Autophagic programmed cell death in the suspensor and endosperm of *Vicia faba*: An ultrastructural study

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Abstract

Programmed cell death (PCD) is a widespread phenomenon among multi-cellular organisms. Without the deletion of cells no longer needed, the organism would not be able to develop in a predicted way. It is now believed that all cells have the capacity to self destruct and that the survival of the cells is depending on the repression of this suicidal programme.

PCD has turned out to show similarities in many different species and there are strong indications that the mechanisms running the programme might, at least in some parts, be evolutionary conserved. PCD is a generic term for different programs of cell destruction, such as apoptosis and autophagic PCD. An important tool to determine if a cell is undergoing PCD is the transmission electron microscope.

The aims of my study were to find out if, and in what way, the suspensor and endosperm in broad bean, *Vicia faba*, which are short-lived structures, undergoes PCD. The endosperm degradation precedes the suspensor cell death and they differ to some extent ultrastructurally. The cell death occurs about 13-14 days after pollination (dap) when the embryo proper is mature enough to support itself. It was found that both tissues are committed to autophagic PCD, a cell death characteristic of conspicuous formations of autophagic vacuoles. It was shown by histochemical staining that acid phosphatases are accumulated in these vacuoles but were also present in the cytoplasm. These vacuoles are similar to autophagic vacuoles formed in rat liver cells, indicating that autophagy is a widespread phenomenon. DNA fragmentation is the first visible sign of PCD in both tissues and it is demonstrated by a labelling technique (TUNEL). In the endosperm nuclei the heterochromatin subsequently appears in the form of a network while in the suspensor it is more conspicuous with heterochromatin that forms large electron dense aggregates located close to the nuclear envelope.

In the suspensor, the plastids develop into chromoplasts with lycopene crystals at the same time or shortly after DNA fragmentation. This is probably due to the fact that the suspensor plastids function as hormone producing organelles and support the embryo.
proper with indispensable growth factors. Later the embryo will be able to produce its own growth factors and the synthesis of these, in particular gibberelins, might be suppressed in the suspensor. The precursors can then be used for synthesis of lycopene instead.

Both the suspensor and the endosperm are going through autophagic PCD but the process differs in some respects. This is probably due to the different function of the two tissues and that the signals that trigger the process presumably are different. The embryo proper is probably the source of the death signal affecting the suspensor. The endosperm, which has a different origin and function, might be controlling the death signal within its own cell. The death might in this case be related to the age of the cell.
List of Papers

This thesis is based on the following papers. They will be referred in the text by their Roman numerals.


In all papers I did the experiments and wrote the manuscripts.
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Introduction

The fact that cell death is a requirement for life and not the other way around might be a new way of looking at development and growth. But during the last decade, programmed cell death, PCD, has proven to be absolutely indispensable in many different situations in almost all organisms. In multicellular organisms, all individual cells have to work together in agreement, to make a tissue or an area function in a predicted way. The cells need to communicate with each other to maintain homeostasis, a delicate balance between failure and success. It is presently believed that all eukaryotic cells are programmed to die but that they are prevented from doing so until the right signal triggers the cell death programme (Ameisen, 2002). This repression is often controlled by neighbouring cells. Many human diseases are a consequence of the wrong signals at the wrong time (McCarthy, 2002). Examples of this are the mass cell death in Alzheimer’s disease, or the opposite, cell proliferation as in many tumours.

PCD has been documented in many different cells and tissues among species from several phyla. There are many examples of this process in plants. In the very beginning of life, three non-functional megaspores are deleted after meiosis in Marsilea (Bell, 1996). This is also seen during initiation and development of somatic embryogenesis in Picea abies (Filonova et al., 2002). It is documented in maize endosperm and in the aleurone layers of different cereals (Wang et al., 1998; Young and Gallie, 1999, 2000; Young et al., 1997; Buckner et al., 1998, 2000), where the aleurone cells are specialized endosperm cells that die after seed germination. An example of PCD at a later stage during tissue differentiation is the formation of tracheary elements, which is perhaps the process that has been studied in greatest detail in plant PCD (Mittler and Lam, 1995).

Some cases of non-developmental cell death are also examples of PCD. Hypersensitive response (HR) is a reaction to pathogen attack (Greenberg et al., 1994; Mittler et al., 1996, 1997), where the cells die in a rapid manner at the site of the pathogen entry, as a way for the plant to prevent spread of the infection. Dictyostelium discoideum is a slime mould that under favourable conditions undergoes cell differentiation and morphogenesis to a multicellular fungus-like structure with a fruiting body and a stalk (Cornillon et al., 1994). The highly vacuolated stalk cells show characteristics of PCD similar to those seen in other eukaryotic cells. There have also been reports of PCD in fungi, algae and bacteria; but most of all in animal cells, a group that totally dominates the research area.
Morphological characteristics
Morphological studies of PCD can be done by light microscopy techniques or by transmission electron microscopy, TEM. To identify single apoptotic cells in a tissue, light microscopy is a sufficient tool. Immunolocalization of different proteins can be done with the help of fluorescence or confocal microscopy as well as with TEM. However, the best and many times the only method to study individual organelles and characterize PCD is through the electron microscope.

Different types of cell death
Programmed cell death was first recognized morphologically by Kerr and co-workers (1972) when they described cell death distinct from necrosis. Necrosis is considered to be a passive death, in contrast to the programmed cell death process. The most important characteristic of necrosis is the loss of membrane integrity (Fig.1). The whole cell, including mitochondria, swells, and the cell death is presumed to be unregulated and not under genetic control (Zakeri, 1998). The cell swells and loses integrity and eventually disintegrates, but the overall shape of the cell is usually maintained. Necrosis occurs when cells are exposed to external threats such as extreme temperature, to high or low pH, or toxic agents.

![Figure 1 Diagram showing different kinds of cell death.](image)

Figure 1 Diagram showing different kinds of cell death. Necrosis to the left. The cell swells resulting in loss of membrane integrity. Apoptosis, in the middle, with cell condensation and formation of apoptotic bodies with intact membranes. To the right, autophagic cell death. Formation of autophagic vacuoles and preservation of organelle membranes.
Programmed cell death

Three types of programmed cell death are recognized; Type 1- apoptosis, Type 2- autophagic (lysosomal) cell death and Type 3 - non-lysosomal cell death (Schweichel and Merker, 1973; Clark, 1990). Non-lysosomal cell death has only been reported in animal cells as a diffuse degeneration. It is a general cytoplasmic cell death without condensation of the nucleus and has not, to my knowledge, been described in plant cells. It is therefore not treated here.

Type 1
Apoptosis is a well established term in cell death research. The word means “dropping off”, like leaves falling from a tree. It was first described as a “shrinkage necrosis” in developmental systems by Kerr (1972). This is a PCD form mostly found in animal cells but hypersensitive response in plants after pathogen attack shows some similarity to apoptosis (Mittler et al., 1996). The classical degradation pattern in apoptosis is characterised by rapid changes in the nucleus, where dense chromatin appears and increases, until the nucleus is totally condensed or pyknotic (Clark, 1990). Large areas of condensed heterochromatin are located in the margins of the nucleus, which at this stage has become highly convoluted. The cytoplasmic organelles are not affected more than that they are closely packed in the cytosol, which becomes denser because of water loss. However, ribosomes are lost from polysomes and ER dilates or disappears (Clark, 1990). Membrane bound apoptotic bodies are formed, which are phagocytosed at a later stage; a feature only found in animal cells. The whole cell has a shrinking appearance, but organelles and apoptotic bodies have intact membranes, which is an important difference from necrosis. Several studies of apoptosis have revealed that this is a cell death type mostly found in individual cells that need to be quickly removed and not in regions were mass death occurs (Clark, 1990; Schweichel and Merker, 1973)

Type 2
Autophagic PCD is characterized chiefly by the formation of autophagic vacuoles (Clark, 1990; Dunn, 1990 a,b). Many different species in several phyla show features of autophagic PCD. This cell death type shows greater heterogeneity between different tissues than apoptosis. The nuclear changes are somewhat similar to apoptosis with a fragmented DNA, even though exceptions occur. However, the typical budding of membrane bounded fragments does not occur. The condensation pattern of the nucleus
differs between species, but most cells show characteristic electron dense areas in the margins of the nuclei. The nuclei are often irregular in shape, but have intact envelopes. The formation of autophagic vacuoles is extensive, and cytoplasmic portions are degraded within the vacuoles. Golgi bodies are often dilated playing a role in the PCD process by discharging vesicles containing hydrolytic enzymes into the vacuoles (Clark, 1990). Mitochondria and plastids keep their membrane integrity in most cases, and are, like in apoptosis, the last organelles to disintegrate. Endoplasmic reticulum usually dilates and takes part in the formation of autophagic vacuoles (Matile, 1975; Deepesh, 2000). Autophagic PCD occurs when a large accumulation of cells is being degraded, such as developing tissues or larger areas after injury, and only seldom when individual cells within a tissue are programmed to die.

**Mechanisms of PCD**

Studies on the nematode *Caenorhabditis elegans* have provided general information on how genes control the programmed cell death process (Horvitz, 1999). 13 cell death genes have been characterized that regulate the cell death in 131 of 1090 cells during the nematode development. One gene, *ced-9* (*cell death*), protects cells from programmed cell death (Horvitz, 1999). Other genes, such as *ced-3* and *ced-4* are required for programmed cell death to occur. Many of these genes are conserved throughout evolution. Most homologous are found in mammals, but there are also counterparts in plants and fungi (McCarthy, 2002).

Regulated cell death can be divided into three phases: initiation, commitment and execution.

**Initiation**

The common mechanisms of apoptosis are negatively regulated by several sets of genes that are both pro- and anti-apoptotic such as the bcl-2 family. This is a large gene family which acts on mitochondria and is homologous to *ced-9* in *C. elegans*. Similar genes have been documented in tobacco (Dion et al., 1997). One bcl-2 family member, bax, binds to the outer mitochondrial membrane and changes its permeability. Large pores are formed resulting in the release of mitochondria bounded proteins out to the cytosol. During inactive phases, bax is controlled by anti-apoptotic bcl-2 genes. They bind to bax in the active conformation site, preventing it from binding to the mitochondrial outer membrane. This ability to interact with each other gives the bcl-2 members control over life and death in a cell (Tsujimoto, 2003).
However, much more investigation must be done to determine what triggers the mitochondrial activation.

Commitment and execution

The commitment phase is not a universal pathway. Instead, it seems to depend on cell type and what kind of stimuli triggers the cell death. A well documented pathway is the cytochrome c release in mammal cells, after bax induction. When cytochrome c is released into the cytoplasm it binds to Apaf-1 (apoptotic protease-activating factor-1) and ATP. This complex then recruits caspases-9 and forms an apoptosome. Caspase-9 belongs to a family of cystein proteases. They are found in animal cells, where all members cleave their substrates after aspartate residues. Caspases were first recognized as a homolog to ced-3 in C. elegans. Some of the 14 caspases that have been identified to date have no participation in PCD, but the majority seems to be involved in the cell death process (Zhang et al., 2003).

Caspase-3, 6, and 7 are recognized as execution caspases. They are located downstream of caspases-9 and are responsible for the proteolytic cleavage of several proteins (Zhang et al., 2003).

The morphological changes characteristic for PCD are a result of proteolysis. True caspases have not been found outside the animal kingdom. However, caspases are a type of proteases, and protease activity has been linked to PCD in plant cells (Woltering et al., 2002). The formation of tracheary elements (TE:s) is a well known plant PCD model (Mittler and Lam, 1995). TE cell differentiation is coupled to secondary wall formation, which in turn is triggered by the secretion of a serine protease (Groover and Jones, 1999). Serine proteases are also involved in the type of cell death that occurs after elicitin-induced hypersensitive response (Woltering et al., 2002). Other caspase-like proteins are paracaspases, found in metazoans and Dictyostelium, and metacaspases found in plant, fungi and protozoa (Uren et al., 2000). Metacaspases found in the yeast Saccharomyces cerevisia, show caspase-typical features when cells are stimulated to undergo apoptosis (Madeo et al., 2002). Metacaspases have also been detected in Trypanosoma and Plasmodium, suggesting that metacaspases resemble the ancestral protease and that paracaspases and caspases have evolved later.

There are other ways for PCD to occur than through the caspase cascade. Apoptosis inducing factor (AIF) is another protein bounded to mitochondria that is released into the cytoplasm upon apoptotic stimuli in the same way as cytochrome c and considered
to take part in a caspase-independent process (Lorenzo et al., 1999). Nevertheless, recent work has revealed that AIF might also be caspase-dependent since the release of AIF can be suppressed by caspases inhibitors (Arnoult et al., 2002). However, AIF is found in plants, fungi and C. elegans, organisms without identified caspase activity, indicating that AIF might function in parallel cell death pathways (Penninger and Kroemer, 2003). AIF is a mitochondrial inner membrane bound protein, and a consequence of this is that, when bax induces permeabilization in the outer mitochondrial membrane, AIF stays unaffected due to its location (Arnoult et al., 2002).

When AIF was added to purified nuclei, DNA fragmentation and chromatin condensation occurred, indicating that caspases are not necessary for nuclear degradation (Candé et al., 2002). AIF has homologues in mammals, C. elegans, Drosophila, plants, fungi and bacteria. The strongest homologues with AIF found in mammals are the plant flavoprotein semidehydroascorbate and ascorbate reductases. Research on how AIF participates in plant programmed cell death has just started, with the aims to show how these proteins interact during PCD.

Structures in the embryo sac
The embryo sac is the female gametophyte in angiosperms. This polar structure has a chalazal end were the antipodal cells are situated and a micropylar end were the egg apparatus is located. The angiosperm embryo sac is in general composed of the egg cell, two synergids, three antipodal cells and a central cell. All individual cells have a single nucleus, except for the large central cell that contains two.

When the megagametophyte has reached maturity the stigma is ready for the pollen grain. The newly developed pollen tube will grow through the style towards the ovule. The pollen tube has to pass through the integument and nucellar tissues to reach the embryo sac, which is possible only through a narrow channel, the micropyle. When the pollen tube reaches the embryo sac it releases its cell contents. One of the generative male cells fuses with the egg cell, which will give rise to the zygote. The nucleus of the second male cell will fuse with the two central cell nuclei, which gives rise to a triploid tissue, the endosperm. Angiosperms are, because of this second fusion, said to have a double fertilization.

The suspensor
After fertilization the zygote usually divides into one terminal and one basal cell. The smaller terminal cell will divide further and give rise to the embryo proper. The larger
basal cell will develop into a highly vacuolated suspensor. The growth rate of the suspensor is often very rapid during earlier stages of embryogenesis (Natesh and Rau, 1984). The suspensor is fully developed in many species when the embryo proper is still in the octant stage. Later, when the embryo proper has reached maturity, the suspensor degenerates.

Morphology
There is no typical angiosperm suspensor since the shape and size of suspensors varies a great deal between different species. A suspensor can consist of one single cell as in the nun orchid, Phaius tankervilliae (Ye et al., 1997), or 200 cells as in Phaseolus coccineus. Fabaceae and Orchidaceae may have the greatest diversity of suspensors within an individual family. While Phaseolus develops quickly to form a large and wide column, other suspensors in Fabaceae consist of just one or two cells. Vicia, Pisum, Lens and Lathyrus has the most distinctive and uniform suspensor (Lersten, 1983). It consists of only four large cells in two tiers, where the two basal cells are long and slender and the two apical cells are spherical. In Brassicaceae most suspensors are filamentous, as in Capsella bursa-pastoris and Arabidopsis thaliana, which have a suspensor of 6-8 cells in a single file (Natesh and Rau, 1984). They can also be grapelike, as in Cytisus laburnum, or tube like as in Cymbidium. In species such as Lycopsis, Tilia and Viola there is no suspensor at all. In families as Rubiaceae, some suspensors produce haustoria, which is an outgrowth that invades maternal tissue and/or the endosperm.

Ultrastructure
Wall ingrowths, characteristic for transfer cells, often develop in the basal part of the embryo sac (Johansson and Walles, 1994; Raghavan, 1997). These structures have been studied in C. bursa-pastoris, where the projections appear in the basal cell during the zygote stage (Schultz and Jensen, 1969). Wall ingrowths will then increase during embryogenesis and the basal suspensor cells will then function as a transfer cell by the time the embryo is in the heart stage. The transfer cells increase the absorption capacity of the suspensor and play an important role in favouring nutrient transport to the embryo proper. There are numerous ER-containing plasmodesmata between the suspensor and the embryo proper as well as between individual cells in the suspensor. However, between the embryo and the endosperm plasmodesmata occur only rarely. Specialized plastids have been found in the suspensor of Pisum (Marinos, 1970), Ipomoea (Ponzi and Pizzolongo, 1973), Stellaria (Newcomb and Fowke, 1974),
Tropaeolum (Nagl and Kuhner, 1976), Medicago (Sangduen et al., 1982) and Vicia faba (Johansson and Walles, 1994). These large sized plastids occur in large numbers. They have lamellar bodies somewhat similar to prolamellar bodies but without the characteristic crystal pattern of true prolamellar bodies (Marinos, 1970). There have not been any reports about these plastids being transformed into chloroplasts.

Function of the suspensor
In a historical perspective, the function of the suspensor was believed to be a device to mechanically push the embryo proper into the embryo sac towards the chalazal end where most nutrients were supposed to be available. Through extensive cytological and physiological studies during the last three decades, a rethinking has been necessary. The suspensor is now seen to play an active role in several ways during embryogenesis. Transport of nutrients from maternal tissues to the embryo proper goes through the suspensor via the frequently appearing plasmodesmata. The wall ingrowths in the micropylar end are increasing the capacity for such transport. Evidence for suspensor transport was shown by Yeung (1980) when \(^{14}\)C labelled sucrose was introduced into the embryo sac of Phaseolus. The labelled solution was directly injected into the endosperm cavity at the heart stage. The radioactivity later appeared in the suspensor and in the basal end of the embryo proper, close to the suspensor. This result shows that substances do not take the shortest way from the endosperm directly into the embryo proper, but rather move to the suspensor and thereafter are distributed to the embryo proper (Yeung, 1980).

Physiology and biochemistry
The suspensor is metabolically active during its life time. It is considered to be the main source of growth regulators to the embryo proper in early embryogenesis (Raghavan, 1997). Auxins (Alpi et al., 1975) gibberelin (Cionini et al., 1976; Picciarelli and Alpi, 1986) and cytokinins (Lorenzi et al., 1978) have all been identified in the suspensor of Phaseolus. The concentration of gibberelin (GA) in P. coccineus is thirty times higher in the suspensor than in the embryo proper at heart stage (Cionini, 1987). However, in the cotyledon stage there is a reversed situation with an increase of GA:s in the embryo proper and a decrease in the suspensor. At this time the GA level in the embryo proper is ten times higher than it was in the heart stage (Cionini, 1987).

When isolated embryos with removed suspensor are grown in vitro at the cotyledon stage there is no retardation in development (Cionini, 1987). However, if the same
treatment is performed on embryos in the heart stage, there is developmental arrest. If gibberellins are added to the medium in early stages, growth continues as normal, but in cotyledon stage the addition of GA retarded the growth (Cionini, 1987). These data indicate that gibberellins are actively synthesized in the suspensor before the embryo is mature.

**Growth potential**

Several studies on the suspensor in different species have demonstrated that the potential development of the suspensor is much greater than its normal fate. These investigations indicate that the suspensor growth is restricted by the normal development of the embryo proper (Haccius, 1963, Yeung and Meinke, 1993). When seeds of different species were irradiated, the actively dividing embryo proper was destroyed while the mature suspensor was left almost unaffected (Yeung and Meinke, 1993). The growth of the remaining suspensors was abnormal in size and shape. The *Eranthis* suspensor did not only increase its growth to become a massive organ, it also developed a new embryo proper (Haccius, 1963).

Mutants provide excellent systems to investigate the connections between different organs. In *Arabidopsis* several suspensor mutants (SUS) have been identified. Investigations on mutants have shown that the suspensor can acquire characteristics normally restricted to the embryo proper such as storage of lipids, proteins and starch (Schwartz *et al*., 1994). The abnormal growth of the suspensor always precedes the abnormality of the embryo proper. It might therefore be a failure of the embryo proper to send inhibitory signals to the suspensor that gives rise to the enhanced development. However, another explanation can be that the mutant embryo proper does not function as a normal sink for nutrients and hormones transported via the suspensor. These substances will then accumulate in the suspensor, resulting in cell proliferation. The SUS mutant embryo proper does serve as a sink in early embryogenesis, and even accumulates storage products, indicating that, in this case, the failure of the signalling system is responsible for the suspensor proliferation (Schwartz *et al*., 1994).

**The endosperm**

The triploid endosperm in angiosperms is, as mentioned above, the result of fertilization between the diploid central cell and a sperm cell. Nuclear division in the endosperm starts early and the development of the endosperm precedes that of the embryo. In *Triticum aestivum* the division of the primary endosperm starts six hours after fertilization while the zygote waits for 22 hours (Raghavan, 1997). Five days
later the endosperm consisted of 5000 cells while the pro-embryo was built up of 96 cells. The fact that there is a time interval between the endosperm and zygote is probably significant for the nutrition of the zygote.

**Development**

The pattern of the nuclear divisions has been the basis for a simple classification of the endosperm into nuclear, cellular and helobial types (Esau, 1977).

In the nuclear type, the nuclear division of the nucleus occurs without cytokinesis. The large central cell will be filled with thousands of free nuclei. However, in some species, cell walls will develop in the final stage of endosperm ontogeny. In the nuclear type of endosperm, a large central vacuole will occupy a major part of the central cell. As a result of this, the nuclei and the rest of the cytoplasm will be pushed towards the embryo sac border and surround the embryo. There are several examples of species with nuclear endosperm. Many investigations have been done in cereals (*Poaceae*), which have a coenocytic stage with free nuclei that later will go through a cellular configuration (Raghavan, 1997). Other families are *Fabaceae*, *Brassicaceae*, *Curcurbitaceae*, *Areaceae* and *Proteaceae*. In some species within these families, such as *Vicia* and *Pisum*, the nuclear stage persists throughout the life time of the endosperm (Vijayaraghavan and Prabhakar, 1984; Johansson and Walles, 1994).

In the cellular type, cell wall formation follows mitosis and continues to do so as long as the endosperm is growing. This type is found in families such as *Gesneriaceae*, *Acanthaceae* and *Loranthaceae*. The formation of an haustorium is common within this type of endosperm, which is a structure that invades the ovular tissue and function as an additional nutrition source for the growing seed. This structure occurs also in the nuclear type, but only rarely in the helobial type.

The helobial type of endosperm is the least frequent type. It occurs mainly in monocotyledons where the group follows more or less the same developmental pattern in early stages, but variations occur in later stages. The division of the first primary nuclei gives rise to two unequal cells with the larger cell located in the micropylar end. The chalazal end cell will develop in a non-cellular manner while cellularization occurs in the cell at the micropylar end.
Endosperm function

The endosperm was early recognized as a tissue to support the growing embryo with nutrients. It is also a link between the maternal tissue and the embryo. In many species such as *V. faba* and *Arabidopsis* (Brown *et al*., 1999) the endosperm is consumed before seed maturity. The embryo and the endosperm are closely associated, but many of the interactions that occur between the two different tissues remain to be solved. However, several specialized structures strengthen the nutrition theory. To enhance the nutrient pathways between the embryo and the endosperm structural adaptations, such as transfer cells with wall ingrowths have developed. These cells are located in the basal part of the suspensor towards the endosperm and are, for example, found in *Vicia faba* (Johansson and Walles, 1993,1994), *Capsella bursa-pastoris*, *Phaseolus coccineus* and *Glycine max* (Raghavan, 1997). The cell surface may increase by as much as twenty times through these invaginations, and thereby a possibility for increased nutrient uptake. The formation of haustoria is another adaptation for increased uptake.

The nutrients stored in the endosperm are mostly starch, lipids and hemicelluloses. In some plants such as mustard, oil is the main storage product and can constitute as much as 40 % of the dry weight (Esau, 1977). In species where the endosperm is consumed during early embryogenesis there is no accumulation of starch, since the cotyledons have taken over the storage function in the seed.

In cereals nuclear endosperm dominates. It is persistent and contains three different cell types at seed maturity. The outermost cells, the aleurone layer, make up a specialised group of living cells filled with lipids and storage proteins. This layer can be one to four cells thick (Raghavan, 1997). The second layer, also called “modified aleurone”, consists of transfer cells which are specialized for uptake and transport of nutrients from the maternal tissue (Becraft, 2001). The innermost part is the starchy endosperm, the cells of which are dead by the time of seed maturity. The germination triggers with the release of GA from the embryo to the living aleurone cells, which respond with a secretion of α-amylase into the starchy endosperm (Crozier *et al*., 2000). This will subsequently result in the degradation of the aleurone cells in a PCD manner (Wang *et al*., 1998; Young and Gallie, 1999). It has been demonstrated by Bethke *et al* (1999) that GA is a signal that initiates PCD in barley aleurone cells, and that ABA opposes that effect. Under the influence of ABA, aleurone cells can remain alive for many years after the genetically identical starch endosperm cells have died. Later, when seed dormancy has been broken, GA will be produced in response to
signals from the embryo, and the aleurone layer will degenerate in a programmed cell death manner (Bethke et al., 1999).

Aims
Programmed cell death is indispensable in multi-cellular organisms. Without the deletion of specific cells the developmental pattern would be disturbed and the organism unable to develop in a predicted way. This type of programmed cell death has turned out to occur in many different taxonomic groups and there are strong indications that the mechanisms running the program in these organisms are similar in many ways. One of the best methods to determine whether a cell or tissue is subjected to the cell death programme is through morphological studies. There are several ways to find out if a tissue or single cells are committed to program cell death. A single characteristic might not be sufficient enough to establish PCD but the diagnosis becomes more certain when several features are taken together.

The objectives of my investigation have been to find out if the suspensor and endosperm of *Vicia faba*, which are short-lived structures, are undergoing a programmed cell death. Important questions have also been if the cell death is synchronized in time and behaviour between the two tissues since they have different origin due to double fertilization and different functions. The study has focused on nuclear and mitochondrial changes, autophagic vacuole formation and plastid transformation. The aim was to study the organelles separately and then bring the data together to get an overall view of how PCD proceeds morphologically in the suspensor and endosperm of *V. faba*.

Results and Discussion
The endosperm of *Vicia faba* consists of a huge, single multinucleated cell. It belongs to the nuclear type without the development of internal cell walls. The suspensor consists of four large multinucleated cells with two elongated basal cells connected to the micropylar end, and two rounded cells towards the embryo proper (Fig. 2). When the embryo has reached the heart stage, degradation of the short-lived suspensor and endosperm starts. These two multinucleated tissues are ideal for morphological studies on PCD since the classical morphological identification of these processes is partly based on various nuclear changes.
**DNA fragmentation**

Heterochromatin condensation is seen in almost all types of PCD (Clark, 1990). The fragmentation of chromosomal DNA is characteristic for apoptosis as well as for some, but not all, types of autophagic PCD. This fragmentation is considered to be one of the most important hallmarks of apoptosis and PCD. One method to detect DNA fragmentation is the *in situ* cell death method, the TUNEL assay (TdT-mediated dUDP fluorescent (FITC) nick end labelling technique). The detection is done *in situ* with help of terminal deoxynucleotidyl transferase (TdT), which adds the labelled dUTP to all available 3’ ends. The more fragmented DNA, the more 3’ ends and thus brighter signal. The nuclei of *V. faba* suspensor and endosperm show DNA fragmentation using this method at 13-15 dap. In some embryo sacs the endosperm nuclei stain heavily as early as 13 dap (Paper I). However at 14 dap, all embryo sacs stain with strong fluorescence in the endosperm nuclei. The same pattern occurs in the suspensor, except that the first sign of fluorescence is seen 14 dap, one day later than in the endosperm. Subsequently, at 15 dap, all suspensor cells have brightly stained nuclei. Embryo sacs younger than 13 days did not show any signal at all when exposed to the TUNEL assay. The endosperm nuclei are easily recognized by their large and oval shape while the suspensor nuclei are at this time rounded or lobed, which makes it easy to distinguish the nuclei between the two cell types.

The nuclear envelopes are still intact at the time of DNA fragmentation and no other visible signs of degradation are detectable ultrastructurally. In a late stage of necrosis it is possible to get a positive result during TUNEL staining because of DNase release.
However, in that case there would be several other symptoms of severe degradation and the nuclear envelope would be broken, features not seen in the suspensor or endosperm cells when TUNEL staining first appears.

**Mitochondrial preservation**

Research on animal and yeast systems has revealed that DNA fragmentation is actually a result of previous mitochondrial activation and not a first event in PCD as it was previously believed (McCarthy, 2002). Substances released from mitochondria such as cytochrome c and AIF are involved in the DNA fragmentation and perhaps also further downstream in the degradation process (Penninger and Kroemer, 2003). The suspensor and endosperm mitochondria of *Vicia faba* are structurally intact during almost the entire disintegration (Paper IV). The mitochondria do not show any signs of destruction until very late in the cell death process. This is consistent with the information animal studies have revealed (Clark, 1990; Waterhouse et al., 2002). ATP production is necessary for a programmed cell death, and the mitochondria membranes have to stay intact for this process. Studies on mitochondria involvement in *Zinnia* tracheary element (TE) PCD showed that, even though cytochrome c was released, it was not sufficient enough to trigger PCD (Yu et al., 2002). PCD in TE:s starts with vacuolar collapse, but mitochondrial membrane potential changes before the collapse, indicating a role in inducing that process. TE PCD is a special form of plant PCD, with the vacuolear collapse, and parallels in other plant systems are not always obvious. However, mitochondria might play the same kind of role in triggering the majority of PCD systems in different cells, but the events downstream of mitochondria activity might differ depending on the cell type. Plant cells lack proteins such as caspases that are responsible for the execution of apoptotic animal cells. Nevertheless, similar proteins exist in plants and these might play the same role during PCD as caspases in animal cells.

In an uncontrolled cell death, as necrosis is considered to be, mitochondria swell with the result of broken envelop and loss of function. The fact that suspensor and endosperm mitochondria retain their integrity for such a long time indicates that the process involved in plant PCD might be similar to the process in animal apoptosis. At about 19 dap mitochondria show precipitates of acid phosphatase (Paper VI). Cristae as well as envelopes are affected, and eventually mitochondria disintegrate in the same way as the rest of the cell content.
Changes in the suspensor nuclei

A few days after DNA fragmentation ultrastructural changes start in the nuclei in the endosperm and suspensor cells (Paper I). The heterochromatin in the suspensor nuclei becomes more electron dense. There are fragments, distributed as scattered aggregates throughout the nuclei but mostly located adjacent to the intact envelope. These features are similar to animal apoptosis (Kerr, 1994), but the heterochromatin in *V. faba* suspensor nuclei is not as segregated and does not form the same sharply circumscribed masses as is formed in animal apoptosis. Also the lysosomal PCD (type 2), has almost the same nuclear degradation pattern as seen in *V. faba* suspensor cells. The nuclei become pyknic, but not as prevalent or striking as in apoptosis (Clark, 1990). Compared to other organisms such as *Dictyostelium* (Cornillon *et al*., 1994) and tobacco plants infected with virus (Mittler *et al*., 1997), the chromatin condensation is more conspicuous.

Shortly before DNA fragmentation, vesicle-like inclusions are seen inside the suspensor nuclei, mainly confined to the euchromatin region. They vary in number and are scattered throughout the nucleoplasm. These types of inclusions are found in many other cells without connections to PCD such as those described in transmitting tissue in *Lilium* (Singh *et al*., 1998). These authors showed that the vesicles are profiles of a reticulum that is continuous with the inner membrane of the envelope. No such connections have been observed in *V. faba*. However, it is still likely that the vesicles originate from the inner membrane.

There are other structures within the nuclei of the suspensor at this stage, such as narrow tubules connected to a bladder-like vesicle. These structures are closely associated with heterochromatin and are often found close to the nuclear membrane. The tubular structures probably originate from the nuclear envelope like the other vesicle-like inclusions. There is good reason to believe that these two structures are connected to each other since the narrow tubules ends in a vesicle. They may perhaps not be involved in PCD, but they increase the surface area of the inner membrane and might facilitate communication within the nucleus and to the rest of the cell.

At a later stage, 17 dap, the suspensor nuclei show considerable signs of degradation. The nuclear envelope loses its integrity and cytoplasm intrudes into the nucleus from areas where the membrane is broken. The nucleolus is still recognizable but swollen and sometimes invaded by small fragments of heterochromatin. At this stage the heterochromatin is more electron dense and mostly distributed in the periphery of the
nuclei. Eventually, only remnants of the nuclei are seen as scattered fragments in the cytoplasm or trapped inside an autophagic vacuole.

Changes in endosperm nuclei

In the endosperm the nuclear changes are somewhat different from those of the suspensor nuclei (Paper I). The most obvious is the heterochromatin that appears as a network distributed throughout the nuclei. Eventually the heterochromatin becomes more and more diluted. Large electron transparent areas appear within the nuclei replacing the euchromatin. The shape of the nuclei in the endosperm remains mostly elongated with a few exceptions. This might be a result of the cytoplasmic distribution within the embryo sac, where the cytoplasm is lying as a thin sheet close to the embryo sac boarder. The nucleoli do not as in the suspensor keep their rounded shape, instead they split into pieces and are scattered throughout the nucleus. Inclusions without a surrounding membrane appear in the endosperm nuclei. They are not similar to those found in the suspensor. Instead, these inclusions consist of large aggregates that might include partly decomposed cytoplasmic material. The inclusions increase in size during the cell death process.

Membrane bounded vesicles are also found in the endosperm nuclei. They are of different shape and size and sometimes fuse, forming large systems. They are vacuole-like and similar to inclusions reported in the nuclei of different plants (Sheffield et al. 1979; Williams and Jordan, 1982; Majewska-Sawka et al., 1990; Yi and Shi-Yi, 1993). Most reports on the formations of these “nuclear vacuoles” concern meiosis (Sheffield et al., 1979, Rowley and Walles, 1985, Yi and Shi-Yi, 1993) but they also include vacuoles fond in degenerating cells of Brassica root tips (Williams and Jordan, 1982). Sheffield et al (1979) showed that some of the nuclear vacuoles originate from the inner membrane and proposed that they are involved in the uptake of material subjected to dissolution within the nuclei. This might be a reasonable explanation why the vacuoles increase in number as the degradation of the endosperm precedes since several substances are transported in different directions during this time.

Acid phosphatases

An interesting result is the fact that after histochemical staining both suspensor and endosperm nuclei show traces of acid phosphatases at about 15 dap (Paper II). This occurs when the cytoplasm is still unaffected, and no other organelles are stained. The nuclear envelope is still intact when small scattered grains, resembling reaction products, are distributed throughout the nuclei. The grains are preferably concentrated
in the heterochromatin but they appear in the euchromatin as well, although in much less amount. Since acid phosphatases are present in vacuoles involved in degradation, it is possible that the nucleus may take an active part in its own degradation. These cells are multinucleated, with a considerable amount of nuclear material that has to be digested before the disintegration is completed. The degradation process proceeds faster in this way and only remnants of the nuclei are finally degraded in the cytosol. This might be a reason why so few nuclei are seen in the cytoplasm at later stages.

As mentioned before, nuclear vacuoles appear just before DNA fragmentation and traces of acid phosphatases is seen shortly after the fragmentation. It is possible that acid phosphatases are transported into the nucleus via ER. Since the nuclear vacuoles are presumed to be formed by the inner membrane, it is a reasonable explanation why acid phosphatases are found inside the nucleus during cell death. The DNA fragmentation indicates a point of no return, since the DNA can not be restored. The nuclear vacuoles are not seen at later stage in the degrading nucleus as euchromatin dilates and eventually disappears. Acid phosphatases could also be transported to the nucleus via Golgi bodies which at this stage are very active and mostly distributed around the nuclei and cell walls. Small Golgi vesicles located close to the nucleus may fuse with its envelope and release their content into the nucleus.

**Autophagic vacuoles**

The large scale formation of autophagic vacuoles in both suspensor and endosperm cells strengthens the conclusion that these cells are subjected to autophagic PCD (Paper II). Autophagy plays a central role in protein and organelle turnover in normal homeostasis. However, the formation of autophagic vacuoles in the *V. faba* embryo sac is far more conspicuous than in ordinary cell processes. The formation of such vacuoles starts with the isolation of a region of cytoplasm captured by cisterns of ER. The same pattern occurs in both suspensor and endosperm. Several concentric layers of ER can form multilayered compounds which will be precursors of autophagic vacuoles. Such formations are described in several plant species as well as in animals (Matile, 1975; Dunn, 1990; Depeech, 2000). However, to become autophagic the vacuoles need acid hydrolysis derived from Golgi vesicles. These vesicles fuse with the former ER membrane and the vesicle content is released into the pre-vacuole. Golgi bodies are located close to the newly formed structure in *V. faba* suspensor and endosperm. Vesicles located inside these vacuoles have the same density and size as Golgi vesicles located outside the vacuoles. The outer membrane remains impermeable to hydrolytic enzymes and will become the tonoplast; the inner
membranes eventually dissolve due to the acid environment (Marty, 1999). Within the autophagic vacuoles, organelles such as mitochondria and plastids may be trapped together with the cytoplasm, and are subsequently degraded. Acid phosphatases are considered to be a marker for autophagic compartments, and when ovules are treated with a lead salt procedure, precipitation is found in the autophagic vacuoles in both suspensor and endosperm cells. The lead salt staining is concentrated to the vacuoles, but is also seen distributed throughout the cytoplasm. Several autophagic vacuoles may fuse to form large areas with strongly degraded material at late stages. Inside these, it is possible to find highly digested remnants of nuclear origin. At a late stage, nuclear remnants are absent in the cytoplasm, with a few exceptions. In several human cells undergoing autophagy, the degradation of Golgi and ER precedes the nuclear destruction (Bursch et al., 2001). In the endosperm and suspensor of V. faba, the degradation of these organelles occurs simultaneously. Plastids and mitochondria trapped in autophagic vacuoles are broken down, but those remaining in the cytoplasm seem to stay intact until only remnants of the cell persist. The cytoplasm in the large endosperm will eventually become vastly diluted. Organelles are scattered throughout the space left in the cell, but mostly concentrated close to the cell wall. The embryo fills the embryo sac more and more and remnants of the endosperm are pressed towards the embryo sac boarder. The suspensor cytoplasm will eventually have the same distribution of cell material as the endosperm cell. It will also, though, have islands of electron dense material resembling condensed cytoplasm, preferable located in the micropylar end where the degradation of the suspensor starts.

Descriptions of autophagic cell death from different organisms have shown that the cellular morphology of the process is basically the same in plants, yeasts and animal cells (Klionsky and Emr, 2000; Dunn, 1990). However, different course of events might be involved in triggering the cell death but the overall morphological process is similar. This is reported in the slime mould Dictyostelium discoideum (Cornillon et al., 1994), the filamentous fungus Podospora anserina (Pinan-Lucarré et al., 2003), Drosophila salivary glands (Baehrecke, 2003) and in the nematode C. elegans (Bursch, 2001). In some mammal cells, such as in rat liver, autophagic vacuoles from ribosome free regions of rough ER (Dunn 1990a), show similarities to the equivalent formations in V. faba suspensor and endosperm cells.

A well studied PCD process in plant systems is the formation of tracheary elements. This is considered to be of the autophagic type of PCD, however, the induction of degradation starts with vacuolar collapse (Kuriyama and Fukuda, 2002). Hydrolytic enzymes are released into the cytoplasm and organelles are attacked and degraded.
Hormones or other triggering substances for this event have not yet been found. However, PCD in trachery elements is tightly coupled to secondary wall formation (Kuriyama and Fukuda, 2002). This type of PCD differs to a large extent from PCD in *V. faba* and many other systems previously mentioned.

In yeast, macroautophagy occurs upon nutrient starvation (Klionsky and Emr, 2000). Double membrane structures form inclusions, termed autophagosomes, containing organelles such as mitochondria. The autophagosomes dock with the existing vacuole and fuse with the tonoplast, releasing their content. The basic machinery for autophagy in yeast and mammals is identical (Reggiori and Klionsky, 2002). Several autophagic controlling genes investigated in yeast are found in *Arabidopsis thaliana* as well (Reggiori and Klionsky, 2002). Also, genes found in the fungus *Podospora* are related to genes involved in autophagy in *Saccharomyces cerevisiae* (Pinan-Lucarré et al., 2003). In *Dictyostelium*, 10 homologues to yeast autophagy genes are known (Otto et al., 2003).

The different processes involved in autophagy seem to be basically related. Considering this, it appears that the type of autophagic PCD depends more on the cell type represented than on the taxonomic group the species belong to. Developmental autophagy might differ in degradation pattern compared to other autophagic cell death processes such as starvation or cellular differentiation. However, the fundamental processes might be the same with a few exceptions. The occurrence of autophagy from yeast to mammals indicates that it might be a phylogenetically older process than apoptosis (Klionsky and Emr, 2000).

**Suspensor plastids**

Specialized plastids occur in suspensors of various plants, and they differ conspicuously from plastids present in embryo and endosperm cells. They are reported preferably in *Fabaceae*, as in *Medicago scutella* (Sangduen et al., 1982), *Phaseolus coccineus* (Yeung and Clutter, 1979), *Pisum sativum* (Marios, 1970) and *Vicia faba* (Johansson and Walles, 1993,1994). These unique plastids have also been found in other taxa, such as *Capsella bursa-pastoris* (Scultz and Jensen, 1969), *Tropeolum majus* (Nagl and Kuhner, 1976) and *Stellaria media* (Newcomb and Fowke, 1974).

The plastids in the suspensor of *V. faba* are quite different from plastids found in the rest of the embryo sac (Paper III). In early developmental stages they are rounded or club-like with small membrane structures forming tubular complex. A well developed reticulum is seen in early stages that is in contact with the inner membrane of the
plastid envelope. Electron transparent vesicles are seen in the periphery of the plastid as a result of sectioning through this reticulum.

A few days later, 6 dap, the shape of the plastids changes and become more elongated or amoeboid. They can either have single thylakoids or bundles of membrane tubules, sometimes with interconnection between different thylakoids. They form structures resembling prolamellar bodies. Prolamellar bodies are typical formations in etioplasts and are also present in some chloroplasts (Walles, 1971). These bodies transform into grana when they are illuminated. However, in the suspensor plastids no transformations into grana-like structures occur. The inability to form grana is in this case not due to insufficient light, as grana have been seen in a few plastids in the embryo proper adjacent to the suspensor. Almost all suspensor plastids contain at least one prolamellar body-like structure. All these features are similar to those described in Pism sativum suspensor plastids, which has the same type of suspensor as V. faba (Marinos 1970).

**Plastids as autophagic compartments**

Acid phosphatase activity in suspensor plastids has been show in some species such as Phaseolus coccineus (Nagl, 1977), P. vulgaris and Tropaeolum majus (Gärtner and Nagl, 1980) and Pism sativum (Marion, 1970). It is preferable seen in late heart stage or cotyledonal stages of the embryo. V. faba suspensor or endosperm did not show any acid phosphatase precipitations in the plastids, when treated with the same lead salt staining at late heart stage (Paper II). The rest of the cytoplasm, with the exception of mitochondria, had precipitates of small grains indicating acid phosphatase activity. Not until very late in the degradation phase, did the plastids, or for that matter mitochondria, show any indication of acid phosphatases staining. The plastids eventually become more electron dense and appeared swollen in a late stage, however, the overall impression is that this is a kind of organelle with continued integrity.

In Phaseolus coccineus suspensor plastids display acid activity in the late heart stage (Nagl, 1977). The plastid transforms into a “plastolysome” which functions as a lytic compartment. A spoon-like configuration caused by polar swelling of the plastids with subsequently invaginations of the cytoplasm is formed. The cytoplasm will be enclosed by double membranes and the spoon-like structures changes to become more ball-like.

Similar structures have also been found in Norway spruce during somatic embryogenesis (Filonova et al., 2000), as well as in P. vulgaris suspensor cells
(Gärtner and Nagl, 1980). Even though the plastids in the suspensor of *V. faba* have cytoplasmic portions, somewhat similar to those just described, they do not appear as plastolysomes. There is no indication of acid activity at all, they seem very well preserved. The portions of cytoplasm sometimes seen inside the plastids are due to the amoeboïd shape of the plastids at this stage. When such a plastid is sectioned cytoplasmic inbuddings will appear as inclusions surrounded by double membranes.

**Chromoplasts**

From 13-14 dap, the suspensor plastids undergo remarkable changes (Paper III). Some of the thylakoids show a conspicuous dilation, and the resulting electron transparent spaces contain undulating membrane fragments, sometimes with electron dense inclusions. Similar structures are present in crystal-containing chromoplasts found in the flowers of *Narcissus poeticus* (Kuhn, 1970), in tomato fruits (Harris and Spurr, 1969) and in leaves of a carotenoid mutant of maize (Walles, 1971). In the living plant, the large electron transparent areas are occupied by crystals of β-carotene or lycopene. During the preparation for TEM, the pigments are extracted during the dehydration process. The resulting electron transparent areas will then indicate where the crystals used to be. In the light microscope the carotenoid crystals in *V. faba* appear bright red. Since β-carotene is orange, the red crystals in *V. faba* suspensor cells presumably consist of lycopene.

Gibberellin (GA) plays an important role for the growing embryo proper, and the suspensor is responsible for synthesis and transport of this hormone (Cionini, 1987). The plastids are presumably the synthesizing site for GA. An early step in GA synthesis is the conversion into ent-Kaurene by terpen cyclase, an enzyme located in the plastids (Crozier et al., 2000).

If the embryo proper sends inhibitory signals to the suspensor when it is mature enough to synthesise its own growth hormones, the synthesis of those substances has to decrease in the suspensor cells. The initial step in the biosynthesis of GA is the conversion of isopentenyl diphosphate to geranylgeranyl diphosphate (Fig. 3).

This is a common pathway, used for the production of several compounds and steroids, among them lycopene. Reorganization of GA biosynthesis into carotenoid production might be an efficient way to get rid of precursors no longer needed for syntheses of GA (Fig. 4).
It happens quickly, the plastids changes to chromoplasts from one day to another when the embryo is about 13 days. This indicates that the suspensor plastids actively participate in the programmed degradation of the suspensor cells. How the communication between the embryo proper and the suspensor take place is still unknown. However, if the embryo proper functions as a sink during development, but seals the connecting plasmodesmata after maturity, there will be no command after GAs, and subsequently no need for more synthesis. Perhaps the precursors of GA production are quickly eliminated by the synthesis of lycopene. That accumulation of lycopene can trigger formation of chromoplasts has previously been shown in a mutant of Zea maize (Walles, 1971).

Some suspensor chromoplasts show round inclusions bounded by double membranes. These structures show similarities to those described in Capsicum annum, and are termed thylakoid sheets (Spurr and Harris, 1968). Observations through serial sections,
Figure 4. Synthetic pathway of lycopene (From Crozier et al., 2000)

indicates that several thylakoid sheets occurs in one chromoplast and that they are connected to each other through a common thylakoid plexus. This plexus occurs preferably in etioplasts and forms an intricate membranous array, or tubular network of the thylacoid system (Spurr and Harris, 1968). This structure has been correlated to plastids that fail to develop chlorophyll or its precursors.

Conclusions
The cell death signal received by the suspensor is probably sent from the embryo proper when it is mature enough to support itself with growth factors. The lack of
demand for gibberellins (GA) by the embryo forces the suspensor to use a precursor for synthesis of GA:s for synthesis of lycopene instead. The accumulation of lycopene results in transformation of the plastids into chromoplasts. When this development of chromoplasts occurs, the PCD process is already running, and the signal from the embryo proper must have been sent at a much earlier stage. There might be some other signal that triggers the PCD process in the suspensor and the change in GA synthesis might be only a secondary effect of that signal. However, taking account of retardation in the system, the decreased demand for GA could well be sufficient as the triggering signal, sent much earlier by the embryo proper.

The endosperm PCD precedes the suspensor cell death and differs in morphology. The triggering of PCD might be controlled by the endosperm itself. This tissue grows quickly after fertilization and is mature when the embryo proper still is in its first stages of development. Perhaps the age of the endosperm is the triggering factor. The structure of the endosperm plastids remains undifferentiated throughout the larger part of degradation and the only change is the increased electron density. However, there are no structural indications of active participation of the endosperm plastids in PCD.

DNA fragmentation occurs at about 13-14 dap, in both the suspensor and the endosperm. In the endosperm nuclei the heterochromatin degradation appears in the form of a network, while in the suspensor the heterochromatin forms large electron dense aggregates that are distributed close to the envelope. In animals, mitochondria are responsible for release of proteins that take part in the fragmentation of DNA. Whether the same occurs in plants is not clear. However, considering the preservation of this energy producing organelle, it is tempting to think that mitochondria plays a similar role in the suspensor and endosperm PCD as it does in animal systems.

The formation of autophagic vacuoles starts shortly after DNA fragmentation in both tissues. The conspicuous formation of autophagic vacuoles in the suspensor cells is similar to the formation of autophagic vacuoles in liver cells. This is an indication that autophagic PCD (type 2) is a model for cell death that works in the same way in plants as in animals. The DNA fragmentation pattern and mitochondria preservation also follows the overall cell death pathway in autophagic PCD (Fig. 5).

The formations of autophagic vacuoles in the endosperm are not as striking as in the suspensor. The autophagic vacuoles are mostly seen close to the border towards the
suspensor. The endosperm belongs to the type 2 PCD due to autophagic vacuole formation, mitochondria preservation and DNA fragmentation. However, even though the formations of autophagic vacuoles are similar and occurs at approximately the same time, none of the ultrastructural features are as striking as in the suspensor autophagic PCD.

![Figure 5. Autophagic programmed cell death in *Vicia faba*. Degradation pattern from an healthy cell to a autophagic cell.](image)

My results show that the suspensor and endosperm have different pathways of programmed cell death. They both undergo PCD in an autophagic manner, but the cell dismantle does not follow the same procedure. Thus the signalling systems are presumably different. The embryo proper is probably the source of the death signal affecting the suspensor. The endosperm, which has a different origin and function, might be controlling the death signal within its own cell. The death might in this case be related to the age of the cell.
References


Singh S, M Lazzaro and B Walles, 1998. The nuclear reticulum in placental cells of Lilium regale is a part of the endomembrane system. Protoplasma 203:144-152.


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