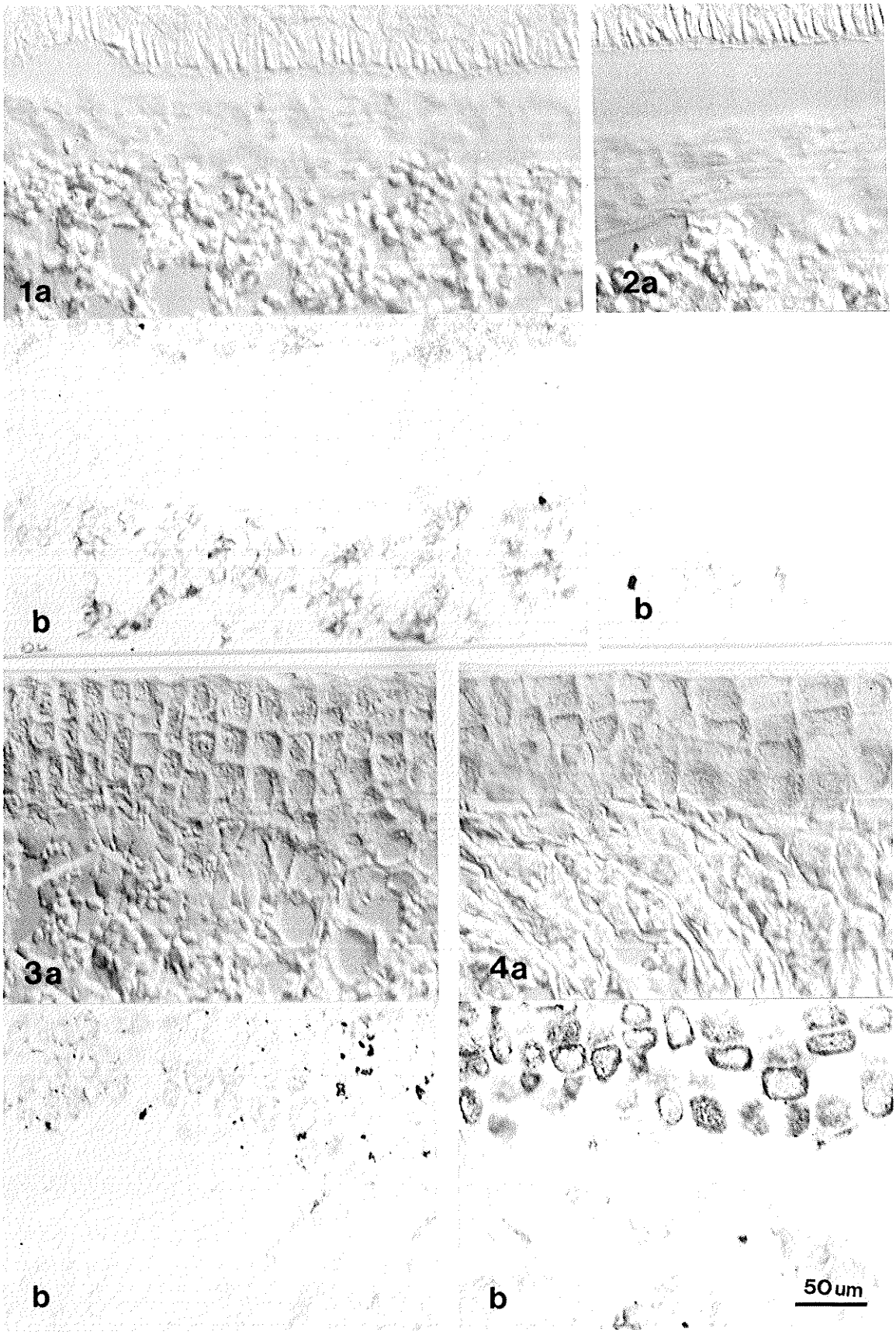

1a
2a
b
b
3a
4a
b
b
50um

Figure 17 : Localisation of BASI and starch granules in the endosperm and aleurone of mature kernels. Endosperm (E) adjacent to (**1**) scutellum (Sc) and adjacent to aleurone (A) on the (**3**) ventral and (**4**) dorsal sides of a mature kernel treated with anti-BASI antibody is illustrated. (**2**) Endosperm and aleurone on the dorsal side of a mature kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy. BASI signal is not observed in cell walls (CW) or nuclei (N).

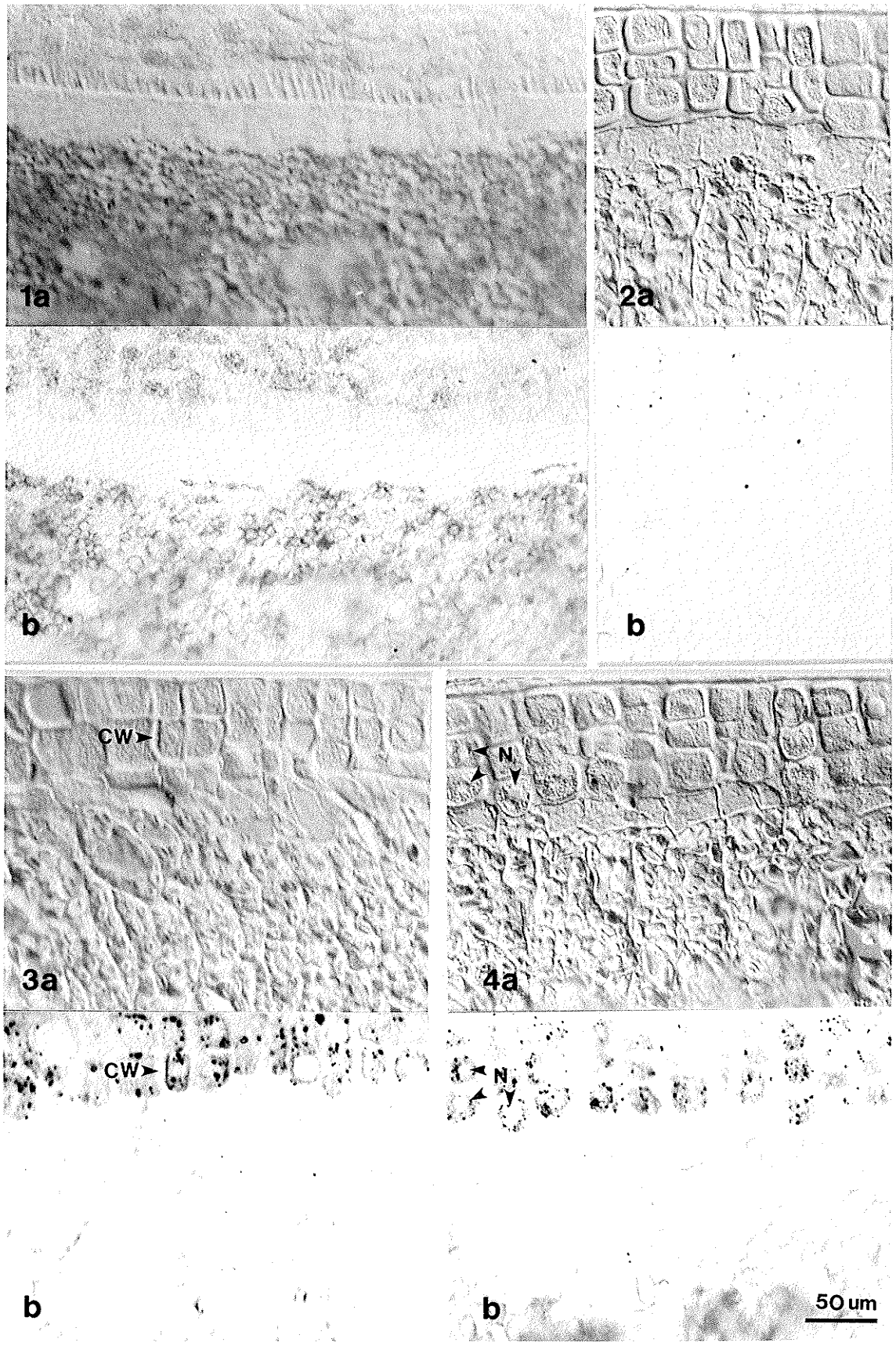

1a
2a
b
b
CW
N
3a
4a
CW
N
b
b
50 um

FIGURE 18 : Localisation of BASI and starch granules in the endosperm and aleurone of 1 DI kernels. Endosperm (E) adjacent to (**1**) scutellum (Sc) and adjacent to aleurone (A) on the (**3**) ventral and (**4**) dorsal sides of a 1 DI kernel treated with anti-BASI antibody is illustrated. (**2**) Endosperm and scutellum of a 1 DI kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.

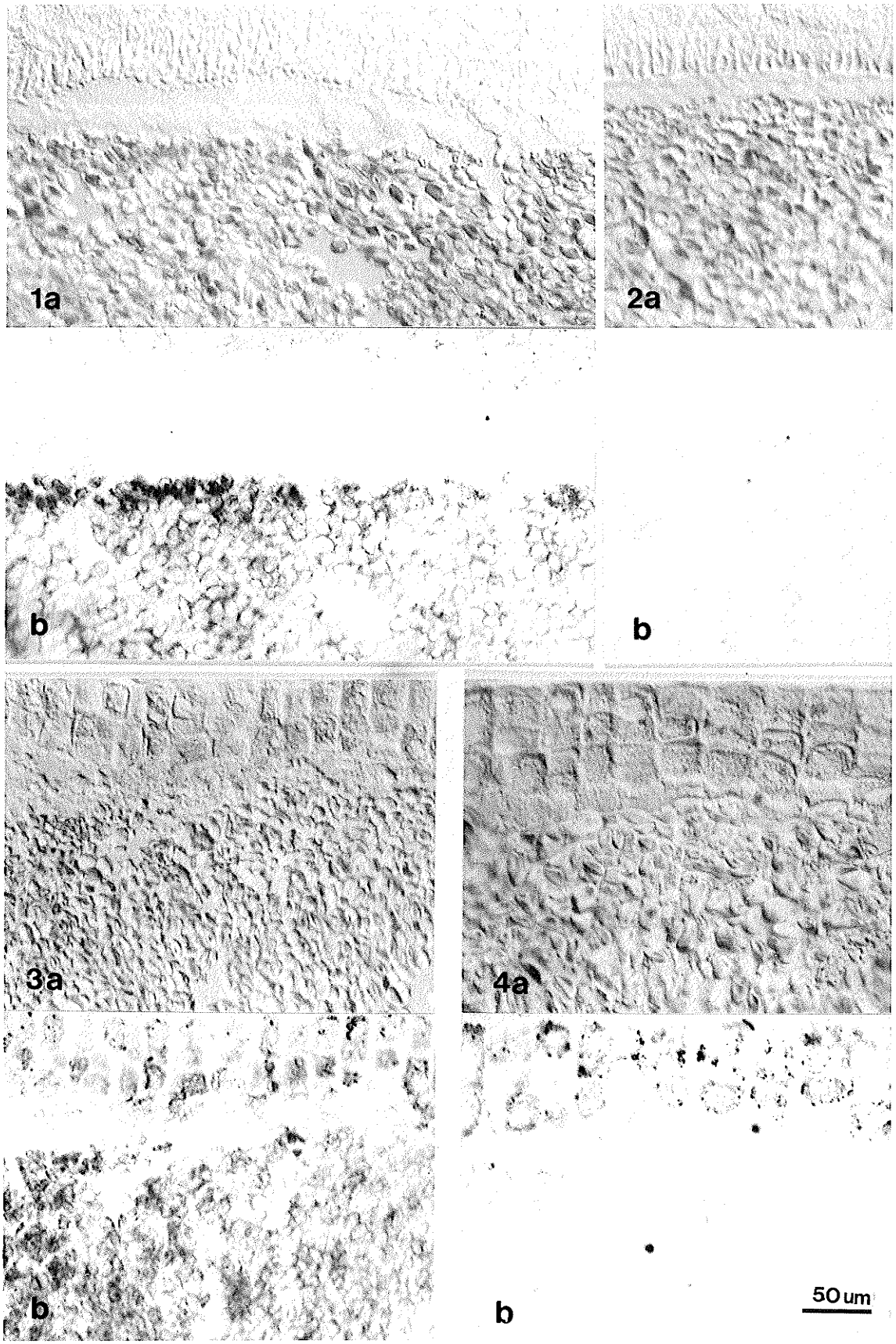

1a
2a
b
b
3a
4a
b
b
50 um

FIGURE 19 : Localisation of α-amylase and starch granules in the endosperm and aleurone of 1 DI kernels. Endosperm (E) adjacent to (1) scutellum (Sc) and adjacent to aleurone (A) on the (3) ventral and (4) dorsal sides of a 1 DI kernel treated with anti-α-amylase antibody is illustrated. (2) Endosperm and aleurone on the ventral side of a 1 DI kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.

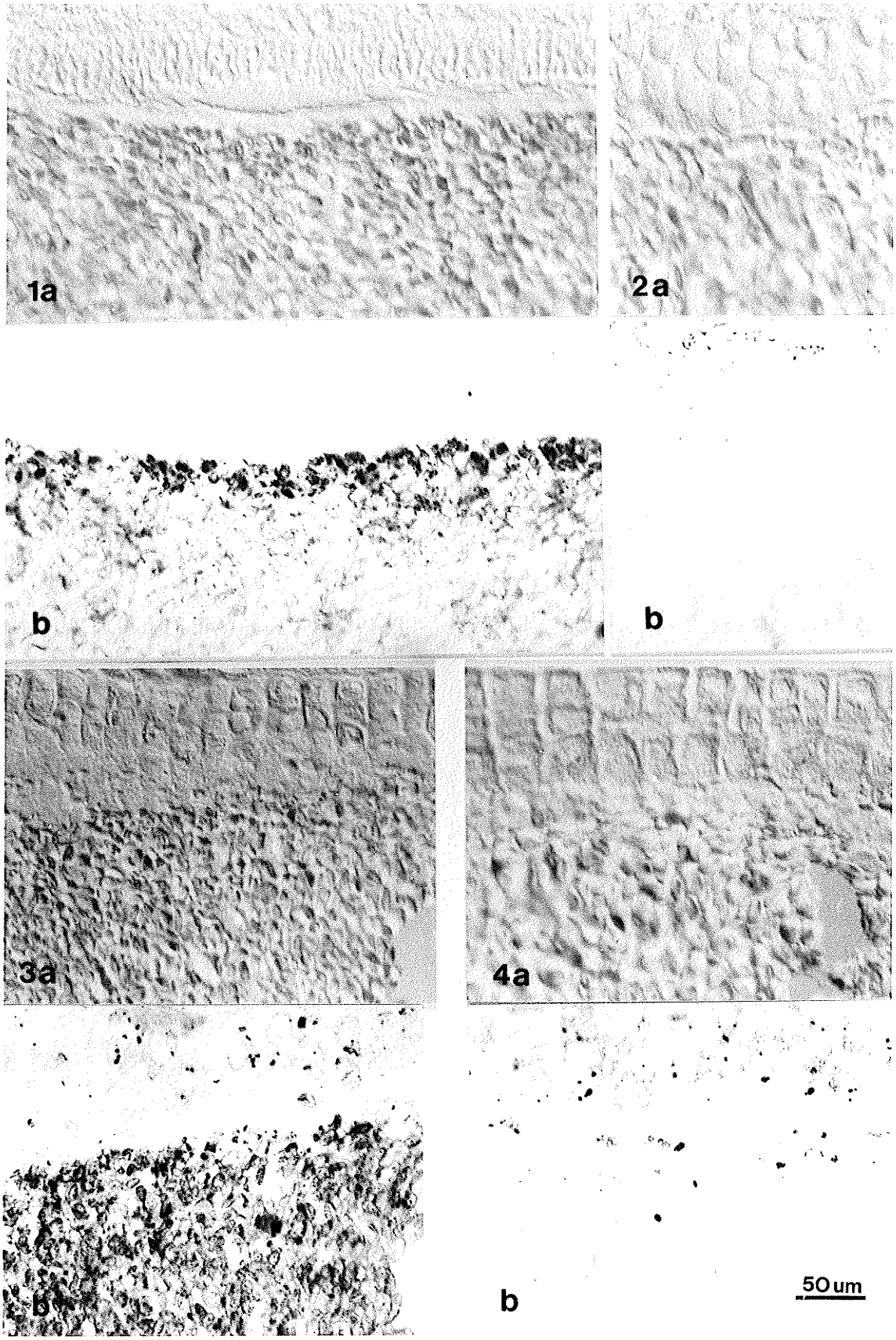

1a
2a
b
b
3a
4a
b
b
50um

FIGURE 20 : Localisation of BASI and starch granules in the endosperm and aleurone of 2 DI kernels. Endosperm (E) adjacent to (**1**) scutellum (Sc) and adjacent to aleurone (A) on the (**3**) ventral and (**4**) dorsal sides of a 2 DI kernel treated with anti-BASI antibody is illustrated. (**2**) Endosperm and scutellum of a 2 DI kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.

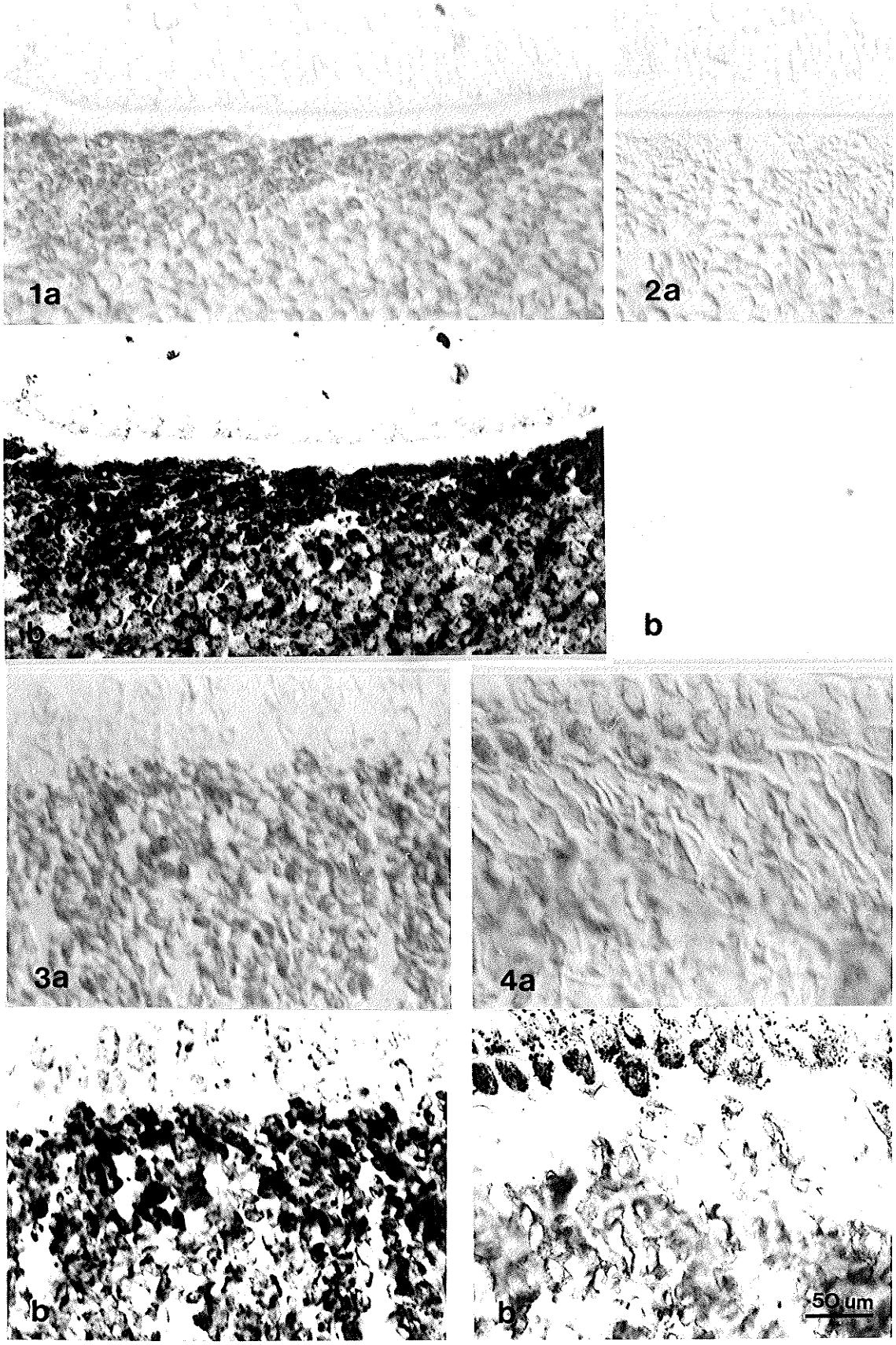

1a
2a
b
b
3a
4a
b
b
50 um

FIGURE 21 : Localisation of α-amylase and starch granules in the endosperm and aleurone of 2 DI kernels. Endosperm (E) adjacent to (1) scutellum (Sc) and adjacent to aleurone (A) on the (3) ventral and (4) dorsal sides of a 2 DI kernel treated with anti-α-amylase antibody is illustrated. (2) Endosperm and scutellum of a 2 DI kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.

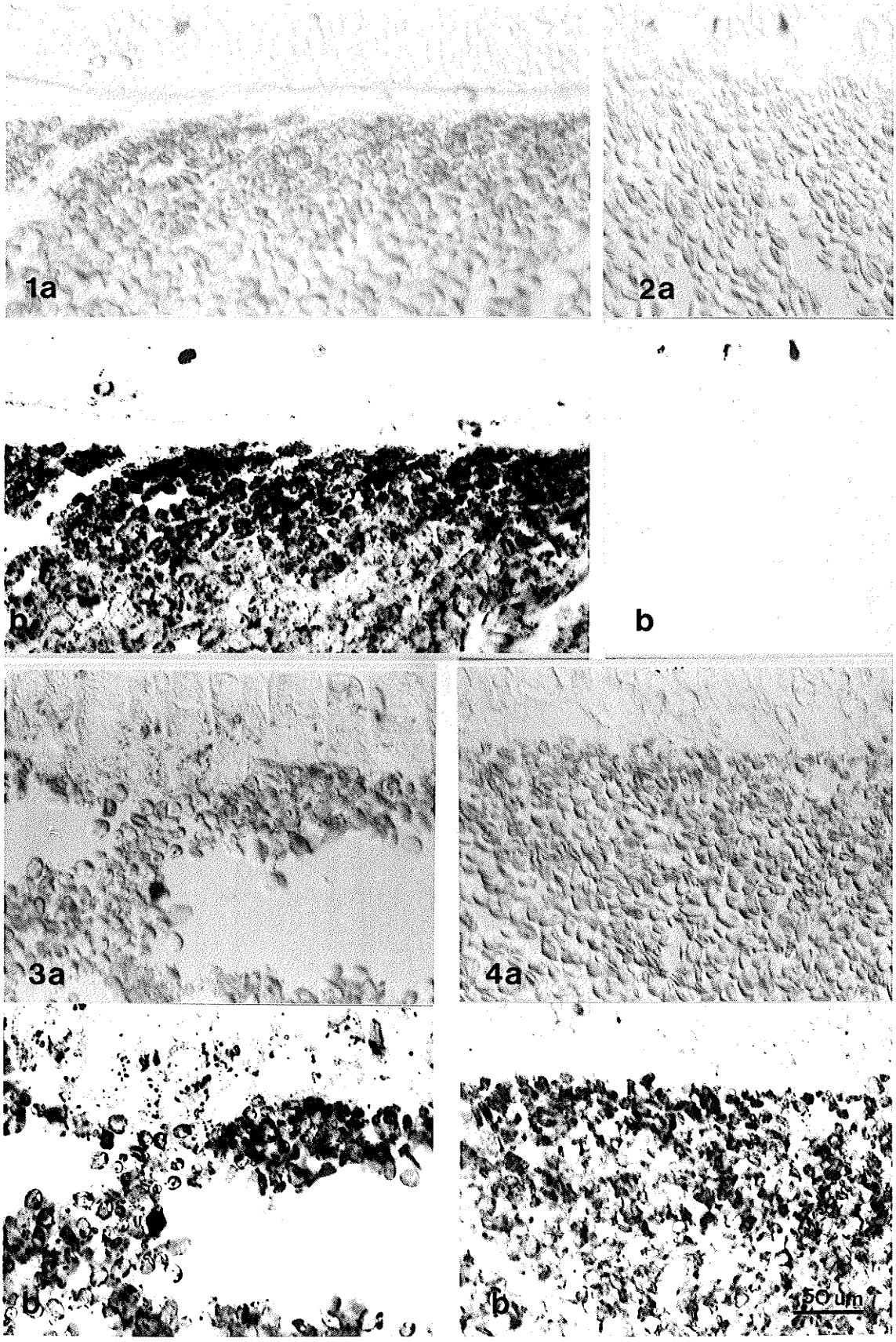

1a
2a
b
b
3a
4a
b
b
150 um

FIGURE 22 : Localisation of BASI and starch granules in the endosperm and aleurone of 3 DI kernels. Endosperm (E) adjacent to (**1**) scutellum (Sc) and adjacent to aleurone (A) on the (**3**) ventral and (**4**) dorsal sides of a 3 DI kernel treated with anti-BASI antibody is illustrated. (**2**) Endosperm and scutellum of a 3 DI kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy. Examples of partially degraded starch granules (SG) are labelled.

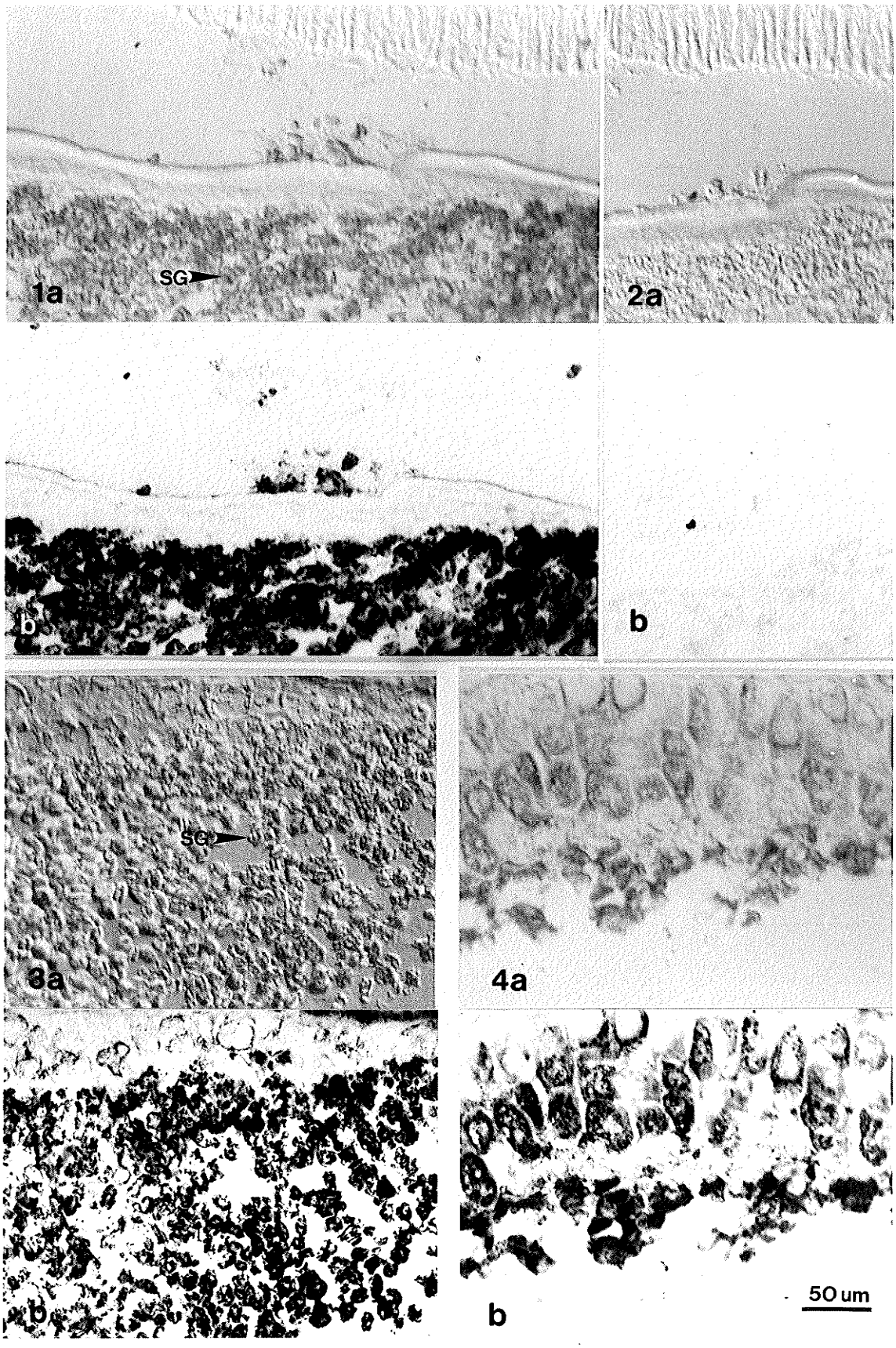
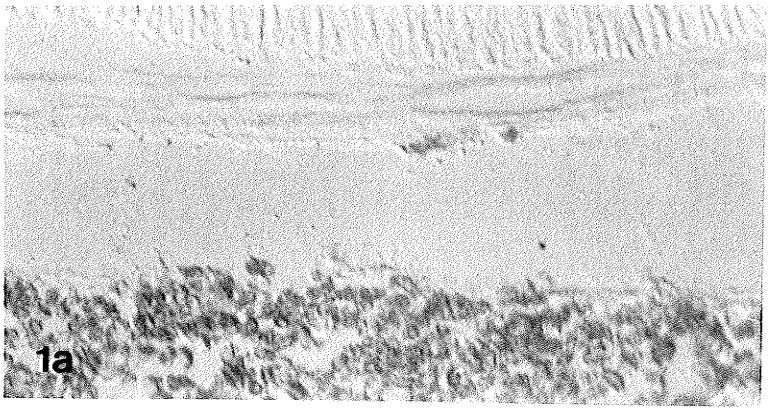
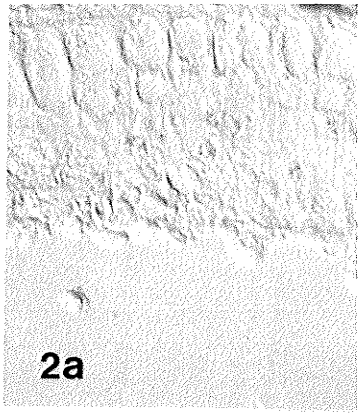
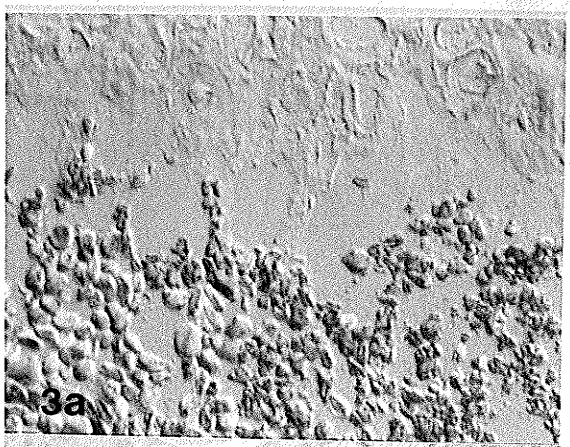

SG
1a
2a
b
b
SG
3a
4a
b
b
50 um

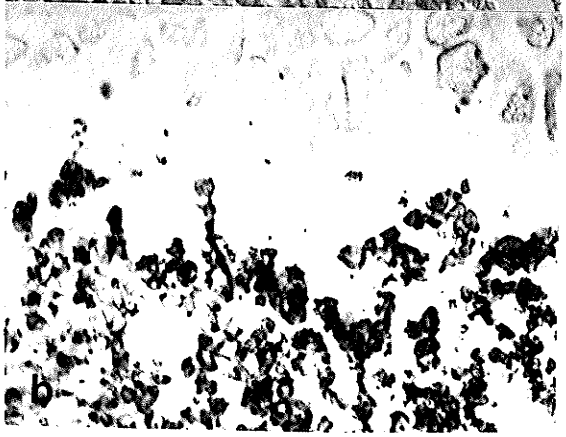
FIGURE 23 : Localisation of α-amylase and starch granules in the endosperm and aleurone of 3 DI kernels. Endosperm (E) adjacent to (**1**) scutellum (Sc) and adjacent to aleurone (A) on the (**3**) ventral and (**4**) dorsal sides of a 3 DI kernel treated with anti-α-amylase antibody is illustrated. (**2**) Endosperm and aleurone on the dorsal side of a 3 DI kernel treated with non-immune (control) antibody is shown for comparison. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy.

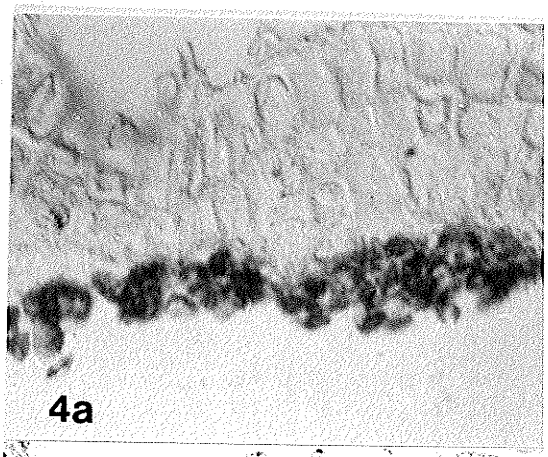




b







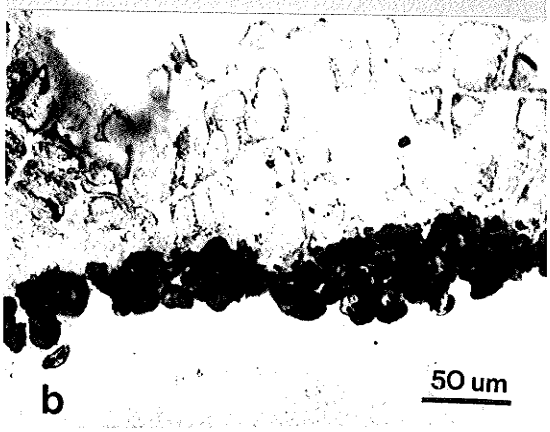


FIGURE 24 : Localisation of BASI and starch granules in the endosperm and aleurone of 4 DI kernels. Endosperm (E) adjacent to aleurone (A) in (**1**) ventral/proximal, (**2**) ventral/distal, (**3**) dorsal/proximal, and (**4**) dorsal/distal regions of a 4 DI kernel treated with anti-BASI antibody. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy. The dorsal photos (3 & 4) are spliced because, due to the softness of 4 DI caryopses, aleurone and endosperm became separated during sectioning.

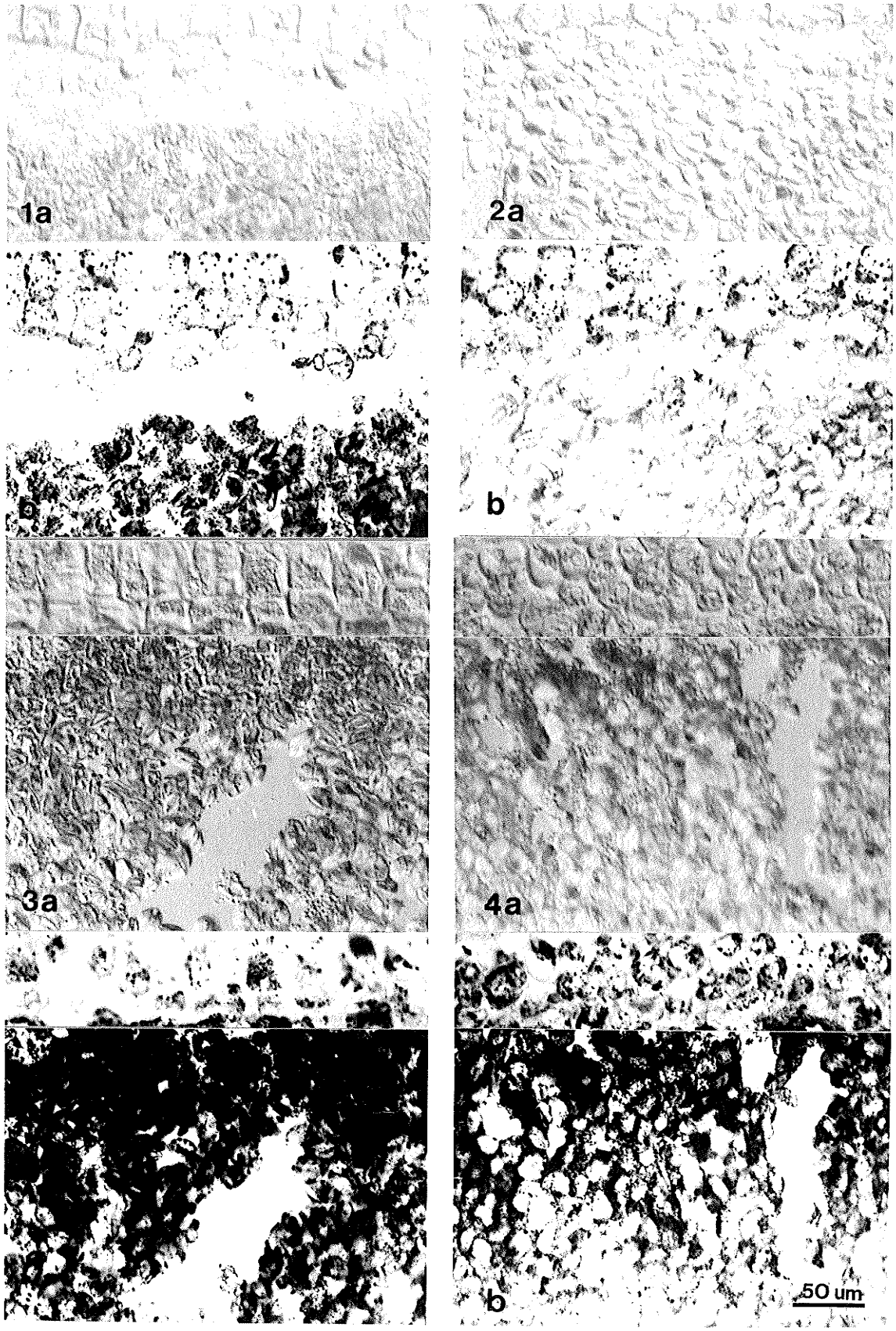

1a
2a
b
3a
4a
b
50 um

FIGURE 25 : Localisation of α-amylase and starch granules in the endosperm and aleurone of 4 DI kernels. Endosperm (E) adjacent to aleurone (A) in the (1) ventral/proximal, (2) ventral/distal, (3) dorsal/proximal, and (4) dorsal/distal regions of a 4 DI kernel treated with anti-α-amylase antibody. Microscopy includes (**a**) differential interference contrast and (**b**) bright field microscopy. The dorsal photos (3 & 4) are spliced because, due to the softness of 4 DI caryopses, aleurone and endosperm became separated during sectioning.

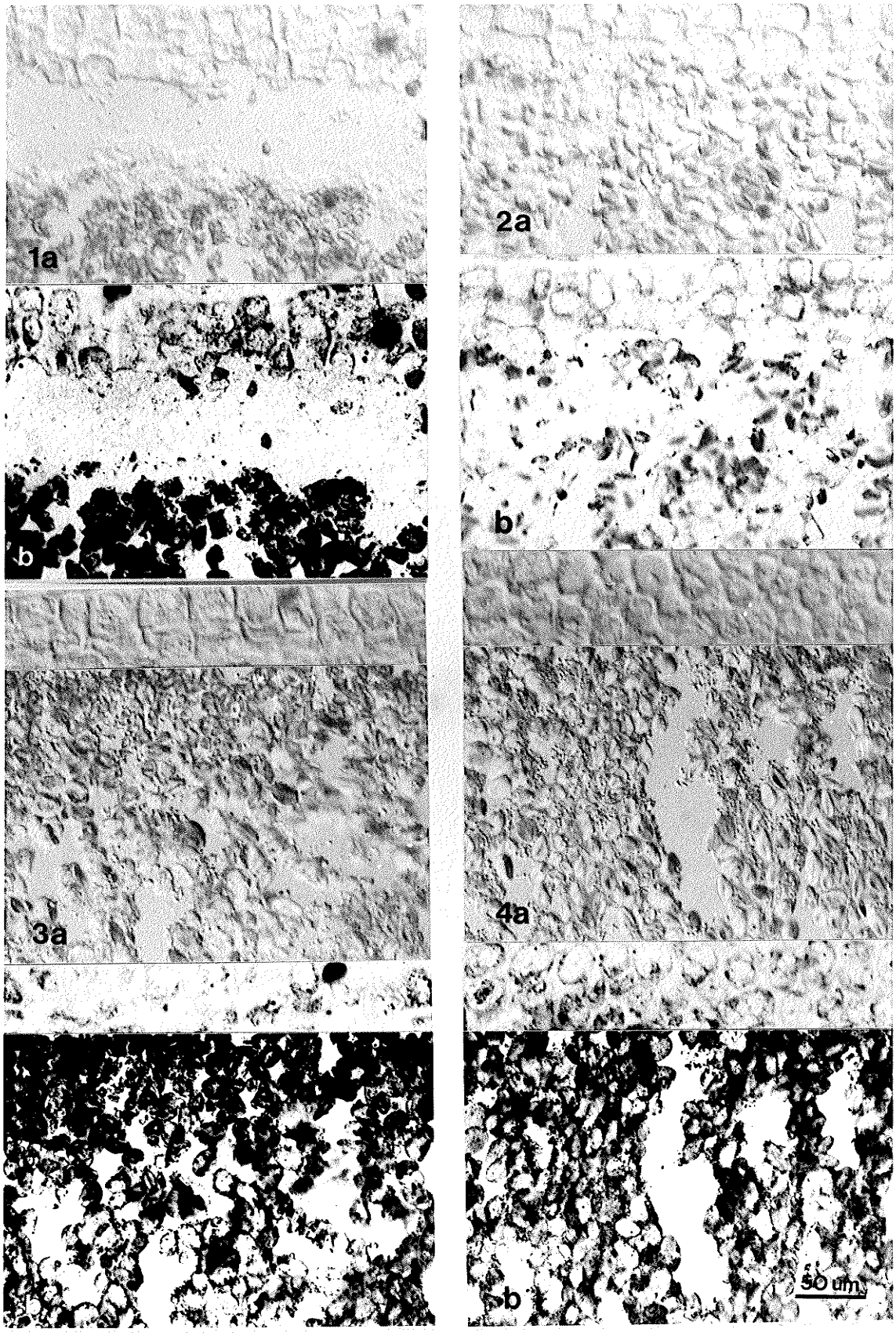

1a
b
2a
b
3a
4a
b
50 um

FIGURE 26 : Immunolocalisation of BASI within protein bodies in the endosperm of 3 WPA barley kernels using transmission electron microscopy. 3 WPA endosperm was treated with (**A**) anti-BASI antibody and (**B**) non-immune (control) antibody. Arrows show examples of immunogold label. Protein bodies (PB), cytosol (C), and inclusions (I) are labelled. have signal peptides, and are located in protein bodies.

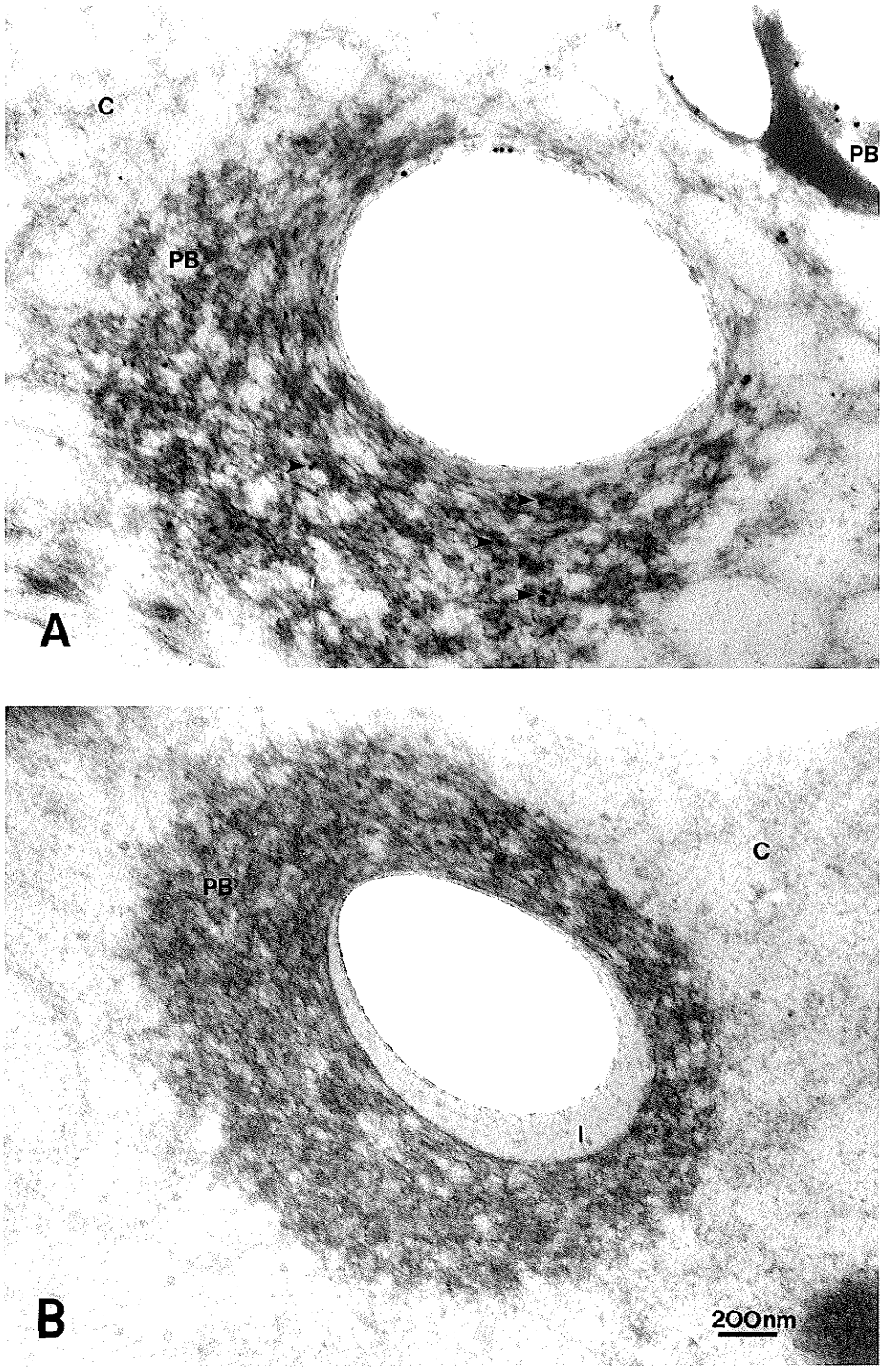

C
PB
PB
A
PB
C
I
B
200nm

Table I Summary of Results

Tissue/Item	1WPA	3WPA	4WPA	5WPA	MATURE	1DI	2DI	3DI	4DI
Basal Scutellum									
Starch Granules	$<1\mu$m	3μm	4μm	$<2\mu$m	absent	1-2μm	2-4μm	2-5μm	$<4\mu$m
Protein Bodies						Dense		Vacuolated	
BASI	+	++	++++	++++	++++	++++	++++	++++	+++
α-Amylase	absent	absent	absent	absent	absent	absent	+	++	++
Coleoptile									
Starch Granules		present				$<2\mu$m			$<4\mu$m
BASI		present				present			present
α-Amylase		absent				absent			present
Endosperm									
BASI	+	++	++++	++++	++++	*	**	***	****
α-Amylase	absent	absent	absent	absent	absent	*	**	***	****
Aleurone									
Starch Granules	absent	absent	absent	absent	absent	absent	absent	absent	absent
Protein Bodies				present	present	present	present		
BASI	+	+++	++++	+++++	+++++	present	present	present	present
α-Amylase	absent	absent	absent	absent	absent	present	present	present	present

NOTE: BASI, α-amylase and starch are absent from the radicle, seminal roots and plumule of developing embryos.
NOTE: BASI was located mainly in the protein bodies of endosperm cells at 3WPA and scutellar cells at 1DI.
NOTE: "*" indicates more intensity than "+++++".

DISCUSSION

Location of BASI, α-Amylase and Starch Granules in the Embryo

BASI was located in all embryonic tissues in which starch granules, the substrate of α-amylase, were located (figure **4**). Both BASI and starch granules were found in the scutellum, coleorhiza and coleoptile of Barley embryos (figures **3** & **4**). The radicle, seminal roots and plumule of the embryo were devoid of BASI and starch granules. Therefore, BASI was located in appropriate tissues to inhibit the activity of α-amylase *in vivo*.

Accumulation of BASI occurred during early to mid development of barley kernels (figures **5** & **6**). The intensity of BASI signal remained constant from 5 WPA through to 3 DI (figures **6** - **9**). This observation was consistent with the conclusions of Chisholm (1989) and Lecommandeur *et al.* (1987) for whole barley kernels. Both groups examined the quantity of inhibitor present in whole barley grains during germination by single immunodiffusion assays. At 4 DI, the intensity of embryonic inhibitor signal appeared to diminish (figure **10**). Lecommandeur *et al.* (1987) found that between 2 DI and 5 DI the amount of BASI in whole kernels (cv. Menuet) decreased by one third. Since BASI is present during the initial days of imbibition, it is possible for BASI to have a role in the early germination process.

The first trace of α-amylase signal appeared in the scutellum at 2 DI (figure **8**). The signal intensity increased over the next 2 days (figures **9** & **10**). This corroborates the work of MacGregor *et al.*(1984), which showed marginal amounts of α-amylase I and II during the first 2 days of imbibition and marked increases in both isozymes in protein

samples extracted from 3 and 4 DI scutellum. The α-amylase signal was found, as was the inhibitor signal, to be located in the tissues containing starch granules. In wheat, starch granules that appear in the scutellum during germination begin to be degraded after 4 DI, according to Swift and O'Brien (1972b). At 4 DI, starch granule breakdown was observed in barley scutellum (figure **10**). At this time, α-amylase was abundant and the level of BASI began to decrease in the scutellum. It is likely that α-amylase contributes to the degradation of the post-germination starch granules. Therefore, BASI may attenuate the action of α-amylase on these starch granules.

It is also possible for BASI to attenuate the degradation of starch granules by α-amylase in coleoptiles. BASI was found only immediately adjacent to starch granules (figure **12**:1&2). After 4DI, α-amylase signal was detected in the same location (figure **12**:3). BASI was positioned in an appropriate location for inhibition of α-amylase.

Pattern of Starch Granule Accumulation in the Scutellum

The observed development of starch granules in the amyloplasts of scutellar tissue closely followed the data presented by Swift and O'Brien (1972a&b), Smart and O'Brien (1979), and Gram (1982) for wheat and barley. The largest starch granules and the greatest concentration of starch granules were found in the basal scutellum (figure **4**). Starch was present during the first few weeks of development, was absent at maturity (figures **5** & **6**) but reappeared in early germination (figures **7** & **8**). Normal starch granule development occurred during development and germination of kernels used for the immunolocalisation studies.

Association of BASI with Starch Granules in the Scutellum

The location of BASI signal changed between 1 and 3 DI (figures **7-9**). At 1 DI, BASI signal was dispersed throughout the cell (figure **7**) but was later found to be adjacent to the starch granules (figures **8 & 9**). This change may be a consequence of cell sub-structure reorganization which occurs in scutellar cells of barley (figure **2A & B**) and wheat (Smart and O'Brien, 1979 and Swift and O'Brien, 1972a&b) kernels during early germination. At maturity, protein bodies are the dominant feature of scutellar cells. There are many small protein bodies distributed throughout the cells. As germination proceeds, storage proteins are broken down and the protein bodies join to form large vacuolar spaces. Starch granules increase in size and number to become the dominant feature of the cell by 3 DI. The granules are fewer in number and larger in size than the protein bodies are in mature embryos.

BASI Synthesis in the Basal Scutellum

The intensity of inhibitor signal in the scutellum was found to increase from a very low level at 2 WPA to a maximum at 4 to 5 WPA (figures **5 & 6**). This observation is consistent with a time course study of BASI synthesis in whole grains (Robertson and Hill, 1989) but contrasts with the work of Robertson *et al.*(1989) which did not find a significant increase in the inhibitor level in barley embryos until after 4 WPA. According to Robertson *et al.*(1989), the time of BASI synthesis in the embryo is more like that of a LEA protein than a storage protein. Robertson *et al.*(1989) used western analysis to compare known quantities of BASI with protein extracted from embryos.

Immunocytochemistry is most valuable for protein localisation; exact quantitation is not possible since known standards can not be compared to test results. However, immunocytochemistry avoids error associated with tissue dissection and protein extraction. As the kernels become harder in the later part of development, it becomes increasingly difficult to dissect embryos from kernels without retaining any endosperm tissue. Since the plumule, radicle and seminal roots do not contain BASI, additional endosperm tissue would increase the proportion of BASI containing tissue in the test sample. Robertson *et al.*(1989) also measured ABA content in the embryo samples. They found that the majority of BASI was produced after ABA increased in the embryo. Thus, it seemed likely that ABA induced synthesis of BASI in the embryo. In the immunocytochemical study, ABA was not measured and it is possible that ABA was synthesized at an earlier date in these samples than in those used in the research involving western analysis.

Localisation of BASI and α-Amylase in Scutellar Epithelial Cells

BASI was detected only in scutellar epithelial cells of mature kernels when anti-BASI/immunogold-treated cryosections of developing and germinating barley kernels were viewed by light microscopy (figure 17). However, when anti-BASI/immunogold treated sections of 1 DI kernels were viewed under a transmission electron microscope, BASI was found in the protein bodies of scutellar epithelial cells (data not shown). Thus, small quantities of BASI may be present in the scutellar epithelial cells of barley at different developmental stages.

α-Amylase was detected in the scutellar epithelial cells of neither developing nor germinating kernels (figures **19, 21, 23, & 25**). Both Ranki (1990) and Pogson *et al.*(1989) reported the presence of α-amylase in the scutellar epithelial cells of 1 - 2 DI kernels. Since α-amylase is synthesized and secreted into the endosperm, it is possible that the quantity of α-amylase present at any one time within epithelial cells was insufficient to be detected using immunocytochemistry and light microscopy.

Localisation of BASI and α-Amylase in the Endosperm

The inhibitor signal was present throughout the endosperm tissue in developing kernels. The immunocytochemical data confirmed the work of Weselake *et al.* (1985b) which determined, by examining pearling fractions, that BASI was evenly distributed in the endosperm of mature kernels. The signal was first detected in small quantities in the endosperm of 2 WPA kernels (figure **13**). Signal intensity increased over the next 2 - 3 weeks of development (figures **14 & 15**). After 4 - 5 WPA, the level of inhibitor signal remained constant until the kernel was imbibed (figures **16 & 17**). This pattern of BASI synthesis corresponds well with the time course of BASI synthesis in barley kernels published by Robertson and Hill (1989). It is also consistent with the time other proteinase/amylase inhibitors and storage proteins are synthesized during grain development (table **2**).

Upon germination, α-amylase signal first appeared in endosperm beside the scutellum and adjacent aleurone, then spread to the central and distal portions of the kernel (figures **19,21,23 & 25**). The progression of α-amylase was identical to that reported by

Gibbons (1979) in immunocytochemical studies of α-amylase in germinating barley kernels.

The quantity of BASI in germinating kernels has been shown to remain constant (Chisholm, 1989) or to decrease slowly after 2 DI (Lecommandeur *et al.*, 1987). However, in this study (figures **17 - 25**), there appeared to be a definite increase in inhibitor signal in the endosperm of germinating kernels at the same location and intensity as had been found for α-amylase. Given that it is unlikely that the level of BASI was indeed increasing during germination, there are two possibilities that may explain the intense inhibitor signal at this time. First, the intensity of signal prior to imbibition may not be representative of the total amount of inhibitor present. This would happen if BASI were not accessible or only partially accessible to the antibody. Reduced accessibility may be due, for example, to the particular way in which BASI is packed in the storage tissue. Upon germination, the action of the α-amylase, cell wall degrading enzymes, or other germination specific enzymes may loosen the endosperm tissues enough to allow access of the antibody to the inhibitor molecules. Also, the inhibitor may be stored in a bound form, as is ß-amylase (Hara-Nishimura *et al.*, 1986), until it is required during germination. The bound form may not be recognized by the anti-inhibitor antibody. Second, the intensity of signal after imbibition may not be representative of the quantity of inhibitor present. The anti-inhibitor antibody may have an increased affinity for the inhibitor due to a change in cellular conditions or to the form of inhibitor, or to the α-amylase-inhibitor complex itself. In any case, both α-amylase and BASI are found in the same location and the reason for the discrepancy in signal intensity, once determined,

may itself support the idea of inhibition of α-amylase by BASI *in vivo*.

Localisation of BASI and α-Amylase in the Aleurone

BASI was detected in aleurone cells during both development and germination (figures 13 - 24). BASI was distributed throughout the cell except in the nucleus and cell wall (figure 17). Since mature aleurone cells are packed with protein bodies (figure 2C), it is probable that BASI is stored in these organelles during kernel development. As expected (Gubler *et al.*, 1986 and Zingen-Sell *et al.*, 1990), α-amylase was detected in the aleurone upon imbibition. Given that the α-amylase synthesized in the aleurone upon kernel imbibition is enroute to the endosperm, BASI would also have to be secreted to be able to inhibit α-amylase.

Subcellular Localisation of BASI

Immunocytochemistry, utilizing transmission electron microscopy, revealed that BASI was located primarily within the protein bodies of developing endosperm (3 WPA: figure 26) and germinating scutellum (1 DI: data not shown). This was expected since many of the α-amylase/proteinase inhibitors have been located in protein bodies (Table 1). The location of BASI within protein bodies confirmed that the leader sequence of BASI is able to act as a signal peptide.

BASI was detected, in very small quantities, in the cytoplasm of basal scutellar cells (data not shown). It is possible that some BASI is destined to remain in the cytoplasm. There may be two genomic copies of BASI (Leah and Mundy, 1989) which encode

different transport signals. Alternately, it is possible that some inhibitor protein was released from the protein body.

Immunocytochemical studies (Gubbels and Hill, unpublished) have subsequently shown both BASI and α-amylase to be located in the vacuolated protein bodies of 3 DI basal scutellar cells. Although there is very little protein remaining in the protein bodies/vacuoles, both BASI and α-amylase remain abundant. Neither α-amylase nor BASI is found within the amyloplasts of scutellar cells at 3 DI. The identical cellular location of BASI and α-amylase suggests that it is possible for BASI to bind and inactivate α-amylase.

Comparison of BASI with other Seed-Specific Proteins

A comparison of BASI to other well characterized seed-specific proteins was conducted to assess possible functions of BASI in barley kernel development (Table 1). LEA proteins, which are thought to be involved in relieving desiccation stress, have the least features in common with BASI. LEA proteins are synthesized later in embryo development, lack signal peptides, and are not located in protein bodies or endosperm tissue. The remaining tabulated seed-specific proteins, including BASI, are all synthesized during early to mid kernel development, have signal peptides, and are located in protein bodies (Table 2). In addition to acting as protein reserves for the embryo, they possibly play roles in caryopsis defence through heterologous inhibitor activities or fungicidal/ insecticidal properties (Garcia-Olmedo *et al.*, 1987, Mishkind *et al.*, 1982, and Janzen *et al.*, 1976).

Table II **Comparison of Representative Seed Proteins**[1,2]

Gene Family	Protein Size (AA)	Signal Peptide (AA)	Time of Synth. (Seed)	Location (Tissues)	Location (Cells)	Function
STI						
BASI	181	22	E-Mid	E,A,S,C,H	PB	α-Amylase/ subtilisin Inhibitor
STI(kTi3)	181	25	E-Mid	Ct, Em	P B, W	Trypsin Inhibitor
Cocoa bean albumin	221	26			MO	
Cereal Tryp/α-AI CM (pUP-13)	123	>13	E-Mid			Trypsin/ α-amylase Inhibitor
Potato Inhib. I Tomato lambda clone 1	69	23	E-Late	Fruit (NOT Seeds)	PA(V)	Chymotrypsin & subtilisin Inhibitor
WGA	186		E-Mid	R,Sr,C,S (NOT E)	PB,Ec	Fungicide
BGA	186	26	E-Mid	R,Sr,S		
Phytohemagglutinin						
PHA-E	254	21		C,Ea	PB	Insecticide
PHA-L	252	20				
LLP/αAI	223	20	E-Mid	C,Ea	PB	α-Amylase Inhibitor
LEA						
EM	93	none	Late	Em (NOT E)	Cytosol	dehydration stress?

Notes: 1. Details and references are found in the Literature Review.
2. Explanation of Abbreviations:

A - Aleurone
AA - Amino Acid residues
C - Coleoptile
Ct - Cotyledon
E - Endosperm
Ea - Embryonic axes
Ec - Electron translucent cytoplasm
Em - Embryo
E-Mid - Early to Mid
H - Coleorhiza
MO - Membrane enclosed Organelle
PA - Protein Aggregates
PB - Protein Bodies
R - Radicle
S - Scutellum
Sr - Seminal root
V - Vacuole
W - cell Wall

The subtilisin inhibitory activity of BASI suggests that it too could be involved in kernel defence. However, BASI was not located in a good position to defend germinating seedlings since it was absent from the radicle, seminal roots and plumule of the embryo. By comparison, barley germ agglutinin, which is thought to have a role in plant defence, is located most prominently in the surface cell layers of the radicle and seminal roots (Mishkind *et al.*, 1983).

Based on sequence and/or structural similarity, BASI is most similar to the families of serine protease/α-amylase inhibitors, in particular the soybean trypsin inhibitor (Kunitz) family. One interesting feature of the serine protease/α-amylase inhibitor families is the hypervariability found in the active site of inhibition (Laskowski and Kato, 1980). The active sites of most enzyme families do not tolerate amino acid substitutions. Mutations of the inhibitor families' active sites are more likely to change the specificity of inhibition rather than general binding ability (Laskowski and Kato, 1980). Modified specificity gives this superfamily the unique ability to provide many new and potentially useful proteins which apparently have been selected and retained by different organisms throughout evolution. α-Amylase specificity is common among the active sites of the inhibitors (Garcia-Olmedo *et al.*, 1987). It is significant that only those cereals that produce the α-amylase II isozyme (Marchylo *et al.*, 1987) contain genes encoding α-amylase II-specific inhibitors (Weselake *et al.*, 1985b). This fact suggests that the α-amylase/subtilisin inhibitor provides a useful biological function in the kernels of these cereal species through inhibition of an endogenous α-amylase.

The Role of BASI in Barley Kernels

A combination of the biochemical information available on the interaction of BASI and α-amylase II and the immunolocalisation data on these proteins suggests that α-amylase II is bound to BASI during the early stages of germination. The low dissociation constant of the α-amylase II : BASI complex (Halayko *et al.*, 1986) indicates that, should they be positioned close together, BASI would easily bind α-amylase II. Given that BASI was located in the same tissues and subcellular organelles as α-amylase, it is likely that BASI does indeed bind α-amylase *in vivo*. The quantity of BASI available is not a limiting factor since about a 2.5 fold molar excess of BASI over α-amylase exists after 7 days germination of the kernel under typical growing conditions (Hill *et al.*, 1992). Thus, BASI likely attenuates starch granule degradation during early germination through the inhibition of α-amylase II.

CONCLUSION

The locations of α-amylase/subtilisin inhibitor, α-amylase and starch granules, in developing and germinating barley kernels, were determined using immunocytochemistry and polarizing microscopy. BASI was first synthesized during early kernel development. BASI was found in the scutellum, coleorhiza and coleoptile of the embryo, as well as, in the endosperm and aleurone. Starch granules were observed in all kernel tissues in which the inhibitor was located. α-Amylase appeared in these same locations upon imbibition of the kernel. BASI was located in the protein bodies of endosperm and scutellar cells. α-Amylase has also been observed in the protein bodies of scutellar cells. Since BASI and α-amylase were located in the same tissues and organelles, it is physically possible for BASI to inhibit α-amylase *in vivo*. This research supports the hypothesis that BASI attenuates starch granule degradation by binding α-amylase.

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