Isolation and characterisation of the bap genes, a new family of maize basal endosperm-specific proteins.

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Vorgelegt von

Antonio Serna Sanz

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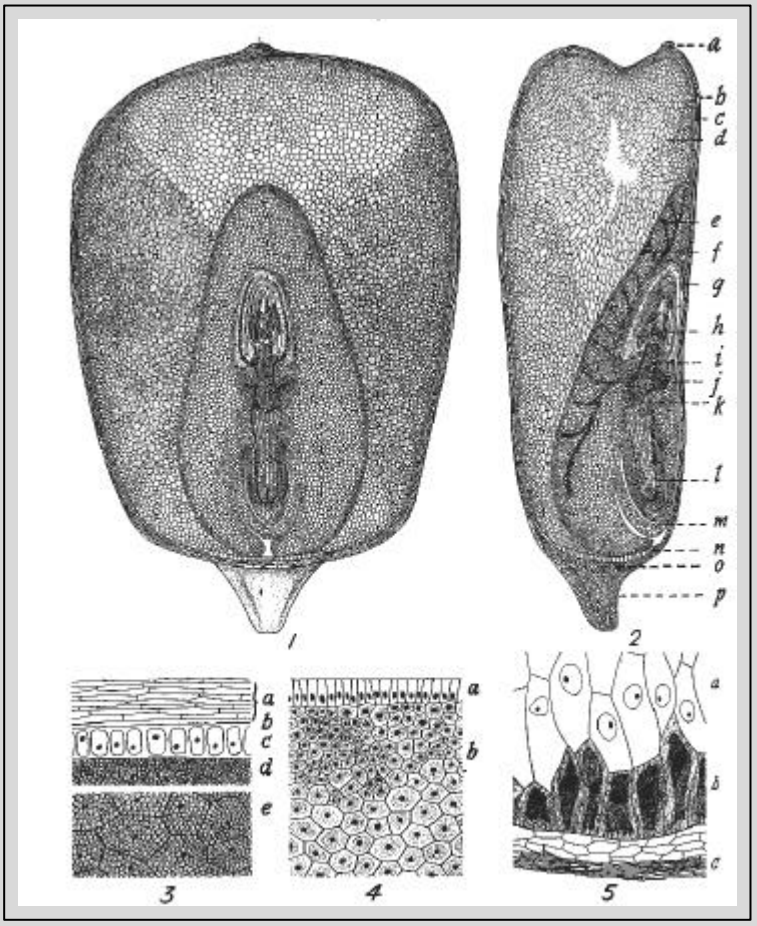
“Work then without disputing, it is the only way to render life supportable."

Candide

Voltaire

A Mireia por todo su apoyo y cariño



Introduction

"…Everyone who enjoy, thinks that fruit is what important from the tree, when indeed it's the seed. Here we have the difference between those who enjoy and who believe…"

Friedrich Nietzsche

1.- Introduction

Plant foods contain all of the organic and inorganic elements necessary for human life. They provide important nutrients for the dietary food chain converting elemental compounds into more complex but assimilable components. Because the representation of these components among the different plant species that normally contribute to the daily diet is heterogeneous, and the concentration of many of the basic dietary components is often low, knowledge about how these components are being produced, absorbed, transported or accumulated could help in the manipulation of crops of agronomical importance and therefore in the improvement of their nutritional value. One of the first steps in this process started with the domestication of cereals more than ten millennia ago. Cereals are one of the most important sources of carbohydrates, proteins, lipids and lipid-soluble vitamins. They are important not only in human nutrition but also for livestock. Cereals belong to the family of annual plants termed the *Gramineae* where the main interest is focused, due to their nutritional importance, on the fruits, known as grains, kernels or caryopses. All cereal grains share many characteristics, like life cycle and grain anatomy, but the value of cereals as the staple carbohydrate source is derived from the endosperm. The endosperm makes up the largest fraction of the grain and is composed of two different types of tissues: *the starchy endosperm and the aleurone*. The nutrients are mainly stored in the starchy endosperm. It possesses a typical composition depending on the species. The aleurone consists of 1 to 3 cell layers, being up to 6 (in rice) in the region adjacent to the conducting tissues. It is important in grain development providing cells to the growing endosperm and in germination when in most species is a site of synthesis of hydrolytic enzymes responsible for solubilising reserves. The aleurone possesses a higher content of protein and lipids per dry weight than the starchy endosperm. As a result of a double fertilization, in most of the cases, the grain will develop maintaining an intimate contact with the parent plant. The starch accumulation is dependent on a high rate of sucrose

transfer from the phloem terminals. This association is a prerequisite for successful grain development. Thus, the understanding not only of storage product deposition in the endosperm but also of the nutrient assimilation, product of this association, could help to improve the grain yield and content.

In this introduction an overview about Endosperm formation and development will be given, as well as its interactions with the embryo and maternal tissues. Maize will be taken as a model cereal species in most of the cases

1.1.- GRAIN ANATOMY

The maize grain is the largest of the grain cereals with the most robust pericarp. It contains a single-cell aleurone layer and an endosperm that occupies about the 80% of the mature kernel almost totally surrounding the embryo. Composition of the grain is graphically shown in figure 1.

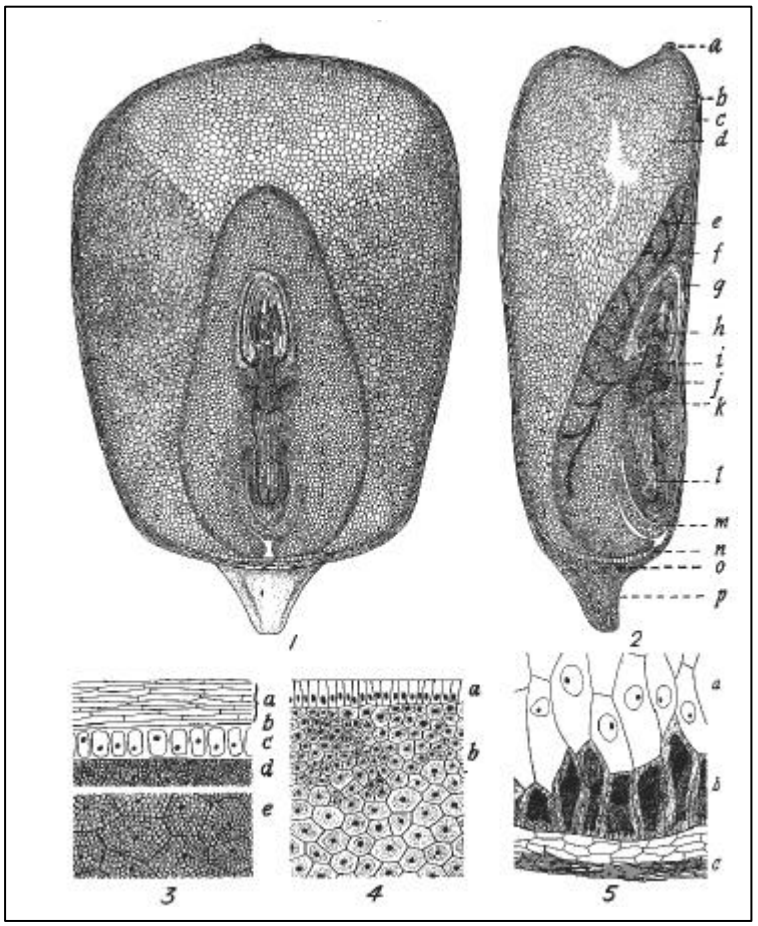


Fig 1.- Sections at different regions from a mature kernel
1-2.-Vertical sections in two planes a, silk scar; b, pericarp; c, aleurone; d, endosperm; e, scutellum; f, glandular layer of scutellum; g, coleoptile; h, plumule with stems and leaves; i, first internode; j; lateral seminal root; k:scutellar node; l, primary root; m, coleorhiza; n, basal conducting cells of endosperm; o, brown abscission layer; p, pedicel or flower stalk.
3.-enlarged section through pericarp and endosperm. a, pericarp; b, nucellar membrane; c, aleurone; d, marginal cells of endosperm; e, interior cells of endosperm.
4.-enlarged section of scutellum. a, glandular layer; b, interior cells.
5.-vertical section of the basal region of the endosperm. a, ordinary endosperm cells; b, thick-walled conducting cells of endosperm; c, abscission layer.

Picture taken from "The structure and reproduction of the Corn" T.A Kiesselach

1.1.1.- Pericarp

The outer grain membrane is derived from the mature ovary walls (fig 1 sketch 2; b). Consists of a multilayered structure of largely empty cells disposed surrounding the kernel and in continuity with the pedicel. The pericarp lies over a thin residual nucellar membrane (fig 1 sketch 3; b) that is in contact with the single-cell layered aleurone. It represents about 6 percent of the maize grain and gives protection and support to the growing endosperm and embryo. At the top of the pericarp in the mature kernel the silk scar is visible (fig 1 sketch 2; a).

1.1.2.- Endosperm

The endosperm is the most abundant tissue of cereal grains. It is comprised of two cell types: the starchy endosperm (fig 1 sketch 2; d) and the aleurone (fig 1 sketch 2 and 3; c). The aleurone, consisting of a single layer of cells, is modified at the base of the endosperm to form conducting cells. This modification forms a layer of transfer cells (fig 1 sketch 2 and 5; n,c respectively) important for the nutrient uptake from the mother plant (Pate *et al.* 1972).

The composition of the endosperm differs depending on the region, so the central endosperm is mainly composed of starch with less protein, within big cells, whereas the cells at the periphery tend to be smaller and contain more protein. The aleurone cells accumulate protein and oils rather than starch.

The function of the endosperm is to nourish the germinating embryo upon hydrolysis of its accumulated components by the aleurone.

1.1.3.- The embryo

Develops to give rise to the young corn plant. The embryo axis (fig 1 sketch 2; h to j) and the scutellum (fig 1 sketch 2; e), form the embryo. The embryo axis is the plant of the next generation and contains shoot, leaf and root primordia it is intimately connected to the scutellum that lies between the endosperm and the embryo. The scutellum behaves as a secretory and

absorptive organ serving the requirements of the embryonic axis. During germination it serves to facilitate solute absorption from the starchy endosperm.

1.1.4.- Pedicel

The tissues of the pedicel maintain the vascular connection with the parent plant. The pedicel merges without clear distinction into the mature ovary walls or pericarp. It serves as symplastic connection interrupted apoplastically only at the junction with the transfer cell layer. This discontinuity is of great importance in the nutrient uptakes of the grain.

1.2.- ORIGIN OF THE ENDOSPERM: *ovule and embryo sac development*

All the different tissues mentioned above give shape and function to the cereal grain. The whole structure is the consequence of the development of the embryo sac, after sexual or asexual reproduction, inside the ovary. The origin of the structures and organs where the grain will be developed should be considered to give more insight into the physiology of the grain development.

1.2.1.- Ovule development

The ovule is the structure where the embryo sac will develop. It will contain the grain formed after fertilization from the embryo sac (reviewed by Grossniklaus U. and Schneitz, K. 1998; Schneitz K. *et al.,* 1998). The ovule consists of three basic structures: nucellus, two integuments and the funiculus, that in the case of maize is not well defined, being completely merged in the placental tissue. The nucellus is derived from the apical portion of the ovule primordium. Shortly after ovule initiation a subdermal cell enlarges and displays a prominent nucleus. This is the archesporium where, after successive cell differentiation processes, the embryo sac will be formed.

Once the structures of the ovary are functionally distinguishable, megasporogenesis takes place. The megaspore will undergo meiosis producing four megaspores disposed end to end in line with the micropyle. The three

megaspores close to the micropyle quickly degenerate. Simultaneously the nucellus will divide periclinally originating a five to six cell layer nucellus (Randolph *et al* 1926). The next step prior to giving a fertile ovule is the formation of the mature embryo sac.

1.2.2.- Embryo sac development.

Once the process of the megaspore formation has taken place (megasporogenesis) the megaspore will undergo a maturation phase (megagametogenesis) in which after several meiosis and subsequent mitosis divisions the embryo sac will be ready for fertilisation. Embryo sac development in maize is of the monosporic type or *polygonum* type, one of the most common forms of embryo sac development observed (Haig D. 1990; Reiser *et al* 1993).

During the first meiotic division a vacuole is formed in between the two nuclei that are disposed at the poles of the cell. A second simultaneous division of the nuclei at the poles will give the four nuclei embryo sac. After the third division, the embryo sac consists of eight nuclei in two groups of four situated at each pole. It is at this stage when the cellularization process of the embryo sac will start.

One nucleus from each group will move toward the center, to be located next to each other nearby the region where the egg cell will form. The nuclei at the chalazal pole will divide till a group of 20 to 40 cells is formed. One of the nuclei of the micropylar end enlarges and becomes the mother cell whereas the others become the synergids. The embryo sac (fig 2) so, without cell division will enlarge, being ready for fertilization. If fertilization does not take place, the embryo sac disorganizes and it is not available anymore.

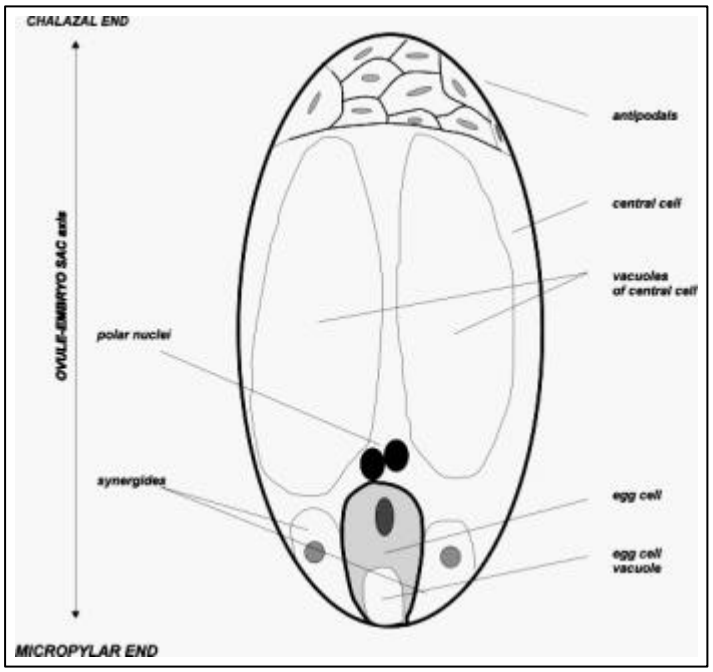


Fig 2.- Drawings of a mature embryo sac in maize

Both polar nuclei are in contact above the egg cell but they won't fuse till fertilization takes place.
Some of the antipodals are binuclear
The embryo sac will be fertile during a period of about two weeks, after that if fertilization did not take place will degenerate.

1.3.- ENDOSPERM DEVELOPMENT

During fertilization, two haploid sperm nuclei fuse to both the egg cell and the polar nuclei respectively. The fertilization of the egg cell gives rise to the zygote and the fusion of the polar nuclei to the second sperm nucleus will originate the endosperm (reviewed by Dumas and Mogensen 1993, Pruitt Robert E. 1994). Why the egg cell will develop into an embryo giving rise so to the new plant and the polar nuclei to the endosperm, is something, which still has to be further investigated. In some plants fertilization does not seem to be necessary for endosperm formation due to the possibility of undergoing apomixis (reviewed by Van Dijk P. and Van Damme J. 2000). Some mutants have been isolated that develop endosperm without the existence of fertilization (Ohad N. *et al* 1999) which could help to understand the mechanisms that regulate this onset in reproduction. Nevertheless, double fertilization is a prerequisite for both embryo and endosperm development in most of the higher plants. (Phenomenon already discovered in the last century) (Odd-Arne Olsen 1999).

After the sperm nucleus is fused to the polar nuclei, the triploid endosperm nucleus so formed, starts to divide. Several types of endosperm development have been described depending on the cellularization pattern

(reviewed by Vijayaraghavan and Prabhakar 1984). In the case of maize the pattern is nuclear (fig 3). The endosperm nuclei start to divide without forming intervening cell walls. The nuclei will be disposed within the embryo sac, around a central vacuole during the first two days after pollination. Afterwards, during the cellularization phase that takes place 3 to 5 days after pollination approximately, cell walls will be formed around the nuclei. After the formation of the cell walls, periclinal divisions begin, so replacing the large central vacuole. Once the endosperm becomes cellular, the cell divisions will progress mainly from the outer cell layers, which will work as meristematic region all around the endosperm except over the hilar region. By approximately 20 days after pollination these outer cells stop their periclinal divisions and start to divide just anticlinally.

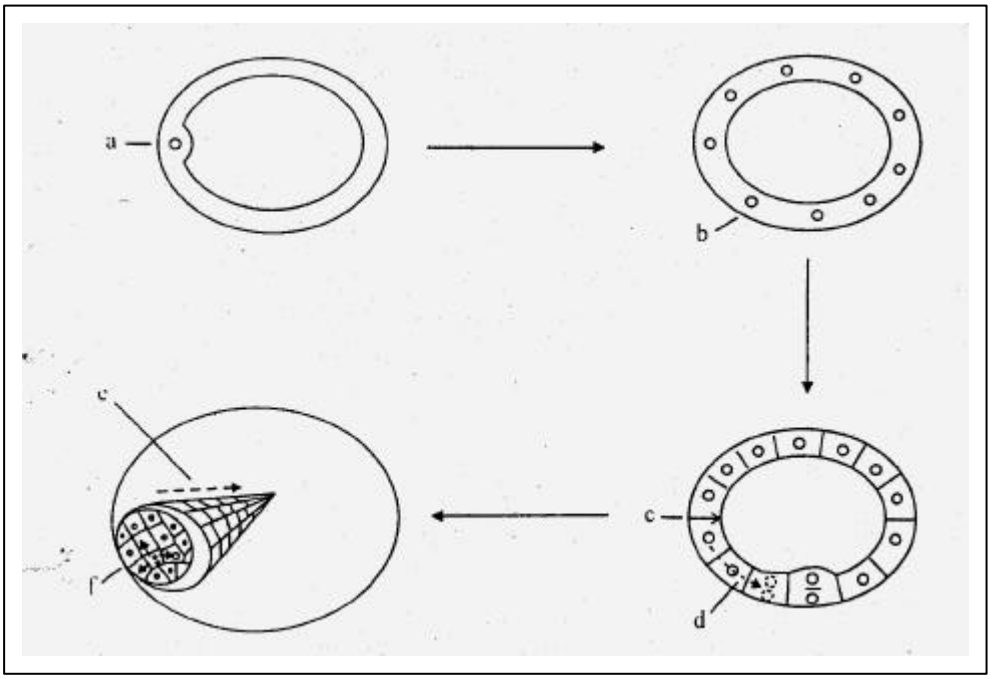


Fig 3.- Model that represents early stages of endosperm development in a nuclear endosperm mode

Model taken from H.A Becker *et al* 1999. a) Primary endosperm nucleus at the micropilar end of the embryo sac; b) phase of nuclei c) Cellularization phase; d) first periclinal division; e-f) fully cellularized endosperm.

1.3.1.- Starch biosynthesis and accumulation

A critical step during endosperm development, due to its importance for the embryo growth, is starch deposition. Starch will be one of the main energy reserves for the developing embryo and germinating plant. It is also an important carbohydrate source and basic to nutrition in developed and developing countries.

Approximately two weeks after fertilization, the starch deposition begins. The first cells to be filled are cells of the crown region, creating so a major starch gradient between the upper and the basal part of the endosperm. There is also lower starch content in those cells nearby the periphery of the endosperm. A prerequisite for starch synthesis and accumulation is sugar assimilation from the maternal tissues (fig 4). Sucrose passes symplastically through the pedicel and enters the endosperm apoplastically through uptake by the transfer cell layer (reviewed by Gunning and Pate 1972 and Thorne 1985). Part of this sucrose is hydrolysed during the uptake process in the transfer layer into glucose and fructose by a cell-wall invertase (Felker *et al* 1980; Doehlert *et al.,* 1987). The importance of sucrose assimilation for good endosperm development, and the role of the transfer cell layer in this process, is indicated by the phenotype of the mutant *miniature-1,* which lacks an endosperm-specific cell wall invertase, CWI-2. *Miniature1* causes a loss of about 70 % dry seed weight at maturity. Another mutant characterised by Maitz *et al.,* (2000), and termed *rgf1* (reduced grain filling 1) is a good example of the important role of the transfer-cell layer during endosperm development. This mutant is characterised by a defect in sugars uptake in the endosperm cells. The levels of free sugars in the endosperm are normal as well as the starch synthesis capacities. This atypical environment greatly affects the normal development of the transfer cell layer and part of the pedicel. The expression of important markers of the basal region of the endosperm is highly reduced. The existence of this mutant establishes a clear connection between the role of the transfer-cells and the normal assimilation of nutrients into the endosperm.

Starch consists basically of two glucose polymers, the highly branched amylopectin and the relatively unbranched amylose. The synthesis of both polymers takes place inside the amyloplast by the starch synthase enzymes (fig

4), at the surface of the starch granule, and through the starch branching enzymes in the soluble fraction of the amyloplast (Smith *et al.,* 1997).

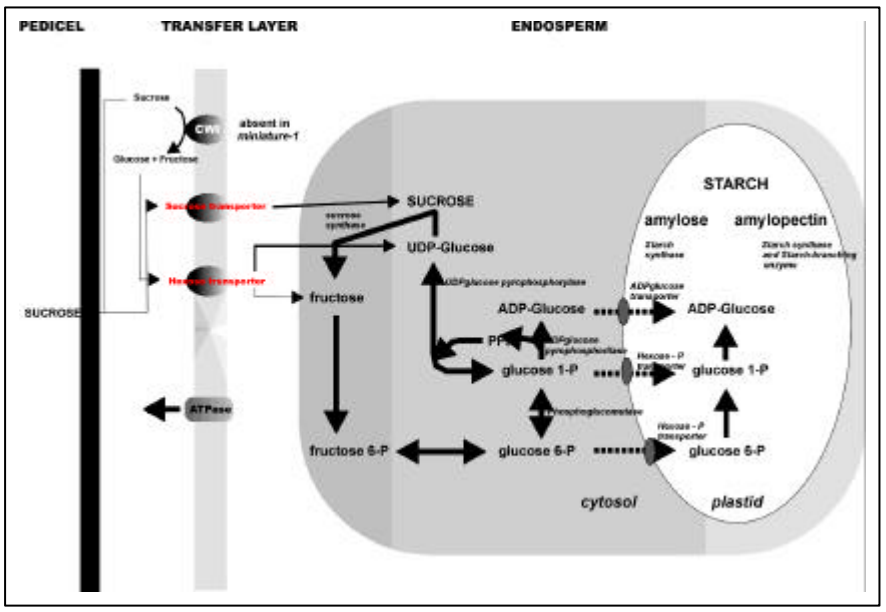


Fig 4.- Diagram showing starch biosynthesis
In maize endosperm the synthesis of ADP-glucose by the ADPglucose phosphorilase (AGPase) is mainly cytosolic (Denyer *et al* 1996). The plastidic presence of AGPase was detected by inmunolocalisation .

1.3.2.- Protein deposition

The endosperm contains a variety of proteins of which the most abundant are referred to as storage proteins. Maize storage proteins are accumulated in protein bodies that develop in the endoplasmic reticulum (Slocombe *et al* 1999). These storage proteins are divided into groups depending on their solubility in different solvents (Osborne *et al* 1924). Thus we have the globulins and certain albumins, they are soluble in saline solutions of different concentrations; and the prolamins rich in proline and glutamine, which are highly hydrophobic and appear only in alcohol fractions or denaturing solvents.

In maize the prolamins (zeins) can be over 50% of the total endosperm protein. These zein proteins from maize are classified in four different groups attending to their molecular weight, so there are α,β,γ, and δ-species. The most abundant group, the α-zeins are products of a large gene multifamily clustered in at least three different loci. The major prolamin storage protein genes are endosperm specifically expressed. Their transcripts start to accumulate twelve days after pollination in maize (Müller *et al* 1995) and the amounts deposited depend on the availability of reduced nitrogen.

The development of the endosperm is the consequence of the coordination of different cellular types or domains. The most evident cell types established during the differentiation of the endosperm are the starchy endosperm, the aleurone and the transfer cell layer.

1.3.3.-Regulation of gene expression in Endosperm Domains

The comparison of different promoter sequences has revealed the existence of a conserved motif, termed the –300, prolamin or endosperm box (Forde *et al* 1985). By *in vivo* foot-printing assays different elements have been identified, a TGTAAAG element is bound by a factor present at 10 DAP, whereas a *GCN*4 element is protected from DNAase activity by a protein present at 14 DAP onwards (table 1). *Opaque*-2, a gene with similarities to the transcription factors of the *bZIP* family, has been cloned (Motto *et al* 1988) that seems to be a good candidate for being part of the *GCN*4-binding motif. *Opaque*-2 protein is able to trans-activate α-zein and b-32 promoters in transient expression experiments in tobacco protoplasts (Lohmer *et al* 1991).

Site of action/factor	Target sequence	Reference
Aleurone-specific		
C1	C/CTAACG/TG	Bodeau and Walbot 1996
R	CACCTG	
VP1	CGTCCATGCAT	Kao et al., 1996
?	CAA/N_{2-9}/TGG	Leah et al., 1994
?	"RY"(CATGCATG)	Dickinson et al., 1988
Central endosperm		
Opaque-2	TCCACGTAGA GATGAC/TG/ATGG/A	Schmidt et al., 1990 Lohmer et al., 1991
Prolamin binding factor	TGTAAAG	Vicente-Carbajosa et al., 1997
Basal layer		
?	"RY" repeats	Guo and Thompson, in prep.

Table 1.- *Cis*-elements and the corresponding *trans*-acting factors conferring cell-type specificity in endosperm cells

A gene encoding for a protein binding to TGTAAAG, the prolamin binding box factor, PBF, has been recently isolated (Vicente-Carbajosa *et al* 1997). The protein is able to interact *in vitro* with *Opaque-2* and binds to prolamin promoters at the endosperm motif. Thus, promoters active in starchy endosperm require at least two factors conferring cell-type specificity.

1.3.3.1.- Aleurone Domain

Due to the role of the aleurone during endosperm development and seed germination, it becomes interesting the finding of specific promoters at this region of the endosperm. The expression of anthocyanin pigments at the aleurone has been studied and different factors from the anthocyanin regulatory loci have been identified, *C1* and *R1,* are relatives of the Myb- and bHLH transcription factors respectively, they act on the *bronze*-2 locus (Bz2) and are directly involved in the regulation of the anthocyanin deposition in the aleurone. The two factors were shown to interact *in vitro* and recognize different elements at the promoter region of the regulated genes. These elements were mapped and identified (Bodeau and Walbot, 1996) as *C1* element TAACTG and *R1* element CACGTG. *C1* and *R1* motifs appear in the promoters as redundant scattered functional repeats. Only the *C1* factor has shown effective DNA binding activity, the *R1* factor does not bind directly to the DNA but could be responsible for the stabilization of the *C1* complex. (As one relative of *R1* factor has been cloned, *In1* (intensifier), that inhibits anthocyanin production by competing *R1* protein in nucleoprotein complex formation) (Burr *et al.* 1996).

Another regulatory gene *Vp1* has been identified as a regulator of anthocyanin deposition. *Vp1* is also involved in the regulation of acquisition of seed dormancy and interacts with *cis* regulatory elements of the *C1* gene, identified as RY repeats or Sph-boxes (Hattori *et al* 1995). The RY repeats are elements consisting of alternating pyrimidine and purine bases that in the case of the Sph-box is CATGCATG. Another prerequisite for wild-type antocyanin biosynthesis is the exposure of the aleurone to the light. *C1* gene expression is also light dependent, this aspect being mediated by *cis*-elements distinct from those that confer *Vp1* responsiveness (Kao *et al*., 1996).

Aleurone differentiation also requires the gene product of the *Crinkly-4* (Cr4) locus, a transmembrane kinase (Becraft *et al.,* 1996) whose precise function remains unknown.

1.3.3.2.- Basal transfer layer Domain

The best established role of this region is nutrient uptake into the endosperm. The presence of the transfer-cell layer is critical not only for normal endosperm development but also for the function of the pedicel, as concluded from the studies of different transfer-cell defective mutants (Maitz and Thompson, 2000). Currently, several genes have been isolated as being specifically expressed in the **b**asal **e**ndosperm **t**ransfer **l**ayer and have been termed *BETL*1 to 4. The transcripts of the *BETL* gene family are detected only in the transfer-cell layer and are absent in adjacent regions corresponding to different domains of gene expression, as in the *embryo surrounding region* (*ESR*) for example (Opsahl Ferstad *et al.,* 1997). The study of the cDNA sequences reveals not clear homology with other sequences currently isolated, although two of them, *BETL*-1 and *BETL*-3 share weak homology with defensins (Hueros *et al.,* 1999). The time course of expression of the *BETL* genes is synchronous for the four genes isolated, all being expressed from 5 DAP to 25 DAP with a peak of expression at 14-16 DAP.

Little is known about trans-acting regulators of the *BETL* genes. The study of the promoter of the *BETL* genes (Hueros *et al.,* 1999) has identified several possible regulatory sequences. Among them several *GATA*-like microsatellite repeats are present in the promoters of the *BETL* genes. The *GATA* motif is recognized by the transcription factors of the zinc finger family termed *GATA* transcription factors (reviewed by Teakle and Gilmartin, 1998). None of these factors so far cloned showed binding activity in the *BETL* promoter (Serna *et al.,* unpublished). In addition, a cDNA encoding a novel transcription factor of the Myb family has been isolated (Hueros *et al.,* unpublished data) and termed *zmMRP1*. The transcript of *zmMRP1* is specifically expressed in the basal endosperm and is detectable already in the coenocytic stage of endosperm development. Moreover, it is able to activate the

BETL2 promoter in transient expression experiments in tobacco protoplasts, suggesting it may regulate BETL expression in vivo.

Another important role of the transfer-cell layer is the *protection* of the embryo from pathogen ingress during endosperm development, allowing so the progression of the plant life cycle. In order to achieve this function different processes recently described take place at this cell layer that make of it a real shield.

1.4.- THE ROLE OF PLANT DEFENSINS

Plants are exposed continuously to pathogens during their lifespan, necessitating them to develop mechanisms to control potential infections. The mechanisms developed by plants in order to avoid the ingress of pathogens can be classified in *inducible* and *non-inducible* systems; among the inducible systems the responses can be grouped in *locally induced defense responses*, where one of the most salient components is the phytoalexins (Frey M *et al.,* 1997; Glazebrook J., *et al.,* 1997). The other major group of induced defense mechanisms are the *systemically inducible resistance* genes, among which two subgroups are recognised: the salicylic acid dependent responses (Mur LAJ, *et al.,* 1996) and the salicylic acid-independent pathways (Pieterse CMJ., *et al.,* 1996).

A mechanism to control the growth of microorganisms, conserved among the plant kingdom is the production of small peptides with biological activity. Based on the structural characteristics of the active peptide, different families have been described, so the first group, the ***cecropines***, present in lymphae of insects (reviewed by Boman *et al.,*, 1987a,b) and in the intestines of mammals (Lee *et al.,* 1989) they transiently form ion channels in the membrane of targeted cells. Other types are the ***maganins*** isolated from amphibians (Nicolas and Mor. 1995). The third and the biggest group is formed by the cysteine-rich peptides with seven subgroups (fig 5): the ***thionins***, the ***defensins***, ***lipid transfer proteins***, the ***Hevein-type***, the ***knottin-type***, the ***Ib-AMPs*** and the ***MBP-1***. The *thionins* correspond to small peptides that possess two antiparallel α-helices and an antiparallel β-sheet, whereas the *defensins* posseses just one antiparallel α-helix but three antiparallel β-sheets. (Broekaert *et al.,* 1997). The

Hevein and *knottin-type* are chitin-binding proteins (Raikhel *et al.,* 1993). *Ib-AMPs* and the *MBP-1* are small peptides that do not share homology with any other antimicrobial peptides so far isolated. These small peptides were cloned from *Impatients balsamica* seeds (Tailor *et al.,* 1997) and maize seeds (Duvick *et al.,* 1990, 1992) respectively.

In plants, the most common peptides are those from cysteine-rich groups. They are detected in different organs and are encoded by different genes displaying organ-specific expression (Garcia-Olmedo *et al.,* 1998). The amino acid sequence comparison reveals that relatively few residues are conserved in all antimicrobial plant peptides. Most of them are synthesized as preproproteins with a signal peptide at the N-terminus followed by the prodomain, whereas plant *defensins* are for the most part synthesized as preproteins with a signal peptide at the N-terminus but without a prodomain. However, some exceptions have been identified in plant *defensins* where the prodomain was present (Gu *et al.,* 1992, Milligan and Gasser, 1995).

Based on the antimicrobial effects two different groups of plant *defensins* have been distinguished, the ***morphogenic*** group and the ***non-morphogenic*** group. The plant *defensins* included in the first group reduce hyphal elongation and hyphal branching of exposed fungi, and the plant *defensins* of the *nonmorphogenic* group, slow down hyphal elongation but do not induce branching or marked morphological distortions. The antifungal activity of both plant *defensins* is reduced when incubated in a medium with increased ionic strength and particularly when incubated in the presence of monovalent or divalent cations, being at least one order of magnitude lower with divalent cations such as Ca^{2+}(Terras *et al.,* 1992, 1993). The mechanisms underlying the antimicrobial activity of the *thionins* and *defensins,* are different. The *thionin* mechanisms are based on permeabilization effects caused by the interaction of the proteins with different domains of phospholipids at the membrane (Vernon *et al.,* 1992), whereas the *defensins* cause dramatic changes in the influx/efflux of Ca^{2+}/K^{+} respectively (Thevissen *et al.,* 1996) causing extracellular alkalization. This ability to modify the cellular influx/efflux of certain cations was shown to require a conserved tyrosine present at between amino acid 35 to 40 of the mature peptide (Thevissen *et al.,* 1996).

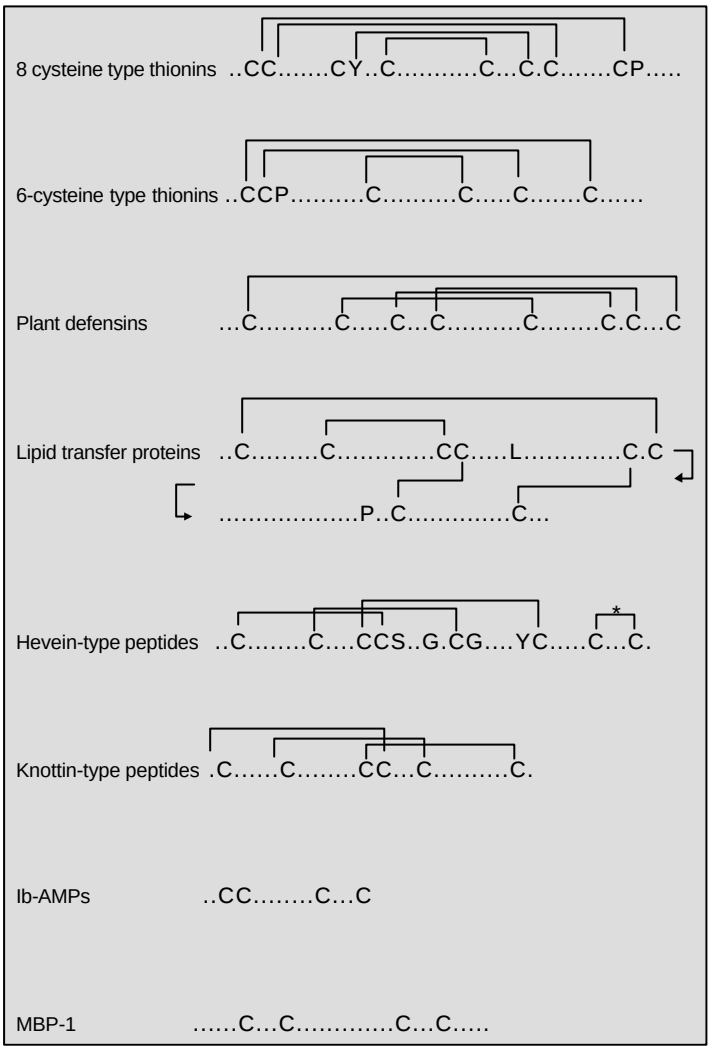


Fig 5.- Conserved residues in primary structures of different antimicrobial peptides.
Cysteins connectivities are indicated by solid lines. Dashed underlining indicates segments that vary in length between different members of an antimicrobial peptide family.
(model taken from *"Antimicrobial peptides from Plants"*, Williem F. Broekaert *et al.,* 1997)

Certain antimicrobial peptides are synthesized de novo upon pathogen challenge and others are expressed constitutively, albeit in some cases in a precursor form. The constitutively expressed class is most commonly found in the peripheral cell layers surrounding plant organs contributing so to the formation of an antimicrobial shield.

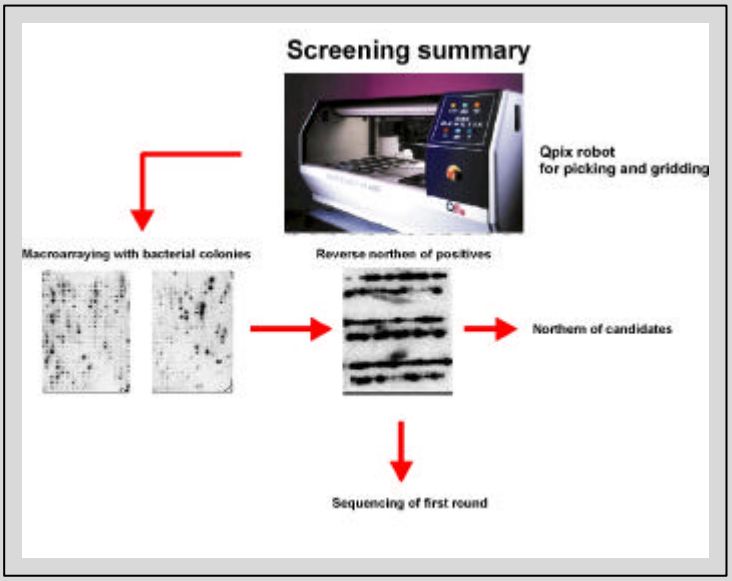
1.5.- Nutrient uptake into the developing seed

During the introduction several steps in storage product accumulation in the developing seed have been described. All of these processes are necessary for successful embryo development and seed dispersion. Before storage product deposition takes place in the endosperm, solutes needed for storage product synthesis have to be assimilated from maternal tissues.

The endosperm receives the nutrients from the phloem terminals, which is highly branched at the end of the pedicel. The nutrients pass via symplastic connections through the placenta-chalaza and cross into the endosperm apoplastically at the site of *the transfer cell layer*. These cells form the interface between the maternal tissue and endosperm, and consist of most basal endosperm cell layer adjacent to the pedicel. They possess cell ingrowths that increase the surface area of associated plasmalemma and thus highly facilitate solute transport (reviewed by Pate and Gunning 1972; Thorne 1985). The function of these cells during endosperm development is not only important to translocate nutrients from the maternal tissues to the growing embryo but also as a natural barrier that avoids pathogen ingress from adjacent tissues, protecting the embryo and ensuring reproduction.

1.6.- Aims of the thesis

Due to the unquestionable importance of the role of the basal transfer cells during endosperm development, several research projects are ongoing towards a better understanding of the genetic regulation and physiology of this layer (Hueros *et al* 1995, 1999). In this thesis the cloning and characterisation of four genes specifically expressed in these cells is presented. The genes cloned are homologues to a previously isolated basal endosperm transfer layer (*BETL*) specific gene termed *BETL2* (Hueros *et al* 1999)(1.4.2.3). The *BETL2* gene shares weak homology with plant *defensins* and with a family of *cobratoxins*. It possesses a signal peptide and a conserved, C-terminal, cysteine-rich region. Evidence for a putative role in pathogen defence has been obtained *in vitro* (Serna *et al* unpublished). *The aim of the work carried out in this thesis was to contribute through the finding of new sequences of special interest to understanding the functions of the transfer-cell layer.*



Materials and Methods

2.- Materials and methods

2.- MATERIALS AND METHODS

2.1.- Abbreviations

Abbreviation	Signification
Amp	Ampicillin
APS	Ammonium persulphate
BAP	Basal Antifungal type Protein
BCA	Bicinchoninic Acid
BETL	Basal Endosperm Transfer Layer
Bp	Base pair
BSA	Bovin serum albumin
CDNA	Complementary deoxyribonucleic acid
Dap	Day after pollination
DATP	Deoxyadenosinetriphosphate
DCTP	Deoxycitidinetriphosphate
DEPC	Diethylpyrocarbonate
DGTP	Deoxiguanidinetriphosphate
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
DTT	Dithiothreitol
DTTP	Deoxythymidinetriphosphate
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FPLC	Fast protein liquid chromatography
HEPES	4-(2-hydroxiethyl)-1-piperazinethanesulfonic acid
IPTG	Isopropylthio-β-o-galactopyranoside
Kb	Kilo base (s)
Kd	Kilo Dalton (s)
Min	Minutes
MOPS	3-(N-morpholino)-propanesulfonic acid
MRNA	Messenger RNA
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonyl fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

2.2.- Materials

2.2.1.- Plant materials

Zea mays L. cv. A69Y

2.2.2.- Bacterial strains, fungal strains, cloning vectors and primers

2.2.2.1.- *E.coli* strains

- **BL21(DE):** *hsdS gal* (cI*ts*857*ind*1 *S*am7 *nin*5 *lac* UV5-T7 gene*1.*
- **DH10B:**F^-, mcrA∆(mrr-hsdRMS-mcrBC)Φ80dlacZ∆M15,∆lacX74,deoR, recA1, endA1, araD139,∆(ara,leu)7607, galU, galK, λ^-rpsl,nupG (GIBCO-BRL)
- **M15:** [pREP4](Nal^S, Str^S, Rif^S, Lac$^-$, Gal$^-$, Mtl$^-$, F^-, RecA$^+$, Uvr$^+$, Lon$^+$)
- **Xl1-Blue MRF´:** ∆(mcrA)183 ∆(mcrCB-hsdSMR-mrr)173 endA1 *supE44 thi-1 recA1 gyrA96 relA1 lac*[F´*proAB lac*^{q}*ZDM15* Tn*10* (Tet^r)]

2.2.2.2.- Fungal strains

Fusarium culmorum
Fusarium moniliforme
Fusarium nagamai

2.2.2.3.- Cloning vectors

VECTOR	Origin
pBluescript® SK(+/-)	(Stratagene)
PQE60	(Qiagen)

2.2.2.4.- Primers

Primer name	Purpose	Sequence
B2ala NsiI	Mutation of BETL2 by substitution of the basic region between the cysteine motif	5´cgt atg cat agc tac tgc cgc gtg cta tgc gag ctt gct gct 3´
B2 thr PstI	Mutation of BETL2 by substitution of the conserved Thr	5´cgt ctg cag aaa ggc aaa taa gtg c 3´
B2 tyr PstI	Mutation of BETL2 by substitution of the conserved tyr	5´cgt ctg cag aaa gac taa taa gtg cgc aaa gag c
Px35 Nco5	Cloning of px35 into the expression vector pQE60 (forward)	5´ggg cca tgg aaa ttg aag atg tta gc 3´
Px68+81 Nco5	Cloning of px68 and px81 into the expression vector pQE60 (forward)	5´ggg cca tgg aga taa taa gag gtg aaa cc 3´
3Px35	Cloning of px35 into the expression vector pQE60 (reverse)	5´ggg aga tct act atg gca gtg ctc caa gc 3´
3px68	Cloning of px68 into the expression vector pQE60 (reverse)	5´ggg aga tct cat act gtt gca atg gca 3´
3px81	Cloning of px81 into the expression vector pQE60 (reverse)	5´ggg aga tct tat act gtg gca aaa ctc 3´
Qere	Sequencing of constructs in pQE vectors (reverse)	5´gtt ctg agg tca tta ctg g 3´
Uni	Sequencing of constructs and PCR	5´gta aaa cga cgg cca gt 3´
Rev	Sequencing of constructs and PCR	5´aac agc tat gac cat g 3´
Race1	Amplification of the 5´cDNA end from pxa8	5´aca cct ttc aac cac ggc 3´
Race2	Amplification of the 5´cDNA end from pxa8. nested primer	5´caa cca cgg caa tgc tcc 3´
RACE polyT	RACE PCR	5´ccc ggg aag ctt aag ct_{14} 3´
Nested RACE polyT	Nested primer for RACE polyT	5´ccc ggg aag ctt aag c 3´

2.2.3.- Chemical and enzymes

The enzymes used in the lab were purchased from Pharmacia, Gibco, Boehringer, New England Biolabs and Fermentas, unless otherwise stated. For standard PCRs homemade *Taq* DNA polymerase was used.

Laboratory reagents were supplied by GIBCO, SIGMA, otherwise stated. The radioisotopes were from Amersham.

2.2.4.- Media

- **Luria Bertani (LB) medium:** 1% tryptone, 0,5% yeast extract, 1% NaCl, and 2% of agar in the case of the solid medium the medium is autoclaved and cooled to 50°C before adding the antibiotic.
- **PDB:** Potato dextrose broth. (used at half-strength: 12g/l). Potato dextrose broth was also used as a preparative with agar (37 g/l)

2.2.5.- Buffers and solutions

- **Alkaline phosphate buffer:** 100mM tris pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$
- **30% Acrylamide:** 29,2 %(w/v)acrylamide, 0,8%(w/v)N-N´-methylene bisacrylamide in de-ionazed water.
- **5 x MOPS:** 0,2M MOPS pH7, 50mM NaOAC, 50mM EDTA. Everything prepared in DEPC water. Autoclave.
- **Denhardts solution (100x):** 2%(w/v) BSA, 2%(w/v)Ficoll, 2%(w/v)PVPP360
- **DEPCwater 0,1% (w/v):** DEPC in de-ionazed water shaking over night at 25°C. Then autoclave
- **DNA extraction buffer:** 100mM TrisHCl pH 8,5, 100mM NaCl, 50mM EDTA pH 8, 2%SDS and 0,1mg/ml fresh added proteinase K.

- **PROTEIN BUFFERS:**

1. ***BLOTTING buffer****:* 25mM Tris-HCl pH 8,3, 192 mM glycine, 20% methanol.
2. ***DIGESTION buffer****:* 200 mM KH_2PO_4 pH 7.0, 1 mM EDTA, 1mg/ml BSA and 5% 15 DAP crude protein extract corresponding to the fraction 9 of a fractionated extract in a 12% superose column by FPLC.
3. ***EXTRACTION buffers****:*

- **For native conditions isolation from plant tissues**: 20 mM KH_2PO_4 pH 6.0, 1 mM EDTA and 10 mM ß-mercaptoethanol.
- **For denaturing conditions isolation from plant tissues***:*1ml 0.5M Tris pH 6.8, 1.6 ml 50% glycerol, 1.6 ml 10% SDS, 0.4 ml ß-mercaptoethanol.

- **For denaturing conditions isolation from bacteria**:
  - **a.-** Buffer B: 100mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea. Adjust pH to 8.0
  - **b.-** Buffer C (wash buffer): 100mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea. Adjust pH to 6.3
  - **c.-** Buffer D (elution buffer): 100mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, 100mM EDTA. Adjust pH to 6.3.
- **For native conditions from bacteria**:
  - **a.-** Buffer 1(lysis buffer): 50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole. Adjust pH to 8.0.(lysozime 200µg/ml, DNAse 100µg/ml, RNAse 100µg/ml, 1mM PMSF)
  - **b.-** Buffer 2 (wash buffer): 50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole. Adjust pH to 8.0 (1mM PMSF).
  - **c.-** Buffer 3 (elution buffer): 50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole. Adjust pH to 8.0.

4. ***LAEMMLI buffer(4x)**:* 0,25M Tris-HCl pH 8,2, 0,4%(w/s) SDS, 767 mM glycine.
5. ***LOADING buffer for protein gels (2x)**:* 160mM Tris pH 6.8, 5% SDS, 20% glycerol, 0.004% Bromophenol Blue, 20% ß-mercaptoethanol (The buffer is prepared without adding the ß-mercaptoethanol, which is added immediately before adding to the sample.
6. ***MOBILE PHASE for FPLC fractionation***: 20 mM MES pH 6.0 and 1 mM EDTA or ultra-pure sterile filtered water.
7. ***SDS-precipitation buffer:*** 3.59 ml of K_2HPO_4, 1.42 ml of KH_2PO_4 and water to 50 ml.
8. ***Tricine gels:***
   - Anode buffer: 0.2M Tris pH 8.9
   - Catode buffer: 0.1M Tris, 0.1M Tricine, 0.1% SDS (pH 8.25; do not need to be adjusted)
   - Gel Buffer: 3M Tris, 0.3% SDS, pH 8.45
   - Stacking gel: 1.6 ml Acrilamide (30% commercial stock), 3.1 ml Gel buffer, 12.5 ml H_2O, 150µl APS and 12µl TEMED.
   - Spacer gel: 10% acrilamide, 10ml gel buffer, H_2O to 30ml, 300µl APS and 12µl TEMED.

- Separating gel: 16% acrilamide, 10ml gel buffer, 4g glycerol, H_2O to 30ml, 300µl APS and 12µl TEMED.

9. ***T-TBS***: 500mM NaCl, 20mM TrisHCl pH 7,5, 0,05% Tween-20. For the blocking of the filters and incubation with the appropriate antibodies 5% of non-fat milk powder was added or 3 % BSA without Tween-20 for the blocking of filters that were going to be probed with anti-HIS antibodies.

- **RNA BUFFERS:**
  1. ***Buffer I***: 0,1M NaCl, 0,05M TrisHCl pH 9, 0,01M EDTA, 2% SDS and 0,2mg/ml fresh added proteinase K
  2. ***Buffer II***: 0.4M NaCl, 0,01 Tris-HCl pH 7,5 , 0,2% SDS
  3. ***Buffer III***: 0,1M NaCl, 0,02M TrisHCl pH 7,5, 0,01% SDS
  4. ***Buffer IV***: 0,01M TrisHCl

  For extraction of total RNA: 0,2M TrisHCl pH 7,5, 0,1M LiCl, 5mM EDTA, 1% SDS. Everything is prepared in DEPC water

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- **20 x SSC:** 3M NaCl, 300mM sodium citrate
- **20 x SSPE:** 200mM disodium hydrogen phosphate, 20mM sodium dihydrogen phosphate, 3,6M NaCl, 20mM EDTA pH8.
- **PSE buffer:** 0.5 M sodium phosphate pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA (pH 8.0) and salmon sperm DNA 50µg/ml. To prepare the stock of phosphate buffer: mix 720 ml of 1 M Na_2HPO_4 + 280 ml of 1 M NaH_2PO_4 and set up the pH to 7.2.
- **STE buffer (1x):** 20mM Tris-HCl pH 7,5, 10mM EDTA pH8, 100mM NaCl
- **TAE buffer:** 400mM Tris-HCl, 200mM NaOAC, 18mM EDTA pH 7,8 with glacial acetic acid
- **TNT buffer:** 100mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.3% (v/v)Triton X-100

2.3.- Methods

2.3.1.- Plasmid DNA extraction

The method used is a modification of the alkaline lysis method of Birnboim and Doly (1979) taken from Maniatis *et al.* (1982). For sequencing quality DNA the QIAGEN miniprep extraction Kit was used

2.3.2.- Plant genomic DNA isolation

The method used was the one developed by Sharp *et al.* (1988).

Material ground in liquid nitrogen is resuspended in DNA extraction buffer at 4ml/g and incubated at 37°C for 30min. The mixture is then transferred to 40ml centrifuge tubes, and sequentially phenol extracted and centrifuged. After RNAase treatment the DNA is precipitated and quantified.

2.3.3.- Isolation of total RNA and messenger RNA from plant tissues

For the isolation of total RNA the liquid nitrogen ground material is resuspended in the extraction buffer and after phenolisation is precipitated with LiCl. All buffers are prepared in DEPC-treated water.

For the messenger RNA extraction (Bartels and Thompson 1983), the material after grinding with liquid nitrogen is resuspended in Buffer I, and after an incubation at 37°C for 30 min., is extracted with phenol/chloroform. The messenger RNA is isolated by binding to oligo d-T cellulose, which is successively washed with the buffers II and III. The RNA is eluted from de cellulose in buffer IV.

2.3.4.- Genomic southern analysis

DNA was digested with different restriction enzymes and electrophoretically separated on 0.8 % agarose gels in TAE buffer. The gels

were treated with a 0.125 N HCl solution for depurination, and denatured for 30 min in 0.5M NaOH and 1.5M NaCl. The neutralisation is done in 0.5M Tris-HCl pH 7.5, 1.5M NaCl for 30 min. The DNA is then transferred and bound to a Hybond N membrane (Amersham) following the standard capillary transfer procedure (Maniatis *et al.*., 1989). The filters were UV-cross-linked and prehybridised and hybridised in 5xSSC, 0,5% SDS, 5xDenhardt´s and 50µg/ml denatured salmon sperm DNA at 65°C.

Following hybridisation, the filters were washed twice in 2xSSC, 0,1% SDS during 10 min and twice in 1xSSC 0,1% SDS during 10 min.. They were packed in plastic bags, sealed and exposed to autoradiography during the required period of time.

2.3.5.- Northern blot analysis

All the instruments for RNA handling were used just for that in order to avoid RNAases contamination. After the isolation of the RNA, this was electrophoresed in a denaturing agarose gel (1,2% w/v agarose 1xMOPs buffer, 2.2M formaldehyde) using 1xMOPS as running buffer. 50 µg total RNA were used and 2 or 4µg poly-A RNA depending on the expression level of the transcript to detect. The RNA is denatured prior to electrophoresis by heating at 65°C during 10 min., in 50% (v/v) deionised formamide, 2.2M formaldehyde, 0.5xMOPS buffer and 1xRNA loading buffer. After electrophoresis, the samples were blotted onto Amershan nylon membranes. The fixation to the filter was done by means a UV cross-linker (120.000 µJoules cm^{-2} for 30s). Pre-hybridisation and hybridisation of the filters was done in 50% deionised formamide, 5xSSPE, 0,5% (w/v) SDS, 5xDenhardt´s solution and 50µg/ml salmon sperm DNA. Wash steps were identical to southern analysis (2.3.4).

2.3.6.- Preparation of radioactive labelled probes

2.3.6.1.- Random prime labelled probe

Probes were prepared by isolating the inserts from plasmids and purifying from agarose gels. The DNA was denatured by heating and labelled using the T7 QuickPrimer® Kit (Pharmacia Biotech) as follows:

34µL OF DENATURED DNA (50 TO 100 NG)
10µl of reaction mix (containing the buffer, nucleotides and random primers)
5µl (3000Ci/mmol) [α^{-32}P]-dCTP
1µl T7 DNA polymerase

The reaction was done at 37°C during 15min. and the probe purified using QIAGEN PCR purification Kit. Prior to use the probe is denatured by boiling for 5 min.

2.3.6.2.- cDNA probes

The labelling was carried out in 25µl of the next reaction mix:

2µG MRNA IN 10µL DEPC WATER
1µl Oligo(dT)$_{14}$ primer (100pM) primer and mRNA are heated 10min at 70°C
5µl 5x First strand Buffer
2.5µl 100mM DTT
1µ dNTP (10mM, lack dCTP)
5µl (3000Ci/mmol)[α^{-32}P]-dCTP
1.5µl (200U/µl) reverse transcriptase (SUPERSCRIPT^{RT} GIBCO BRL)

The reaction was incubated at 42°C during 1h and the non- incorporated nucleotides were removed using the QIAGEN PCR purification kit. Prior to use, the probe is denatured by boiling 5 min.

2.3.7.- PCR amplification and other polymerisation techniques

2.3.7.1.- Genomic PCR and RT-PCR (Innis *et al.,* 1990 modified)

The amount of DNA template depended on the abundance of the transcript to amplify in the case of the RT-PCR (first strand cDNA) and in this case varied between 20 and 100 ng. For genomic PCR, 50ng of pure DNA from *Zea mays* were used.

The reaction was done in 50µl of final volume with the next amounts of components:

20 pm for each primer
50 µM dNTPs (dATP, dGTP, dCTP, dTTP)
taq buffer to 1x with a final concentration of $MgCl_2$ of 2 mM
2.5U home-made taq polymerase.

The reaction was overlaid with mineral oil and the amplification was carried out in a Biometra® Thermal Reactor as follows:

2 min 30 s at 85 °c	initial denaturation
40 s at 94°C	denaturation
1 min at 60°C	annealing
1 min at 72°C	extention

The last three steps were cycled 35 times, followed by an extension of 5 min to ensure the completion of the reactions.

2.3.7.2.- Plasmid PCR amplification

The amplification was done either from 10ng plasmid or from single colonies. The reaction was done in 20µl of final volume and under the same

conditions as before. The program contained a longer denaturing initial cycle (3 min at 94°C) and the number of cycles was decreased to 25.

2.3.7.3.- RACE PCR

Reverse transcription is done as follows with 2µg of total RNA in 10 µl of DEPC water.

Denaturation of the RNA during 1 min at 94°, then quickly on ice. The following reaction mixture was prepared:

5X BUFFER	5
100 mM DTT	2.5
RNAsin (40u/µl)	1
Primer race1(1-10pm)	1 specific primer for the sequence to RACE
DdH_2O	2
55°C 15 min., 42°C 1h (after 5min. add 1µl of retrotranscriptase) and 3min. 94	

The result is purified using a PCR purification kit from QIAGEN, and the ssDNA is eluted in 30µl. The volume is reduced to 9.8 in a PCR block. The next step is the tailing of the ssDNA at the 5´ end as follows:

5 X TDT BUFFER	4
1mM dATP	4
25mM $CoCl_2$	1.2
CDNA	9.8
terminal transferase	1 (25U) The program is 15 min at 37°C and 5 min. at 72°C

After another purification step as before, the first PCR is performed using as nested primer race-2 and the RACE polyT modified primer. The PCR protocol was already described (2.3.9.1).

In some cases it could be necessary to perform a second nested PCR in which it is possible to use the nested polyT RACE primer.

2.3.7.4.- Sequencing

The sequencing reactions were done using the kit T7 Sequencing from USB based on the incorporation of dideoxy-nucleotides described by Sanger, F. *et al* 1977.

2.3.8.- Cloning methods

2.3.8.1.- Preparation and transformation of *E.coli* competent cells

a.- *Preparation of E.coli electro competent cells:*

A single colony is used to inoculate a 10ml LB culture that is grown over night. The next day a dilution 1/100 is done and the medium is kept growing till an OD_{600} of 0.6 is reached. The culture is pelleted during 10 min at 2000xg and gently resuspended in ice-cold sterile water. The process is repeated twice. After the washing, the cells are gently resuspended in a 1/100 volume of the initial culture in 10% sterile glycerol, again pelleted as before and resuspended in 1/1000 volume of glycerol. The cells can be already aliquoted and stored. The aliquots should be immediately frozen in liquid nitrogen.

Transformation of E.coli electro competent cells:

20 to 50 ng of salt-free ligation DNA is mixed with the competent cells, and so pipetted to the electroporation cuvete. A pulse of 9 kv/cm; capacitance 25 µF; resistance 400-600 Ù. Immediately after the pulse, the cells are resuspended in 1 ml of LB and incubated at 37°C before plating.

b.- *Preparation of $CaCl_2$ competent cells:*

A single colony is used to inoculate a 10ml LB culture that is grown over night. The next day 100ml of medium are inoculated with 1ml of the o.n culture and the medium is kept growing till an OD_{600} of 0.6 is reached. The medium is pelleted at 2000xg in a cold centrifuge and the pellet is resuspended in 10 ml of ice-cold 0.1M $CaCl_2$ and kept in ice during 20 min. The cells are pelleted again under the same conditions and resuspended in

1ml of 15% glycerol 0.1M $CaCl_2$. 100 µl aliquots are prepared and immediately frozen in liquid nitrogen.

Transformation of $CaCl_2$ competent cells:

The cells are thawed on ice during 20min. Meanwhile the ligation is diluted twice with ice-cold 0.1M $CaCl_2$. Afterwards the ligation is carefully mixed with the cells and kept in ice during 1h. After this period the cells are heat-shocked at 42°C during 2min and resuspended in 300-500 µl of LB before plating.

2.3.8.2.-Ligation of double stranded DNA fragments.

For this purpose the enzyme used was the ligase purchased by GIBCO-BRL®. One unit of enzyme was used per reaction. The vector was dephosphorilated and 10ng were used for the reaction in the presence of different ratios of insert DNA.

2.3.9.- Libraries screening

2.3.9.1.- Differential screening Qpix system

10368 colonies were screened with this method. A cDNA library constructed in the phagemid insertion vector λZAPII (*Hueros et al.* 1995) from 10 days after pollination endosperm messenger RNA, was plated. Single colonies were picked and spotted using a robot system *Qpix* from Genetix® (http://www.genetix.co.uk/QPix.htm). For colony hybridisation the standard method was used (*Maniatis et al.* 1989). The probe was prepared as explained on 2.3.6.2.

2.3.10.- Protein analysis

2.3.10.1.- Protein extraction

For that purpose, the material is grounded in liquid nitrogen and immediately resuspended in extraction buffer (see buffer and media 2.2.5). The

protein extraction was done from maize kernels under native conditions (see buffers) and the extracts were used for protein processing of the recombinant *E.coli* obtained proteins.

Extractions for SDS-PAGE gels were done under denaturing conditions. Under these conditions, the grounded material is resuspended in the extraction buffer, vortexed for 2 min and next then boiled during 5 min. The proteins are recovered after centrifugation during 5 min at top speed. To determine the protein concentration the SDS present in the buffer is precipitated as follows:

- 20µl of the protein extract are diluted to 100µl with water
- 400µl of the SDS precipitation buffer are added.
- Shake and keep at room temperature during 10 min
- Centrifuge at top speed during 5 min and take 100 µl of the supernatant without disturbing the pellet for Bradford determination.

2.3.10.2.- Expression and purification of recombinant Histidine-tagged proteins

The expression constructs were prepared in pQE60 vectors from QIAGEN, in which the poly-histidine region is at the N-terminus of the fusion protein. The expression was carried out in M15 *E.coli* induced with 1 mM ITPG afterwards the proteins were extracted in *buffer B* and purified by affinity chromatography (Stüber *et al.* 1990; Bush *et al.* 1991). The elution was done in buffer C with 100mM EDTA solution.

After the purification, the protein is refolded by dialysis using a cellulose benzoylated dialysis tubing (SIGMA). The dialysis is done twice against 4M urea, 100mM NaH_2PO_4, 10 mM Tris; twice against 100 mM NaH_2PO_4 10 mM Tris; and twice against ddH_2O. Each dialysis step is carried out during 12 hours with gently shaking at 4 °C. After the refolding process the protein concentration is determined by BCA (P.K Smith, *et al.,* 1985) reaction which is read at OD_{562}.

2.3.10.3.- Processing of recombinant *E.coli* proteins

After the purification of the recombinant proteins, the protein is digested in the presence of native kernel protein extracts. For that purpose 800 µl of the recombinant protein in water is mixed with 200 µl of *digestion buffer* (see buffers) and so is incubated at 37 ° C during 72 h. The process is followed by Western blot analysis and when the efficiency of the reaction reach a completion over 90 %, the fragments are fractionated in a Superose 12 % column by FPLC. The protein-containing fractions are detected by Western blot assay and the fractions dialysed three times against water in the same dialysis tubing as before explained.

2.3.10.4.- Western blot analysis (Maniatis *et al.* 1989)

Protein was run overnight on a discontinuous SDS-PAGE gel electro-blotted in blotting buffer onto supported nitrocellulose membrane and blocked overnight at 4°C in 5% milk TTBS. The first antibody was diluted in the blocking solution and incubated together with the filter at room temperature during 2h. After three washes of 10 min in TTBS the secondary antibody diluted 5000 fold in blocking solution, is added to the filter and incubated as before during 45 min. After three washes of ten min in TTBS, the detection is done by means of the ECL system from Amersham, followed by autoradiography.

2.3.11.- Histological techniques

2.3.11.1.-*In situ* hybridisation

Kernels 10 DAP were fixed in 4% paraformaldehyde, 0,1% glutaraldehyde in 0.1M sodim phosphate buffer pH 7.2 during 12 to 24 hours depending on the tissue volume. The samples were dehydrated through different ethanol concentrations and included in fibrowax (Plano, Marburg, Germany). The sections were prepared at the microtome (Reischler-Jung, Nussloch Germany) with a 10µm thickness. The sections were transferred to 3 –aminopropyltriethoxylane pre-treated glass slides. The hybridisation was

carried out with the probes in sense and antisense orientation in-vitro transcribed by the T3 and T7 RNA polymerises and digoxigenin-labeled. Prior to hybridisation the probes are partially digested with sodium carbonate. The hybridisation was performed essentially as described by Block and Debrouwer (1993). Before hybridisation the sections are deparaffinized and treated with protease (Merck) at 5µg/ml. Mounted slides were incubated at 50°C during 16h with 40 to 80 ng of RNA per slide. After hybridisation the slides are treated with RNAase A at 37°C and washed with 2xSSPE. For inmunological detection, the sections were subsequently treated with 0.5% blocking reagent form Boehringer and 1% w/v BSA in TNT buffer. After blocking of the sections, were incubated with antidigoxigenin-alakaline phosphate conjugate (Boehringer-Manheim, 1:3000 dilution) in 1% BSA solution at room temperature during 1 hour with gentle shaking. Afterwards the sections are washed several times in alkaline phosphate buffer. The colour reaction is carried out in alkaline phosphate buffer containing nitroblue tetrazolium chloride/5-bromo-4-chloro-indolyl phosphate. Sections were later on stained with 0.01% Safranin, dehydrated in grade ethanol and mounted with DPX mounting medium (Agar scientific, Stansted, UK).

2.3.12.- Antifungal assays.

The assays were performed as described by Broekaert *et al.,* 1990.

2.3.12.1.- Collection of fungal spores

An agar plug containing the mycelium of the desired fungi is placed in the middle of a PDB agar plate. The plate is sealed and incubated at room temperature under white fluorescent light. Meanwhile the fungal growth is checked at the microscope till the phase of spore production is reached. At this point each petri dish is covered with 5 to 10 ml of sterile water allowing the spores to transfer to the liquid phase. The spores containing water is filtered through a sterile glass wool. The spores are washed several times and the spore density is determined in a Thoma chamber. The density is adjusted at 4 x

10^7 / ml. They can be stored frozen in a medium containing 25% glycerol. The spore yields obtained vary between 10^7 and 10^9 every 10 petri dishes.

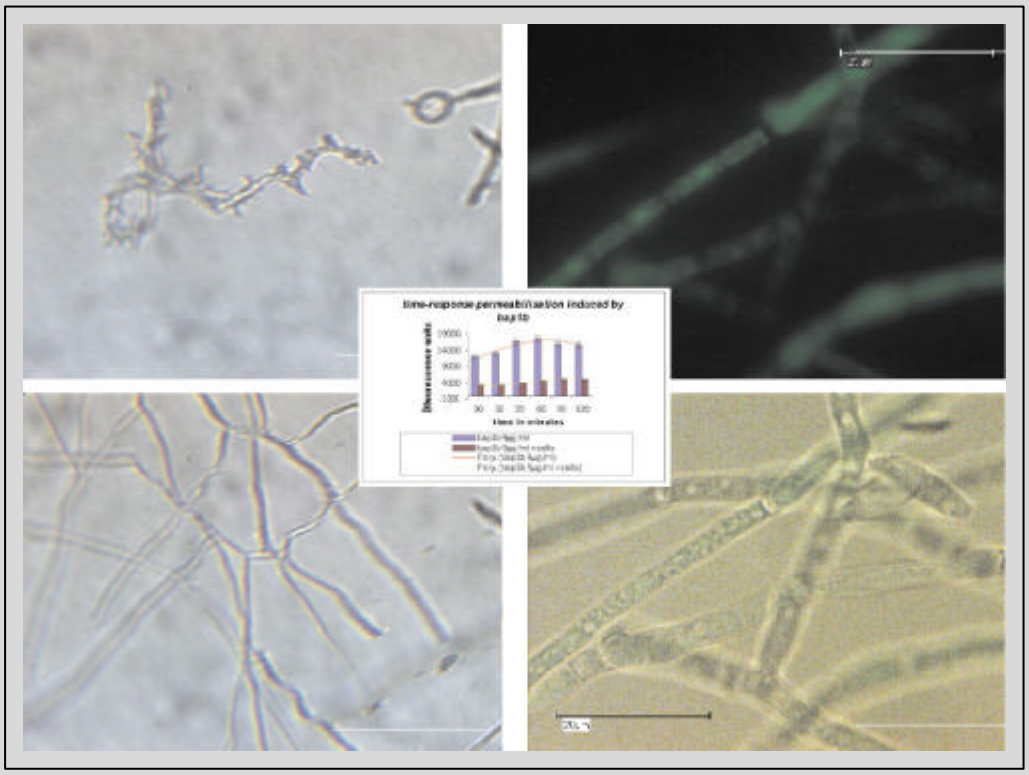
2.3.12.2.- Test for antifungal activity

The spore suspension is prepared by diluting the stock of spores 1000 folds in ½PDB medium containing 10 µg/ml of tetracycline and 100µg/ml of cefotaxime. The test is carried out in a microtiter plate of 96 wells with flat bottom. The protein to be tested is diluted sequentially from well number 1 to well number 8, thus creating a range of concentrations. To the protein distributed on the microtiter plate are added 80 µl of the medium containing the spores previously prepared. In parallel another test is performed with the rest of medium to which $CaCl_2$ to a final concentration of 1 mM and KCl to a final concentration of 50 mM were added.

The antifungal activity is determined by measuring the optical density at 620 nm, and the IC_{50} are calculated in the intervals where the OD_{620} is half of density where the fungi were growing under normal conditions.

2.3.12.3.- SYTOX Green uptake assay

Fusarium culmorum was grown in 50 ml PDB at an inoculum density of $3x10^5$ spores/ml for 20 h at 22°C. The hyphae after growth were collected and washed several times with 100µM HEPES-KOH pH 6.5. After the washes the hyphae are resuspended in the same initial volume used for growth in 100µM HEPES-KOH pH 6.5 supplemented with 0.2µM SYTOX Green. The assay is performed at 22°C, with shaking for 30min. The dye uptake is assayed in the presence of the peptide to be assayed at different concentrations in the presence or absence of salts. The SYTOX Green uptake efficiency is measured in a fluorescence spectrometer at an excitation wavelength of 488 nm and an emission of 540 nm. For kinetic studies the fluorescence was measured at 30 min. intervals during 2 hours.


time-response permeabilisation induced by
Time in minutes

Results

3.- RESULTS

3.1.- Characterisation of a 10 DAP endosperm library

As it is likely that the endosperm transfer cell layer plays an important role in endosperm development but the extent and details of the physiological mechanisms regulated by these cells is not fully clarified (Pate *et al.* 1972, Lyznik *et al.* 1985, Wang *et al.* 1994, Hueros *et al.* 1995) a library screening was set up in order to identify genes specifically expressed in this region and whose function could help to give a better insight into the mechanisms that govern these cells.

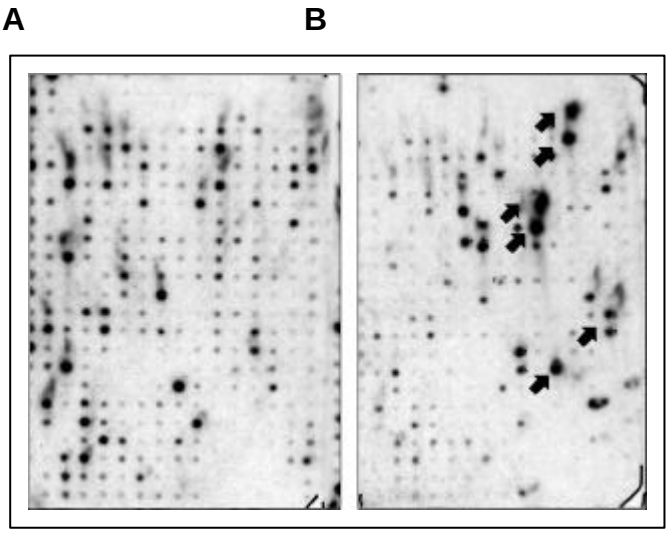


Fig6.- An example of the hybridization of one array.
The filter was sequentially hybridized with two different probes: top(a) (cDNA probe from TOP half of the kernel) and bottom half of the kernel (b)(cDNA probe from the bottom of the kernel) right. Arrows indicate clones differentially expressed

The library was plated and the clones were picked using a robot (Qpix from Genetix, materials and methods pg 32). 10368 clones were distributed in 27 plates of 384 wells and spotted onto nylon filters. The filters had a density of 2304 clones distributed in 6 fields of 384 spots. A total of 5 filters were prepared. For the preparation of the cDNA probe, 10 DAP maize kernels were dissected into two halves, an upper one containing the crown of the

endosperm, the storage tissue, and most of the embryo and a bottom part, containing basically of the transfer cell layer and the basal part of the embryo. Before dissecting the kernels, the pedicel was discarded. Messenger RNA was isolated from the dissected parts as explained in materials and methods (section 2.3.3 pg 26), and cDNA probes were prepared for the screening. After hybridisation, comparison of the relative signal strengths with upper and lower cDNA probes (Fig 6), led to the isolation of 144 putative positive clones. From these initial clones, basal endosperm transfer layer specific genes (*BETL*s) previously cloned were identified by hybridisation with a complex probe containing the *BETL*-1 to 4 genes (Hueros *et al.,* 1995, 1999). Those clones that did not cross hybridise with the complex *BETL* probe, were rechecked by reverse Northern. For this purpose, the inserts were amplified by PCR with flanking universal and reverse primers, fractionated on a 1% agarose gel, blotted onto a nylon filter and hybridised the cDNA probes prepared from TOP and BOTTOM messenger RNA(fig 7) The clones that showed clear specificity for RNA from the BOTTOM part of the kernel, region where the transfer-cell layer is present, were rechecked by Northern blot. For further investigation the clones selected in the first selection round were sequenced. A summary of the results is depicted in table 2.

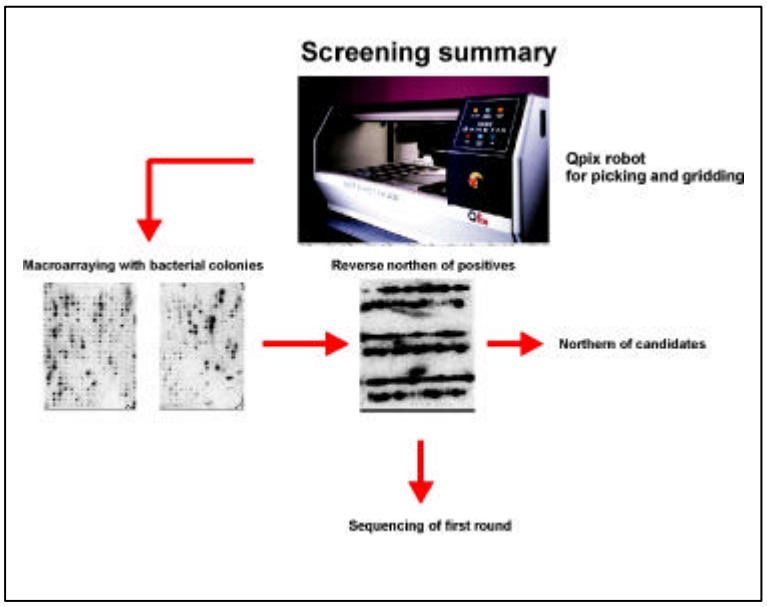


Figure 7.- Graphic summary of the screening of the 10 DAP library

Characteristics of the DATABASE accessions					
CLONE	**Accession number**	**Species**	**Protein**	**Probability***	**Insert length (bp)**
px22			NSH		365
px23	ACO15450	A.thaliana	Unkown protein contains a zinc-finger	1e-37	535
px24			NSH		444
px26			NSH		361
px27	AJ133529	Z.mays	*BETL*-2	3e-10	470
px28	AJ133531	Z.mays	*BETL*-4	2e-07	539
px29	AC006931	A.thaliana	Phosphatase	9e-88	535
px31			NSH		410
px32	AJ133529	Z.mays	*BETL*-2	3e-10	476
px33	U13148	P.ciliae	Apomixis related	2e-70	520
px34			NSH		345
px35	AJ133529	Z.mays	*BETL*-2	3e-07	389
px36	U67327	M. musculus	WD40repeat	2e-06	441
px37			NSH		407
px38			NSH		401
px39	AJ133531	Z.mays	*BETL*-4	1e-07	511
px40			NSH		366
px41	AC002328	A.thaliana	SEC14 like	3e-12	681
px42			NSH		350
px43			NSH		466
px44			NSH		405
px45	AC007369	A.thaliana	Unknown protein	1e-37	603
px46	AC010927	A.thaliana	Unknown protein	2e-46	585
px47	M64737	R.comunis	ATPpyruvateKinase	2e-94	646
px48			NSH		382
px49	AC004077	A.thaliana	Unknown protein	2e-09	642
px50	AF136004	T.aestivum	Initiation factor 4B	1e-21	676
px51			NSH		262
px52			NSH		360
px57			NSH		349
px58			NSH		374
px59			NSH		355
px60			NSH		722
px61			NSH		488
px63			NSH		405
px64			NSH		501
px65	AJ133529	Z.mays	*BETL*-2	1.8	339
px66	Aco31394	A.thaliana	Unknown protein	2e-14	470
px67			NSH		404
px68	AJ133529	Z.mays	*BETL*-2	5e-10	610
px69			NSH		301

Table 2a.- Isolated clones bottom-specific candidates from differential screening

NSH: no significant homology was found. *Random matching probability according to the stochastic model of Karlin and Altschul (1990).(Database comparison performed with BLAST program, Altschul *et al.,* 1990ab)

Characteristics of the database accessions					
CLONE	Accession number	Species	Protein	Probability*	Length (bp)
px71	AF153277	N.tabacum	RNApase II	2e-34	423
px72	AL161563	A.thaliana	Unknown protein	2e-58	623
px73	AJ133531	Z.mays	*BETL*-4	2e-07	588
px74			*BETL*-1		421
px75	AJ133529	Z.mays	*BETL*-2	7.5	269
px76	AC007369	A.thaliana	Cystein-rich	1e-37	656
px77			NSH		394
px79	AF114486	D.melanogaster	Msx-1 interacting	1e-12	658
px81	AJ133529	Z.mays	*BETL*-2	3e-08	590
px83	AJ133529	Z.mays	*BETL*-2	5e-10	607
pxa1			NSH		360
pxa2	AP000836	O.sativa	Isocitrate DHase	1e-09	271
pxa3	AC018848	A.thaliana	Unknown protein	1e-70	570
pxa4	Z49203	Z.mays	*BETL*-1	1e-18	420
pxa5			NSH		399
pxa6			NSH		241
pxa7	AF114171	S.bicolor	Unknown protein	2e-28	484
pxa8	AJ133529	Z.mays	*BETL*-2	2e-06	249
pxa15	AC009894	A.thaliana	Elongation factor 2	1e-100	716
pxa16			NSH		197
pxa17	S66339	Z.mays	Elongation factor 1	1e-138	718
pxa18	C29960	C.tentas	Balbiani Ring	0.35	325
pxa20	AL035356	A.thaliana	Unknown protein	5e-11	860
pxa21	AF015522	Z.mays	Ribosomal protein	4e-94	660
pxa22	U62750	Z.mays	Ribosomal protein	0.78	323
pxa23	AC016662	A.thaliana	Ribosomal protein	1e-30	399
pxa25	X92491	S.tuberosum	TOM20	2e-18	565
pxa26	U45857	Z.mays	G3PDHase	3e-51	505
pxa27			NSH		338
pxa28	P12783	T.aestivum	PGkinase	7e-70	600
pxa30	AC080318	A.thaliana	Sti1 like	6e-46	540
pxa34	U08226	Z.mays	Histone 2B	5e-38	626
pxa35			NSH		260
pxa37	S22323	T.aestivum	Histone 2B1	0.006	628
pad3	AF039573	O.sativa	ABA inducible	1e-09	540
pad7	AB003324	O.sativa	MADs box protein	2e-36	622
pad31	U83245	A.thaliana	ARF-1	2e-27	632

Table 2a.- Isolated clones bottom-specific candidates from differential screening
NSH: no significant homology was found. *Random matching probability according to the stochastic model of Karlin and Altschul (1990). (Database comparison performed with BLAST program, Altschul *et al.,* 1990ab)

3.2.- Top-specific or enriched sequences

The identification of sequences with different expression patterns in the endosperm can be of great interest for the isolation of promoters, which could be used for the directed expression of genes of agronomical importance. Genes being expressed in regions other than the transfer cell layer were identified in the screening of the endosperm library, however they were not further characterised, being outside of the main interest of the project.

The clones pxa18 and pxa25 whose main characteristics are summarised in table3, presented an expression mainly localised in the upper part of the endosperm (fig 8).

Clone	Length without poly-A tail	Size observed in Northern-blots	Identity of closest homologue	Probability
Pxa18	325	≈ 1.25 kb	Balbiani ring	0.35
Pxa25	565	≈ 1,1 kb	TOM20	2e-18

Table 3.- Characteristics of top-enriched sequences isolated during the screening

Random matching probability according to the stochastic model of Karlin and Altschul (1990).

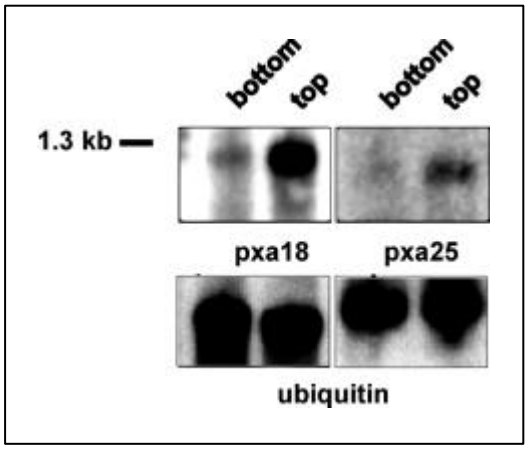


Figure 8.- Northern blots of endosperm halves.

Messenger RNA was isolated from endosperm halves dissected into TOP and BOTTOM. 2 µg of mRNA was separated in a denaturing formaldehyde gel, blotted onto nylon filters and the resulting Northern blots hybridized with the corresponding probes indicated in the picture. The filter was subsequently probed with a polyubituitin cDNA as a loading control.

Both transcripts were preferentially expressed in the top part of the endosperm although expression in other tissues was not investigated. Transcript accumulation was in both cases low, at least at the stage of endosperm development where it was tested (10 DAP). In neither of the case did the sequence available correspond to the full-length transcript predicted from Northern experiments, so the polypeptides encoded were not studied.

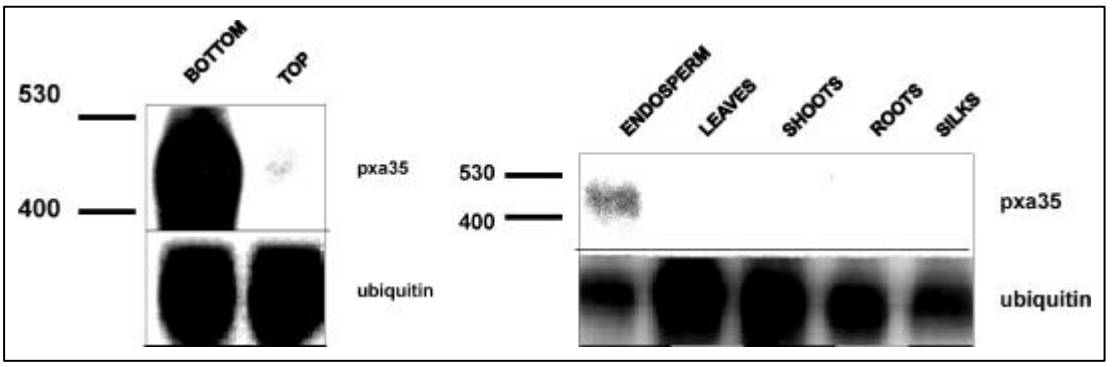
3.3a.- Isolation of transfer cell specific clones: *Novel sequences specifically expressed at the transfer cell layer.*

The sequence of the clone pxa35 whose main characteristics are summarized in table 4 was specifically expressed in the transfer cell layer (fig 9). A database comparison did not give any significant homology with currently available sequences.

Clone	Length without poly-A tail	Protein	Predicted Signal peptide	Predicted Isoelectric point
Pxa35	260	58 AA; 6451d 63 AA; 7434d	At position 9-10 SSG-EG (frame 3)	6.81 (frame 3) 7.03(frame 2)
Frame3	MSEISLSSGEGKQLHCTIVSFHSQISSYISHLFTINRTEKPILILTNLYLLLVSVLSS			
Frame2	LIARGNITLHELNQIIGRDSGSDDISPWYERDFFVLWRGKTAALYHRIVSFSDFKLYFSFVHN			

Table 4.- Summary of the main characteristics for pxa35 and predicted sequences.

Fig 9.- Site of expression of the clone pxa35 (down)
2µg of messenger RNA from the indicated tissues were fractionated in a formaldehyde denaturing gel, blotted onto nylon filters and the resulting Northern Blots hybridized with the radioactively labeled pxa35 probe.



The pxa35 transcript is localized exclusively in the bottom part of the endosperm, giving clear hybridization signal. The transcript observed on Northern blots in longer than the cDNA isolated. Therefore as the starting ATG is not known with certainty, two different reading frames are possible (table 4), of which Frame 2 is incomplete at the N-terminal region. The two reading frames have no homology to other database sequences.

3.3b.- Isolation of transfer cell specific clones: *Further representatives of previously identified BETL sequence types*

As depicted in table 2a,b, some of the sequenced clones possessed homology with other characterised bottom-specific clones (Hueros *et al.,* 1995, 1999). The first step was to identify the homology between them to discard possible doubly represented clones. That was the case for clones px73 and px39, homologous to *BETL*-4, and also for px32, px83 and px68, related to the BETL-2 gene. The results of the alignments at protein level are depicted in figure 10.

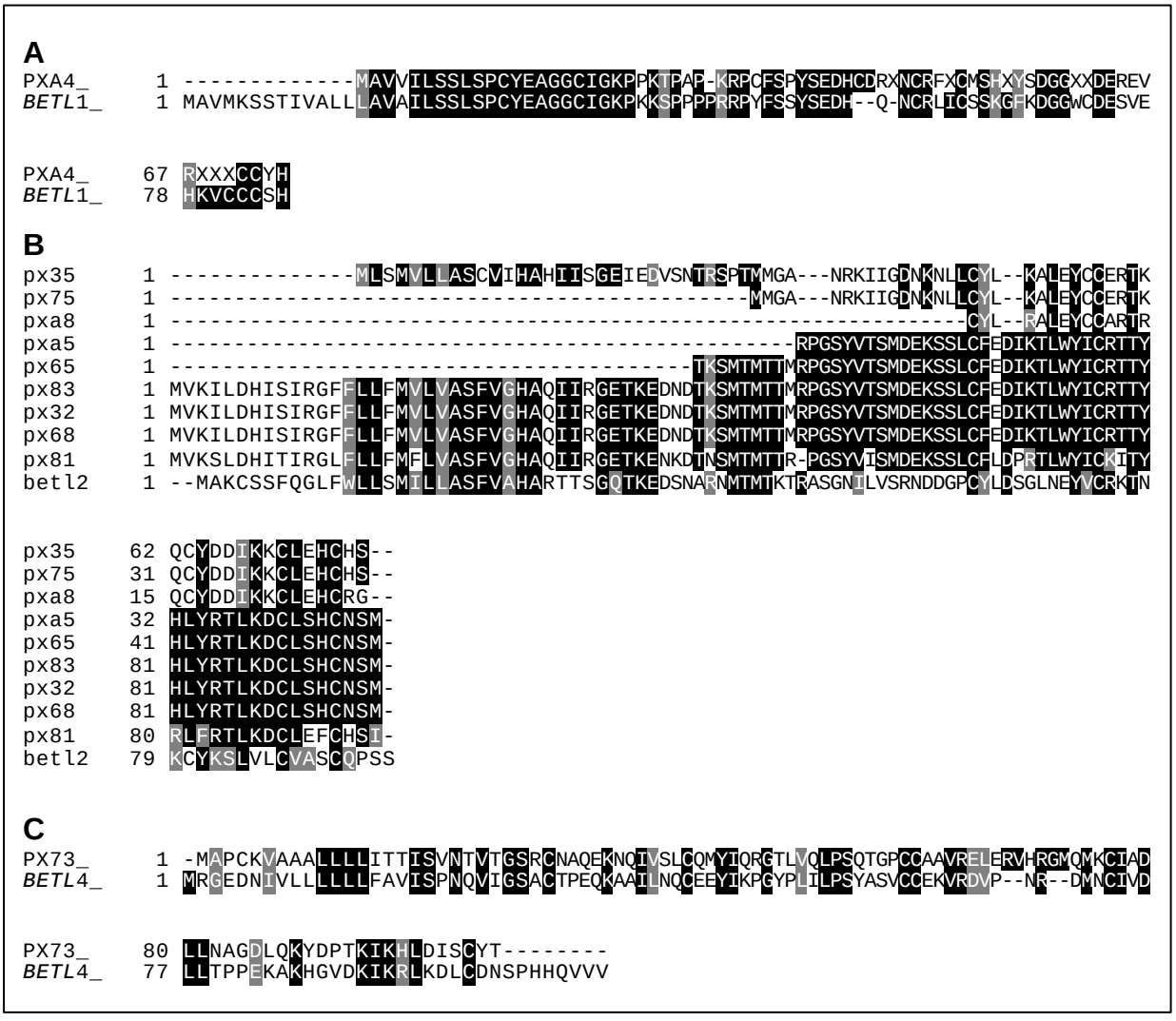


Figure 10.- Alignment of *BETL* relatives obtained in the screening.

Analysis performed by CLUSTALW program (Thompson *et al* 1994a,b). Black shaded letters indicates identical amino acids and gray shaded letters indicates homologous amino acids.

A: amino acid alignment of *BETL*1 relative; B: amino acid alignment of *BETL*2 relatives; C: amino acid alignment of *BETL*4 relative

The isolation of new sequences with similarities to other transfer-cell layer specific clones currently characterised permits the identification of motifs, which might give more insight into the function of these proteins during endosperm development. For this purpose *the group of sequences with similarities to the BETL2 gene (fig 11) that defines by simple homology a new family of sequences, were further characterised in this thesis.*

A

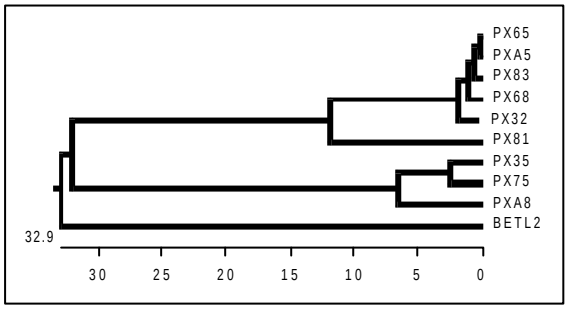


Fig 11.-a.- Dendrogram of relationships among the different clones of *BETL*2 homologues.

Graphic derived from the matrix of sequence similarities calculated with the CLUSTALW program (Thompson *et al* 1994a,b).

b.- Pairwise comparison of one member of each group with *BETL*2 at protein and DNA level.

The genes depicted in this table will be further characterised. Probabilities according to the stochastic model of Karlin and Altschul (1990).(x means no significant match).

B

	Pxa8		Px35		Px68		Px81		BETL-2	
	DNA	protein	DNA	protein	DNA	Protein	DNA	protein	DNA	Protein
Pxa8			e^{-141}	$2e^{-35}$	x	$3e^{-10}$	2.1	$4e^{-10}$	0.002	$1e^{-08}$
Px35					x	$4e^{-08}$	1.4	$2e^{-07}$	0.007	$1e^{-07}$
Px68							0.0	$5e^{-38}$	$9e^{-11}$	$1e^{-10}$
Px81									x	$7e^{-09}$

There are five different groups of sequences in the *BETL*-2 homologous group (fig 11a). The sequences px32, px65, px68, px83 and pxa5 are different lengths derived from the same transcript; as is the case with px35 and px75. To complete the studies, a representative of each group was chosen. The selection was mainly based on the availability of the full-length clone, thus the sequences px35, px68, pxa8 and px81 were selected for the next studies.

The *BETL-2* gene cloned by Hueros *et al* (1999) shows weak homology with two other sequence types, the *cobratoxin* group and the anti-pathogenic group of genes termed *plant defensins* (Broekaert *et al.,* 1995) (Fig 12), although based on the alignment obtained with the new group of sequences under study, and the closest database accessions, we can conclude that the homology of the BETL2 group of sequences is limited to a few cysteines and that the protein motifs described for the plant defensins are not conserved in BETL2 related sequences.

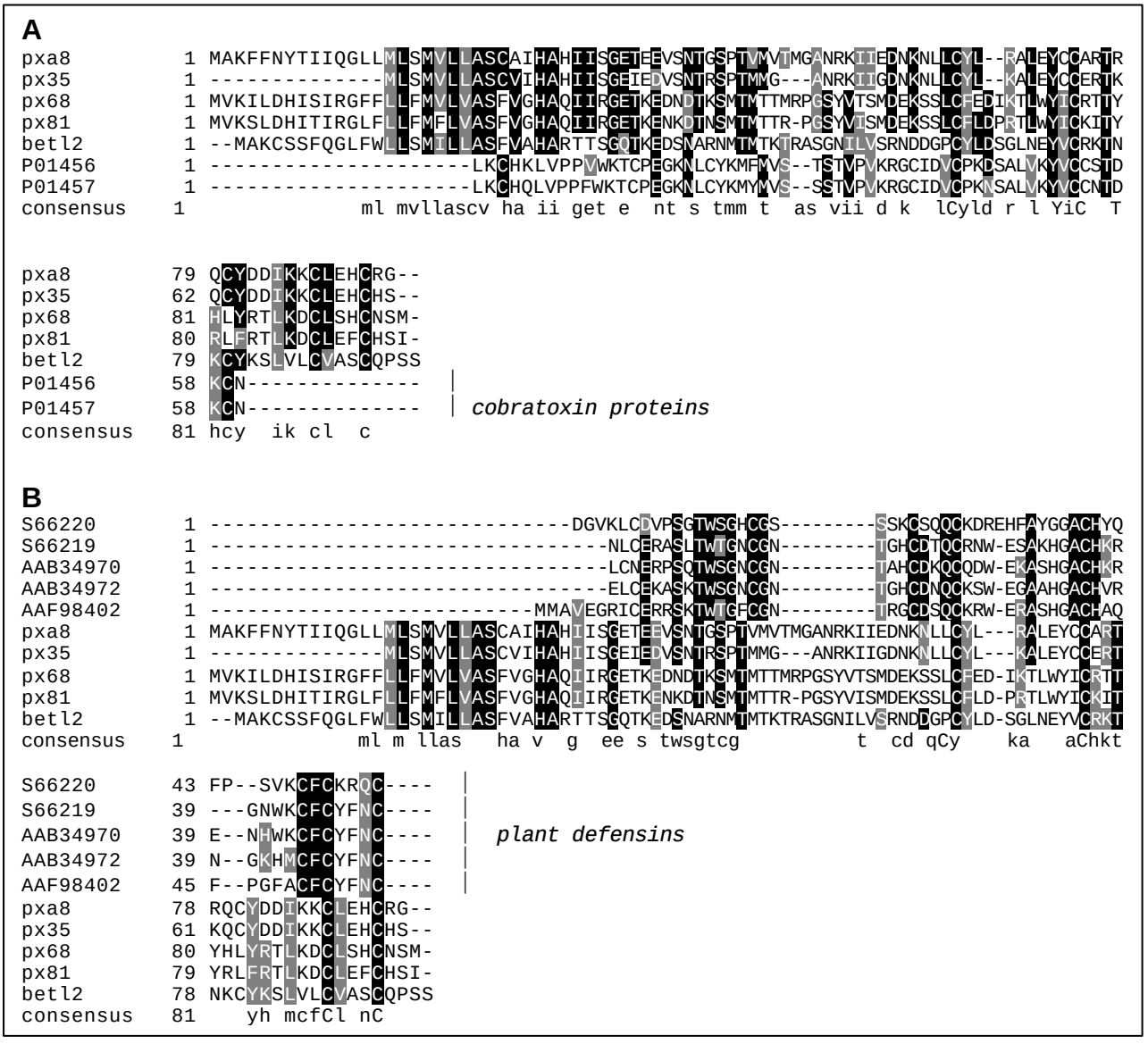


Fig 12.- Amino acid alignment between the *BETL2* relatives and different representatives from cobratoxin (a) and plant defensins (b)

Analysis performed by CLUSTALW program (Thompson *et al* 1994a,b). Black shaded letters indicates identical amino acids and gray shaded letters indicates homologous amino acids.

A: Amino acid alignment of *BETL2* relatives and cobratoxin proteins; B: Amino acid alignment of *BETL2* relatives and plant defensin related database accessions. (the sequence of the clone pxa8 presented in the alignment was completed by RACE PCR)

3.4.- Characterisation of the isolated clones belonging to the BETL2 relatives: *expression and genomic distribution*

3.4.1.- Sequence characterisation

Clone	Length without poly-A tail	Molecular weight of the predicted protein	Predicted isoelectric point
Pxa8	547bp (After RACE PCR)	12.1 kd	7.62
Px35	377 bp	8.6 kd	6.87
Px68	592 bp	11.1 kd	7.7
Px81	576 bp	11 kd	8.78
Betl2		10.4 kd	9.04

Table 5.- Main characteristics of the clones in study and the predicted proteins

All the clones presented here encode small proteins, and all possess a common cysteine motif localised in the carboxyl terminal region. Other regions were predicted from the alignments that were identified in other previously isolated BETLs. The clone pxa8 was clearly partial, so the sequence was completed by RACE PCR.

The basic structure of the *BETL-2* sequence (Hueros *et al* 1999), the closest homologue to the new group under study, is a small protein with a putative signal peptide at the amino terminus (fig 13) linked by a hydrophilic domain to a novel cysteine motif. The existence of an amino terminal signal peptide for the BETL genes was first described by Hueros *et al.,* (1995, 1999). To predict the possible existence in the BETL2 related sequences of a putative signal peptide, the program SIGNAL-P from the CBS at http://www.cbs.dtu.dk/services/SignalP/, was used (fig 14, table6). The other region of the basic structure of the BETL genes, the highly variable hydrophilic region, has been shown to be an area of protein maturation by protease cleavage (Serna *et al.,* in press, appendix). The cysteine motif (fig 15, table7) of the sequence group under study was highly conserved at the carboxyl terminus but with differences in the most amino proximal cysteines, so all clones possessed four cysteines in the same region; two of them (px81 and

px68) lacked a fifth cysteine present in *BETL*-2. In px35 and pxa8 an extra cysteine is present, which was not present in the other proteins of the group.

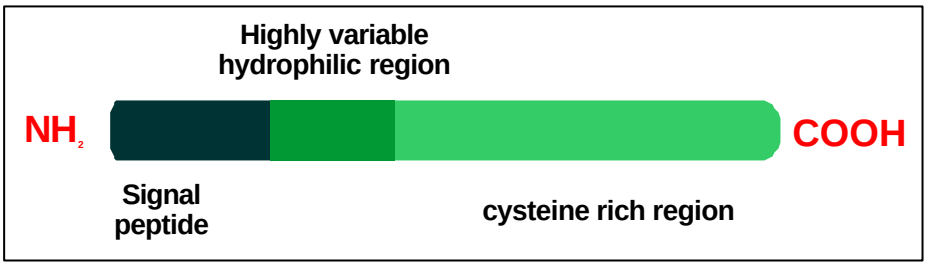


Fig 13.- Graphic representation of the *BETL* family structure.

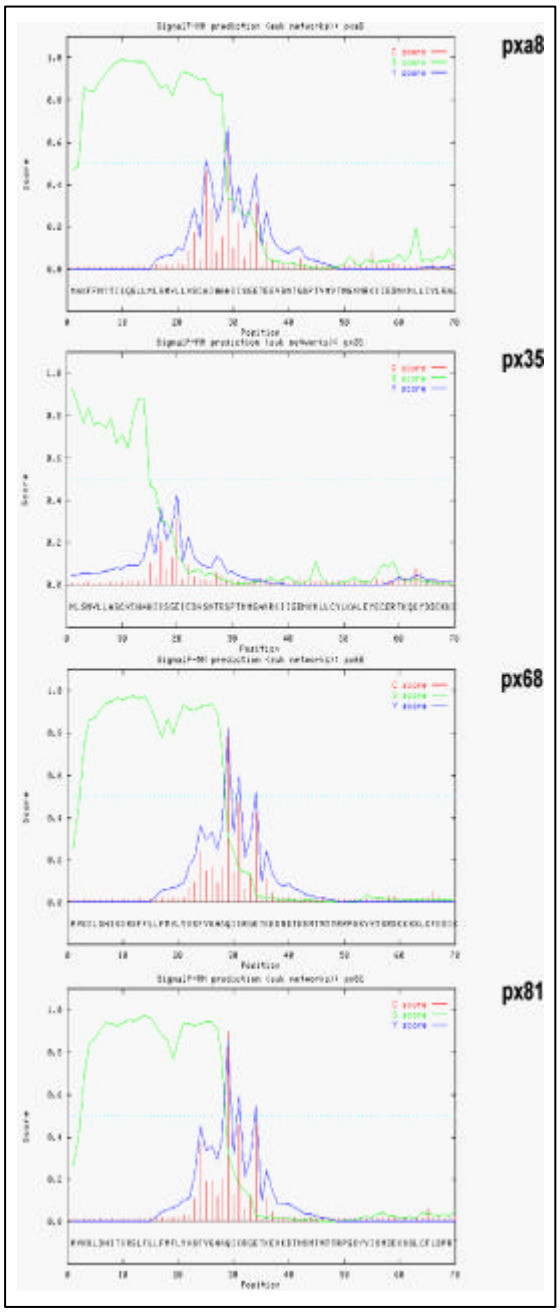


Fig 14.- Signal peptide prediction of the BETL2 relative sequences. It is possible to see how in all of the cases but in pxa35, the program gives a high probability of having a cleavage site in the region around the amino acid 20th, as it was already postulated for other *BETL*s

The program here used will return three types of scores:

- ***C-score (raw cleavage site score)***

This score gives an idea about the most probable site where the cleavage can take place, thus:

High at position +1 (immediately after the cleavage site)
low at all other positions.

- ***S-score (signal peptide score)***
  The score serves to predict the position of the putative signal peptide, thus:

High at all positions before the cleavage site
low at 30 positions after the cleavage site and in the N-terminals of non-secretory proteins.

- ***Y-score (combined cleavage site score)***

The prediction of cleavage site location is optimized by observing where the C-score is high and the S-score changes from a high to a low value. The Y-score formalizes this by combining the height of the C-score with the slope of the S-score.

Sequence	Most likely signal peptide cleavage site	N° Aa in Signal peptide	N° Aa in proprotein	N° Aa in prodomain (by comparison with BETL2)
Px35	19 and 20: ISG-EI	20	57	22
Pxa8	28 and 29: IHA-HI	28	66	30
Px68	28 and 29: GHA-QI	28	69	X
Px81	28 and 29: GHA-QI	28	68	X

Table 6- Summary of the results obtained with the program at the CBS and main characteristics of protein sequences. (X non-determined)

a

```
px35          1 --------------MLSMVLLASCVIHAHIISGE
pxa8          1 MAKFFNYTIIQGLLMLSMVLLASCAIHA------
BETL2         1 MAKCS--SFQGLFWLLSMILLASFVAHA------
px68          1 MVKILDHISIRGFFLLFMVLVASFVGHA------
px81          1 MVKSLDHITIRGLFLLFMFLVASFVGHA------
consensus     1 m k      i g wlLsMvLlASfvaHA
```

b

```
px68          1 QIIRGETKEDNDTKSMTMTTMRPGSYVTSMDEKSSLCFEDIKTLWYICRTTYHLYRTLKD
px81          1 QIIRGETKENKDTNSMTMTTR-PGSYVISMDEKSSLCFLDPRTLWYICKITYRLFRTLKD
px35          1 ------IEDVSNTRSPTMMG---ANRKIIGDNKNLLCYL--KALEYCCERTKQCYDDIKK
pxa8          1 HIISGETEEVSNTGSPTVMVTMGANRKIIEDNKNLLCYL--RALEYCCARTRQCYDDIKK
BETL2         1 RTTSGQTKEDSNARNMTMTKTRASGNILVSRNDDGPCYLDSGLNEYVCRKTNKCYKSLVL
consensus     1  ii getke sntksmTmt    g  vii dnk  lCyld k leYiCrrT hcyrtlk
                                                   -          -

px68         61 CLSHCNSM-
px81         60 CLEFCHSI-
px35         50 CLEHCHS--
pxa8         59 CLEHCRG--
BETL2        61 CVASCQPSS
consensus    61 ClehChs
                -   -
```

Fig 15.- Alignment at amino acid level of the putative signal peptides (a) and propeptides (b)

Analysis performed by CLUSTALW program (Thompson *et al* 1994a,b). Black shaded letters indicates identical amino acids and gray shaded letters indicates homologous amino acids.

Arrows indicate the position of conserved cysteines.

The analysis of the alignments performed with the protein family defined the existence of two protein motifs, one located in the signal peptide region and the other in the carboxyl terminal region where the cysteines are present. (fig 15, table 7).

Region	Motif
Signal peptide	MxKxx-xxxxxxxxLxMxLxASxxxHA
Carboxyl terminal region	Cxxx-x-xxYcCxxTxxcyxxxxCxxxC

Table 7.- Predicted motifs from the alignment of the new protein family

The BETL2 class of peptides share some properties with plant antifungal proteins such as defensins and thionins, including their extracellular location, site of expression and possession of a few conserved cysteine residues in the C-terminal region. Therefore, the possibility that BETL2 might possess antifungal activity was tested by incubation with a variety of filamentous fungi strains like *Fusarium* and *Neurospora,* showing that BETL2 possesses strong antipathogenic activity (Serna *et al.,* in press; appendix). Based on the biological activities described for *BETL2*, and its restricted localisation of the expression the new family of proteins defined by *BETL2* and the new sequences presented here will be termed *BAP* for Basal layer type Antifungal Proteins. The family was divided in three groups based on the sequence homology observed from alignments and further analysis presented here (fig 15 and fig 16), thus the corresponding proteins of the family were termed *bap*1, *bap*2 and *bap*3 (see summary on table 8).

Clone working name	Locus terminology
Pxa8	*Bap*1-a
Px35	*Bap*1-b
BETL2	*Bap*2
Px68	*Bap*3-a
Px81	*Bap*3-b

Table 8.- Nomenclature adopted for the new family of proteins.

The existence of three different groups inside the family was shown using a dot blot experiment (fig 16). Four different filters were prepared by blotting 10 ng of plasmid inserts of the genes to be tested.

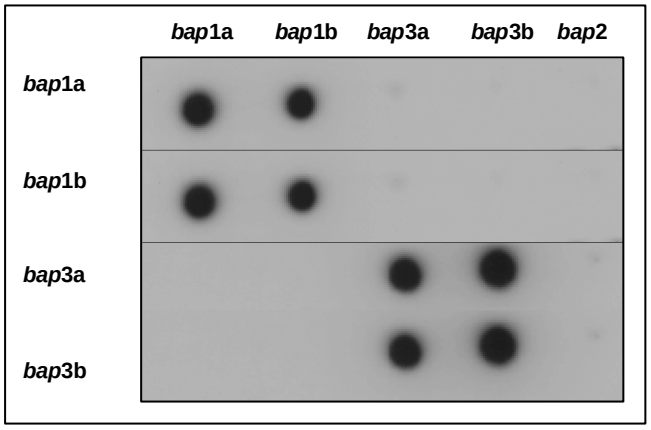


Fig 16.- Cross-hybridisation assay of all members of the BAP family

10 ng of plasmid insert (indicated at the top of the picture) were spotted onto four different nylon filters. The resulting Southern blots were hybridised with the radioactive labelled DNA probes from the BAP members indicated in the left side of the picture

The result confirmed the existence of three different groups inside the family. The first group is composed of the *bap*1 genes (*bap*1a and *bap*1b), the second group of *bap*2 (previously termed *BETL*2) and the third group of *bap*3 genes (*bap*3a and *bap*3b) (table 8).

3.4.2.- Expression analysis of the sequences

The previously termed BETL2 gene (*bap*2) is a gene specifically expressed at the transfer-cell layer with a characteristic time course of expression during endosperm development (Hueros *et al.,* 1999). The spatial and temporal localisation of transcripts was analysed for the new BAP genes. The first studies were performed through Northern blot experiments, first localising the expression of the genes in different maize tissues in order to confirm endosperm specificity (fig 17) and next detecting the transcripts on RNA isolated from different endosperm regions (figs 18,19). The cell-type specificity was definitively confirmed by *in situ* hybridisation experiments (fig 20).

When messenger RNA isolated from different tissues is fractionated on a formaldehyde denaturing gel, blotted and the corresponding Northern blot is hybridised with probes from each gene, the signal is exclusively detected in the endosperm mRNA (fig 17).

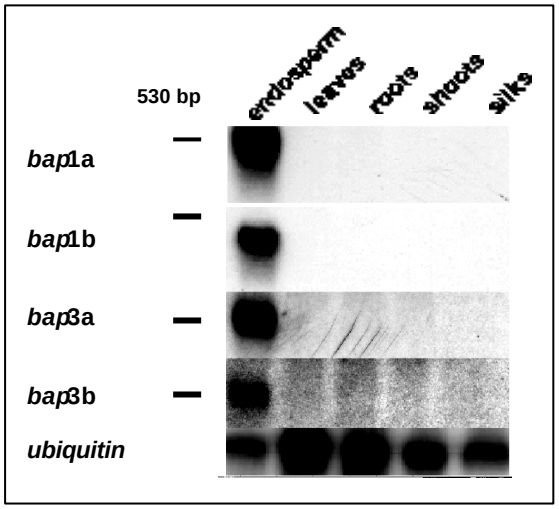


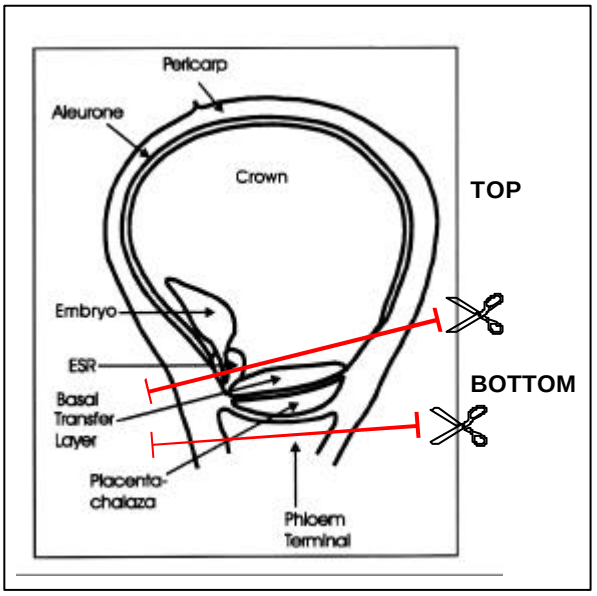
Fig 17.- Site of expression of the BAP family in *Z.mays* line A69Y+

Messenger RNA was isolated from the tissues indicated at the top of the figure. 2µg were fractionated in a denaturing formaldehyde gel, blotted onto a nylon filter and the resulting Northern blot sequentially hybridized with the radioactive DNA probes from the genes indicated in the left border of the picture.

For the localisation of the region of the endosperm where the transcripts were being deposited, the kernels were dissected into two halves following the strategy employed for the screening: the *top* half of the endosperm that mainly contains the crown of the endosperm, aleurone, pericarp and the most upper part of the embryo; and the *bottom* part of the kernel where is mainly present the transfer-cell layer and contaminating parts of the most basal region of the embryo, part of the aleurone and basal endosperm. In the preparation the pedicel is discarded (fig 18). After dissection messenger RNA is isolated and

Fig 18.- Scheme of a longitudinal section from a maize kernel.

The picture shows the parts obtained after kernel-dissection from which mRNA is isolated for further experiments



(Taken from Slocombe et al., *1999)*

fractionated on a denaturing agarose gel, blotted and the resulting Northern blot hybridised with a probe from each gene (fig 19).

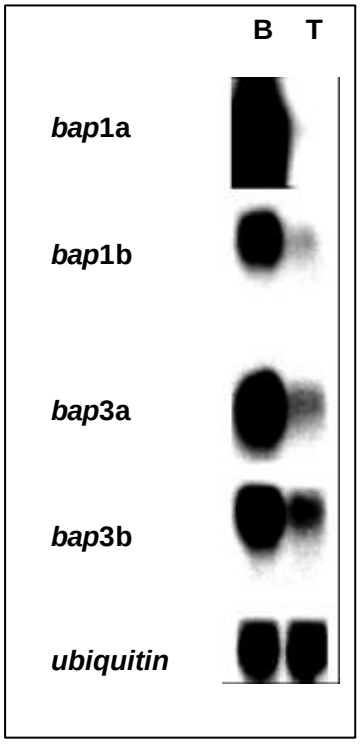


Fig 19.- Northern blots of endosperm halves.
mRNA from endosperm of *Z.mays* line A69Y+ kernel-halves was isolated, and 2µg were fractionated in a formaldehyde denaturing gel, blotted onto a nylon filter and the resulting Northern blot hybridized with probes from each member of the BAP family.
B: mRNA from the bottom half; **T:** mRNA from the Top half

The signal in the four cases tested was mainly detected in the mRNA prepared from the *bottom* half of the endosperm. There was also some signal to be detected in the *top* part in three cases: in the case of *bap*1b, *bap*3a and *bap*3b. The reason why signal was detected in TOP mRNA when these probes are used could be due either to cross-hybridisation with distantly related sequences that could be present in other cell-types of the endosperm, or simply due to the relative unspecific methodology employed in the dissection of the tissues.

In order to confirm the cell-type specificity and rule out possible wrong interpretations from the Northern experiments, *in situ* hybridisation experiments were performed (fig 20). Maize kernels from 12 days after pollination were embedded in paraffin and 10µm sections cut with a microtome. The hybridisation was performed using *in vitro* transcribed

digoxigenin-labelled RNA probes from one gene of each group either in sense or antisense orientation.

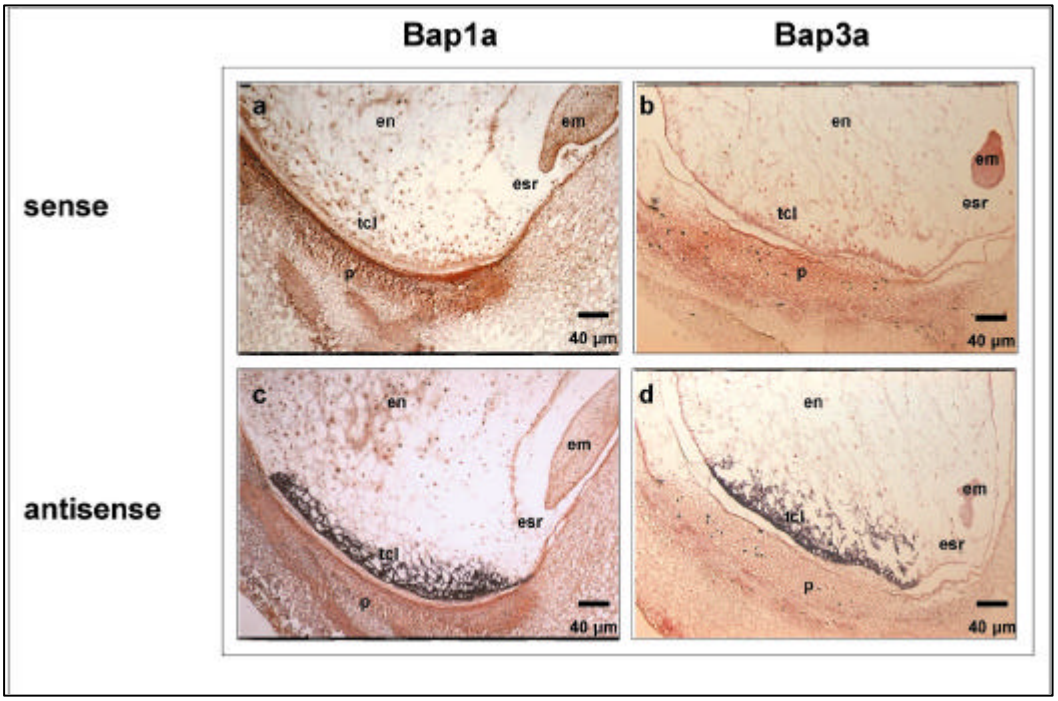


Fig 20.- Cell specificity of the BAP family

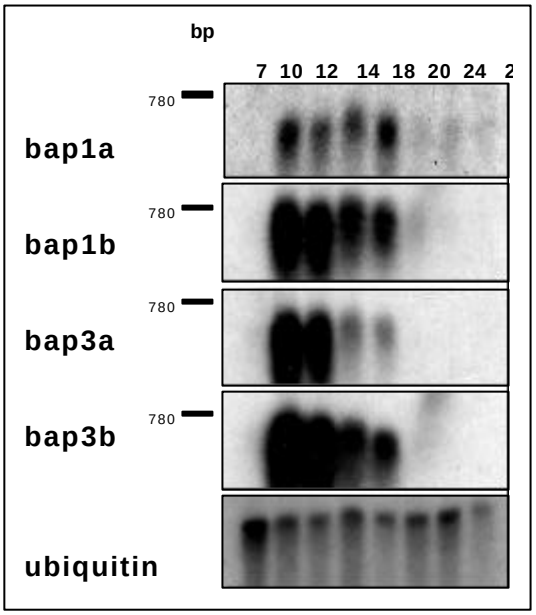
Longitudinal sections of 12 DAP endosperm from *Z.mays* line A69Y+ were probed with the *in vitro* digoxigenin labelled RNA probes transcribed either in sense (a,b) or antisense orientation (c,d). The probes were obtained from *bap*1a (a,c) and *bap*3a (b,d). Pictures **a and c** were kindly provided by Dr Geraldina Santandrea. Black bars in the lower right border equal 40 µm. The symbols, Esr: embryo surrounding region; Em: embryo; En: endosperm; TCL: transfer cell layer; P: pedicel.

The *in situ* hybridisation experiments performed with one member of every group demonstrated that the transcripts of the *BAP* genes are present only in the cells of the transfer cell layer. No signal differences in first cell layers, as seen for BETL-1 (Hueros *et al.,* 1995) were observed between the antisense probes employed in the experiments apart from weak changes in the intensities of hybridisation most probably due to the quality of the probe rather than to differences in the levels of the expression. It should be noticed that the transcript accumulation of the transcripts under study seems to be relatively

higher in the most peripheral cells. The sense probes did not give a detectable signal.

When filters containing messenger RNA from kernels at different stages of endosperm development were hybridised with bap1 and bap3 cDNA probes, the transcripts (fig 21) were seen to be co-ordinately expressed, appearing at 10 DAP and declining by 18 DAP. This expression profile is similar to that seen for BAP2 (BETL2)(Hueros *et al.,* 1999).

Fig 21.- Time course of expression of the genes in study.
2 µg of mRNA from seeds of at different stages of endosperm development from the wild-type line A69Y+ was fractionated in a denaturing gel and blotted onto a nylon filter. The filter was sequentially hybridized with the radioactively labeled probes.



The expression of the different sequences takes place between 8 and 18 days after pollination. The transcripts are detected from 10 DAP and from here onwards, decrease, being present up to 18 DAP. The mRNA abundance defined two patterns of accumulation during endosperm development. Thus, the transcripts corresponding to the *bap*1 group presented a more homogeneous distribution expression profile, whereas the transcripts of the *bap*3 group exhibited a clear reduction after 12 DAP. In earlier stages of endosperm development there appeared to be no significant differences in the mRNA amounts. The relative differences in intensity observed here between the blots for each gene are most probably due to the quality of the probes employed in the experiment, rather than real differences in mRNA amounts.

In order to quantify the differences observed after 12 days after pollination between the three groups of genes members of the *BAP* family, Northern blot experiments were performed and the signal intensity quantified using a STORM 840 Phosphoimager® and ImageQuant® software (Molecular Dynamics). For the experiment different quantities of messenger RNA were fractionated in a denaturing formaldehyde agarose gel, blotted into nylon filters and the corresponding Northern blots hybridised with radioactive labelled probes from one gene of each group (fig 22).

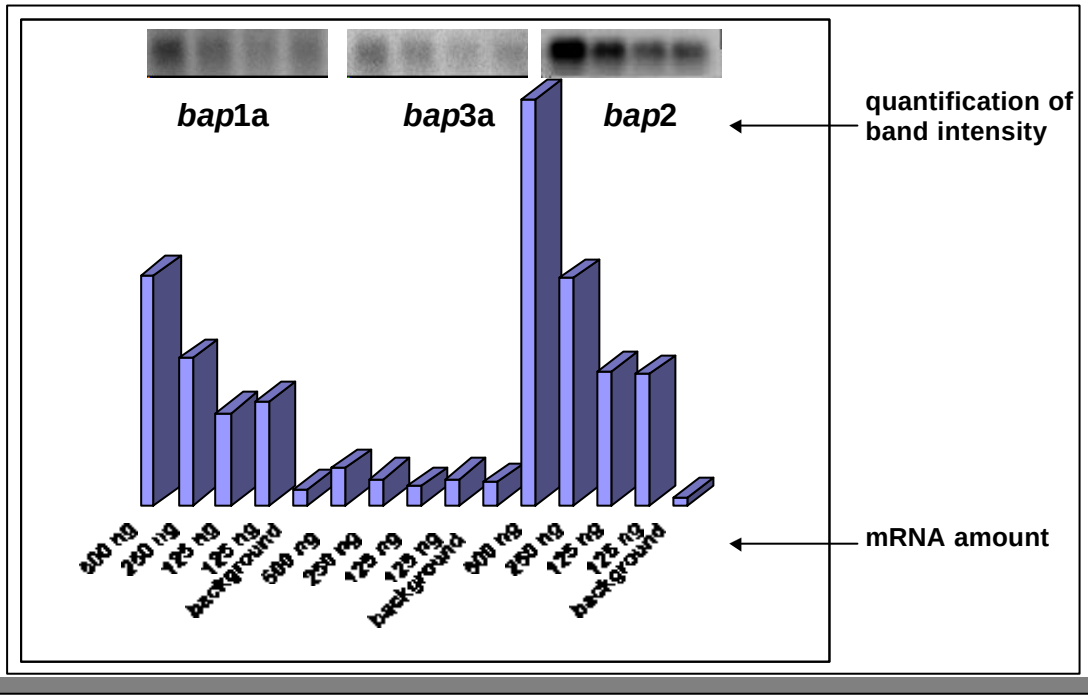


Figure 22.- Levels of expression of the different group sequences 15 days after pollination.

Different amounts of endosperm mRNA from the maize line A69Y+ were fractionated on a denaturing formaldehyde gel, blotted onto a nylon filter, hybridized with the correspondent probes and quantified using a phospho-imager system.

As was observed in previous experiments, the expression of the transcripts corresponding to the *bap*3 proteins are dramatically reduced in later stages of endosperm development being 10% of the expression observed for *bap*2, the gene of this family with the highest level of expression at this point of endosperm development. The expression of genes of the *bap*1 group is also lower than for *bap*2, corresponding to 60% of the mRNA abundance observed for *bap*2, whose mRNA amounts are the highest

observed of the genes specifically expressed in the transfer-cell layer currently isolated (Hueros *et al.,* 1999).

3.4.3.- Genomic distribution of the sequences

Genomic DNA from the maize line A69Y was digested with different endonucleases, fractionated in an agarose gel, blotted and the resulting Southern Blot hybridised with the corresponding radioactive labelled probes. The results indicate that the genes here presented are organised in the maize genome as a low copy gene family (fig 23). The existence of two bands with *BamH*I and *EcoR*I, both enzymes that do not cut within the sequences, indicates a minimum of two genes are present for each probe. The upper bands in Bap1a/*Bam*HI and the *Xba*I digest are due to partial digestion.

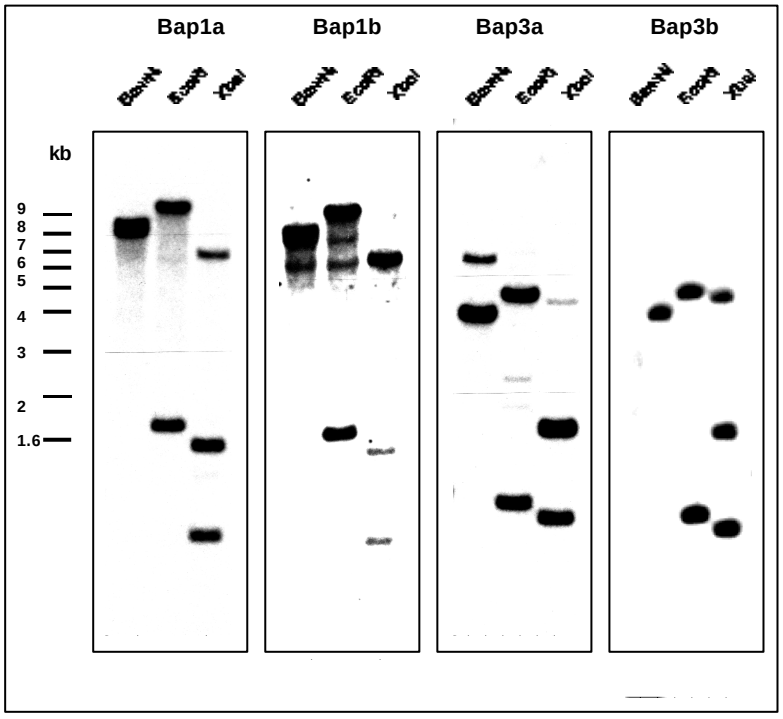


Fig 23.- Southern blot analysis of the *BAP* family

10µg of genomic DNA from the wild-type line A69Y+ were digested over-night with *Hind*III (H), *EcoR*I (E) and *Xba*I (X) then next fractionated in an agarose gel (0.8 %). The DNA was blotted onto a nylon filter and probed with the radioactively labeled correspondent probe. As predicted, *bap*1a and *bap*1b gave the same pattern; in the same way for *bap*3a-b

In the case of the *bap*1 group of genes the hybridisation gave one single band in the region of high molecular weight (between 7 and 8 kb) when the DNA was digested with *Bam*HI, two bands of 1.6 and 9 kb for the DNA digested with *Eco*RI and three bands for the digestion with *Xba*I (cuts in both genes of the *bap*1 group). The fact of having a single band in the case of the *BamH*I enzyme, suggest the proximity of both loci in the maize genome, although no predictions can be precisely done without employing double digestions. The investigation on the genomic organisation of the *bap*3 group gave, surprisingly, a quite similar hybridisation pattern in spite of the non-cross-hybridisation of both groups. This could suggest a similar organisation of both genomic regions.

The investigation of the genomic distribution of the BAP family in different species could contribute interesting data about the evolution of the family. In order to proceed with this experiment, the sequences related to the *bap* family were studied in different species of *Gramineae* (fig 24).

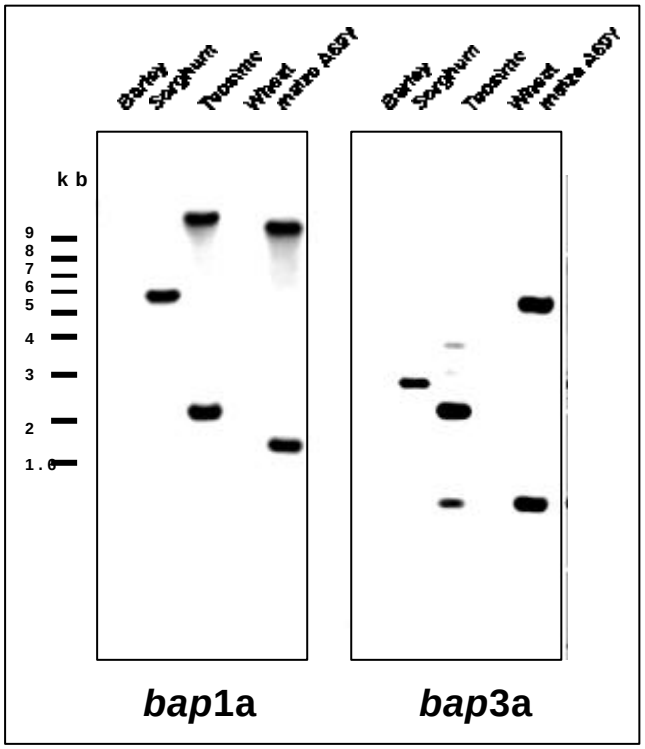


Fig 24.- Genomic distribution of members of the *bap* family in different species.

10µg of genomic DNA proceeding from the species at the top indicated, were digested overnight with *Eco*RI, fractionated in a 0.8% agarose gel, blotted onto a nylon filter and the resulting Southern blot hybridized with the radioactively labeled DNA probes from members of the groups Bap1 and Bap3

The investigation of the genomic distribution of the members of the *Bap* family on different species suggested a similar genomic organisation for both group of genes. Thus, when genomic DNA from different species (fig 21) was digested with *EcoR*I, fractionated on an agarose gel, blotted onto nylon filters and the resulting Southern blots hybridised with probes prepared from the BAP genes, signal was only detected in Sorghum (*Sorghum bicolour*), Teosinte (*Zea luxurians*) and *Zea mays* (line A69Y) for both sequence groups, whereas no detectable signal was observed in DNA from barley or wheat. In the case of *bap*1a a single clear hybridisation band in the region of 6 kb appeared on DNA proceeding from Sorghum; a 2 kb band and a 9 Kb band hybridised in Teosinte DNA; when the *bap*3a gene was used as a probe to hybridise the same Southern blots, also a single band appeared in Sorghum DNA in the region of intermediate molecular-weight (4kb) and two bands in Teosinte DNA.

3.4.4.- Mapping of the BAP family

For genetic mapping Recombinant inbred lines from the cross Co159xTx303 were used (Burr B. *et al.,* 1988). In order to screen for polymorphic bands, genomic DNA from the parents Co159 and Tx303 was extracted. 10µg of each parent was independently digested by *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III, the digested DNA was fractionated in an agarose gel, blotted and the resulting Southern blots hybridised with probes originating from one member of the groups BAP1 and BAP3 of the BAP family (fig 25). Polymorphisms were obtained with all the four enzymes employed in the experiments. In both cases the enzyme of choice for the digestion of the recombinant inbred-line was *Eco*RI which for the *bap*1a gene gave two polymorphic bands, one band of about 4 kb (in Co159) and a second band (in Tx303) close to 3 kb. Other bands were detected in the region of high molecular weight probably due to the cross-hybridisation with non-digested DNA. When the filters were hybridised with the probe originating from *bap*3a one band of about 4 kb is present in the Co159 parent and one of about 9kb in Tx303. A band of about 5 kb common to both parents was also detected.

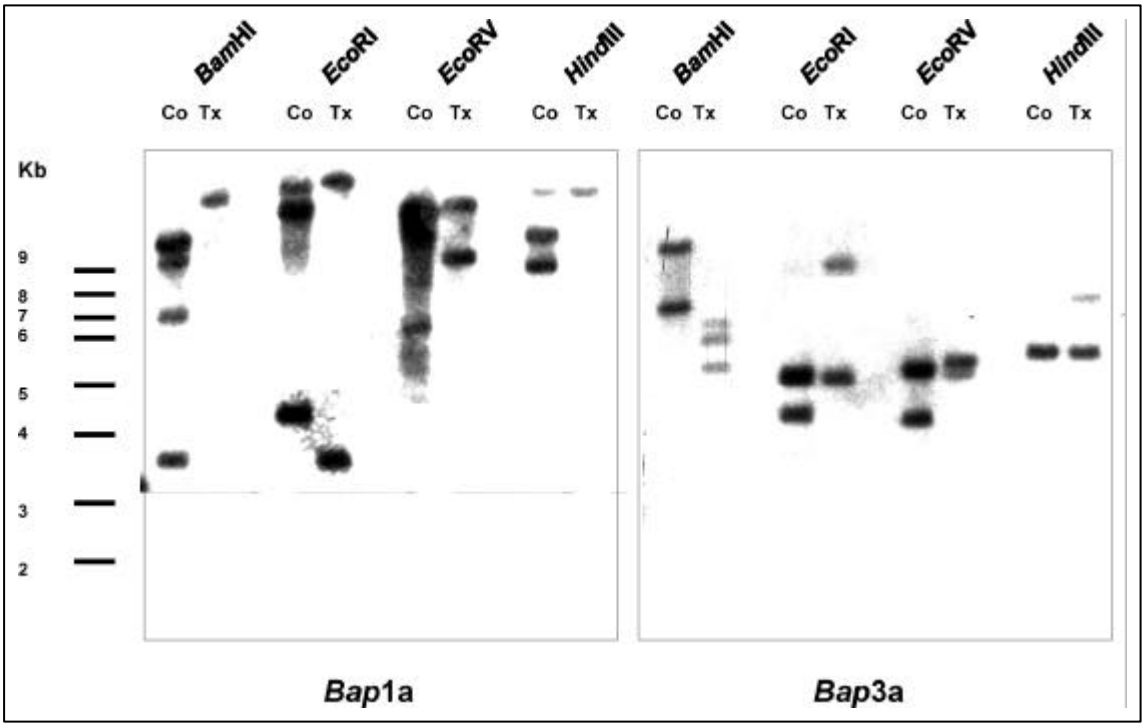
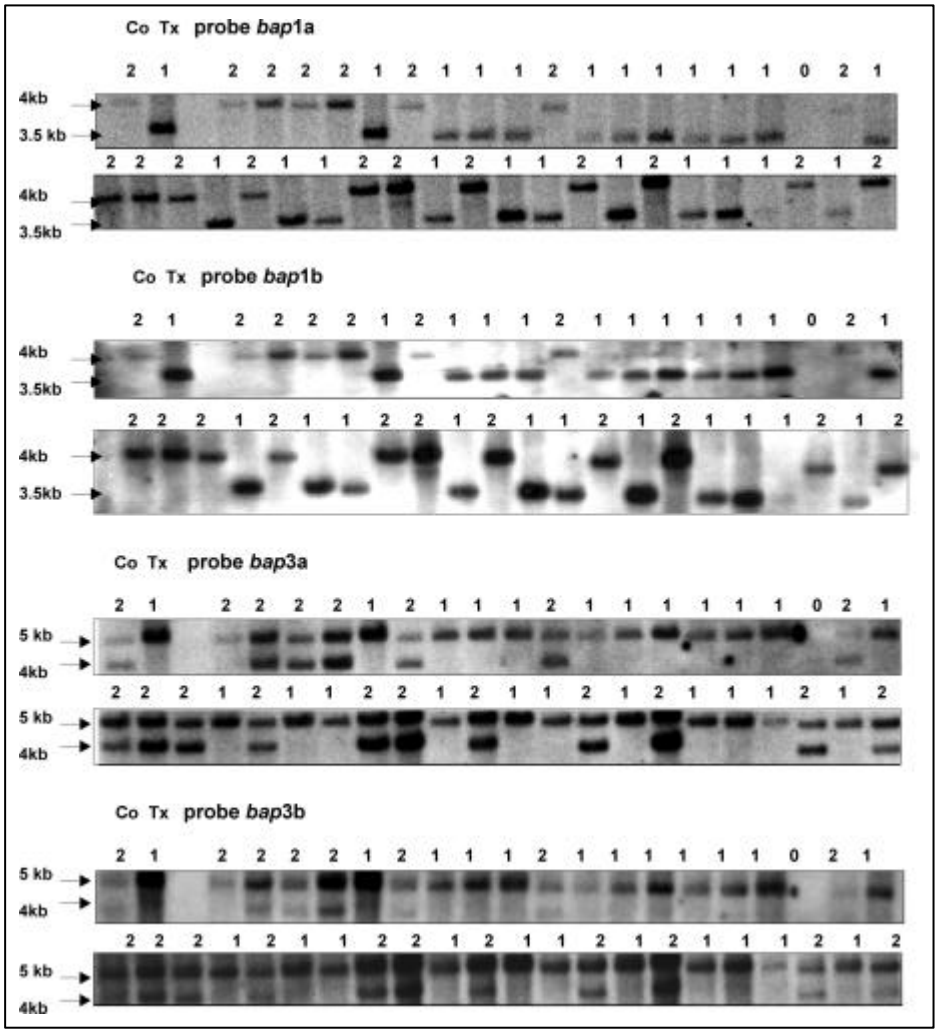


Fig 25.- Southern blot analysis of the Co159 and Tx303 parents probed with *bap*1a and *bap*3a

10µg of genomic DNA from the Co159 and Tx303 parent inbreeds, were digested overnight with the enzymes indicated at the top, fractionated in a 0.8% agarose gel, blotted onto a nylon filter and the resulting Southern blot hybridized with the radioactively labeled DNA probes from one member of each group of the BAP family.

Genomic DNA was isolated from 41 individual recombinant inbred-lines and together with the parental DNA was digested over-night with *Eco*RI, fractionated in a 0.8 % agarose gel, blotted onto a nylon filter and the resulting Southern blot hybridised sequentially with the different probes originated from the genes in study. The results are showed in figure 26 together with the allele scoring, such that Tx303 alleles receive a value of 1 and the corresponding alleles from Co159 a value of 2. The analysis of the mapping population revealed that the *bap*1 and *bap*3 members co-segregate on chromosome 10L (a summary of the results is presented in table 9; the data were analysed by Prof. B. Burr, Brookhaven National Laboratory, New York).

A



B

Score: 22221211121111110212221211221211212111212

Fig 26.- Mapping results of the members of the BAP family.

10µg of genomic DNA were digested overnight with *Eco*RI, fractionated on a 0.8% agarose gel, blotted onto a nylon filter and the corresponding Southern blot hybridized with the radioactively labeled DNA probe from every member of the family. The probes used and parents as well as the scores are indicated at the above the lanes**.**

Locus unit	locus	map	m	diff	R
0.1094	Umc152	10S 48.6	39	7.0	0.1795
0.0556	Npi105A	10S 51.8	40	4.0	0.1000
0.0417	Php06005	10L 53	39	3.0	0.0769
0.0135	Npi445	10L 54.2	38	1.0	0.0263
0.0278	Umc155	10L 55.4	38	2.0	0.0526
0.0270	Ncsu2	10L 57.2	39	2.0	0.0513
0.0862	Npi305B	10L 69.5	34	5.0	0.1471
0.0405	Npi264	10L 72.7	40	3.0	0.0750
0.088	Npi563	10L 83.7	38	4.0	0.1053
0.0968	Npi232A	10L 86.4	37	6.0	0.1622

Table 9.- Scoring results of the *bap*1 and *bap*3 probes.

M: number of non-zero comparisons
Diff: Total of absolute differences between allele distributions
R: Recombinant fraction
Unit: Estimated distance in Morgans

3.5.- Protein analysis: *protein expression and in vitro studies*

3.5.1.- Purification of the *E.coli* expressed recombinant protein

A method has been described for testing in vitro the anti-microbial activity of proteins with putative roles in the prevention of pathogen ingress in plants (Broekaert *et al.,* 1990; Terras *et al.,* 1992). The method is a bioassay in which the effect of added protein on hyphal growth rate is followed spectrophotometrically. In this thesis the experiments were performed with recombinant proteins expressed in *E.coli* (Harrison SJ *et al.,* 1999).

In order to test the putative anti-pathogenic activity of members of the bap family the cDNA of one member of each group was cloned into the pQE60 vector from QIAGEN® as a carboxyl terminal poly-histidine fusion. The construct was used for the transformation of the *M15 E.coli* cells. South-western technique was employed to detect colonies that could express the

recombinant proteins (data not shown). Protein expression was only detected for the proteins of the *bap*1 group, so one member of this group, *bap*1 b, was chosen for further experiments.

Small-scale cultures were used to test the protein solubility. The protein could be efficiently extracted only under denaturing conditions, strongly suggesting that the expressed protein is in inclusion bodies.

For the large-scale purification of the protein, 3 litres of LB medium containing the clone expressing the protein were induced with IPTG (1mM) during 1h and the cell-pellet obtained was resuspended in extraction buffer (8M urea, 100mM NaH_2PO_4 and 10mM Tris pH 8, 5ml/g of cells). The protein was bound to a nickel-agarose matrix (Janknecht, R. *et al.,* 1991) and the washes were performed in the same buffer as the binding but at pH 6.3. The elution was done in the presence of 100mM EDTA (Fig 27, b).

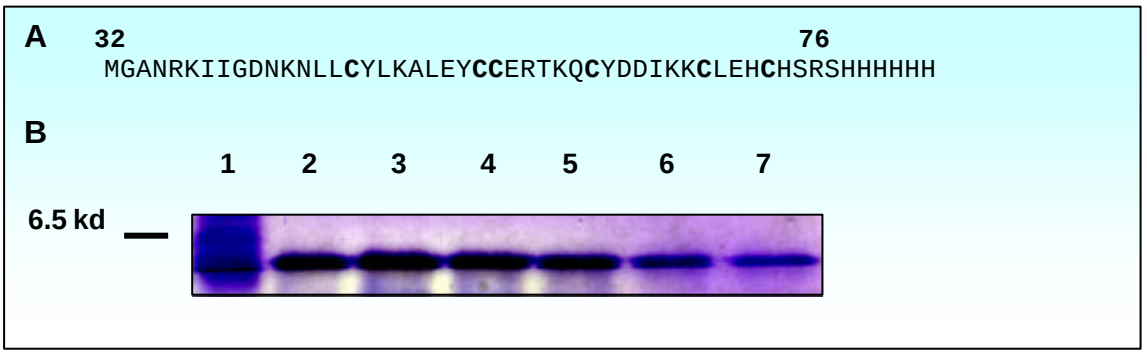


Fig 27.- Purification of *bap*1b recombinant protein from *E.coli*

a.- Sequence of the protein purified from *E.coli,* in bold are indicated the cysteines

b.- Stepwise elution of the recombinant protein from a nickel-agarose column; 1:unbound proteins, 2 to 7 fractions collected during the elution in 8M urea, 100mM NaH_2PO_4, 10mM Tris pH 6.3, 100mM EDTA (the amounts loaded in the gel correspond to a 1/50 of the total. The protein is eluted from the column with 6 ml of elution buffer (see material and methods) and alicuots of 1 ml are collected).

3.5.2.- Processing of the histidine-tagged proteins

3.5.2.1.- Protein refolding

The first step necessary prior to proceeding with further modification steps is the refolding of the protein. As has been explained before, the protein was produced in *E.coli* where it was accumulated in inclusion bodies. For that reason denaturing conditions were used for its isolation. The urea-eluted protein is denatured, and needs to renature before use in the assays. The refolding of the protein was done in benzoylated dialysis tubing from SIGMA. Benzoylated dialysis tubing has smaller pore size with a Mr cut off of 1.2 kd than conventional tubing (cut off Mr is 12-15 kd), thus allowing the efficient dialysis of the *bap*1b (Mr of 8.6 kd). The process was performed first at half stringency, which corresponds to 4M urea concentration to encourage refolding. Dialysis was performed against 4 M urea, 0.1 M NaH_2PO_4 and 10 mM Tris for 12 hours and repeating the process twice; the next dialysis step was performed against 0.1 M NaH_2PO_4 and 10 mM Tris, again 12 hours twice in order to remove urea present; and the final dialysis step was done against ultra-pure water for 12 hours. This step was also repeated twice (see scheme fig 28). No reducing agents were employed in the protein refolding in order to not to disrupt the disulphide bridge formation. After dialysis the protein was freeze-dried, resuspended in 1 ml of ultra pure water and quantified using the BCA method (P.K Smith, *et al.,* 1985). The protein was stored in aliquots of 0.5 mg/ml for further experiments.

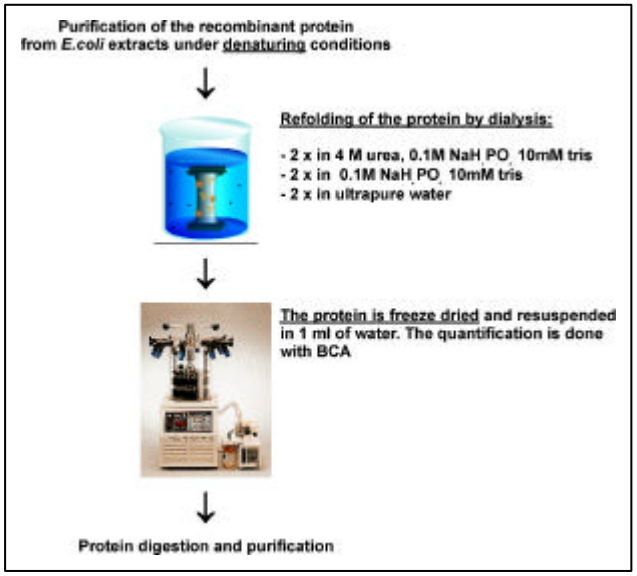


Fig 28.- Scheme of the process of protein refolding

3.5.2.2.- Protein digestion

A characteristic of the BAP group of proteins is that they are synthesised as immature forms and processed later. *bap*2 (previously termed *betl*2) for example, can be detected in endosperm extracts as two different products (Maitz *et al.,* 2000). The largest product of about 8 kd would correspond to the immature protein product and the shortest of about 4 kd, to the mature peptide, which is proposed to be the active form of the protein. Other anti-pathogenic proteins have been described to be processed in a similar manner, by cleavage of the pro-peptide region (Yount *et al.,* 1999; Tao *et al.,* 1990; Romero *et al.,* 1997). The process of maturation could be carried out by a protease or a protease complex that recognizes the specific sequence present at the pro-peptide / mature-peptide junction (table 10).

Sequence	Signal peptide predicted cleavage site	Pro-peptide/mature peptide junction	Size of pro-peptide
Bap1a	28 and 29: IHA-HI	Predicted from alignment with BAP2 **ED-NKN**	30
Bap1b	19 and 20: ISG-EI	Predicted from alignment with BAP2 **GD-NKN**	22
Bap2	26 and 27 HA-RTT	Predicted by sequencing of peptides (Serna et al., appendix) **SR-NDD**	31
Bap3a	28 and 29: GHA-QI	Non-predicted	
Bap3b	28 and 29: GHA-QI	Non-predicted	

Table 10.- Sequences predicted to be the region of cleavage in the BAP family

The next step in the task of protein processing would be the digestion of the protein at the specific site, and purification of the active peptide for future analysis. To proceed with the analysis, a method of digestion was developed based on the use of crude endosperm extracts (originally done by Monika Maitz). As we did not know which protease or proteases are

responsible for the maturation of this family group, a protein extract from 15 days after pollination endosperm was size-fractionated on a Superose-12 column in order to obtain a non-denaturing fractionation of total endosperm proteins (in: 20 mM MES pH 6.0, 1mM EDTA) by Fast Protein Liquid Chromatography (FPLC). Aliquots of 1 ml were collected every 4 min. at a flow of 0.25 ml / min. 20 µl of the fractions so obtained were incubated in the presence of 1 µg of the recombinant protein, 13 µl of 1M potassium phosphate pH 7.0, 1mg/ml BSA and 1 µl of 0.25 M EDTA pH 8.0, over-night at 37°C. After incubation, the samples were fractionated in a 15 % acrylamide-tricine gel (Schägger and Jagow, 1987). The gel was electro-blotted to Immobilon-PVDF membranes (Millipore) and the resulting Western blot incubated with the anti-histidine tag antibodies (fig 29).

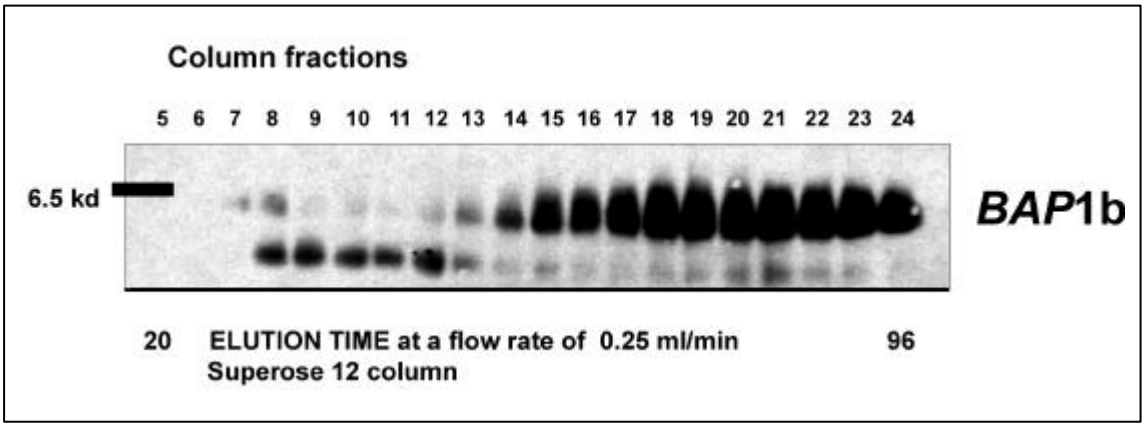


Fig 29.- Western blot analysis of *bap*1b recombinant protein digested with different endosperm fractions.

1 µg of *E.coli* expressed BAP1b protein was incubated in the presence of different protein fractions from endosperm. The result was fractionated on a tricine-SDS gel and blotted into an immobilon-PVDF membrane. The resulting Western blot was incubated with anti-histidine tag antibody for quantification of the digestion.

The fractions between 8 and 12 (indicated in the top of figure 29) showed protease activity, therefore one of these fractions was chosen to process the recombinant protein expressed in *E.coli*. Fraction 9 was chosen and concentrated by lyophilisation.

The large-scale digestion mix consisted of: 1ml of the protein (the concentration varies between 0.5 mg/ml and 1mg/ml depending on the

purification yield), 0.2 ml of 1M KH_2PO_4, 48 µl of **Fraction 9** and 1 mM EDTA. However, due to the large amounts of protein processed (up to 1mg), the time of digestion was increased to 96 hours and the process monitored by Western blot (fig 30,31). When the digestion was 90 % complete, the resulting mature protein was purified by size-exclusion chromatography.

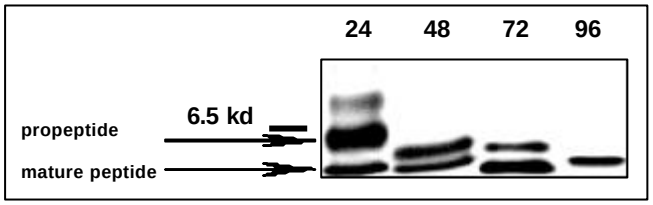


Fig 30.- Composition of the different stages observed during the protein digestion of *bap*1b protein. On the top is indicated incubation time

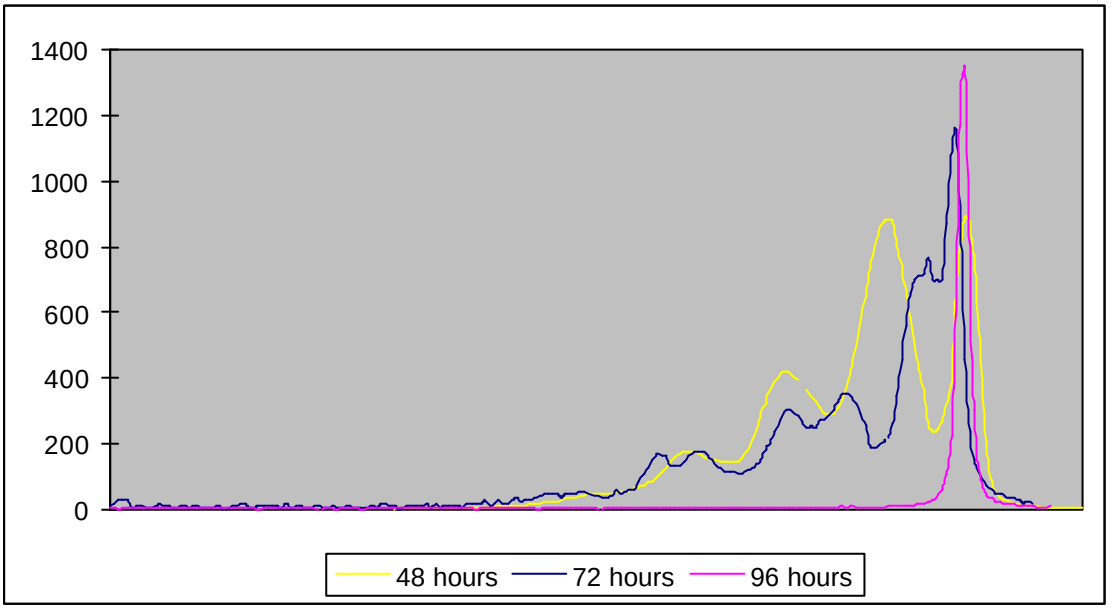


Fig 31.- Example of a quantitative analysis of the process of digestion of the *bap*1b protein.

The analysis was performed using the Lumi Analyst 3.0 from Biorad.

After size fractionation a Western blot was performed to detect the protein (fig 32), the fractions containing the protein were freeze-dried and the dried pellet resuspended in a volume such that the final concentration of protein is approximately 0.5 mg/ml.

In order to provide a negative control for further experiments, column fractions corresponding to those where the *BAP*1b mature protein was being eluted were taken from a column run of **the protease extract alone**, (fraction 9) without added *E.coli* BAP1b protein (fig 32).

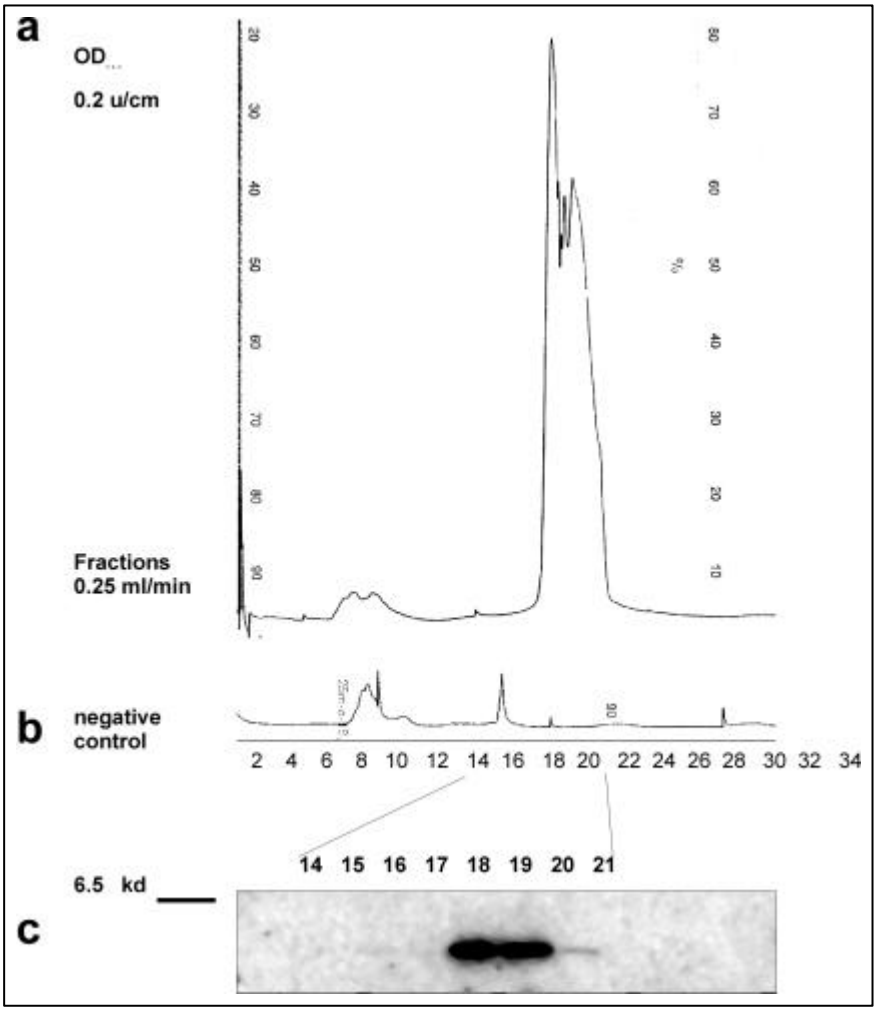


Figure 32.- Scheme of the purification process of the digested *bap*1b protein.

a.- Profile of a run of fraction 9 + BAP1b. Two peaks: the first corresponding to the fraction with protease activity and the second to the digested protein.

b.- Diagram where is shown the methodology to obtain the negative control. Those fractions, 18 and 19, where the protein is being eluted, are collected from a column run where only the protease fraction is loaded.

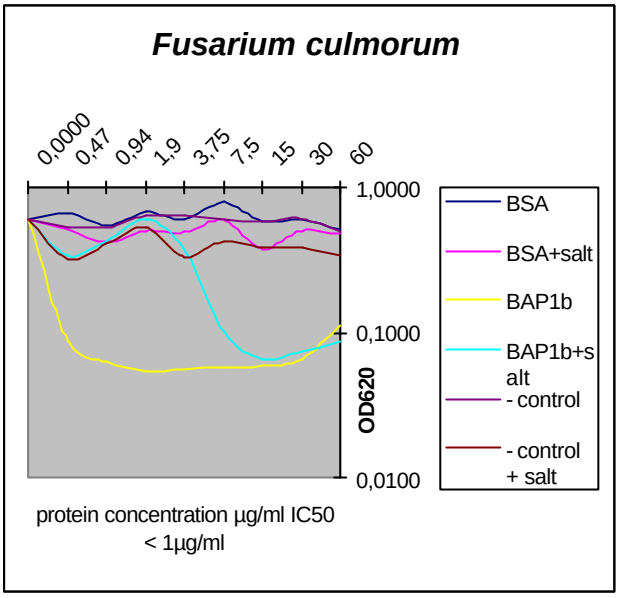
c.- Western blot resulted from the fractionation on a 15% acrylamide gel of the fractions 14 to 21 and blotting to immobilon-PVDF membranes. The filter was probed with anti-HIS antibody.

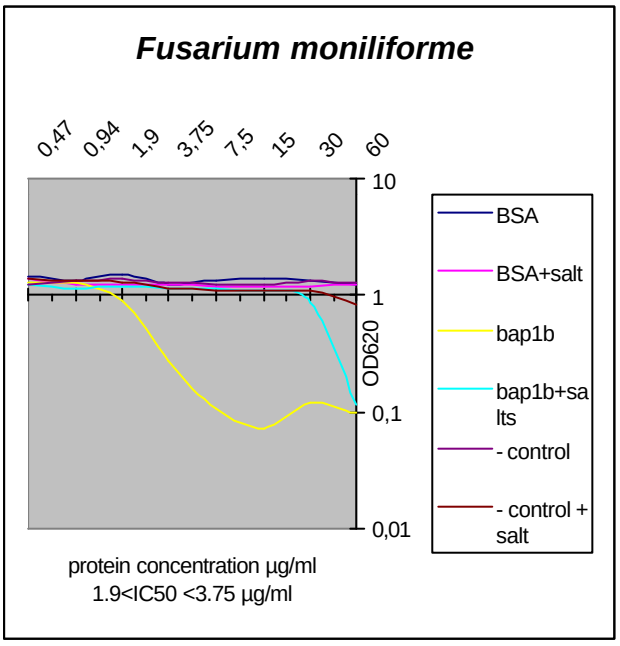
3.5.3 *In vitro* antifungal activity assay

When a protein that exhibits antifungal activity is added to a fungal culture, two main effects can be observed: the first effect is a reduction in fungal growth this is usually represented as the IC_{50}, the amount of protein necessary to reduce growth rate by 50% (Broekaert *et al.,* 1990; Terras *et al.,* 1992; Osborn *et al.,* 1995). The second concomitant effect is a possible modification of hyphal morphology. According to this effect, a classification into *morphogenic* and *non-morphogenic* antifungal proteins has been made (Broekaert *et al.,* 1997). In the first group are those proteins that are able to induce morphological distortions in hyphal elongation. The activity of both classes of antipathogenic proteins has been shown to decrease when the cultures are supplemented with cations, for example K^+ or Ca^{2+} (Terras *et al.,* 1992). The resulting decrease in activity is typically at least two orders of magnitude when incubated with a divalent cation like Ca^{2+}.

Based on these characteristics, different methods have been developed to test *in vitro* the possible antifungal activity of proteins. The method used here was first described by Broekaert *et al.,* (1990) and involves growing the fungi in two types of media: ½PDB and ½PDB supplemented with 1mM $CaCl_2$ and 50 mM KCl. With this method the mature BAP1b was tested. Serial dilutions of the active peptide from 60 µg / ml to 0,45 µg / ml were done in order to determine the IC_{50}. The test was done against three different types of fungi: *Fusarium moniliforme, Fusarium nagamai* and *Fusarium culmorum.* Of these fungi *Fusarium moniliforme* is a maize pathogen that causes the Fusarium Ear Rot, the symptoms are quite characteristic and seldom involve the whole ear. The rot usually occurs at the tip of the ear or in scattered areas. The colour of fungal mycelium around infected kernels varies from white to faint pink to reddish brown. Infected kernels vary in colour from light to dark brown.

The effects on fungal growth of the presence of the protein were determined by measuring the OD_{620} in multi-well plates (fig 33) and by observing under the microscope whether the protein affects the fungal morphology or not (fig 34). A summary of the process is graphically represented in figure 35.





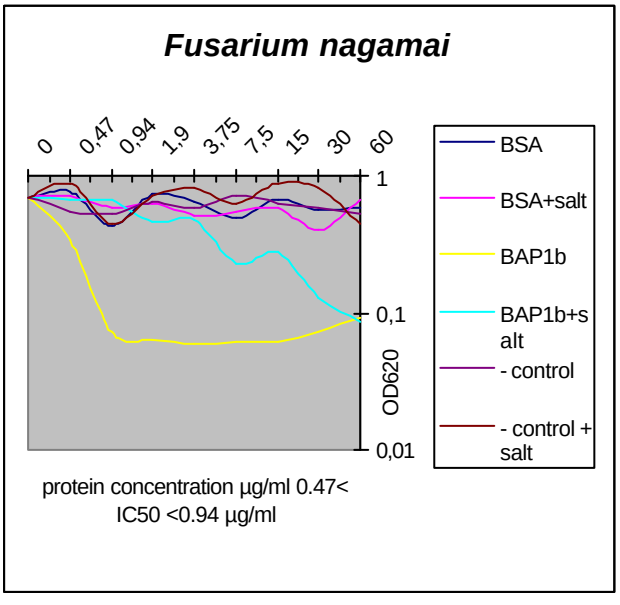


Fig.- 33 Fungal growth curves obtained in the presence of *bap*1b.

Series of dilutions of the proteins from 60 to 0.45 µg/ml, were done and the fungi cultures in PDB medium exposed to these protein concentration in order to determine the IC_{50} values for each of the fungi strains tested.

The fungal strain analysed is indicated at the top of every graph. The amounts of protein necessary to reduce fungal growth to half were calculated in the interval where the OD at 620 nm was half of the optical density obtained from the fungal cultures growing in the absence of the tested protein. The calculated IC_{50} values and the intervals are indicated at the bottom of each graph.

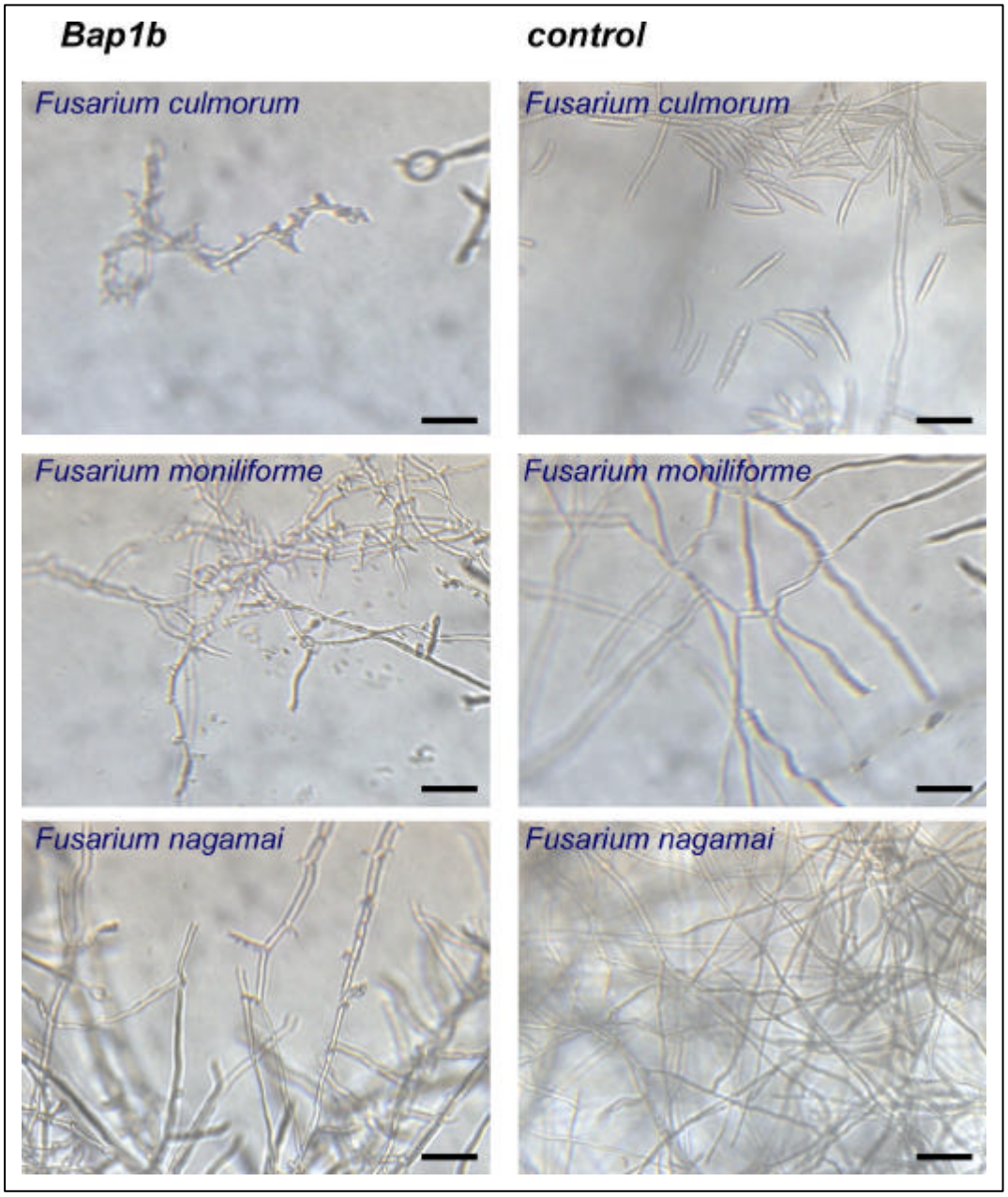


Fig 34.- Effects of *bap*1b protein on fungal morphology

Panels on the right show the growth of fungi grown under normal conditions.

The left panels show the effects caused by the presence of *bap*1b protein on growth of different fungal strains.

The pictures were taken with a digital camera attached to an inverted microscope with ZEISS lens. The bar depicted in the lower-right border of the pictures equals 40 µm.

BAP1b protein was shown to be able to induce morphological changes in *Fusarium culmorum.* When the fungal morphology is observed under the microscope it is possible to distinguish a hyper-branching effect characterised by shortened and swollen hyphae (fig 34). This phenomenon has been described for the antifungal proteins of the morphogenic type, so *bap*1b protein can be considered as morphogenic antifungal protein in the presence of *Fusarium culmorum.* In the case of *Fusarium moniliforme* small distortions in hyphal elongation can be seen (fig 34), although these are not as pronounced as in the case of *Fusarium culmorum*. For *Fusarium nagamai* the mode of action is limited to the reduction of mycelial growth (table 11).

Antifungal activity of *bap*1b			
Fungal strain	**IC_{50} (µg/ml) in medium A**	**IC_{50} (µg/ml) in medium B**	**Mode of action**
Fusarium culmorum	<1	4.62	+++ morphogenic
Fusarium moniliforme	2.5	37.6	+ morphogenic
Fusarium nagamai	<1	6.4	- Non-morphogenic

Table 11.- Summary of the antifungal activity obtained for *bap*1b

The medium A correspond to PDB at half of concentration, the medium B is the same as A but containing 1 mM $CaCl_2$ and 50 mM of KCl. Both media contain also antibiotics to avoid a possible growth of bacteria

The crosses indicate the extent of mycelial growth changes seen. (+++ high, ++ medium, + low, - none).

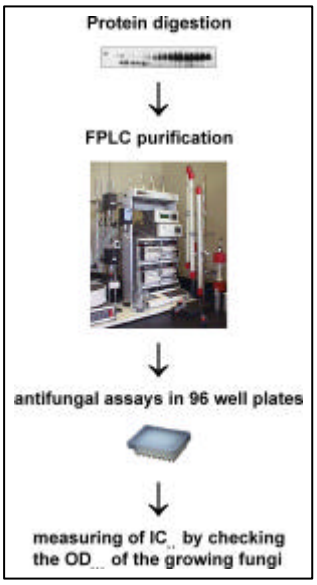


Figure 35.- Summary of the procedure for testing the anti-pathogenic activity of the transfer-cell specific endosperm proteins.

3.5.4.- Detection of Fungal membrane permeabilization based on the uptake of SYTOX green

The ability of the BAP1b protein to permeabilize fungal membranes fungi was tested using SYTOX green. SYTOX is a dye which fluoresces upon interaction with nucleic acids. It emitts fluorescence at 540 nm after excitation at 488 nm (Thevissen *et al.,* 1999). Because a prerequisite for the emission of fluorescence is the interaction with nucleic acids; the method is useful for detecting compromised membranes, which allow the uptake of the dye into the nucleus.

For the test, BAP1b protein at different concentrations was incubated in the presence of *Fusarium culmorum* hyphae in 100µM HEPES pH 6.5. The permeabilisation of SYTOX was assessed 1 hour after the addition of the hyphae (fig 36). The main peak of fluorescence concomitant with fungal membrane permeabilisation is observed at concentrations of BAP1b protein close to 4µg/ml, which is slightly higher than those concentrations calculated for the IC_{50} (1µg/ml), although weak permeabilisation is observed at values similar to the IC_{50}. When the peptide concentrations are increased above 10µg/ml, the fluorescence measured decreased to 50% of the maximum.

Similar effects have been described for other anti-pathogenic proteins (Thevissen *et al.,* 1999).

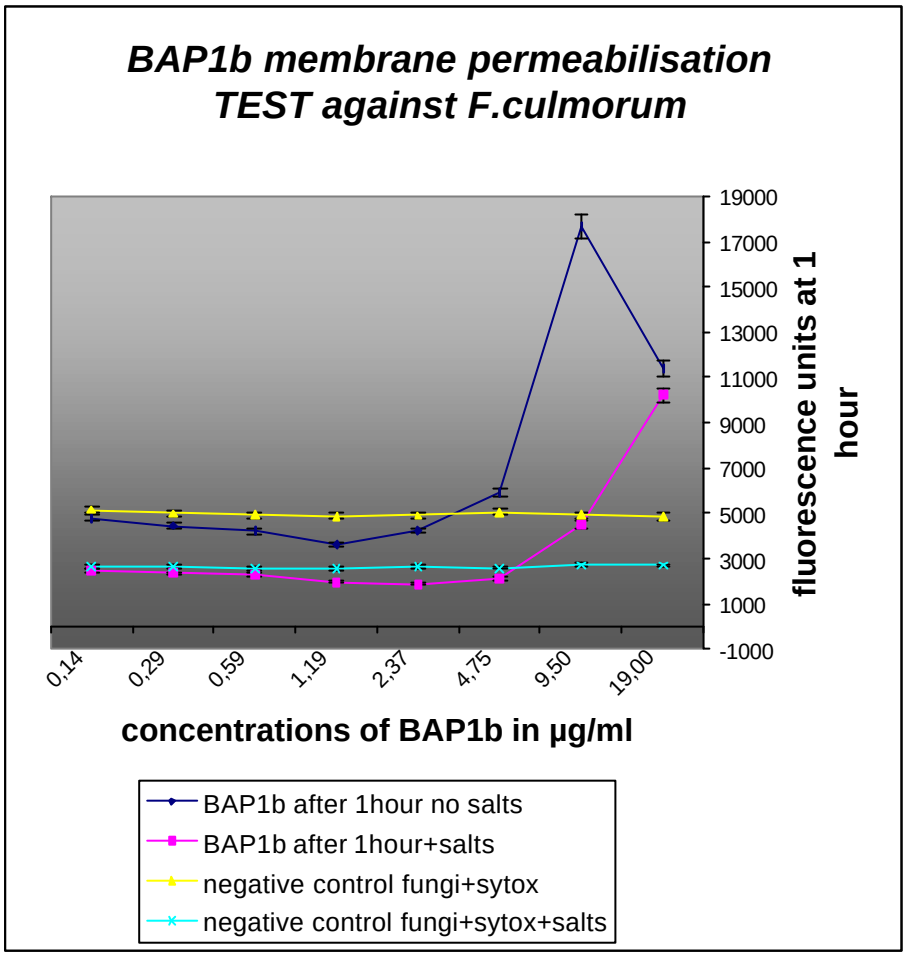
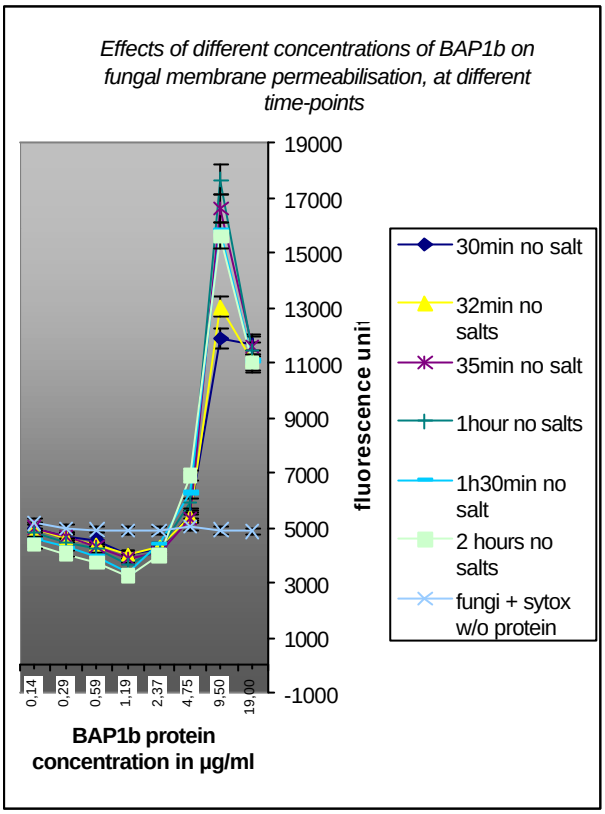


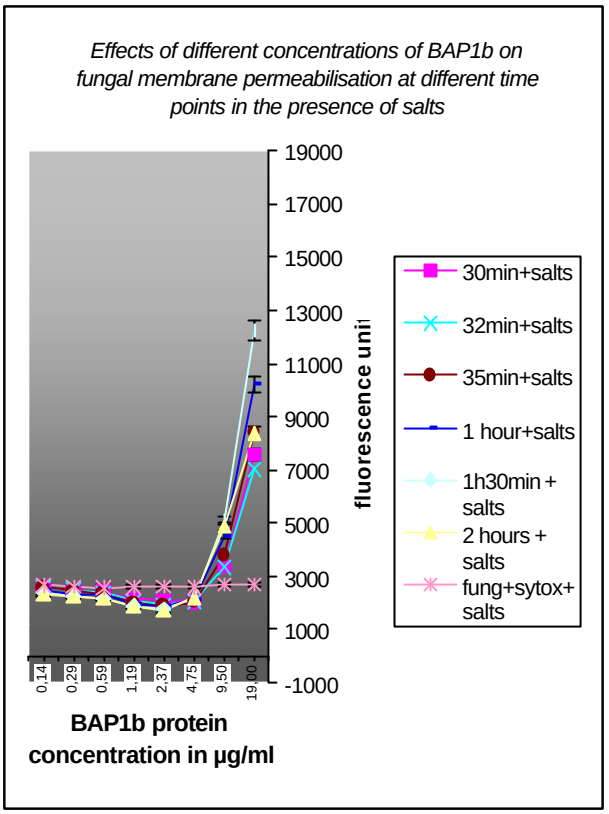
Fig 36.- Dose-response curves of membrane permeabilisation measured by SYTOX green fluorescence induced by BAP1b.

Different amounts of BAP1b protein were incubated with *Fusarium culmorum* hyphae in the presence of SYTOX. Membrane permeabilisation was determined by measuring the fluorescence at 540nm after excitation at 488nm.

When the effect of BAP1b on fungal membrane permeabilisation is investigated at different time points, the maximum effect is reached after 1 hour of incubation of the fungi with the protein (fig 37a and c). 30 minutes after the initiation of the experiment the levels detected are close to the 50% of the maximum observed at 1 hour similar to those levels of fluorescence measured by concentrations over the maximum peak (9µg/ml).



A



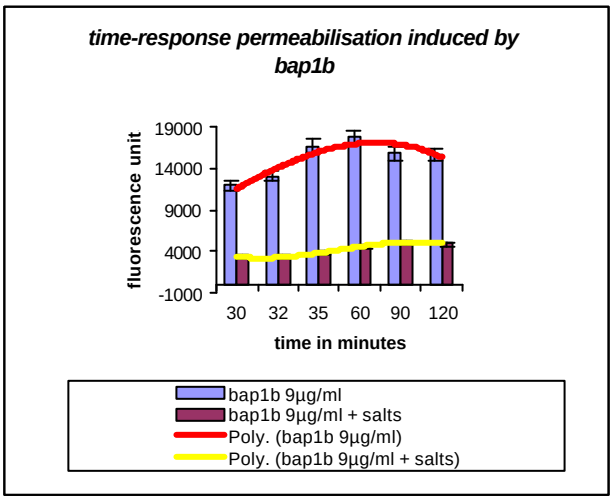
B in the presence of 1mM $CaCl_2$ and 50mM KCl

C

Fig 37.- Kinetics of permeabilisation of *Fusarium culmorum* membranes induced by BAP1b.

Different amounts of BAP1b protein were incubated with *Fusarium culmorum* in the presence of SYTOX, without (A) or with salts (B). The time-response permeabilisation of maximum peak is represented apart (C).

(poly: predicted function).



The fungal cultures of *Fusarium culmorum* incubated in the presence of BAP1b and SYTOX, were observed under the fluorescence microscope (fig 38). Green fluorescence was observed in the cytosol of the compromised cells but especially in the nuclei where the dye interacts with DNA.

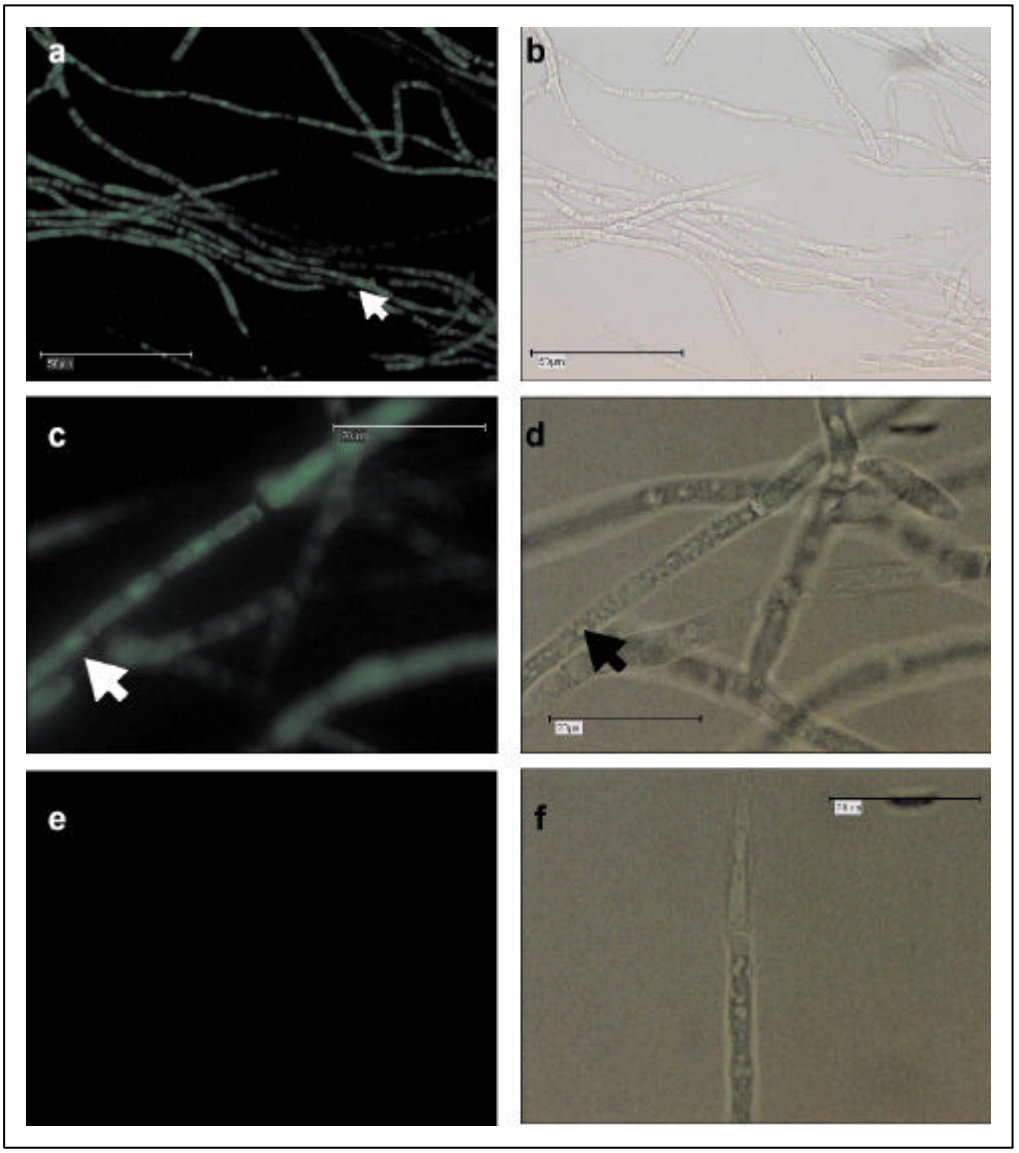


Fig 38.- Fluorescence microscopy of fungal hyphae in the presence of SYTOX green and BAP1b.

Fusarium culmorum hyphae were incubated in the presence of SYTOX and BAP1b protein. Right panels correspond to light microscopy images, whereas the images on left side are fluorescence microscopy images. Bars in upper panels (a,b) equal 50µm and 20µm in lower panels. (arrows indicate the position of nuclei).

Panels e and f show SYTOX green staining of hyphal cultures without BAP1b

These observations taken together confirmed that the protein here investigated is able to cause, at least under the conditions here used, intracellular uptake of the SYTOX dye, due to membrane permeabilisation.

3.5.5.- Mapping of protein function by PCR *in vitro* mutagenesis

As the BAP family constitutes a novel antifungal protein type, it is of interest to determine which features of the protein are required for its biological effect. The bioassay chosen lends itself to rapid screening of variant proteins created by *site directed PCR mutagenesis.*

Based on the conserved residues detected from the alignment performed for the *BAP* family (fig 39) and considering the residues found to be important for activity of defensins (De Samblanx *et al.,* 1997), three different types of mutations were introduced in the protein to be studied (fig 40,41):

a.- The conserved tyrosine at position 44/55 *(position varies depending on the BAP protein, so in BAP1b is at 44, BAP2 at 55) present in the middle of the helix, with a putative role in the conformation of the structure and present in 4 of the 5 members of the family;

b.- The conserved threonine at 43/50 *;

c.- The amino acids around the tyrosine and threonine conferring to the region a basic environment that has been described as an important factor (De Samblanx *et al.,* 1997).

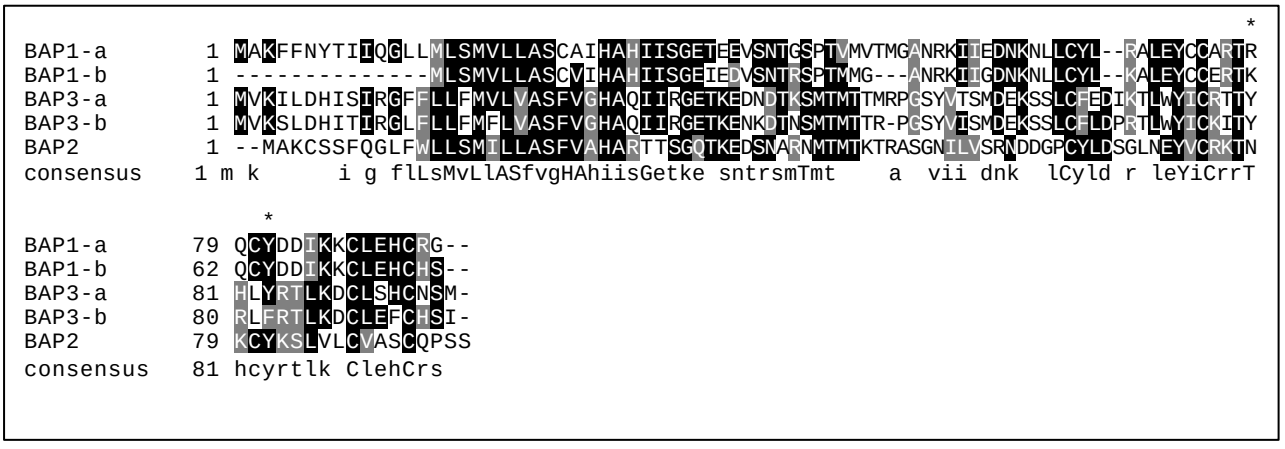


Fig 39.- Alignment at amino acid level of the BAP family

Analysis performed by CLUSTALW program (Thompson *et al* 1994a,b). Black shaded letters indicates identical amino acids and gray shaded letters indicates homologous amino acids.

The conserved amino acids changed are indicated by asterisks.

As a model for mutagenesis experiments, *BAP*2 was chosen as it possesses a *Pst*I site immediately before the cysteine motif which facilitated the cloning steps necessary for the amino acid substitutions.

The process of cloning began with double digestion of the pQE60-*BAP*2 containing plasmid with *Pst*I/*Hind*III. The *Hind*III site is in the plasmid immediately after the histidine-tag. The PCRs were performed with the primers depicted in figure 42 and the reverse primer present in the plasmid downstream of the *Hind*III site.

```
-42 gcacgagtattagactattgtagctcatatcatctgtcacccatggcgaagtgcagcagc
                                              M  A  K  C  S  S    6
 19 ttccaaggattattctggttgctttccatgattcttctagcatcctttgttgctcatgca
    F  Q  G  L  F  W  L  L  S  M  I  L  L  A  S  F  V  A  H  A   26
 79 cgcacaacaagtgggcaaaccaaagaggacagcaatgctaggaacatgacgatgaccaag
    R  T  T  S  G  Q  T  K  E  D  S  N  A  R  N  M  T  M  T  K   46
139 acgagggcatcaggcaacatacttgttagccgtaatgacgacgggccatgctatctagat
    T  R  A  S  G  N  I  L  V  S  R  N  D  D  G  P  C  Y  L  D   66
199 tccggtcttaatgagtacgtctgcagaaagactaataagtgctataagagcttggtgctc
    S  G  L  N  E  Y  V  C  R  K  T  N  K  C  Y  K  S  L  V  L   86
259 tgcgtggcgagttgtcaaccatcatcatgaattcatgatactgcggagacatcatgatac
    C  V  A  S  C  Q  P  S  S                                     106
319 tgcggagacagacggcga
```

Figure 40.- *BAP*2 sequence

Nucleotide and amino acid sequence of *BAP*2. In bold is indicated the *pst*I site employed in the mutagenesis strategy. Red letters show the amino acids substituted by Ala in the process.

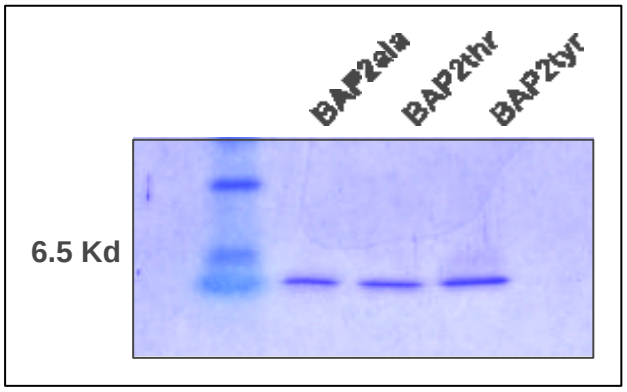


Fig 41.- *BAP*2 varieties mutated proteins generated by site-directed PCR mutagenesis

1µg from each mature protein was fractionated in a 15% SDS-PAGE gel and stained with coomassie blue

The mutated proteins were expressed (fig 41) (constructs depicted in figure 42) and processed under the same conditions already described and with the refolded proteins the antifungal assays were repeated (figure 43).

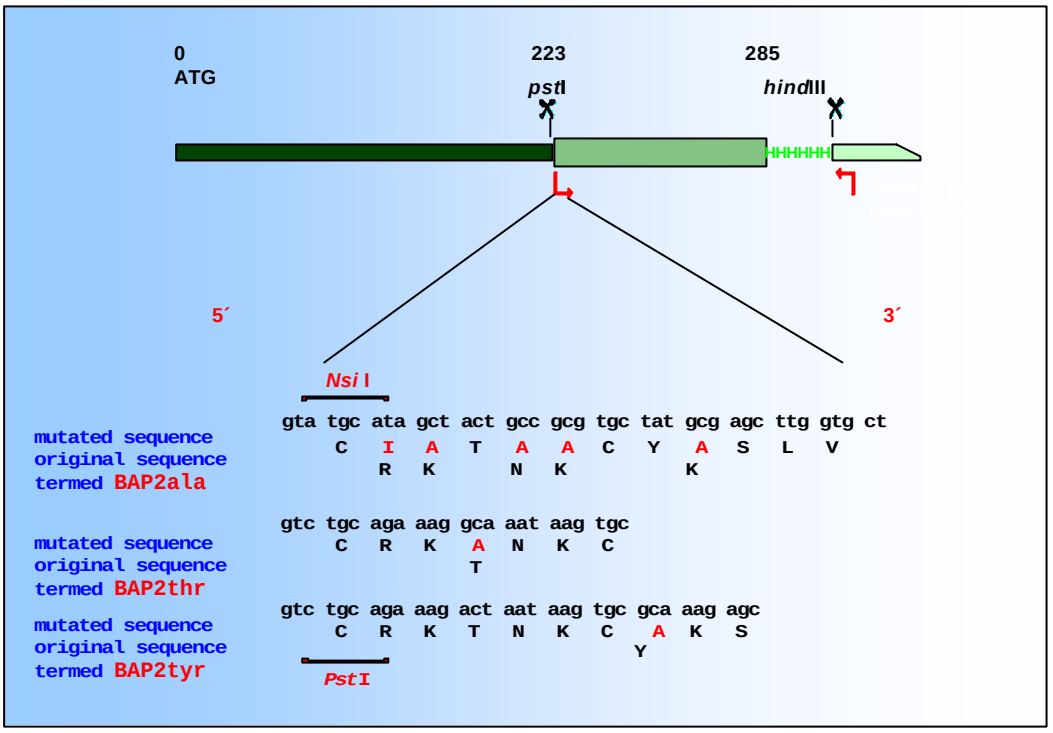


Figure 42.- Diagram showing the strategy employed for *BAP*2 transformation.

The PCRs were performed with the primers indicated and the reverse primer for the pQE60 plasmid. The incorporation of an enzyme target in the primer with a compatible end with *pst*I was sufficient to introduce an additional change.

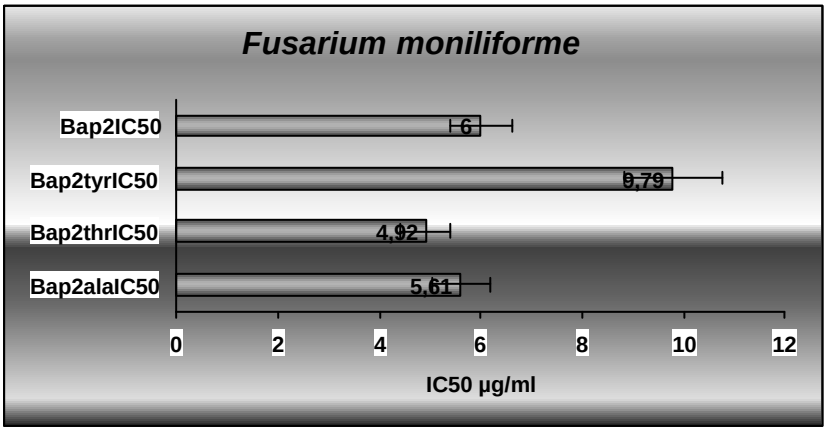


Fig 43.- Values obtained from the antifungal experiments for each of the mutated proteins.

The values represent the average of the IC_{50} values calculated from 2 independet experiments.

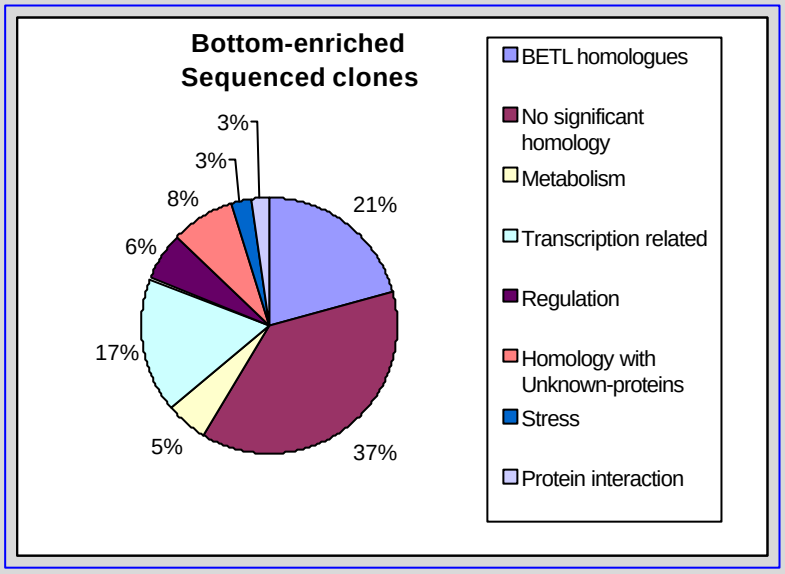
From the results obtained (fig 43, summary table 12) we can conclude that with the construct where the tyrosine at position 55 was changed to alanine, the antifungal activity of the protein was significantly reduced, increasing the amount of protein necessary to reach the IC_{50} value to 163 %.

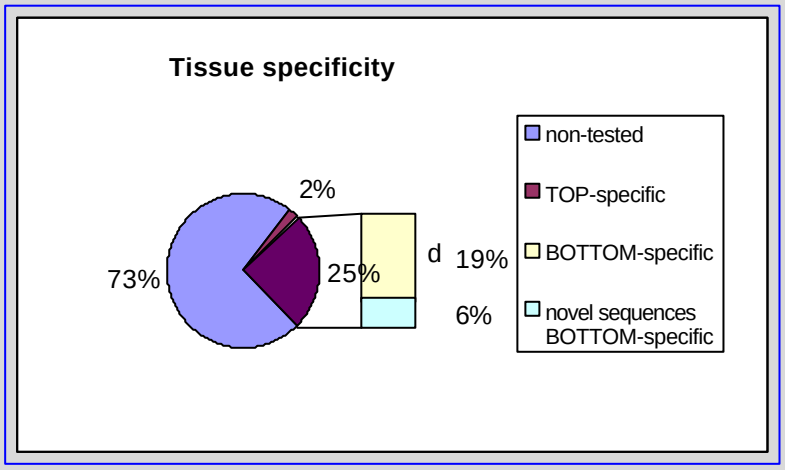
The other constructs show IC_{50} values only slightly different from that calculated for the wild type protein, being a 6% lower for the protein *BAP*2ala and 18% for the protein *BAP*2thr.

PROTEIN	IC_{50}		Relative activity
	Medium A	Medium B	
*BAP*2	6	n.d	100
*BAP*2ala	5.61	32	106.9
$BAP2_{thr50 \rightarrow A}$	4.92	25.8	121.9
$BAP2_{tyr55 \rightarrow A}$	9.79	62.6	61.2

Table 12.- Summary of the activities obtained from the different *BAP*2 peptide classes

The IC_{50} values are expressed in µg/ml and the relative activity is the percentage of activity for every peptide assuming the value of the wild type protein as 100%.


Bottom-enriched
Sequenced clones
3%
3%
8%
21%
6%
17%
5%
37%
BETL homologues
No significant homology
Metabolism
Transcription related
Regulation
Homology with Unknown-proteins
Stress
Protein interaction


Tissue specificity
2%
73%
25%
d
19%
6%
non-tested
TOP-specific
BOTTOM-specific
novel sequences BOTTOM-specific

Discussion

4.- DISCUSSION

In maize endosperm a basal layer of cells is modified to facilitate the solute transfer into the endosperm, forming the *transfer layer*. These cells facilitate the uptake of sugars and amino acids to the endosperm and play an important role in water assimilation during germination. After 2 days after pollination in the first stages of endosperm cellularization it is already possible to distinguish molecularly the region of the transfer cell layer by the expression of a transfer layer-specific transcription factor (Hueros *et al* unpublished). Genes specifically expressed in this region have been isolated and termed *BETL* 1 to *BETL*4. They encode unrelated small proteins of currently unknown function, all of which possess relatively conserved cysteine rich regions. The alignment of *BETL*1 to *BETL*3 with current database accessions showed weak homologies with plant defensins (S66218, S66219 and AAB34974.1). *In vitro* experiments using the *BETL*2 recombinant protein expressed in *E.coli*, showed that this protein possesses antifungal biological activity (Serna *et al.,* unpublished).

In order to facilitate the assignment of a function to the currently isolated *BETL* proteins and so to understand more of the role of the transfer layer during endosperm development, the work reported here focused on the characterisation of BETL2-related sequences: *The BAP gene family.*

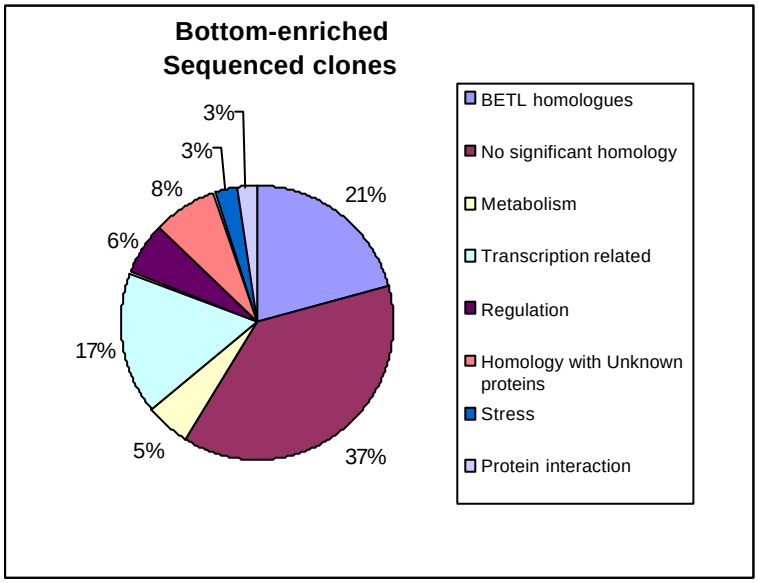
4.1.- Cloning of new members of the *BETL* family

One approach to investigate the specialised features of endosperm transfer cells is to characterise genes expressed specifically in this cell layer. This strategy was first employed by Hueros *et al.* 1995 and has been extended in this thesis. Different methodologies were used.

A critical step for the screening is the preparation of the differential probe. For the probe preparation, young maize kernels ten days after pollination (10 DAP) were dissected into two halves, one containing the transfer-cell layer and the basal part of the embryo and the other containing the upper part of the embryo and the endosperm crown. Messenger RNA was isolated and reverse-

transcribed in the presence of radioactively labelled nucleotides. This cDNA probe was used to screen a 10 DAP cDNA library. PCR based methods like DDRTPCR were tested with unsatisfactory results, none of them identified transfer-layer specific clones, most probably due to the relatively unspecific dissection of the kernels.

A problem common to all methods currently employed to display differentially expressed genes, is the identification of low-abundance differentially expressed transcripts. The differential screening method offers the possibility of distinguishing different expression levels, although the detection limit lacks sufficient sensitivity. Using this technique, 16 clones out of 88 were isolated showing high homologies with other transfer-cell specific genes and showing specificity for the transfer-cell layer. Other sequences isolated during the screening showed different expression patterns (fig 44).



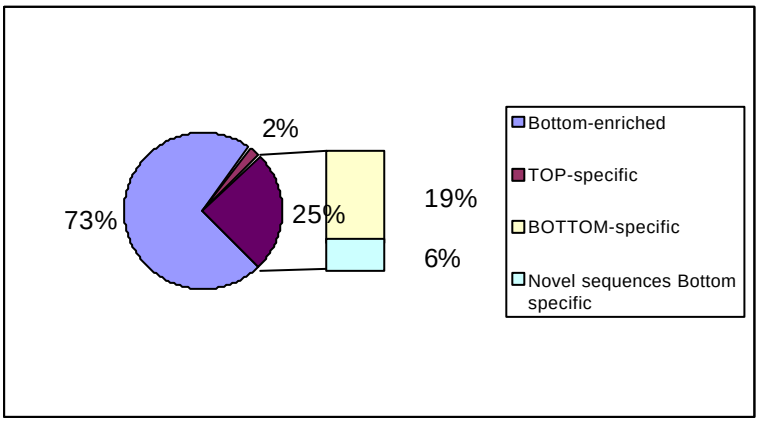


Fig 44.- Schematic diagram showing the type of sequences found during the screening. The upper panel shows type of sequences and the lower panel tissue specificity of the tested clones

The isolated sequences will be discussed in the following categories:

4.2.-Bottom-specific clones:

4.2.1.- Further representatives of previously identified *BETL* sequence types (*BETL*-1/3 and *BETL*-4 related genes)
4.2.2.- The BAP gene family
4.2.3.- Other novel sequences

4.3.- Top-specific or enriched sequences:

4.2.- Bottom-specific clones isolated during the screening

4.2.1.- *Representatives of previously isolated BETLs*

Different cDNAs from gene transcripts being specifically expressed at the transfer cell layer have been currently isolated. The genes identified, termed *BETL* for being basal endosperm transfer layer specific, lack close relatives among the database accessions, what makes the isolation of new relatives an important task in the attempt to describe a function for this family. During the differential screening performed in this thesis two new genes were isolated, pxa4 and px73 sharing homology with *BETL*-1 and *BETL*-4 respectively (Fig 10, pg 47). The function of these proteins is not clear for the moment, they share weak homologies to defensin-like, extensin motifs in the case of *BETL*-1 and bi-functional trypsin á-amylase inhibitors in the case of *BETL*-4 (Hueros *et al.,* 1999).

The currently isolated *BETL* genes encode for small proteins. The expression pattern of these proteins is similar during endosperm development; they start to be accumulated at early stages of endosperm development (Hueros *et al,* 1995,1999) and decline in expression, being practically undetectable before maturation and during germination. The transcripts are detectable exclusively at the transfer cell layer by in-situ hybridisation (*BETL*-1, *BETL*-2 and *BETL*-4 Hueros *et al.,* 1995, 1999) and the proteins are localised in

cell-wall extracts (*BETL*-1) or associated to membranes and cell wall (*BETL*-2). With respect to the function, little information is available to assign a role to these proteins in maize endosperm, however it seems to be obvious that one possible function of these genes could be protection against pathogen ingress. Some other functions could also be assigned to these proteins, such as membrane permeabilisation and cell wall structure conformation. However, the limited area of expression could support more evidence for a role in defence than in any other more general function. Because of dramatic crop yield losses caused by pathogen infections (Oerke, E-C *et al.,* 1994) the investigations to make clearer the function of this protein family becomes a challenge.

4.2.2- Bottom specific clones: The BAP *gene family*

4.2.2.1.- Sequence analysis

Alignments of *BETL*2 suggest from weak homologies that *BETL*2 might belong to a family of anti-pathogenic proteins (Hueros *et al.,* 1999). This weak evidence led to *in vitro* experiments with the recombinant *E.coli* expressed *BETL*2 protein, which have demonstrated that *BETL*2 is an active inhibitor of fungal growth (O´Connell and Thompson personal communication). One of the most salient features of small antifungal proteins is the existence of a characteristic pattern of cysteine residues in the primer sequence, which serves to differentiate groups inside this protein class (reviewed by Broekaert *et al.,* 1997; Olmedo *et al.,* 1998). BETL2 possesses a novel distribution of cysteines that does not fit to any other so far described (fig 45), therefore we can conclude that *BETL*2, defines a new family of anti-pathogenic proteins. In light of their putative function and their restricted distribution of the expression, the new family was renamed BAP for Basal layer type Antifungal Proteins, and the corresponding loci as *bap*1*, bap*2 (corresponding to the previously termed *BETL*2) and *bap*3. From the alignment of the BAP family with their closest plant relatives, the defensin like proteins (fig 46), it is possible to see how only the cysteines in the carboxyl terminus are conserved although without matching the plant defensin conserved sequence motif (Cornet B. *et al.,* 1995). At the amino

terminus a block of highly conserved serines and threonines might point to a conserved function, this is present in both compared families.

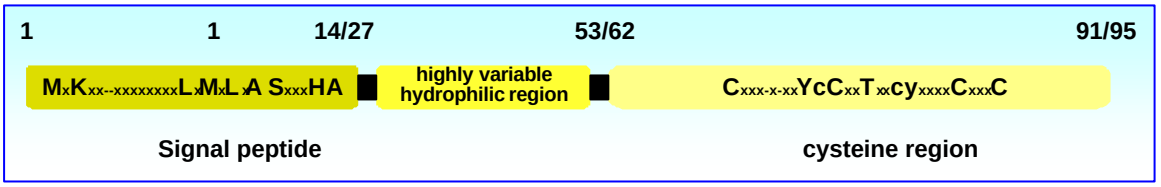


Fig 45.- Scheme of the protein structure for the BAP family and motifs defined by every region

The consensus sequence was obtained from the alignment performed by CLUSTALW program (Thompson *et al.,* 1994a,b) of the *bap*2 sequence and its relatives reported in the thesis. Three regions can be distinguished.

The pattern defined by the BAP family, observed from multiple sequence alignments, defines a motif composed by four cysteines with two additional ones appearing in some members of the group (*BAP*2 has five, bap1b and bap1a six). Inside the cysteine region there are also some other conserved residues like a threonine and a tyrosine that could indicate an important function for these amino acids.

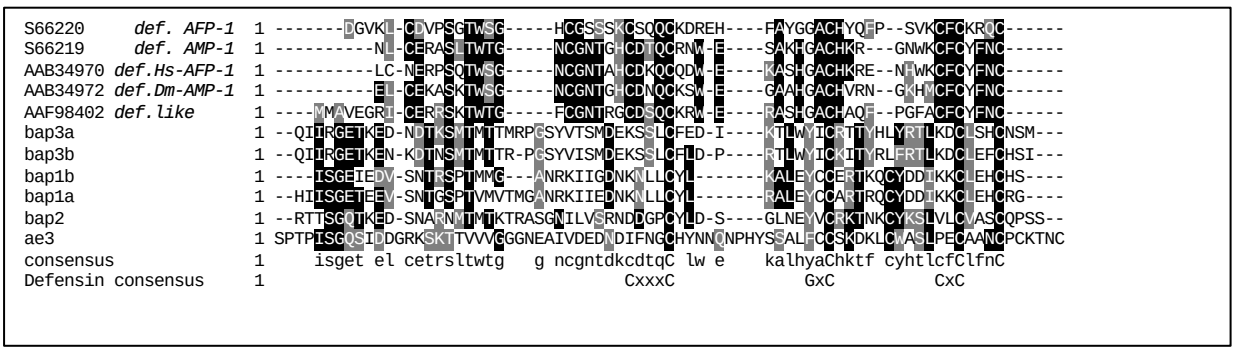


Figure 46.- Alignment of the BAP family with plant defensins data base accessions.

Analysis performed by CLUSTALW program (Thompson *et al* 1994a,b). Black shaded letters indicates identical amino acids and gray shaded letters indicates homologous amino acids.

Another characteristic that has been highlighted from other cysteine-rich anti-pathogenic proteins is their secondary structure (Fant F. *et al.,* 1996) in which the cysteine pattern of the protein plays an important and critical role. The cysteines are normally connected in pairs by disulfide bridges giving to the secondary structure a stable conformation. The importance of these bridges in

stabilising the á-helix is such, that the motif formed by the cysteines is known as *cysteine-stabilised á-helix motif* (Kobayashi *et al.,* 1991). This motif is also present in different anti-microbial protein types (Broekaert *et al.,* 1995) and a similar secondary structure has been predicted for the BAP family in which the pattern seems to be composed by one á-helix and three â-sheet antiparallel strands (fig 47). A similar pattern has been also described for *plant defensins* (Bruix *et al.,* 1993; Fant *et al.,* 1998, 1999). In the *BAP* family the cysteines at the carboxyl terminus are highly conserved and present in a C-terminal coil linked through disulfide bridges to the cysteines in the á-helix, whereas in the case of bap1b and bap1a, two adjacent cysteines are present. Two immediately adjacent cysteines have not been described so far in plant defensins but have been described in ã-thionins, lipid transfer proteins, hevein-type peptides and knottin-type peptides (fig 5, pg 15). Multiple alignments of plant antimicrobial proteins show that the homology is highly restricted to a few residues. The non-structural residues are highly variable, which may contribute to the divergence in biological activities.

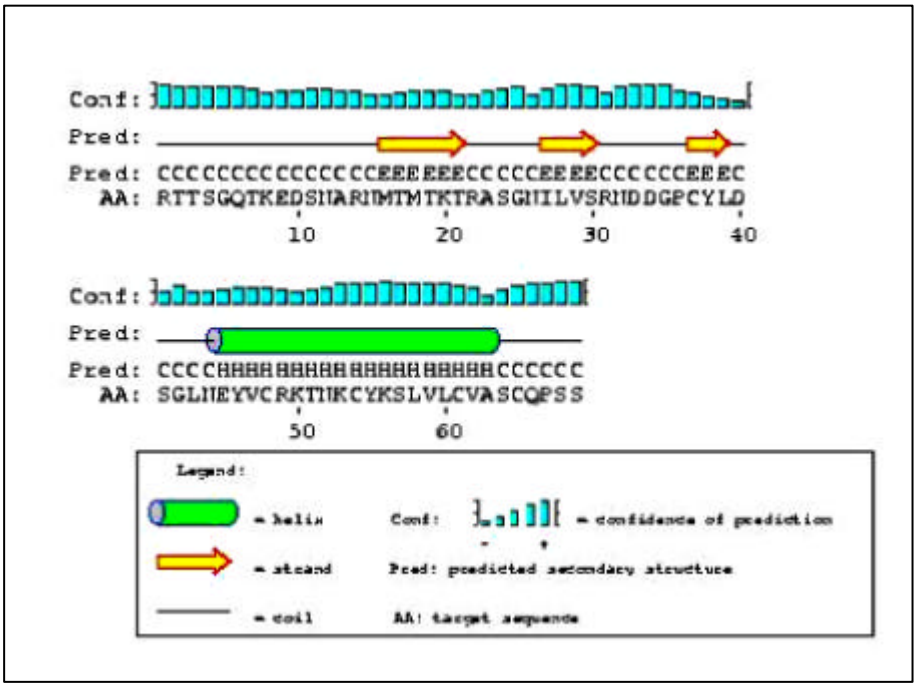


Fig 47.- Secondary structure prediction of BAP2 with the PSIpred V2.0 programm
The graphics were obtained at (http://insulin.brunel.ac.uk) (Jones D.T *et al* 1999)
Protein secondary structure prediction is based on position-specific scoring matrices

A signal peptide has been also predicted from computer analysis of the protein sequences. *BAP*2 protein can be detected in plant extracts as two different lengths (Maitz *et al.,* 2000) to which correspond the propeptide and mature peptide, regions that have been precisely determined by sequencing of the affinity purified peptide versions.

4.2.2.2.- Characteristics of the expression: *Expression Pattern and localisation*

One of the main features of the BAP family that also gives name to the group is its characteristic type of expression. All transcripts tested from this family were specifically expressed at the transfer-cell layer during a particular gap of time during endosperm development, although constitutively without attending at any type of apparent external induction. The expression pattern of other cysteine-rich peptides lies also in a particular form of expression being localised basically in special types of tissues, or in a particular region of different organs. Thus the thionins have been identified mostly as genes displaying organ-specific expression (Garcia-Olmedo *et al.,* 1992), being present not only in monocotyledonous (Ponz *et al.,* 1983, 1986) but also in dicotyledoneus (Schrader-Fischer and Apel, 1993). These proteins have shown to be induced after pathogen stress in a pathway dependent on methyl jasmonate as an endogenous signal transducer (Andressen, *et al.,* 1992; Molina and Olmedo, 1993). The subcellular localisation of this protein family takes place in over 90% of the thionins known in vacuoles, although a minor fraction has been also detected in cell walls (Bohlman *et al.,* 1988). In the case of the BAP family, BAP2 has been found to be associated to cell walls. The protein has been also detected in membraneous inclusions corresponding to the rough ER or Golgi vesicles (Serna *et al.,* unpublished). Other important group of anti-pathogenic proteins, the plant defensins, are accumulated preferentially in the peripheral cell layer upon induction by pathogen stress in a pathway methyl jasmonate dependent, salicylic acid independent (Penninckx *et al.,* 1996) and systemically (Terras *et al.,* 1995). A series of small cysteine-rich antimicrobial peptides isolated from *Impatiens balsamica* and derived from a single precursor with different prodomains has been shown to be expressed in dry seed and in the

stage of development immediately prior to this (Tailor *et al.,* 1997). These proteins presented activity against a wide range of pathogens including Gram+ bacteria.

Small cysteine-rich proteins with specific pattern of expression have been also described with roles other than pathogen defence. Thus, proteins involved in pollen recognition have been isolated whose secondary structure is quite similar to plant defensins (Adrew G. Stephenson, *et al.,* 1997; James Doughty *et al.,* 1998). These proteins were termed PCP (Pollen coat protein) and are involved in the interaction with Stigmas receptors normally of the glycoprotein class. The interaction of the PCP proteins and the receptor, allows pollen recognition and fertilisation. In mammals, cysteine-rich proteins have been isolated with different functions, such as cell-differentiation (Khoo C., *et al.,* 1997) and chromatin condensation in spermatids (Lee CH., *et al.,* 1987). Although it has been shown *in vitro* that proteins belonging to the BAP family present anti-pathogenic activity, a role in the regulation of the ionic balance (Serna *et al.,* unpublished) product of the high activity of co-transporters present at the region of the basal endosperm and pedicel, has been also postulated, although not shown experimentally. This hypothesis lies in the assumption that proteins of this family cause *in vitro* fungal-growth inhibition by disturbing the ionic traffic, which normally takes place at the fungal membrane.

4.2.2.3.- Protein processing of members of the BAP family

Although the transcripts are detected in the basal part of the endosperm a protein translocation of the mature peptide version occurs from this part of the kernel to more distanced regions present in the pedicel, as it has been experimentally shown in the case of BAP2 (Maitz *et al.,* 2000). Possibly the proteins can develop more effectively their role of inhibition of fungal infection located in this maternal tissue, which on the other hand, is also a region with a high risk of pathogen infection.

*BAP*2 protein is expressed as a pre-proprotein. After cleavage of the signal peptide and a further processing of the amino terminal prodomain, the 4.5 kd mature polypeptide is formed. The same protease extract has been shown to be active also on other members of the BAP family (results 3.3.2.2;

*BAP*1b) what points to a similar processing of these proteins and also supports the hypothesis that all could be members of the same protein family.

Similar processes of maturation have been described already for other anti-pathogenic proteins of the defensin class (Broekaert *et al.,* 1997). Most of the defensins are synthesised as pre-pro-proteins: In mouse, for example, has been described a similar processing for defensin proteins of the cryptdin family located in testis (Grandjean *et al.,* 1997). In humans, has been shown that proteins from the defensin family present in the intestinal surface undergo into a similar mechanism of maturation (Porter *et al.,* 1998). These proteins are, in general, synthesized with different amino termini and accumulated in vesicles. Immediately prior to pathogen challenge, the proteins are N-terminal processed and secreted, as it has been clearly established for defensin proteins secreted by blood cells (Ganz *et al.,* 1993).

In plants, anti-pathogenic proteins of the *Thionin* class are synthesised as a precursor form (Ponz *et al.,* 1983), and then processed to the mature form. In this group of anti-microbial proteins, the prodomain seems to be more conserved than the thionin domain itself (Bunge *et al.,* 1992), indicating that it might play an important role. In the case of the BAP family something similar is observed with some amino acid blocks conserved in the prodomain region, and with a low degree of conservation in between the predicted mature peptides of the isolated BAP members, where only 6 cysteines, a threonin and a tyrosine can be aligned. The other group of plant anti-pathogenic proteins, *the plant defensins* are mostly synthesised without prodomain (Terras *et al.,* 1995), although some exceptions have been described (Milligam and Gasse, 1995). From *Impatiens balsamica*, have been isolated a closely related small peptide which possesses anti-microbial activity against a wide range of pathogens (Ravi H. Tailor *et al.,* 1997). What these peptides have in particular is the way in which they are processed. These peptides are derived from a precursor, which consists of a pre-peptide followed by 6 mature peptide domains, each flanked by propeptide domains. The way in which these proteins are being processed is still unknown.

Proteases related to pathogen defence have been previously reported (Pablo Tornero *et al.,* 1996a). They could play a role not only in directly acting on pathogen targets, but also in activation and processing of pathogen-related

proteins. The protein described by Tornero and collaborators was isolated from tomato and was shown to be inducible upon pathogen challenge, moreover this protease was being synthesised also as a precursor form (Tornero *et al.,* 1996b). Possibly the members of the BAP family are processed by a protease of this type, although the processing of members of this family is not pathogen dependent.

4.2.2.4- The BAP*2 relatives isolated are linked in the maize genome*

When the recombinant inbred line of the cross Co159xTx303 (Burr B. *et al.,* 1988) was used for mapping the position of the BAP genes, the result showed that out of 41 lines no recombination events were detected between BAP1a and BAP3a, this predicts a clear proximity of the loci coding for these proteins with the exception of *bap*2 that has been already mapped to chromosome 4. This evidence suggests that the genes isolated belonging to the *BAP* family could correspond to a type of *gene cluster.* Clusters of anti-pathogenic genes have been identified in different species spanning different lengths in the genome. An example of a cluster of anti-pathogenic cysteine-rich proteins is constituted by the antimicrobial peptide human beta-defensin1 (Hutner KM., *et al.,* 1997; Linzmeier *et al.,* 1999), which has been mapped in chromosome 8, spanning 450 kb. In plants not so much information about genomic organisation of anti-pathogenic proteins of the cysteine-rich group has been reported; in maize another defence related gene with a cysteine-rich region, *betl*-1 (Hueros *et al.,* 1995, 1999) has been described to be organised in the maize genome as a cluster of three short tandem repeated copies. The genes isolated from maize belonging to the BAP family could be organized in a similar manner. However, and assuming that this new family of anti-pathogenic plant proteins could be divided in several groups of genes, *BAP*2 is not present at the same chromosome as its relatives isolated and here presented, and therefore is not part of the same cluster.

The origin of the putative gene cluster to which four members of the BAP family belong is of interest as it may relate to the processes involved in evolution of the gene family. Unequal crossing-over following meiotic mispairing, and gene conversion have been described as the major ways in

which novel resistance specificities are generated (Pryor and Ellis 1993; Hammond-Kosack and Jones 1997). Such processes are the responsible of the generation of new multi-gene families in general, such as vertebrate globins (Scott, *et al.,* 1984). However, the phenomenon of crossing-over occurs at a low frequency insufficient to generate new specificities to allow co-evolution with pathogen populations (Michelmore and Meyers, 1998). Another theory that has been proposed to explain the existence of clusters of gene resistance and that explains an increment in the frequency of occurrence is that based on a *Birth-and-death* model (Richard W. Michelmore and Blake C. Meyers, 1998). This theory is based on *intragenic recombination* as the major source of changes, although admits also during the process of fixation of the new genes in the haplotypes the phenomena of crossing-over. The main difference between these theories lies in the type of cluster that both processes generate; the process of mispairing during meiosis will originate, after gene fixation, duplication events and so a gene cluster where the genes of the cluster are more similar to one another than between other homologue genes, conversely the second method originates gene clusters with genes more distantly related. Thus, to the question *How could the BAP gene cluster have been generated?*, With the data available for the BAP family it is not possible to predict which one of these events was the responsible of the genomic reorganisation that could have given rise to the gene family. Because no other relatives have been detected in the maize genome and the genes of the BAP family have been described as single copy genes, most likely the cluster could have been originated by two successive duplication events. The first event formed the ancestor of BAP2 and the ancestor BAP1-3 (fig 48) and because chromosomes 4 and 10, where the members of the BAP family have been mapped are not syntenic the gene duplication does not correspond to an alloploidy event, so most likely a translocation has taken place between these two chromosomes. What it cannot be predicted is whether the first duplication could have occurred in chromosome 4 or in chromosome 10. A second duplication event, most probably of the whole area where the ancestors were present, and subsequent evolution, could have originated the actual genomic organisation, although a third independent duplication of the BAP1-3 ancestors could also have happened, is less likely.

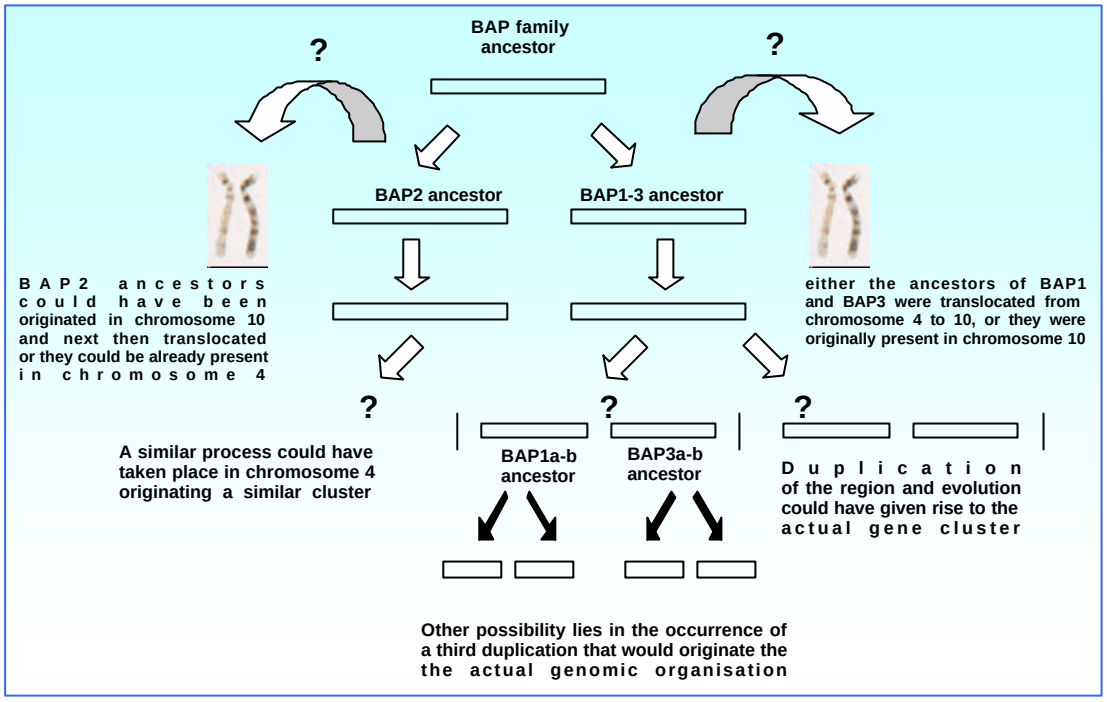


Fig 48.- Graphic representation of different events that could have originated the actual genomic organisation of the BAP family.

As BAP1, 2 and 3 do not cross-hybridise to one another, it is possible there are more related sequences that are similarly divergent and not yet discovered as the maize genome is not completely sequenced, and a model for the evolution of the gene family should take this into account. This is supported by the observation that the homologies between members of distinct group are quite similar (table 13), so the comparison of bap1 with bap2 and bap3 gave rise to similar values. It is for the moment unknown if the *bap*2 gene could be organised in a similar manner in chromosome 4, although it could be possible because other weakly cross-hybridising bands are observed in Southern blots (Hueros *et al.,* 1999) allowing the suggestion of the presence of other relatives (*bap*2 does not cross-hybridise with members of the BAP family currently isolated).

	*Bap*1a		*Bap*1b		*Bap*3a		*Bap*3b		*Bap*2	
	DNA	Protein	DNA	protein	DNA	protein	DNA	protein	DNA	Protein
***Bap*1a**			e^{-141}	$2e^{-35}$	x	$3e^{-10}$	2.1	$4e^{-10}$	0.002	$1e^{-08}$
***Bap*1b**					x	$4e^{-08}$	1.4	$2e^{-07}$	0.007	$1e^{-07}$
***Bap*3a**							0.0	$5e^{-38}$	$9e^{-11}$	$1e^{-10}$
***Bap*3b**									x	$7e^{-09}$

Table 13.- Pairwise comparison of the members of the BAP family at protein and DNA level.

Probabilities according to the stochastic model of Karlin and Altschul (1990).(x means no significant match)

4.2.2.5.- The BAP *genes encode an Orphan class of Andropogoneae-specific proteins*

When the copy number of the new *BAP*2 relatives is tested in different species of grasses, including barley, wheat, sorghum (*Sorghum bicolor*) and teosinte (*Zea luxurians*), clear signals are detected only in sorghum and teosinte under high stringency conditions.

Maize most probably originates from an ancestor belonging to the tribe *Andropogoneae*, a tribe that includes sugar cane and sorghum (reviewed by Mangelsdorf, 1986) and from which have descended *perennial Teosinte* and a primitive cultivated corn. The cross between these two species could have originated the annual *teosinte* and the modern corn after several rounds of selection (fig 49).

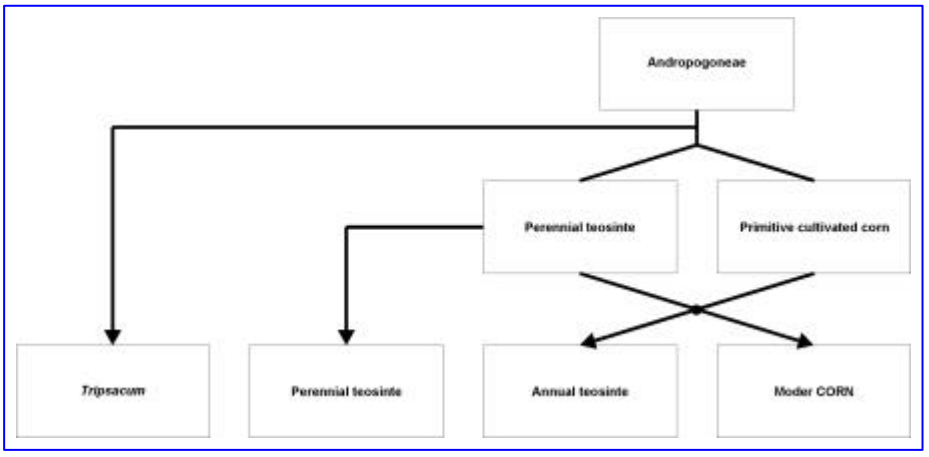


Fig 49.- Genealogy of maize. (taken from *"The origin of the Corn" by Paul C.Mangelsdorf* Sci.Am. 255,72-78)

The BAP gene class ancestor most probably originated in a common progenitor of the Zea and sorghum groups, which belong to the tribe *Andropogoneae.* It is of interest that the genomic distance between the *bap*1 and *bap*3 loci has been maintained, although the gene family presents an extensive sequence divergence what could be the product of positive selection.

4.2.2.6.- Biological activity of the new family of maize proteins

The main function that currently has been described for the transfer cell layer, and to my knowledge the only one, is a function directly involved in the solute transfer to the developing endosperm (Maitz *et al.,* 2000; Slocombe *et al.,* 1999; H.A. Becker *et al.,* 1999; Odd-Arne Olsen *et al.,* 1998; John Thorne, 1985). A mutant has been isolated exhibiting alterations in grain filling. This mutant was termed *rgf1* (reduce grain filling1)(Maitz *et al.,* 2000) The phenotype observed in this mutant is mainly caused by a defect in sugar uptake present at the endosperm cells, the free endosperm sugar levels seem to be normal and also the starch biosynthesis capacities. The background created by this atypical situation most probably is one of the factors that affect transfer-cell and pedicel normal development. In this mutant the expression of transfer-cell specific markers is affected. The existence of this mutant with a clear connection between the integrity of the transfer-cell layer and normal endosperm nutrient uptake supports the importance of the role of the transfer cell layer during normal endosperm development.

A role in protection has been also postulated with the cloning of genes with weak similarities to antimicrobial proteins (Hueros *et al.,* 1999).

During evolution, different cells have been specialised to ensure the continuity of the plant genome during the next generation, solving not only nourishment problems but also protecting the growing embryo or plant of the next generation from possible external invasions. Thus, the cell walls of the maternal embryo sac are thickened after fertilisation. The embryo sac during maturation protects the embryo against desiccation and also plays important roles in pathogen defence (Heslop Harrison *et al.,* 1999). Plant defensin-like proteins associated with the synergid cells have been described (Cordts *et al.,* unpublished), which might function as a barrier to possible pathogens present at

the pollen surface. The expression of antimicrobial proteins seems to be organised in the female gametophyte in what has been termed, *domains of gene expression,* (Reviewed by Slocombe *et al.,* 1999), due to the organisation of gene expression in spatial and temporal compartments. Different genes from these domains have been described which are expressed before and after fertilisation. In one of these domains, the *embryo surrounding region,* proteins with structures similar to other anti-pathogenic related proteins have been described (Opsahl-Ferstad HG, *et al.,* 1997) although no function has been assigned to them. During the period in which the transfer cell layer is more active, transporting solutes to the growing endosperm and embryo, the only barrier between the maternal tissues and the endosperm is the transfer-cell layer. It seems to be obvious that this region could be an important route for pathogen invasion. Therefore, a role not only in nutrient assimilation but also in the formation of an *anti-pathogenic shield* seems to be plausible for the transfer-cell layer. The fact of having proteins, like the BAP family, with a demonstrated strong anti-pathogenic activity and a restricted area and timing of expression, supports this theory and enlarges the role of the transfer cell layer during endosperm development.

Assuming the function of these proteins is in the protection of the developing kernel, the next question to be answered would be the mode of action of these new anti-pathogenic proteins. Unlike the anti-microbial proteins described in other organisms, plant defensins (the closest family by sequence homology to the BAP family) do not form ion-permeable pores in artificial membranes (Thevissen *et al.,* 1996,2000a) as it is the case of insect defensins (Cociancich, S. *et al.,* 1993) or mammalian defensins (Kagan, B. L., *et al.,* 1990), but induce changes in ion fluxes through the membrane and so altering the membrane potential (Thevisen *et al.,* 1996). The mode of action that has been proposed by Thevisen and collaborators for plant defensins lies in an interaction with membrane components that either activates a signal cascade that induces the membrane potential distortion, or allows the integration of the defensin in the membrane activating in that way ion channels. In the case of the protein BAP1b from the BAP family, it has been shown here that it confers *in vitro* membrane distortions sufficient for the internalisation of SYTOX green. Because the binding of proteins of the BAP family to the fungal membrane

seems to be reversible (O´Connell and Thompson personal communication) and susceptible to competition, the interaction with the membrane must lay in the recognition by the BAP proteins of an external membrane component. The reason why fungal growth is stopped in the presence of these proteins must lay in the distortion of the membrane potential as can be predicted from the physiology of fungal growth. It has been proposed (reviewed by Money N.P, 1997), that in hyphal extension without turgor, the cytoskeleton plays an important role. Three different phenomena have been shown to allow the progression of fungal membrane during growth: polymerisation of actin filaments, sliding of actin filaments one over the other by the action of myosin and gel pressure produced by hydration of spaces between filaments. All together increases the turgor pressure that keeps the right form in the hyphae of fungi and allows the progression of the vesicles involved in cell-wall growth. In the polymerisation and orientation of actin monomers to form the actin filaments, play an important role the intracellular concentration of Ca^{2+} and the pH. Elevated intracellular levels of Ca^{2+} can also stimulate protein phosphorilation via calmodulin, which may affect to the conformation of the F-actin network by phosphorilation of actin-binding proteins or direct phosphorilation of actin, disrupting the actin-net (Pollard, T.D, 1990). One of the main effects observed on fungal membranes challenged with plant defensins (the closest relatives to the BAP family) is an efflux of K^+ and influx of Ca^{2+} (Thevissen *et al.,* 1996), that could explain why not only fungal growth is affected but also the morphology in many of the cases.

The range of anti-pathogenic activity observed in proteins of the cysteine-rich class varies depending on the type of protein (Table 14). Normally all exhibit activity against fungi, although some exceptions have been also reported (Cammue *et al.,* 1995), as is the case of maize and barley lipid transfer proteins (LTPs), which were shown to be almost inactive against this type of pathogens. Antimicrobial activity against bacteria and yeast has also been described (see table 14). In most of the cases tested, the proteins did not cause lysis of human erythrocytes, fibroblast or plant cells.

Protein class	References	Pathogen			IC_{50}
		Fungi	Bacteria	Yeast	
Thionins	Stuart and Harris, 1942; Fernandez et al., 1972;Molina et al., 1993;Florack *et al.,* 1994ab.	Include about 20 different phytopa-thogenic fungi	Include several GRAM+ and – phytopathogenic bacteria	Tested against *S.cerevisiae*	1 to 15 µg/ml
Plant defensins	Colilla et al., 1990; Mendez et al., 1990	Active against a wide range of fungi including phytopathogenic	In general are less active against bacteria with some exceptions *(B.subtilis, P.slanacearum* and *C.michiganensis*)	Tested against *S.cerevisiae*	2 to 9 µg/ml
Lipid transfer proteins	Terras et al., 1992; Molina et al., 1993)	Depends highly on the protein so, some are active against a wide range, some others are almost inactive.	Proteins isolated from barley and spinach, are 10 fold more effective on bacteria than thionins, either Gram+ or -	No-information available	1 to 6 µg/ml
Hevein and knottin types	Reviewed by Raikhel et al.,1993	Active against a wide range of fungi	They were shown also to be active against Gram+ bacteria	No information available	Below 10µg/ml
Four cysteine-type	Duvick et al., 1992; Tailor et al., 1997	Active against a wide range of fungi	Active against Gram +	No information available	1 to 12 µg/ml and to 30µg/ml against bacteria
BAP family	Serna et al., in press, 2001 Anhang	Active against a wide range of fungi, some of them maize pathogens like *F.moligniformae*	BAP2 was show to be active against *Xanthomonas campestris*	Non-tested	1 to 6µg/ml or to 20 in the case of *Botrytis cinerea*

Table 14.- Summary of main features of anti-patthogenic proteins so far characterised.

4.2.2.7.- Amino acid residues that confer activity to the proteins: A model to explain BAP action

A comparison of the primary sequences of BAP1-3 reveals relatively few conserved amino acids. The divergence may be due to distinct functions for the different family members, or it may reflect selection for structural diversity to overcome plant-pathogen recognition, as is seen for alleles of race-specific resistance genes, or self incompatibility locus alleles. As the fraction of conserved residues is very low, mutations may be readily introduced at these positions to check their effect, if any, on the biological activity.

The first approach to identifying those residues important for conferring activity to the BAP family of proteins was the mutation of residues through site directed PCR mutagenesis. Based on the multiple alignments those conserved residues appropriate for being changed with the strategy that we developed were mutated and the proteins tested *in vitro*. Similar techniques have been described for the study of the fungal activity in plant defensins (De Samblanx *et al.,* 1997). Surprisingly when one of the conserved residues, the Thr50 was changed to alanine, the activity of the protein was slightly increased. This mutated threonin is conserved within the BAP family. Because the molecular size of the changed amino acids is quite similar, it is unlikely that major secondary structure distortions occur. The main difference between the substituted residue and the incorporated lies in a more aliphatic side chain present in the alanine that could point to an interaction with a non-polar group in the membrane of the fungi tested.

The other residue that was changed by mutagenesis was tyrosine55. This change clearly affected the activity of the protein by decreasing the activity by about a 65 %. The change of an amino acid like tyrosine with a complex side chain for an amino acid where the chain is just a methyl residue could compromise the secondary structure of the protein and so affect interactions of the stereo-specific type. This was supported by the observation that the loss of activity obtained for the protein variant was seen both with and without salt in the buffer, and therefore indicating that was not merely electrostatic.

The tyrosine is located in the middle of the predicted á-helix (fig 50) at the carboxyl terminus. It is possible that the secondary structure was compromised with this mutation and that this affects the activity of the protein.

```
Conf: Confidence (0=low, 9=high)
Pred: Predicted secondary structure (H=helix, E=strand, C=coil)
  AA: Target sequence

Conf: 863101162035663002688988876504677 89
Pred: CCEECCCCCCCHHHHHHHHHHHHHHHHHHCCCCCC
  AA: GPCYLDSGLNEYVCRKTNKCYKSLVLCVASCQPSS
              70        80*        90
```

Figure 50.- Secondary structure prediction for *BAP*2.

The prediction was done using the program available at http://insulin.brunel.ac.uk. Based on the method described by Jones D.T *et al* 1999. Protein secondary structure prediction is based on position-specific scoring matrices
The tyrosine mutated in *BAP*2tyr is marked in red and asterisk

No differences were observed when the basic region present between the cysteines situated at the carboxyl terminal region was substituted. This region has been described to be important for conferring anti-pathogenic activity in other anti-microbial related proteins (De Samblanx *et al.,* 1997), although this basic region is not present in all members of the BAP family, what could suggest that the secondary structure is more important than net charge for BAP action. The lack of enough information about protein structure and sufficient number of family members to predict motifs, was the reason why no more detail studies about protein function by mutagenesis could be performed. In previous works where proteins of the cysteine-rich anti-pathogenic class belonging to the plant defensins family were mutagenised (De Samblanx *et al.,* 1997), conserved residues were predicted by multiple alignments of proteins of different groups within the plant defensins family. This together with available information about protein secondary structure facilitated the identification of residues that could be important in conferring activity. From the mutated residues in plant defensins, more precisely in Rs-AFP2 (50 residues), was determined that 15% of the residues were involved in some type of electrostatic interaction. The substitution of the 32 % of the residues caused a reduction in the biological activity of the protein, from which only the 8% caused a reduction higher of the 200% of the calculated IC_{50}.

What remains in the case of the BAP family to be explained is the way in which the proteins permeabilise or induce the membrane potential distortions. In the case of plant defensins it has been shown that they are able to interact with a lipidic component of the sphingolipid type present in plasma membrane (Thevissen *et al.,* 2000a). Upon this interaction the protein presumably internalises in the membrane originating the distortions that cause inhibition of fungal growth. This type of binding has been shown to be in the case of plant defensins irreversible (Thevissen *et al.,* 2000b) what is consistent with the theory of internalisation in plasma membrane. In the case of the BAP family, it has not been shown so far which type of membrane component may be the responsible of the interaction, although it is known that the interaction is reversible in contrast to that seen for plant defensins (O´Connell and Thompson personal communication), possibly due to an interaction with an external component of the membrane without internalisation event.

4.2.3.- Bottom specific clones: Other novel sequences

During the screening procedure one sequence that was isolated and show to be basal endosperm transfer cell-specific lacked database relatives. The sequence information available does not correspond to the full-length transcript observed in Northern experiments, so no prediction can be made about the encoded protein. The encoded protein could belong to a new family of proteins being specifically expressed at the transfer-cell layer.

Other proteins were isolated during the screening that turned out not to be bottom specific but that showed higher levels of expression at this zone of the endosperm (see table 1 from results). This increment of expression of proteins that normally present a constitutive expression, could give more information about the high activity of these cells of the endosperm during the development of the seed.

4.3.- Top-specific or enriched sequences

As the aim of the research work was exclusively the identification of clones specific expressed at the transfer-cell layer, no further characterization of other clones was done. During the screening two sequences were more highly expressed at the top part of the endosperm, although the results were confirmed just by Northern blots and not by in situ hybridisation. More experiments should be performed to determine endosperm specificity. One of the sequences, termed pxa25, gave strong homology with a mitochondrial protein-import-receptor from potato (CAA63223 TOM20; value 2e-18) in spite of the lack of complete sequence information it could be predicted with a 85% certainty that the location of the protein could be endoplasmic reticulum based on the model described by Jackson M.R, *et al.,* (1990) which is based in the existence of retention motifs in the cytoplasmically exposed tails of the proteins. The other sequence pxa18, did not show any clear homology with the database accessions currently available.

At the top part of the endosperm is present the crown of the kernel where most of the storage products, like zeins, are being accumulated. Genes expressed at this area are higher expressed in the region of the sub-aleurone and their expression declines towards the center of the endosperm (H.A Becker *et al.,* 1999). In view of the high concentration of mitochondria in the transfer cells (B.E.S Gunning and J.S. Pate, 1972), it is surprising the TOM20 related transcript is not detected in basal endosperm RNA. There are few if any examples of tissue-specific transcripts of mitochondrial proteins. However there are three reported mitochondrial import carrier pathways in *Neurospora crassa*, and in yeast a Tom20 mutant was viable, so perhaps there is gene redundancy. Alternatively, there might be more than one non-cross-hybridising TOM-20 homologous gene, one expressed in apical the other in basal endosperm cells.

Summary / Zusammenfassung

5a.- Summary

The basal region of maize endosperm has been described as a specialised region involved in the uptake of nutrients into the maize endosperm (Pate and Gunning 1972, Hueros *et al.,* 1995, Slocombe *et al.,* 1999, Becker *et al.,* 1999, Maitz *et al.,* 2000). The region is characterised by the presence of specialised cells which posses high concentrations of cell wall ingrowths increasing cell surface, thus facilitating their function in solute transfer. Genes specifically expressed at this region of the endosperm have been identified (Hueros *et al.,* 1995, 1999) and characterised (Maitz *et al.,* 2000).

Another role postulated for the transfer cells lies in their putative protection against pathogen ingress, which is supported with the identification in this thesis of genes related to anti-pathogenic proteins. The genes isolated and here described were identified as members of a novel gene family termed BAP (basal layer type anti-fungal proteins). The expression profile of this new family in maize endosperm occurs during endosperm development. The transcripts are detected early in development (from 7 DAP) and following an early increase expression declines till 20 DAP. The gene family belongs to an orphan class of *Andropogoneae* specific proteins which co-segregate in a gene cluster present in chromosome 10 and a single locus present in chromosome 4.

Members of the BAP family showed *in vitro* a strong anti-pathogenic activity against a wide range of fungal strains (Serna *et al.,* in press), amongst them are maize pathogens as *Fusarium moniliforme*. Activity against bacteria has been also detected in the case of BAP2 against some strains of *Pseudomonas* (O'Connell and Thompson, personal communication).

A mode of action has not been established for members of the BAP family, although it was shown *in vitro* that these proteins appear to be causing fungal membrane permeabilisation.

Identification of proteins related to defence in the transfer cell layer supports the importance of this region for normal endosperm development and enlarges the role of the transfer cell layer.

5b.- Zusammenfassung

Die basale Region der Mais-Endosperms ist als Bereich beschrieben worden, der für die Aufnahme löslicher Nährstoffe ins das Endosperm spezialisiert ist (Pate and Gunning 1972, Hueros *et al.,* 1995, Slocombe *et al.,* 1999, Becker *et al.,* 1999, Maitz *et al.,* 2000). Charakteristisch für diese Region sind Zellen, die in hohem Maß Zellwand- einwachsungen zeigen, die die Zelloberfläche vergrößern, und so die Funktion des Transfers von Nährstoffen unterstützen. Gene, die spezifisch in dieser Region des Endosperms exprimiert werden, sind identifiziert und charakterisiert worden (Hueros *et al.,* 1995, 1999; Maitz *et al.,* 2000) .

Eine andere Rolle, die man für die Transferzellen postuliert hat, der mögliche Schutz gegen pathogenen Befall, wird im Rahmen dieser Arbeit mit der Identifizierung von Genen, die mit anti-pathogenen Proteinen verwandt sind, unterstützt. Die in dieser Arbeit isolierten und beschriebenen Gene wurden als Mitglieder einer neuen Genfamilie, die mit BAP benannt wurde (basal layer type anti-fungal proteins), identifiziert.

Das Expressionsprofil dieser neuen Genfamilie läuft während der Endosperm-Entwicklung ab. Die Transkripte lassen sich schon früh in der Entwicklung des Endosperms (von 7dap) mit einem frühen Anstieg nach dem die Expression bis zu 20 DAPs abfällt, nachweisen.

Die Genfamilie gehört zu einer Waisen-Klasse von *Andropogoneae*-spezifischen Proteinen, die in einem Gencluster auf Chromosom 10 co-segregieren und auf Chrosomom 4 von einem Einzellocus repräsentiert werden.

Mitglieder der BAP Familie zeigen *in vitro* eine starke anti-pathogene Wirkung gegen eine große Auswahl von Pilzstämmen (Serna *et al.,* in press) zu denen auch Mais-Pathogene - wie *Fusarium moliniforme* - gehören. Ebenfalls konnte im Falle von BAP2 eine anti-bakterielle Wirkung gegen einige Stämme von *Pseudomonas* nachgewiesen werden (O´Connell and Thompson personal communication).

Die Wirkungsweise von Mitgliedern der BAP-Familie konnte bislang nicht geklärt werden, obwohl *in vitro* gezeigt werden konnte, daß diese Proteine eine Permeabilisierung von Membranen bei Pilzen zu verursachen scheinen.

Die Identifizierung von Proteinen in der Transferzellschicht mit Verwandtschaft zu antipathogenen Proteinen und der Nachweis ihrer Funktionalität unterstützt die Bedeutung dieser Region für die normale Endospermentwicklung und unterstreicht die Rolle des basalen Teils des Endosperms.

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6.- References

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Appendices

Appendix 1

Maize endosperm secretes a novel antifungal protein into adjacent maternal tissue

Antonio Serna[1], Monika Maitz[#], Timothy O`Connell[1], Geraldina Santandrea[1], Karin Thevissen[2], Koenraad Tienens[2], Gregorio Hueros[#], Claudia Faleri[3], Giampiero Cai[3], Friedrich Lottspeich,[4] Richard D. Thompson[1*]

[1]MPI für Züchtungsforschung, Köln, D., [2]Kathioleke Universiteit Leuven, B, [3]Dept. Environmental Sciences, Univ. Siena, I., [4]MPI für Biochemie, Martinsried, D.

*To whom correspondence should be addressed at: MPI für Züchtungsforschung, Carl-von-Linné Weg 10, D-50829 KÖLN, Germany, (Tel. 0049-221-5062-440, Fax –413, thompson@mpiz-koeln.mpg.de)

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#Present addresses: KWS Saat AG, Einbeck, D. (M.M.), Dept. Cell Biology and Genetics, Univ. Alcala, ES, (G.H.). Sequences reported in this paper have been deposited in the EMBL nucleotide sequence database under the following accession numbers: AJ297901(bap1a), AJ297900(bap1b), AJ297902(bap3a), and AJ297903(bap3b).

Summary

A series of endosperm transfer layer-specific transcripts has been identified in maize by differential screening of a 10 days after pollination (DAP) cDNA library. Sequence comparisons revealed among this class of cDNAs a novel small gene family of highly diverged sequences encoding Basal layer Antifungal Proteins (BAPs). The *bap* genes mapped to two loci on chromosomes 4 and 10. *bap*-homologous sequences have thus far only been detected in maize, teosinte and sorghum, and are not present in grasses outside of the *Andropogoneae* tribe. BAP2 is synthesized as a preproprotein, and is processed by successive removal of a signal peptide and a 29-residue prodomain. The proprotein can be detected exclusively in microsomal membrane-containing fractions of kernel extracts. Immunolocalization reveals BAP2 to be predominantly located in the placentochalazal cells of the pedicel, adjacent to the Basal Endosperm Transfer Layer, (BETL) cells, although the BAP2 transcript is only found in the BETL cells. The biological roles of BAP2 propeptide and mature peptide have been investigated by heterologous expression of the proprotein in *E. coli* and tests of its fungistatic activity, and that of the fully processed form, *in vitro*. The mature BAP2 peptide exhibits potent broad-range activity against a range of filamentous fungi, including several plant pathogens.

Keywords: endosperm, transfer layer, antifungal, defensin, protease, transport.

Introduction

The developing cereal grain is adapted for rapid nutrient assimilation by the development of a transfer layer. In maize, these cells, which form a disc in the basal endosperm corresponding to the area of attachment to the pedicel, are highly modified by the development of cell wall projections on the basal cell surface (Davis et al., 1989). A series of basal endosperm transfer layer-, specific cDNA clones (termed BETL1-x) have been isolated by differential screening of immature kernel cDNA libraries (Hueros et al., 1995,1999). The four most abundant BETL clones, BETL1 to -4, encode small polypeptides with Mr´s between 8 and 10 kDa. These proteins have no close relatives in database accessions, but two of them, BETL1 and BETL3 show some similarity to the defensin supergene family of antimicrobial peptides (Terras et al., 1995, Hueros et al., 1999). Of the remaining BETL clones, BETL4 has some similarities to the Bowman-Birk family of trypsin inhibitors (Garcia-Olmedo et al., 1987, Hueros et al., 1999), whereas BETL2 lacks close databank relatives.

In an extended screen for maize endosperm transfer layer-specific clones, we have now identified additional basal endosperm-specific cDNAs related to BETL2, which together define a novel gene family. In the light of their restricted distribution and biological properties, we have called these proteins BAPs (Basal layer type Antifungal Proteins), and the corresponding loci *bap1*, *bap2*, and *bap3*. BAP transcripts accumulate between 8 and 20 days after pollination (DAP). BAP2, (previously termed BETL2), protein follows a similar time course of appearance (Hueros et al., 1999); it turns over by mid-term development, and is absent from mature kernels, suggesting a role in development rather than in maturation or germination. We report investigations of the biological function of BAP2 which show it to be a potent antifungal agent that is secreted into the intercellular matrix of the basal endosperm and accumulates predominantly in the adjacent thick-walled cell layer of the pedicel, the placentochalaza. BAP2 presumably protects the zygote from pathogen ingress through the nutrient-rich basal endosperm cells. BAP2 is a member of a new family of peptides having a putative antimicrobial role, with a structure and mode of activation distinguishing it from the previously characterized families (Garcia-Olmedo et al., 1998).

Results

Classification of a family of transfer layer-specific transcripts

10358 cDNA clones from a 10 DAP endosperm library were gridded onto nylon filters and screened with ^{32}P-labelled cDNA prepared from 10 DAP top- and bottom- endosperm half mRNA. Sequences more highly expressed in bottom- than in top-endosperm RNA were sequenced and compared to existing sequence database accessions. As a result of sequencing 100 clones preferentially or exclusively expressed in bottom as compared with top endosperm, 6 cDNAs were identified that were related to BAP2 using BLAST searches. Pairwise comparisons of the 6 selected sequences were carried out in BLAST2, using the default conditions (Tatusova and Madden, 1999). These sequences were also aligned using the programme CLUSTALW (Thompson et al., 1994), as shown in Figure 1. The corresponding proteins were termed BAPs (Basal layer specific Antifungal Proteins), and the loci corresponding to the three sub-groups of clones were termed *bap1, bap2* previously BETL2), and *bap3* (see Table 2). Inspection of the aligned sequences showed a block of conserved residues in the signal peptide and adjacent prodomain region, with three further conserved residues (Leu , Tyr , Thr) clustered around four cysteines in the COOH-terminal 35 amino acids. An attempt to align BAP sequences with plant defensins indicated, despite the presence of 4 aligned cysteine residues, that the characteristic defensin motif (Conceicao and Broekaert, 1999), CXXXC---GXC---CXC, was not preserved (not shown). BAPs are also unrelated in sequence to a previously reported antimicrobial peptide from maize kernels (Duvick et al., 1992).

The distribution of BAP family members among members of the Poaceae was investigated by Southern hybridiations with BAP1a and BAP3a probes (Figure 2). As both the (BAP1a and BAP1b) and (BAP3a and BAP3b) pairs have 95% DNA sequence homology with one another and cross-hybridize at high stringency, only BAP1a and BAP3a were used as probes. A single hybridizing band was seen for each probe in Sorghum (*Sorghum bicolor*) DNA, whereas Teosinte (*Zea luxurians*) contained two bands for each probe, as did

domesticated maize, *Zea mays* (line A69Y). Neither barley nor wheat DNA gave detectable signals with either probe. Similar results have been obtained for BAP2 (Hueros et al., 1999).

Accumulation of BAP family transcripts during endosperm development.

2µg mRNA samples from kernels harvested between 7 and 27 days after pollination (DAP) were fractionated by electrophoresis in denaturing formaldehyde gels, blotted onto nylon filters and hybridised with BAP 1a, 1b, 3a, and 3b probes (Figure 3a). Transcripts were evident from 10 to 18 DAP with quantitative variations between the probes. From 20 DAP onwards, transcripts were either undetectable or present only in trace amounts. The specificity of expression of BAP1a, 1b, 3a and 3b genes was monitored by Northern analysis of RNAs from endosperm, leaf, root, shoot and silk tissues (Figure 3b). In all four cases, signal was only seen in the endosperm RNA sample. The timing and specificity of expression of BAP1 and BAP3 transcripts therefore corresponded to that seen previously for BAP2 (Hueros et al., 1999).

Cell-type localization of BAP transcripts and BAP2 protein

All the BAP family members identified are restricted in their expression to the endosperm, and accumulate only during mid-term endosperm development according to Northern blot analyses using RNAs from different tissues (Figure 3). Using one member of each of the newly identified sequence classes,(*bap1* and *bap3*) for *in situ* hybridization revealed expression of both to be exclusively in the basal endosperm transfer layer (Figure 4), as also reported for *bap2* (Hueros et al., 1999). Thus, both the site and the timing of expression in the kernel are similar for all three BAP-class cDNAs.

Cell-type and subcellular localization of BAP2

The localization of BAP2 protein was carried out to check whether the site of deposition coincides with that of the mRNA. Wax-embedded sections from

different developmental stages were immunodecorated with preimmune serum (Figure 5a) or with anti–BAP2 antibody (Figure 5b), and detected with peroxidase/DAB staining. The protein was detected as expected in the basal endosperm cells. However, a strong signal was also seen in the placentochalazal layer of the pedicel. Control sections stained with preimmune serum gave no signal in these cell layers. The results indicate that BAP2 protein is synthesized in the basal endosperm transfer layer cells and quantitatively deposited in the placentochalaza.

To more precisely locate the BAP2 protein, and gain insight into the post-translational route taken during deposition, the protein was detected by immunogold labelling and analysed by transmission electron microscopy. one hundred nm thin sections of 15 DAP kernels embedded in LR-white resin were visualized without OsO4 shadowing (Wells, 1985). BAP2 antibody was detected with a secondary antibody coupled to 15nm gold particles. The strongest signal was in the pedicel, at the site of the placentochalazal cells, as shown by light microscopy. These cells displayed little intracellular structure and only rarely was a nucleus seen after 12 DAP. The cell walls were thickened, and cytoplasmic structure was absent, i.e., the cytosol is probably metabolically inactive. Within the endosperm, the first transfer cell layer, which is largely occupied by cell wall material by 15 DAP, was relatively weakly labelled. The second layer of endosperm cells labelled much more heavily. These cells possessed a dense cytoplasm lacking large vacuoles. The cells were rich in mitochondria and cell wall ingrowths, although the latter were less developed than in the first cell layer. The BAP2 protein was located principally at the cell wall/plasma membrane interface of the wall ingrowths (Figure 5, right hand panel). It was also detected more weakly in membraneous inclusions corresponding to the rough ER or golgi vesicles.

Distribution of BAP2 protein forms in basal kernel tissues

The BAP2 open reading frame minus the putative signal peptide (residues 27–95 in Figure 1) was expressed with a COOH-terminal His Tag fusion in pQE60. The construct was used to prepare BAP2 protein to use as an antigen for

raising an antiserum in rabbits. This antiserum recognizes two polypeptides on immunoblots (arrowed in Figure 6a), of apparent sizes 8 and 4.5 kDa. The 8 kDa band can be resolved into a doublet of two closely migrating polypeptides. Immunoaffinity chromatography-purified peptides were gel-purified and submitted to NH2-terminal sequencing. The 8 kDa peptide yielded the sequence: RTTSGQTK, and the 4.5 kDa peptide possessed the sequence: NDDGPCYLDS. From the location of these peptides in the predicted coding sequence (beginning at the arrowheads in Figure 1), we deduced that BAP2 is post-translationally processed in at least two steps: the removal of the signal peptide gives the 8kDa propeptide, and the cleavage of the 31-residue prodomain from this peptide gives the 4.5 kDa mature peptide.

To obtain biochemical evidence for the localization of BAP2 protein in the pedicel, basal endosperm and pedicel tissues were hand-dissected under the binocular microscope, proteins extracted, and analysed by immunoblotting (Figure 6a). As a control for the separation of pedicel and basal endosperm cells, the filter was probed with BETL1 antiserum. BETL1 is synthesised and located in basal endosperm cells, where it is tightly cell-wall bound, and is not synthesized or relocated in the pedicel (Hueros et al., 1995). Only traces of BETL1 were detected in the pedicel protein extract (Figure 6a). The 8 kDa BAP2 propeptide is much more abundant in the basal endosperm extract than in the pedicel, whereas the 4.5 kDa mature peptide is more highly represented in the pedicel extract. Presumably the pedicel accumulates only the fully processed form of BAP2, which partitions into the placentochalazal zone after secretion into the apoplast by the basal endosperm cells. This would be consistent with the lack of BAP2 mRNA in pedicel cells.

The predominant location of BAP2 in the cell wall (Figure 5), is supported by analysis of subcellular fractions from cell lysates (Figure 6b). The mature BAP2 peptide was predominantly in the fraction corresponding to a crude cell wall preparation (CW in Figure 6) and was not recovered in 1000g (crude nuclear, P1) or 6000g (crude organellar, P2) pellets. The propeptide, on the other hand, was enriched in the 80,000g microsomal pellet, whereas the supernatant from this centrifugation step (S3) contained only the mature form of BAP2.

Evidence for a BAP2 processing enzyme in maize endosperm

To characterize the protease responsible for removal of the prodomain from the BAP2 propeptide, the protein was synthesized in *E. coli* (with a C-terminal His-tag), and purified for *in vitro* protease incubations. As the *E. coli* His-tag fusion protein was deposited in inclusion bodies, the protein was extracted under denaturing conditions with urea, eluted from nickel-Agarose columns with a gradient of increasing urea concentration (Shi et al. 1997), and renatured by dialysis.

The recombinant propeptide was incubated in a size-fractionated (>200 kDa) endosperm extract to bring about processing to the mature peptide form. By testing pH values in 0.5 pH unit increments, a pH optimum of 7 for protease activity was determined (data not shown). The effect at pH7 of different classes of protease inhibitors on propeptide cleavage was then investigated (Figure 6c). The protease was inactivated by boiling, but was not affected by most of the inhibitors tested, with the exceptions of a commercial inhibitor mix containing phenylmethylsulphonylfluoride (PMSF) or this reagent alone. Inhibition by PMSF indicates a serine-type protease is responsible for the cleavage. The lack of inhibition by Tosyl-L-phenylalanine-chloromethylketone (TFCK) and Tosyl-L-lysine-chloromethylketone (TKCK), however, suggested the protease was not of the trypsin or chymotrypsin class of serine proteases.

Antifungal properties of the purified BAP2 protein

The antifungal properties of the purified BAP2 protein and its undigested form BAP2 propeptide were compared by determining protein concentrations required for 50% inhibition of fungal growth (IC_{50} values). Eight fungi were included in the assay, namely the saprophytic soil fungus *Neurospora crassa* and the phytopathogenic fungi *Alternaria brassicicola*, *Aspergillus flavus, Botrytis cinerea, Fusarium culmorum, Fusarium moniliforme, Plectosphaerella cucumerina* and *Verticillium dahliae*. *A. flavus* and *F. moniliforme* are maize pathogens. The antifungal activity assays were carried out in two media: ½ potato dextrose broth (PDB) and ½ PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl. The results are shown in Table 3. In the medium without the

addition of salts, IC_{50} values of BAP2 were generally in the 2–5 µg/ml range, except for *B. cinerea*, which was sensitive to BAP2 at concentrations starting from 20 µg/ml. In addition, BAP2 propeptide showed no antifungal activity at concentrations up to 80 µg/ml, except on *N. crassa* and *P. cucumerina*, for which BAP2 propeptide showed a very weak antifungal activity starting from 80 µg/ml. The effect of salts on the antifungal activity of the BAP2 protein was largely dependent on the test fungus. In general, addition of salts to the medium decreased the activity of the BAP2 protein by a factor of 4 to 6-fold. Sensitivity of antifungal activity to inorganic cations has been reported for a number of proteins including the plant defensins (Terras et al., 1992; Osborn et al., 1995).

Membrane permeabilization induced by BAP2 protein

To investigate the mechanism of fungal growth inhibition by BAP2, fungal membrane permeabilization in the presence of BAP2 was studied. To this end, an assay based on the uptake of SYTOX green was used, as described by Thevissen et al. (Thevissen et al., 1999). SYTOX green is an organic compound that fluoresces upon interaction with nucleic acids and only penetrates cells with compromised plasma membranes (Matsuzaki et al., 1997; Roth et al., 1997). Membrane permeabilization to SYTOX in the presence of BAP2 and BAP2 propeptide was assessed for *Neurospora crassa* and *Fusarium culmorum*. As can be seen in Fig 7, SYTOX green uptake in *F. culmorum* and *N. crassa* rose significantly upon treatment with BAP2 at concentrations above 2 µg/ml and 5 µg/ml, respectively, which correlates well with the concentrations required for growth inhibition. In contrast, treatment with BAP2 propeptide did not inhibit growth of these fungi, and also failed to cause permeabilization to SYTOX green.

Discussion

BAP2 belongs to a highly divergent family of basal endosperm-specific proteins

A comparison of the predicted coding sequences of BAP2-related clones indicates that the most extensive homologies lie in the COOH-terminal half of the signal peptide and the prodomain. The mature peptides, extrapolated from the processing site demonstrated for BAP2, contain only 6 residues, 4 cysteine, one tyrosine and one threonine, which are present in all the BAP sequences compared. It was not possible to align these residues unambiguously with those of plant defensin sequences, and in particular, the defensin motif was absent. The extensive sequence divergence seen for the *bap* family is commonly seen within families of plant antipathogenic peptides (Broekaert et al., 1997, Garcia-Olmedo et al., 1998, Conceicao and Broekaert, 1999) in which sequence conservation appears to reflect maintenance of a secondary structure without a catalytic function. A comparable extreme sequence divergence exists among families of non-plant cytotoxin genes, for example, those encoding conotoxins (Duda and Palumbi, 1999).

The newly isolated *bap1a* and *bap1b*, and *bap3a* and *bap3b* gene family members were mapped to chromosomal loci using recombinant inbred (RIL) lines (Burr et al., 1994). All four cDNAs cosegregated to a locus on chromosome 10, unlinked to *bap2* itself, which has previously been mapped to Ch. 4 (Maize genome database at: http://www.agron.missouri.edu/cgi-bin/sybgw_mdb/mdb3/Map/64506). If we assume *bap1a* and *bap1b* clones are allelic variants at one locus, and that the same applies to *bap3a* and *bap3b*, two cosegregating genes, *bap1* and *bap3*, must be represented. As all 4 cDNAs arose from an inbred line (B73), however, and two fragments are seen in Southern blots with each probe, it is likely that the cDNAs represent four different cistrons at the locus. Multiple closely related cistrons seem to be typical for defensin-like peptides. The BETL1 locus (Hueros et al., 1999), encoding a maize defensin-like protein, contains 3 tandem gene copies, and a cluster of five defensin genes is located within 450 kb on human chromosome 8p23 (Linzmeier et al., 1999).

It is of interest to speculate how the BAP gene family has arisen, and, in view of the putative role of BAPs in plant defence, to try to establish the overall family size and extent of diversity represented in it. The restriction of BAP sequences to maize, sorghum, and teosinte implies that the founder member of the BAP gene class arose in a common progenitor of the Zea and Sorghum groups in the tribe *Andropogoneae*. In this connection, it is intriguing that the proximity of *bap1* and *bap3* on chromosome 10 has been maintained, although sequence divergence between the two sequences is extensive (random matching probabilities of $1.e^{-7}$ to $1.e^{-10}$). It is possible that the divergence is a consequence of positive selection for resistance to evolving fungal pathogen populations.

BAP2 protein is detected in two cellular locations

An immunocytochemical localization indicated that BAP2 is accumulated at two sites in the developing caryopsis; the transfer layer and the placentochalaza. Presumably the placentochalazal cell walls preferentially bind apoplastic BAP2 which diffuses from the site of synthesis. It may be that BAP2 can more effectively carry out its role of inhibition of fungal infection if it is located in the maternal tissue, as the orientation of the placentochalazal cell walls perpendicular to the direction of hyphal growth into the endosperm may be a more effective physical barrier and ensure prolonged contact with the cytotoxic proteins. By sequestering the protein in the sieve element-like placentochalazal cells, possible damage to the plasma membranes of other cells may also be reduced. Turnover of the protein, which takes place well before kernel maturity, may also be facilitated by this location.

Cells of the transfer layer contain secretory vesicles, and other proteins are secreted at high concentrations into the cell walls, where they may be tightly bound, as seen for BETL1 (Hueros et al., 1995). BAP2 protein, in contrast, is not tightly bound. Although accumulating in the cell wall fraction, it can be readily eluted from œll wall preparations under low salt conditions (Figure 6b). Sugar and amino acid uptake into transfer cells occurs via H^+-importing symporters. It is possible that release of high concentrations of BAP2 by these cells contributes to the regulation of extracellular pH, and thereby solute flux.

Processing of BAP2

Antiserum raised to E.coli-expressed BAP2 protein detects two peptides, of 8 and 4.5 kDa, in endosperm extracts. Protein sequencing revealed the 4.5 kDa peptide is derived from the 8 kDa species by proteolytic cleavage. Subcellular fractionation of endosperm homogenates showed the 8 kDa peptide to be restricted to microsomal membrane fractions (Fig. 6b). In contrast, the mature peptide was recovered from the 80,000g supernatant, i.e., it is not membrane-bound. These findings suggest the processing event giving rise to the mature 4.5 kDa species takes place within the trans-Golgi network (TGN). Similar processing events are seen for other secreted antimicrobial proteins including 4-Cys peptides (Tailor et al. 1997) and proteinase inhibitors (Atkinson et al., 1993). In contrast, vacuolar processing events are involved in the maturation of vicilin-class seed storage proteins (Jung et al., 1998), although some of the products can also be further cleaved during germination to yield antimicrobial peptides (Chung et al., 1997, Marcus et al. 1999).Thionin precursors were processed in barley leaves by a vacuolar proteinase to give a 5 kDa N-terminal peptide with antifungal properties, and a 6.8 kDa C-terminal peptide which is degraded in vivo (Romero et al., 1997). The proteinase responsible differed in its site specificity and inhibitor sensitivity to that reported for vicilin maturation.

Incubation of endosperm extracts with a series of protease inhibitors (Fig. 6c) indicates a serine-type protease is responsible for propeptide cleavage. In contrast to reported substrates for the TGN-localized Kex2-like protease of plants, which is also a serine protease (Jiang and Rogers, 1999), BAP2 does not possess two adjacent basic residues at the predicted cleavage site (Fig. 1). There is little conservation of sequence among BAP proteins at the BAP2 proteolytic cleavage site, suggesting that site specificity may be conferred at the tertiary structure level, for example, by the formation of an exposed loop prone to cleavage.

Mechanism of action of BAP2

The production of a mature peptide by propeptide processing has been directly demonstrated for BAP2 and BAP1 (Serna, unpublished) of the BAP proteins analysed to date. Synthesis as a propeptide may protect the host cells from possible cytotoxic action; the processing event may also provide a regulatory step in response to pathogen ingress. A pathogen-induced form of the Kex-like plant protease has been identified (Tornero et al., 1996), which could mediate such a regulation. The effect of the maturation step on the biological activity of BAP2 (Table 3) is dramatic, converting it to a potent plant antifungal protein. We have estimated a BAP2 concentration of 10µM in the transfer layer at 16 DAP, which is comparable to the IC_{50} values for susceptible fungal species in the bioassay. The biological activity *in vivo* is also likely to be further potentiated by combinatorial effects with other transfer layer-specific antimicrobial proteins such as BETL1 and BETL3.

Evidence that BAP2 acts by disrupting plasma membrane integrity was provided by incubation of BAP2-treated fungal cultures in the presence of SYTOX green, a dye that is only taken up by cells possessing permeabilized membranes (Roth et al., 1997). The mode of action of BAP2 therefore involves damage to the fungal plasma membrane, as is seen for other antifungal plant proteins, such as the plant defensins (Thevissen et al., 1999). Experiments reported here do not allow us to further define a membrane target or mechanism. Fusarium species are reported to spread *in planta* by movement of conidia through phloem vessels. Therefore, secretion of an antifungal protein into the placentochalaza may prevent passage of conidia to the endosperm and be an effective way of preventing the initiation of an infection there.

Experimental procedures

Molecular biological methods

Standard DNA and RNA methods were carried out as described in Sambrook et al (1989).

Western blotting and immunoblot detection

Proteins were fractionated by SDS-PAGE electrophoresis according to Laemmli (1970), and electroblotted onto polyvinylidine difluoride (PVDF) membranes in a tank blotter. Proteins were detected using enhanced chemiluminescence (ECL, Amersham) and either exposure to NAR-5 X-ray film (Kodak) or for quantitation, image capture using the Lumi-imager (Boehringer).

Purification of BAP2 forms by immunoaffinity chromatography and NH2-terminal sequencing

To extract cytoplasmic proteins, 12-15 DAP maize kernels were ground in a Waring blendor with a minimum volume of extraction buffer (20 mM sodium phosphate pH 7.1, 1 mM EDTA, 1 mM phenylmethylsulphonylfluoride (PMSF), 1µg/ml proteinase inhbitors (Pepstatin, Leupeptin, and Aprotinin). The debris was removed by centrifugation at 100,000 x g, 4°C for 1 h.

´BAP2 antiserum and the corresponding preserum were bound to AffiGel-10 beads (BioRad), according to the suppliers recommendations. 1.5 ml of antiserum was used for 1 ml of resin. For immunoprecipitation, solutions were brought to 200 mM NaCl and 0.2% Triton X-100. Affigel-10-bound antisera were

added at a 1:50 fold dilution, and proteinase inhibitors to 1µg/ml, as before. The mixture was shaken gently at 4°C for 3 h. After incubation, the resin was packed into a column and washed with 80 volumes of buffer I (20 mM sodium phosphate, pH 7, 1 mM EDTA, 1 mM PMSF, 200 mM NaCl, 0.2% Triton X-100, 1µg/ml proteinase inhibitors as before), followed by 100-200 column volumes of buffer II (20 mM sodium phosphate pH 7, 1 mM EDTA, 1 mM PMSF, 1µg/ml proteinase inhibitors), until the A_{280} of the eluant was zero. Bound proteins were then eluted with 1 ml aliquots of ice-cold 200 mM glycine, pH 2.7, and immediately neutralised with 1/20th volume of 2M Tris base. Proteins were separated by electrophoresis on a 16% SDS-PAGE gel, and electroblotted onto Immobilon-PVDF membrane (Millipore). The membrane was stained with Coomassie Blue, and destained for 10 min. with 10% v/v acetic acid before excising bands for sequencing.

BAP2 isolation, refolding and digestion

BAP2 was purified denatured using the COOH-terminal His tag. The protein in inclusion bodies was redissolved in 6 M guanidine.HCl / 0.2 M acetic acid. To refold the protein, the solution was first diluted 1:1 with 8 M urea, 0.1 M Na_2HPO_4 and 0.01 M Tris pH 8.0. Then the preparation was dialysed against: (4 M urea, 0.1 M Na_2HPO_4 , 0.01 M Tris pH 8.0), 2 changes, for 16 hrs, (4 M urea, 0.1 M Na_2HPO_4 , 0.01 M Tris pH 8.0), 2 changes for 8 hrs, 3 x 0.1 M Tris pH 8.0 for 16 hrs, and 3 x H_2O for 16 hrs. Digestion was carried out in 0.8 ml of 0.2 ml 1 M potassium phosphate pH 7.10, 4 µl 0.25 M EDTA pH 8.0 containing BAP2 at 1 to 3 mg.ml^{-1}. To this was added 40 µl aliquots of a >200 kDa size fraction from a non-denaturing extract of 15-16 DAP maize kernels prepared in 20 mM Potassium Phosphate pH 6.0, 1 mM EDTA, 10 mM 2-mercaptoethanol. The solution was incubated at 37°C until the digestion had reached > 90%. The preparation was purified by size-fractionation on a Superose-12 column in the same buffer. Fractions containing BAP2 were located by immunoblotting, pooled, dialysed against water, and concentrated by lyophilization.

Immunolocalization

Maize kernels were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.2, for 12 to 24 hr depending on the tissue volume. Samples were dehydrated and embedded in lowycryl for electron microscopy or fibrowax for light microscopy (Plano GmbH, Marburg, Germany). For light microscopy immunolocalization, sections (8 to 10 µm) were deparaffinized and blocked with 1% bovine serum albumin in PBS (10mM sodium phosphate, 150mM NaCl, pH 7.4) for 20 min at room temperature and incubated overnight with anti-BAP2 antiserum or preimmune serum (both diluted 1:400). The immunoreaction was detected with using horseradish peroxidase-coupled second antibody (Sigma A6154; diluted 1:800) and 3-3´-diaminobenzidine as the substrate. For immunolocalization by electron microscopy, grids were incubated for 1 hr with anti-BAP2 or preimmune serum (both diluted 1:1000). The second antibody was coupled to 10nm gold particles (Sigma G7402) and sections were counterstained with saturated Uranyl-acetate for 30 min.

In situ hybridisation

Immature (15 DAP) maize kernels were fixed in 4% formaldehyde, 0.1% glutaraldehyde in 0.1M sodium phosphate, pH 7.2 for 16 h. Seeds were dehydreted in a graded ethanol-xylene series and embedded in Fibrowax (Plano GmbH, Marburg, Germany). Thick sections (10µm) were cut using a microtome (Reichler-Jung, Nussloch, Germany) and then transferred to glass slides that had been treated with 3-aminopropyltriethoxylane.

Digoxigenin-labeled antisense and sense probes were synthesized using T3 and T7 polymerases (Boehringer-Mannheim, FRG) and partially hydrolyzed (200 bases) with sodium carbonate. In-situ hybridization was carried out essentially as described by De Block and Debrouwer (1993). Before hybridization, the sections were deparaffinized and treated with 5µg/mL proteinase K (Merck). Mounted slides were incubated with 40 to 80 ng of RNA per slide for 16 h at 50°C. After hybridization, the sections were incubated with 40µg/mL RNase A at 37`C for 10 min and washed several times in 2 x SSPE.

For immunological detection, the sections were subsequently treated with 0.5% (w/v) blocking reagent (Boehringer-Mannheim) and 1% (w/v) BSA in TNT (100 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.3%(v/v)Triton X-100). Following the blocking reaction, sections were incubated with antidigoxigenin-alkaline phosphatase conjugate (Boehringer-Mannheim, 1:3000 dilution) in 1% BSA solution at room temperature for 1h, washed several times, and rinsed with alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5,100 mM NaCl and 50mM $MgCl_2$). The colour reaction was performed in alkaline phosphatase buffer containing nitroblue tetrazolium chloride/5-bromo-4-chloro-indolyl phosphate. Sections were than stained with 0.01% safranin, dehydrated in graded ethanol and mounted with DPX mounting medium (Agar Scientific, Stansted, UK).

Subcellular fractionation

Fractionation of subcellular components was performed according to Nagahashi and Hiraike (1982). Eight g B37 endosperm, harvested at 14 days after pollination (14 DAP) was ground in liquid nitrogen in a pestle and mortar, and suspended in 20ml extraction buffer, (EB), (0.5M sucrose, 5 mM EDTA, 5 mM DTT, 30 mM Tris/MES pH7.7). The suspension was filtered through 1 layer of Miracloth (Calbiochem), the retained fraction being treated as crude cell walls (cw), and the filtrate was centrifuged at 1000g for 5 minutes to yield pellet 1 (P1) and supernatant 1 (S1). S1 was pelleted at 6000g for 20 min to give pellet 2 (P2) and supernatant 2 (S2). S2 was pelleted at 80,000g for 2 h. giving rise to pellet 3 (P3) and supernatant 3 (S3). The P3 pellet was resuspended in 1 ml pellet buffer (0.25M sucrose, 1 mM DTT, 3 mM Tris/MES pH7.2).

***In vitro* antifungal activity assay**

Microorganisms. Fungal strains used in this study are *Alternaria brassicicola* MUCL 20297, *Aspergillus flavus* var*. flavus* CBS 111.45, *Botrytis cinerea* MUCL 30158, *Fusarium moniliforme* CBS 218.76, *Fusarium culmorum* MUCL 30162, *Neurospora crassa* FGSC 2489, *Plectosphaerella cucumerina* (provided by Dr.

B. Mauch-Mani, Université de Fribourg, Switzerland) and *Verticillium dahliae* MUCL 19210. Filamentous fungi were grown on six-cereal agar, and conidia were harvested as described previously (Broekaert et al., 1990). Conidia spore stocks in 20% (vol/vol) glycerol were at a final concentration of $2x10^7$ conidia/ml.

Antifungal activity assay. Antifungal activity of BAP proteins against fungi was assayed by microsprectrophotometry of liquid cultures grown in microtiter plates as described previously (Broekaert et al., 1990; Terras et al., 1992). Briefly, in a well of a 96-well microplate, 10 µl of the protein sample was mixed with 90 µl of either half-strength potato dextrose broth (1/2 PDB; Difco, Detroit, MI) or ½ PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl, containing fungal spores at a concentration of $2x10^4$ conidia/ml. Growth was recorded after 24h of incubation at 22°C. The absorbance at 595 nm served as a measure for microbial growth (Terras et al., 1992). IC_{50} values (i.e. the concentration of the antifungal protein required to inhibit 50% of the fungal growth) were calculated from dose-response curves with twofold dilution steps (Terras et al., 1992).

SYTOX Green uptake

Fungal membrane permeabilization was measured by SYTOX Green uptake as described previously (Thevissen et al., 1999). Fungi were grown at an inoculum density of 2×10^5 conidia/ml in 12 g of potato dextrose broth per liter (Difco) with continuous shaking (200 rpm) at 22°C. After 16 h of incubation, hyphae were washed with SMF1 (SMF1 is synthetic medium for fungi, containing 50 µM $MgSO_4$, 50 µM $CaCl_2$, 5 µM $FeSO_4$, 0.1 µM $CoCl_2$, 0.1 µM $CuSO_4$, 2 µM Na_2MoO_4, 0.5 µM H_3BO_3, 0.1µM KI, 0.5 µM $ZnSO_4$, 0.1 µM $MnSO_4$, 10 g/l glucose, 1 g/l asparagine, 20 mg/l methionine, 2 mg/l myo-inositol, 0.2 mg/l biotin, 1 mg/l thiamine-hydrochloride, 0.2 mg/l pyridoxine-hydrochloride, 0.5 mM K_2PO_4). Ninety µl aliquots of the fungal suspension, supplemented with 0.2 µM SYTOX Green, were mixed with 10 µl of the same medium containing BAP proteins, and incubated in white 96-well microplates (PE white, Perkin Elmer, Norwalk, CT). After incubation for 30 min at 22 °C with periodic agitation, fluorescence emitted by the cells in the microplates was measured using a Perkin Elmer fluorescence spectrometer LS 50 B at an excitation wavelength of

488 nm (slit: 10 nm) and an emission wavelength of 540 nm (slit: 5 nm). Fluorescence values of the samples were corrected by subtracting the fluorescence value of a culture in the same medium in absence of the peptides but in presence of SYTOX Green. Absolute values of fluorescence did not differ more than 50% in independent tests performed under identical conditions.

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Tables and Figures

Table 1. Random matching probabilities for pairwise matched BAP amino acid sequences using BLAST2 (Tatusova and Madden, 1999)

	Bap2	bap1a	bap1b	bap3a	bap3b
bap2	0	6e-07	1e-07	1e-10	6e-09
bap1a		0	2e-28	2e-07	3e-07
bap1b			0	3e-08	3e-07
bap3a				0	5e-38
bap3b					0

Table 2. Suggested nomenclature for the Basal layer-type antifungal protein family

Clone working name	locus terminology	locus position
pXa8	bap1-a	Ch. 10L, interval 53-55
pX35	bap1-b	Ch. 10L, interval 53-55
BETL2	bap2	Ch. 4, coordinate 62-60
pX68	bap3-a	Ch. 10L, interval 53-55
pX81	bap3-b	Ch. 10L, interval 53-55

Table 3 Antifungal activity of BAP2 and BAP2 Propeptide against various fungal strains

	IC_{50}, (µg/ml)[a]			
	Medium A[b]		Medium B[c]	
Fungal strain	BAP2	BAP2 Propep.	BAP2	BAP2 Propep.
Alternaria brassicicola	3	> 80	20	> 80
Aspergillus flavus	2	nd	nd	nd
Botrytis cinerea	20	> 80	20	> 80
Fusarium culmorum	2	> 80	4	> 80
Fusarium moniliforme	6	nd	nd	nd
Neurospora crassa	5	80	20	> 80
Plectosphaerella cucumerina	3	80	20	> 80
Verticillium dahliae	2	> 80	10	> 80

[a] IC_{50} values (i.e.concentration of the BAP protein that is required to inhibit 50% of the fungal growth) were determined from dose-response curves with two-fold dilution steps. nd = not determined

[b] Medium A = ½PDB

[c] Medium B = ½PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl

Figure legends

Figure 1

Multiple sequence alignment of the primary translation products of BAP proteins

Analysis performed using CLUSTALW (Thompson *et al* 1994). Black shaded letters indicate identical amino acids and gray shaded letters indicate homologous amino acids. The shading sequence fraction corresponds to 50%. Arrowheads denote the first residue C-terminal of cleavage sites determined for protein maturation. The sequences have been deposited in the EMBL nucleotide sequence database under the following accession numbers: AJ297901(bap1a), AJ297900(bap1b), AJ297902(bap3a), AJ297903(bap3b), and AJ133529 (bap2).

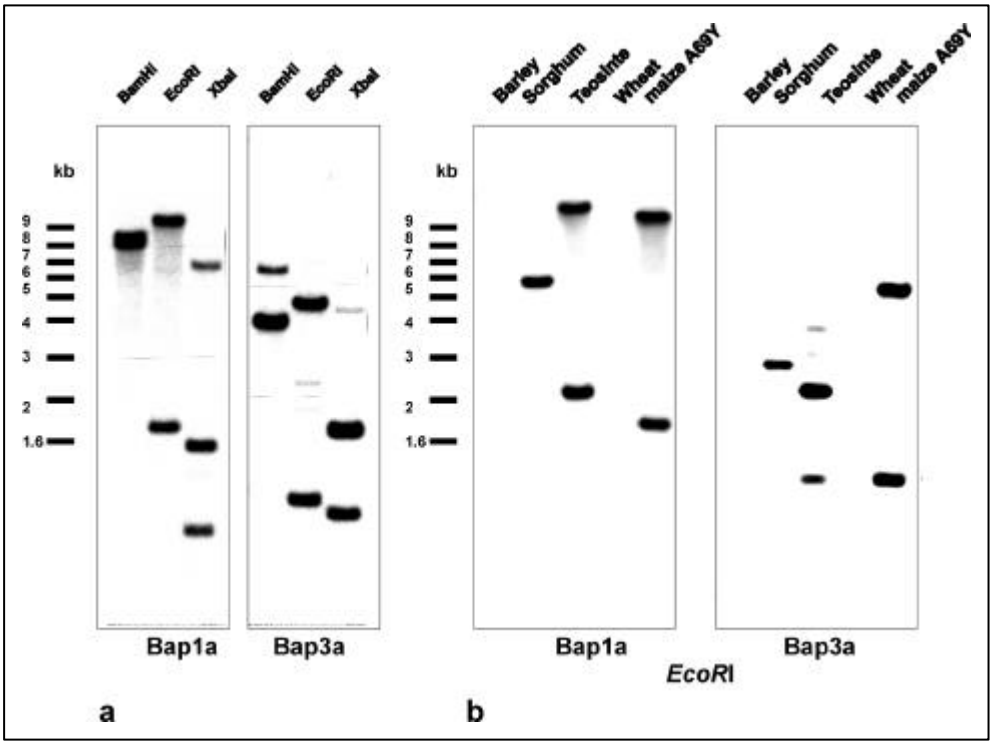


Figure 2

Genomic organisation of *bap* loci

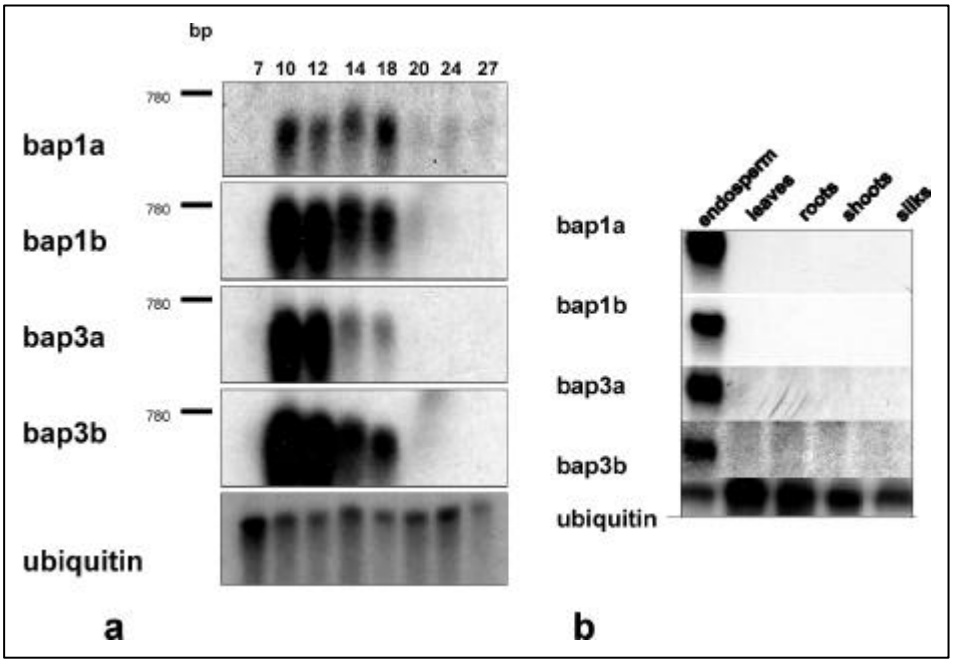
a.- The BAP genes constitute a small gene family in maize.

Maize genomic DNA from the variety A69Y (10µg per lane) was digested with the enzymes indicated, and fractionated in a 1% agarose gel. Southern blots were hybridised either with Bap1a, or Bap3a cDNA inserts, as indicated. The positions of size markers are given in Kb

b.- Presence of BAP-related sequences in other cereals

Genomic DNA from different species (10µg per lane): barley (*Hordeum vulgare*), Sorghum (*Sorghum bicolor)*, Teosinte (*Zea luxurians),* Wheat (*Triticum aestivum)* and Maize (*Zea mays* line A69Y), was digested with *EcoR*I and fractionated on a 1% agarose gel. The resulting Southern blots were hybridised with Bap1a or Bap3a, as indicated. The positions of size markers are given in kb.

Figure 3



Sites of expression of BAP transcripts

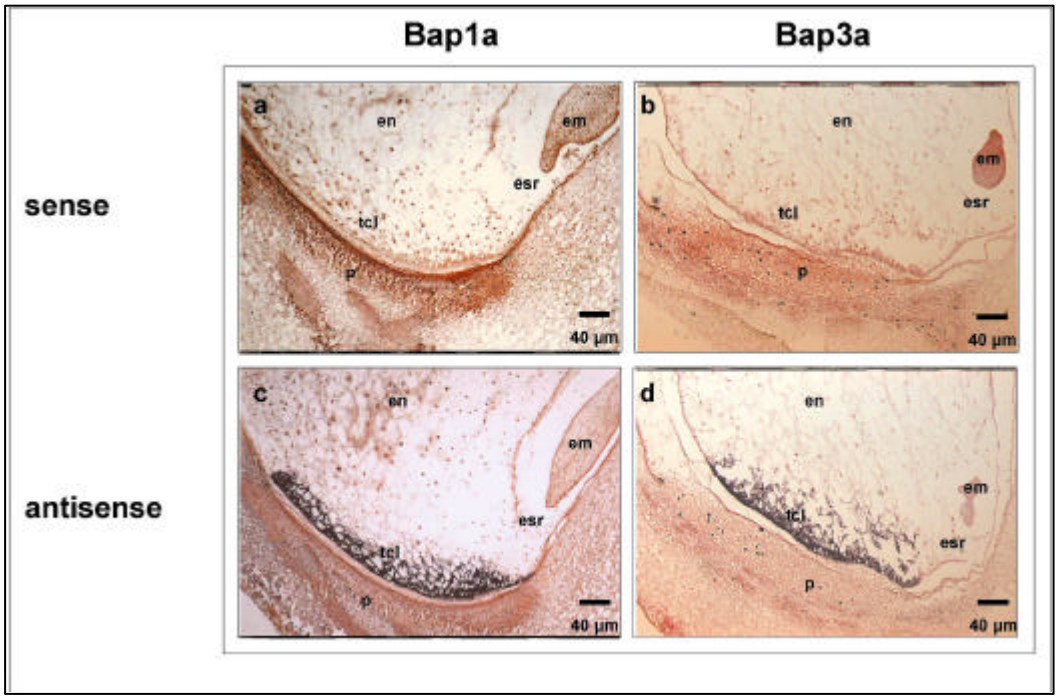
a.- Accumulation of BAP family transcripts during endosperm development.

2µg mRNA samples from kernels harvested at 7 to 27 DAP , as indicated, were fractionated on a denaturing formaldehyde gel. The Northern blot was hybridised with the BAP probes indicated on the left.

b.- Tissue specificity of expression of BAP transcripts.

2 µg of messenger RNA from different tissues as indicated, were fractionated on a denaturing formaldehyde gel. The resulting Northern blot was probed sequentially with the indicated BAP probes

Figure 4 *in situ* hybridisation of *bap* probes to 15 DAP A69Y kernel sections of (a)sense and (c) antisense BAP1a cDNA probes, and (b) sense and (d) antisense BAP3a cDNA Probes; en, endosperm; esr, embryo surrounding region; tcl, transfer cell layer; em, embryo; p, pedicel.



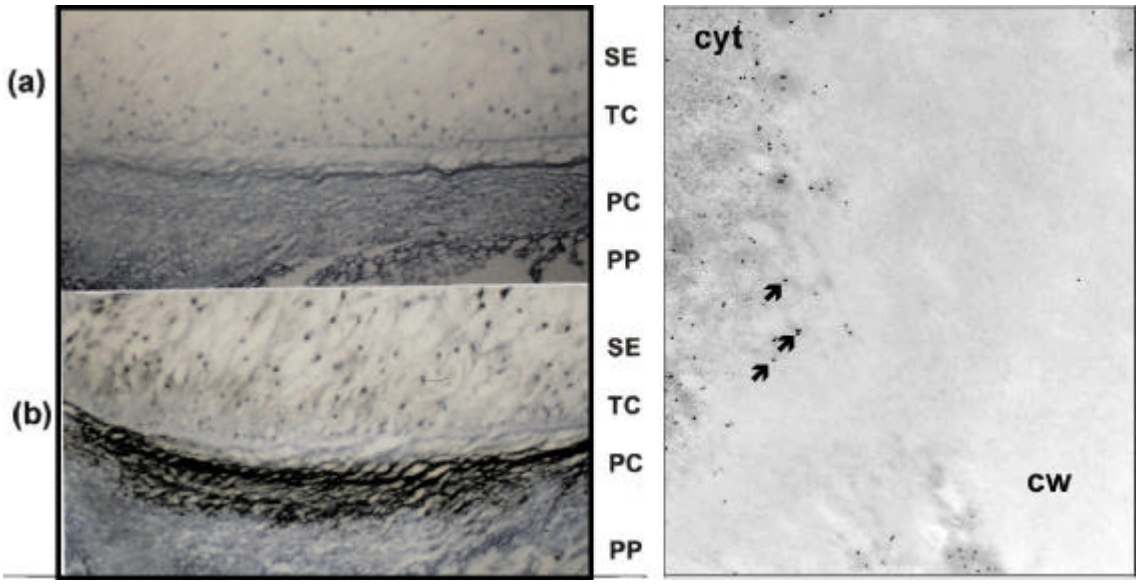


Figure 5. **Immunolocalisation of BAP2 protein.** Left-hand panel) Immunochemical localisation of BAP2 protein on paraffin wax sections of developing maize kernels. (a), incubation with preserum, (b), incubation with anti-BAP2 serum. Black staining indicates the presence of BAP2 antigen. SE starchy endosperm, TC transfer cells, PC placentochalaza, PP phloem parenchyma.

Right-hand panel) Immunogold localisation of BAP2 protein by transmission electron microscopy. Gold particles (15 nm) were located in close proximity to the plasma membrane, not within the matrix of the cell wall (cw). cyt = cytoplasm.

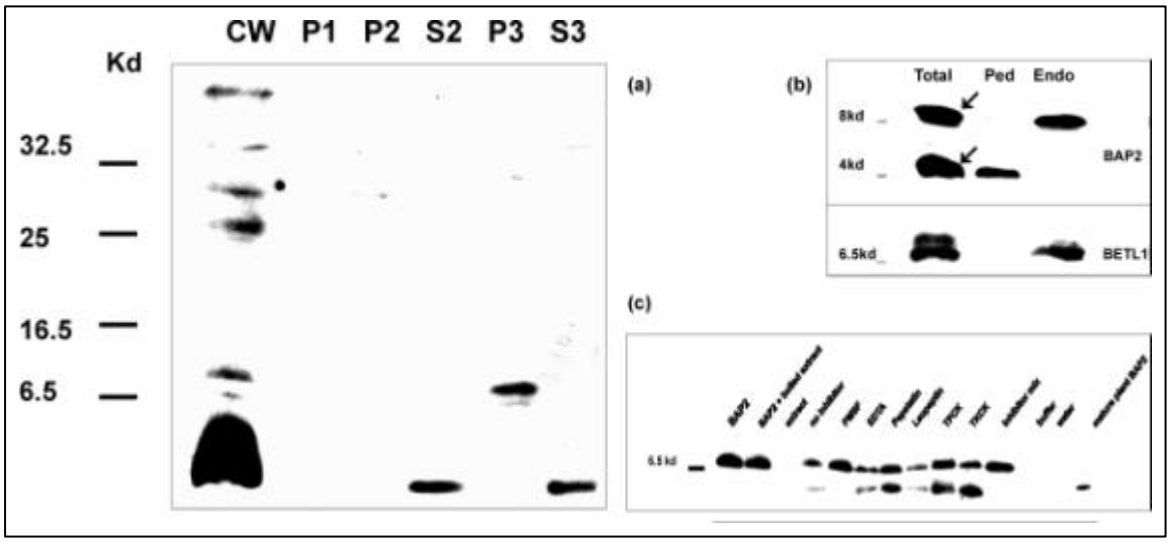


Figure 6(a).**Conversion of BAP2 propeptide to mature peptide form.** Localisation of BAP2 protein forms by subcellular fractionation. Immunoblot probed with anti-BAP2: from left to right, 50µg protein from cw, cell wall fraction, P1, 1000 x g pellet, S1, 1000 x g supernatant, P2, 6000 x g pellet, S2, 6000 x g supernatant, P3, 80,000 x g pellet, S3, 80,000 x g supernatant. (b) **Distribution of BAP2 protein forms in pedicel and basal endosperm**. Immunoblot probed with anti-BAP2: from left to right: 50µg protein from whole endosperm. (c) ***In vitro* protease cleavage of BAP2 propeptide**: Effect of different proteinase inhibitors on propeptide cleavage.

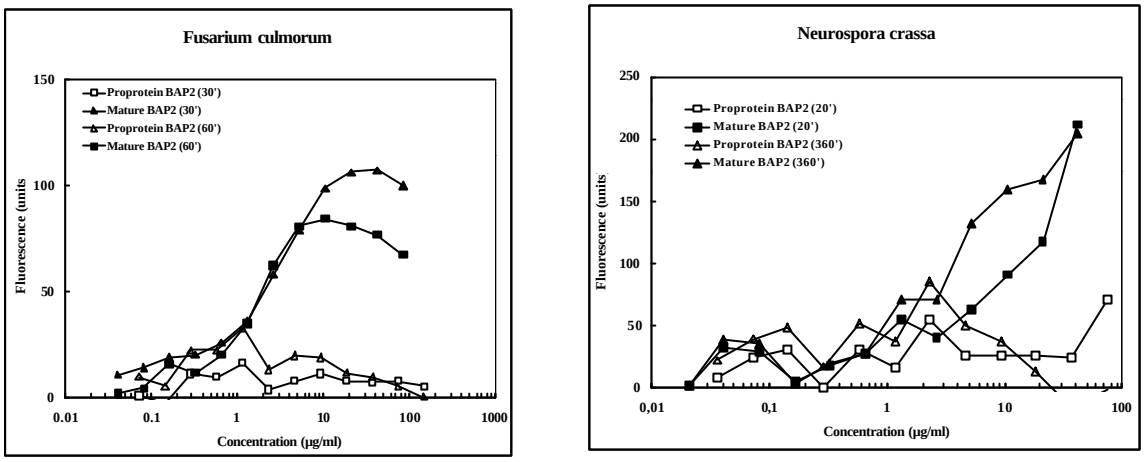


Figure 7. **Membrane permeabilization induced by BAP2 and BAP2 Propeptide on *Fusarium culmorum* and *Neurospora crassa*.** Dose-response curves are presented for membrane permeabilization, measured by SYTOX green fluorescence, of *A)*, *F*. *culmorum* and *B)*, *N. crassa* incubated with BAP2 (Δ) and BAP2 Propeptide (□). Fungi were suspended in SMF1 supplemented with 0.2 µM SYTOX green. After incubation in the presence of BAP proteins for 30 min, fluorescence was measured. Data are means of duplicate measurements and correspond to one representative experiment of two.

Appendix 2

BAP family sequence DATA

(Analysis performed with the Programme Package *"Wisconsin Package Interface"* (Version 10.0-UNIX,1999) (Devereux *et al.,* 1984)

```
Bap1a
-42 taaacacattgttagtattagacactttctagttcatcacccatggcaaagttcttcaac
   1                                              M  A  K  F  F  N
19  tacaccatcatccaaggactcttgatgctttccatggtacttctggcatcgtgcgctatt
   7 Y  T  I  I  Q  G  L  L  M  L  S  M  V  L  L  A  S  C  A  I
79  catgcacacataataagtggggaaactgaagaggttagcaacacagggagcccgacagtg
  27 H  A  H  I  I  S  G  E  T  E  E  V  S  N  T  G  S  P  T  V
139 atggtcacgatgggggcaaaccgaaagataattgaagataataaaaatttattgtgctat
  47 M  V  T  M  G  A  N  R  K  I  I  E  D  N  K  N  L  L  C  Y
199 ctaagggctctagagtactgttgtgcaaggaccagacaatgctatgatgacataaagaaa
  67 L  R  A  L  E  Y  C  C  A  R  T  R  Q  C  Y  D  D  I  K  K
259 tgcttggagcattgccgtggttgaaaggtgtaataaaggatatgctttctttggatctta
 107 C  L  E  H  C  R  G
319 gttggtgatcagaaaaatgttctaccaattcatgcatgtaccaaaggataatataagctg
379 ctcaaaaaatcgtggttaattagagtttttgttggaaaaatgataacttccttacaaac
439 atataccaaattaaagagaccctcttaatgcatgatctatcttttaataaagaacatatt
499 ccatagt

>Bap1b
-39 ttcaactacaccatcgtccaaggactcttaatgctttccatggtacttctggcatcatgt
   1                                          M  V  L  L  A  S  C
 22 gtcattcatgcacacataataagtggggaaattgaagatgttagcaacactaggagcccg
   8 V  I  H  A  H  I  I  S  G  E  I  E  D  V  S  N  T  R  S  P
 82 acaatgatgggggcaaatcgaaagataattggagataataaaaatttgttgtgctatcta
  28 T  M  M  G  A  N  R  K  I  I  G  D  N  K  N  L  L  C  Y  L
142 aaggctctagagtattgctgtgaaaggaccaaacagtgctatgatgacataaagaagtgc
  48 K  A  L  E  Y  C  C  E  R  T  K  Q  C  Y  D  D  I  K  K  C
202 ttggagcactgccatagttgaaaggtgtaataaaacgatatgctttctttggatcttagt
  68 L  E  H  C  H  S
262 tggtgattagaaaaatgttctaccaattcatgcatgtaccaaaggataatataagctgct
322 caaaaaatcatggtt

>Bap3a
-36 ttcagacaaacacgccatttgtattattattagctcttatccatcacaatggtgaagatc
   1                                                  M  V  K  I
 13 ctagatcacataagcatccgagggttctttctacttttcatggttcttgtggcatccttt
   5 L  D  H  I  S  I  R  G  F  F  L  L  F  M  V  L  V  A  S  F
 73 gttggtcatgcacagataataagaggtgaaaccaaagaggacaacgacaccaagagcatg
  25 V  G  H  A  Q  I  I  R  G  E  T  K  E  D  N  D  T  K  S  M
133 acgatgacaacaatgagaccaggaagctatgtaactagcatggatgaaaaatctagcttg
  45 T  M  T  T  M  R  P  G  S  Y  V  T  S  M  D  E  K  S  S  L
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  65 C  F  E  D  I  K  T  L  W  Y  I  C  R  T  T  Y  H  L  Y  R
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  85 T  L  K  D  C  L  S  H  C  N  S  M
313 aactaataagaggccatgctttctcaagattgtagattgagatggtgaagatgttctacc
373 caatcatccatgcatgtaccaagggataaaaaactataagattaattattagttatcatt
433 aagtgttcccagctttaggaaatgtcattttctcaagagtattttcgttgtgtgcgatta
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>Bap3b
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   1                        M  V  K  S  L  D  H  I  T  I  R  G
 37 ctctttctacttttatgtttcttgtggcgtcctttgttggtcatgcacagataataaga
  13 L  F  L  L  F  M  F  L  V  A  S  F  V  G  H  A  Q  I  I  R
 97 ggtgaaaccaaggagaataaggacactaacagcatgacgatgacaacaagaccaggaagc
  33 G  E  T  K  E  N  K  D  T  N  S  M  T  M  T  T  R  P  G  S
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  53 Y  V  I  S  M  D  E  K  S  S  L  C  F  L  D  P  R  T  L  W
217 tacatctgcaaaataacatatcgccttttaggacattgaaggattgcttggagttttgc
  73 Y  I  C  K  I  T  Y  R  L  F  R  T  L  K  D  C  L  E  F  C
277 cacagtatatgatgcaccatgttggaaaaaaagactaataagaggccatatgctttctca
  93 H  S  I
337 agattgtagatggagatggtgaagatgttatacccaatcatccatgcatgtaccaaggga
397 taataaactataagacaacttatcacttattattaagtgttccctattttaggaaatgtc
457 attttctcaagagtattttctttgtatgcgattatgaaagtttcaaatttcatagatgag
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PAIRWISE COMPARISON OF BAP1a and BAP3a DNA sequences

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 22 tattattattag.....ctcttatccatca.caatggtgaagatcctaga 65
              .         .         .         .         .
 57 ctacaccatcatccaaggactcttgatgctttccatggtacttctggcat 106
      |||  | ||||| |||  ||||  | |||| |||||| ||| ||||||
 66 tcacataagcatccgagggttctttctacttttcatggttcttgtggcat 115
              .         .         .         .         .
107 cgtgcgctattcatgcacacataataagtggggaaactgaagaggttagc 156
    | |  | |  ||||||||| |||||||| || |||||  ||||||  | |
116 cctttgttggtcatgcacagataataagaggtgaaaccaaagaggacaac 165
              .         .         .         .         .
157 aacacagggagcccgacagtgatggtcacgatgggggcaaaccgaaagat 206
     ||||   ||||  |||   ||||   || ||| |  ||    |  |  |
166 gacaccaagagcatgac...gatgacaacaatgagaccaggaagctatgt 212
              .         .         .         .         .
207 aa.ttgaagataataaaaattta..ttgtgct......atctaagggctc 247
    || | | |   |  |||||| ||  |||||||      || |||   ||
213 aactagcatggatgaaaaatctagcttgtgctttgaggatataaaaactt 262
              .         .         .         .         .
248 tagagtactgttgtgcaaggaccagacaatgctatgatgacataaagaaa 297
    ||  ||||   ||   ||  ||    ||    |||       | ||| |
263 tatggtacatctgcagaacaacttatcacctttataggacattgaaggat 312
              .         .         .         .         .
298 tgcttggagcattg.................ccgtggttg....aaaggt 326
    ||| ||  ||||||                 || || | |    ||||
313 tgcctgtcgcattgcaacagtatgtgatgcaccatgttggaaaaaaagaa 362
              .         .         .         .         .
327 gtaataa.aggatatgctttctttggatcttagttggtgatcagaaaaat 375
     |||||| |||  |||||||||   |||  ||| | | |||    || ||
363 ctaataagaggccatgctttctcaagattgtagattgagatggtgaagat 412
              .         .         .         .
376 gttctacc....aattcatgcatgtaccaaaggataatata 412
    ||||||||     || ||||||||||||| |||||| | |
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PAIRWISE COMPARISON OF BAP1a and BAP3b

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                .         .         .         .         .
  60 gctctttctacttttatgtttcttgtggcgtcctttgttggtcatgcac 109
      |||||  | ||||  ||| | ||| |||| || |  | |  ||||||||
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                .         .         .         .         .
 110 agataataagaggtgaaaccaaggagaataaggacactaacagcatgac. 158
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 159 ..gatgacaacaagaccaggaagctatgtaattagcatggatgaaaaatc 206
       ||||   || |     | || |      || |     ||| | |||
 175 gtgatggtcacgatgggggcaaaccgaaagataattgaagataataaaaa 224
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                .         .         .         .         .
 256 acatatcgccttttaggacattgaaggattgcttggagtttgccacag 305
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 269 ccagacaatgctatgatgaca.taaagaaatgcttggagcattgcc.... 313
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 314 ..............gtggttgaaaggtgtaataaagg...atatgctt 344
                .         .         .         .         .
 356 tctcaagattgtagatggagatggtgaagatgttatacccaatcatccat 405
     |||   |||  ||| ||| |||    || ||||| ||||     || |||
 345 tctttggatcttagttggtgatcagaaaaatgttctacc....aattcat 390
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 406 gcatgtaccaagggataataaa 427
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PAIRWISE COMPARISON OF BAP1b and BAP3b DNA sequences

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  8 acaccatcgtccaaggactcttaatgctttccatggtacttctggcatca 57
              .         .         .         .         .
 94 tttgttggtcatgcacagataataagaggtgaaaccaaggagaataagga 143
    | |||   ||||||||| |||||||| || ||||   | ||   ||   |
 58 tgtgtcattcatgcacacataataagtggggaaattgaagatgttagcaa 107
              .         .         .         .         .
144 cactaacagcatgacgatgacaacaagaccaggaagctatgtaattagca 193
    |||||  |||  ||| |||   |   |  ||    |  |  ||||| |
108 cactaggagcccgacaatg...atgggggcaaatcgaaagataattgg.. 152
              .         .         .         .         .
194 tggatgaaaaatctagcttgtgctttctggatccaagaactctatggtac 243
      ||| | |||  |   |||||||      ||| ||   |||||  |||
153 .agataataaaaatttgttgtgct......atctaaaggctctagagtat 195
              .         .         .         .         .
244 atctg.caaaataacatatcgccttttaggacattgaaggattgcttgg 292
      |||  |||  | || |  |   | | | |||| | ||| | |||||||
196 tgctgtgaaaggaccaaacagtgctatgatgaca.taaagaagtgcttgg 244
              .         .         .         .         .
293 agttttgccacagtatatgatgcaccatgttggaaaaaaagactaataag 342
    ||   ||||| |||                     ||| |  ||||||
245 agcactgccatagt...................tgaaaggtgtaataaa 274
              .         .         .         .         .
343 aggccatatgctttctcaagattgtagatggagatggtgaagatgttata 392
    |  | ||||||||||   |||  ||| ||| |||    || ||||| ||
275 a..cgatatgctttctttggatcttagttggtgattagaaaaatgttcta 322
              .         .         .
393 cccaatcatccatgcatgtaccaagggataataaa 427
    ||     || |||||||||||||| |||||||| |
323 cc....aattcatgcatgtaccaaaggataatata 353
```

PAITWISE COMPARISON BAP1a and BAP1b DNA sequences

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     Length Weight:      3   Average Mismatch: -9.000

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```

```
                .         .         .         .         .
     52 ttcaactacaccatcatccaaggactcttgatgctttccatggtacttct 101
        ||||||||||||||| |||||||||||| ||||||||||||||||||||
      1 ttcaactacaccatcgtccaaggactcttaatgctttccatggtacttct 50
                .         .         .         .         .
    102 ggcatcgtgcgctattcatgcacacataataagtggggaaactgaagagg 151
        |||||| || |  |||||||||||||||||||||||||| |||||| |
     51 ggcatcatgtgtcattcatgcacacataataagtggggaaattgaagatg 100
                .         .         .         .         .
    152 ttagcaacacagggagcccgacagtgatggtcacgatgggggcaaaccga 201
        ||||||||||  ||||||||||         | ||||||||||| |||
    101 ttagcaacactaggagcccgaca.........atgatgggggcaaatcga 141
                .         .         .         .         .
    202 aagataattgaagataataaaaatttattgtgctatctaagggctctaga 251
        |||||||||| |||||||||||||| |||||||||||| |||||||||
    142 aagataattggagataataaaaatttgttgtgctatctaaaggctctaga 191
                .         .         .         .         .
    252 gtactgttgtgcaaggaccagacaatgctatgatgacataaagaaatgct 301
        ||| || |||| |||||||| ||| |||||||||||||||||| ||||
    192 gtattgctgtgaaaggaccaaacagtgctatgatgacataaagaagtgct 241
                .         .         .         .         .
    302 tggagcattgccgtggttgaaaggtgtaat.aaaggatatgctttctttg 350
        ||||||| |||| | |||||||||||||| ||| ||||||||||||||
    242 tggagcactgccatagttgaaaggtgtaataaaacgatatgctttctttg 291
                .         .         .         .         .
    351 gatcttagttggtgatcagaaaaatgttctaccaattcatgcatgtacca 400
        ||||||||||||||| ||||||||||||||||||||||||||||||||
    292 gatcttagttggtgattagaaaaatgttctaccaattcatgcatgtacca 341
                .         .         .
    401 aaggataatataagctgctcaaaaaatcgtggtt 434
        |||||||||||||||||||||||||||| |||||
    342 aaggataatataagctgctcaaaaaatcatggtt 375
```

PAIRWISE COMPARISON OF BAP3a and BAP3b DNA sequences

```
        Gap Weight:     50      Average Match: 10.000
     Length Weight:      3   Average Mismatch: -9.000

           Quality:   4342             Length:    564
             Ratio:  7.754              Gaps:      4
Percent Similarity: 90.485   Percent Identity: 90.485

       Match display thresholds for the alignment(s):
                   | = IDENTITY
                   : =   5
                   . =   1
```

```
                .         .         .         .         .
  32 agctcttatccatca.caatggtgaagatcctagatcacataagcatccg 80
       || ||||||||||| | |||||||||| |||||||||||||| ||||||
     7 agatcttatccatcacctatggtgaagagcctagatcacataaccatccg 56
                .         .         .         .         .
    81 agggttctttctacttttcatggttcttgtggcatcctttgttggtcatg 130
       |||| |||||||||||| ||| |||||||||| ||||||||||||||||
    57 agggctctttctacttttatgtttcttgtggcgtcctttgttggtcatg 106
                .         .         .         .         .
   131 cacagataataagaggtgaaaccaaagaggacaacgacaccaagagcatg 180
       |||||||||||||||||||||||| ||| | || ||||| || ||||||
   107 cacagataataagaggtgaaaccaaggagaataaggacactaacagcatg 156
                .         .         .         .         .
   181 acgatgacaacaatgagaccaggaagctatgtaactagcatggatgaaaa 230
       ||||||||||||   |||||||||||||||||| |||||||||||||||
   157 acgatgacaaca...agaccaggaagctatgtaattagcatggatgaaaa 203
                .         .         .         .         .
   231 atctagcttgtgctttgaggatataaaaactttatggtacatctgcagaa 280
       ||||||||||||||  ||||  || |||| ||||||||||||||| ||
   204 atctagcttgtgctttctggatccaagaactctatggtacatctgcaaaa 253
                .         .         .         .         .
   281 caacttatcacctttataggacattgaaggattgcctgtcgcattgcaac 330
        ||| |||| ||||| |||||||||||||||||| ||  |  |||| ||
   254 taacatatcgccttttaggacattgaaggattgcttggagttttgccac 303
                .         .         .         .         .
   331 agtatgtgatgcaccatgttggaaaaaaagaactaataagaggcc..atg 378
       ||||| |||||||||||||||||||||| ||||||||||||||  |||
   304 agtatatgatgcaccatgttggaaaaaaag.actaataagaggccatatg 352
                .         .         .         .         .
   379 ctttctcaagattgtagattgagatggtgaagatgttctacccaatcatc 428
       |||||||||||||||||| |||||||||||||||| ||||||||||||
   353 ctttctcaagattgtagatggagatggtgaagatgttatacccaatcatc 402
                .         .         .         .         .
   429 catgcatgtaccaagggataaaaaactataagattaattattagttatca 478
       |||||||||||||||||||| ||||||||||  | |||| | |||| |
   403 catgcatgtaccaagggataataaactataagacaacttatcacttatta 452
                .         .         .         .         .
   479 ttaagtgttcccagctttaggaaatgtcattttctcaagagtattttcgt 528
       |||||||||||||   |||||||||||||||||||||||||||||||| |
   453 ttaagtgttccctattttaggaaatgtcattttctcaagagtattttctt 502
                .         .         .         .         .
   529 tgtgtgcgattatggaagtttcaaatttcttatatgagacaataaaaaaa 578
       ||| |||||||||| |||||||||||||| || ||||||| |||||||||
   503 tgtatgcgattatgaaagtttcaaatttcatagatgagacgataaaaaaa 552
                .
   579 ctttgtcagtcttc 592
        |||||| ||||||
   553 gtttgtctgtcttc 566
```

Acknowledgments

9.- Acknowledgments

"…nothing can move itself…"
St Thomas Aquinas' Five ways

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Antonio Serna Sanz

• 1.- Datos personales / Personal data / Persönlichen Daten

Nombre / Name / Vorname: **Antonio**
Apellidos / Surname / Nachname: **Serna Sanz**
Nacido / Date of birth / geboren am: 10 / 09 / 72
Dirección / Present address / Anschrift : Kolibriweg 14, 50829 Köln Deutschland
Ocupado en / working place / Beschäftigt im:
Max-Planck-Institut für Züchtungsforschung
Carl-von-Linne Weg 10 50829 Köln DEUTSCHLAND
serna@mpiz-koeln.mpg.de
07 49 221 5062462/442 fax 413

• 2.- Formación / Academic Qualifications / Akademische Ausbildung

1976/1985	Graduado escolar / Basic school / Grundschule
1985/1989	Bachiller / Secondary School / Gymnasium
1989/1995	Ldo en CC Biológicas por UAH con grado en el dpto Biología Celular y genética Degree course in Biology at the UAH, graduate at the genetics Department Diplom-Biologie in der Genetischen Abteilung von UAH
1995	Certificado de aptitud pedagógica Pedagogic aptitude certificate Erstes pädagogisches Zertifikat für Lehrtätigkeit
1995/1996	Curso Master „Garantía y Control de Calidad de productos alimentarios” curso financiado por UAH y FSE Master degree in Food technology supported by UAH and FSE Techniker-Kurs “Qualität und Kontrolle von Nahrungsmitteln “
1997	Curso EMBO / EMBO course / EMBO Kurs „Genome diversity and Genome Expression in Plants” en Universidad de Gante / at Ghent University / in der Universität zu Gent
1997/2001	En la actualidad, doctorando en Max-Planck-institut por la Universidad de Colonia At the present, Phd at the Max-Planck-institut by Cologne University Derzeitig: Doktorand am MPI Köln ***„Isolation and characterization of the bap genes, a new family of maize basal endosperm-specific proteins.”***