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GENETIC DISSECTION OF SEED DEVELOPMENT IN MAIZE

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One of the most interesting aspects, that is still poorly characterized from the genetic point of view, of the development of the higher plants is the formation of the seed.

In this thesis several works conducted on the maize seeds are presented with the aim to better understand some of the genetic processes that lead to the formation of the seed and of the two main compartiments of it: the embryo and the endosperm.

The choice of this plant is due to the fact that it's one of the model species most utilized to study the role of the genes in biosynthetic pathways and plant morphogenetics: thanks to its dimensions, the practices of dissection are easier, and thanks to the abundance of tissues in the embryo, leaves and ear, great quantities of material are available for biochemical and molecular analyses.

Two features of maize plants helped largely genetic studies: the ear size and the development of separated male and female inflorescence.

A normal plant can produce about 4-700 seeds. The plants that sprout from them can easily be crossed or selfed producing a wide progeny particularly useful in genetic studies. This progeny can be obtained in a relative short period, between 100 and 150 days from the sowing.

Moreover the localization of male flowers in the tassel and female flowers in the ear allow to avoid pollutions with undesirable pollen, simply hooding the ear without emasculation.

In this thesis, five different works about maize seeds are described and discussed in different chapters.

Chapter 2 of this thesis is about a work conducted on a collection of nine *emp* (*empty pericarp*) mutants of maize characterized by the estreme reduction of the endosperm tissue and embryo lethals at maturity. With the test of allelism we identified those non complementing (*i.e.* allelic) and those complementing (*i.e.* not allelic) in the F_1 generation. Most results in the F_1 were concordant to those obtained in the F_2 generation with the exception of three cases where the F_1 results suggest allelism (*i.e.* one gene) whereas those in the F_2 suggest segregation of two genes.

These intriguing results seem to suggest an interaction between different *emp* mutants, attributable to a phenomenon that is often referred to as second site non-complementation

(SSNC). In some cases of 9 to 7 segregation in the F_2 generation we recovered two different phenotypes of mutant seeds suggesting the presence of an epistatic interaction between the two mutants explained in detail in the same chapter.

Chapter 3 is about the finding that in some ears segregating for a single *emp* mutant in different genetic backgrounds (A636, W23 and Mo17) some mutant seeds were identified, exhibiting a more abundant endosperm tissue, and occasionally an embryonic axis. In some cases we observed a recovery of germination, although in a low %.

This fact is probably due to one of these two phenomenons: these *emp* mutants probably uncover a cryptic variability and the second possibility is that the improved endosperm of the mutant is the result of an interaction between the mutant and a second factor originally present in the inbred line. To identify the genetic modifier/s we made appropriate crosses: in this way we were allowed to identify a dominant and a recessive modifier.

Chapter 4 deals with a work on a maize mutant that shows defects in the embryogenetic program due to the lack of a Brassinosteroid C-6 Oxidase (Makarevitch *et al.*, 2012). The mutant is ascribable to the *Lil1-1* gene.

The purpose of this work is to find a correlation between brassinosteroids (BRs) production and ear development and to test if this hormones might be involved in controlling the number of seeds per ear and/or the kernel.

Our study shows that *Lil1-1/Lil1-1* homozygous plants produce longer ears with a higher number of seeds if compared to plants that carry the recessive allele in heterozygosis while the data collected for the seed weight did not show any effect of the mutation.

Although these results are preliminary we could hypothesise that the reduction in the levels of brassinosteroids in the heterozygous plants affects ear development and the number of seeds per ear.

The last two chapters (**Chapter 5** and **Chapter 6)** regard two works on two different types of insects that attack the stored products: *Plodia interpunctella* a Lepidoptera Pyralidae and *Rhyzopertha dominica* a Coleoptera Bostrichidae.

Abstract

Two different analysis were conducted: one on whole kernels and another one on longitudinally sectioned kernels in order to test which is the compartment of the seed more attacked, *i.e.* the embryo or the endosperm.

For the tests with *Plodia interpunctella* four genotypes of maize were employed:

B73 reference line, RAlex0 line, two viviparous mutants *vp2* and *vp5* and the *emb*-8908* mutant; while in the tests with *Rhyzopertha dominica* five genotypes were employed: B73 reference line, RAlex0 line, the *sugary1* mutant, the *yellow endosperm1* mutant and the ACR line that segregates colored and colorless seeds.

The results obtained in all the tests indicate that both *Plodia interpunctella* and *Rhyzopertha dominica* prefer the embryonic axis and, obviously, the longitudinally sectioned kernels in which the cutting has broken the resistance of the pericarp tissue that represents a hard barrier for the penetration of the young larvae of both insects.

In conclusion the approach used in these last two works provides good indications about the accessibility as well as the suitability of genetic variants to the study of insect attack and development.

Chapter 1

General introduction

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Among the Monocots the family of *Poaceae* is the one of most interesting both in genetic and alimentary terms.

Maize (*Zea mays* L.) belongs to this family and is, among the cereals, the only species that has a terminal inflorescence, known as tassel with only male spikelets. In this monoecious plant, the female inflorescence is located along the stem of the plant. Each female flower has a single functional ovary that ends with silk, made from the stylus and stigma. After the germination of the pollen grain on silk, pollen tube moves downwards, carrying three haploid nuclei: a vegetative one, and two generative (sperms).

In the embryo sac 8 nuclei are present, three at one pole (egg and two synergids) two at the centre (polar nuclei), and three at the opposite pole (antipodal).

Fecundation consists in the fusion of a generative nucleus with the egg to form a zygote from which the diploid embryo generates, and in the fusion of the second generative nucleus with the two polar nuclei to give origin to a triploid cell that, by continuous mitosis, produces the endosperm. This process, typical of the angiosperm, is called double fecundation (Fig.1).

The mature seed is attached to the cob, the entire ear is protected by modified leaves called bracts. Maize does not have effective systems for the dispersal of seeds. The indehiscent dry fruit, is called caryopsis.

From a genetic point of view, the corn is a diploid species with x = 10 chromosomes and 2n = 2 x = 20. It is one of the model species most utilized to study the role of the genes in biosynthetic pathways and plants morphogenetics: thanks to its dimensions, the practices of dissection are easier, and thanks to the abundance of tissues in the embryo, leaves and ear, great quantities of material are available for biochemical and molecular analyses.

Two features of maize plants largely helped genetic studies: the ear size and the development of separated male and female inflorescence.

A normal plant can produce about 4-700 seeds. They are a crucial step in the lifecycle of the plant, representing the beginning of the new sporophytic generation.

The seed needs to achieve successfully the development of the embryo and subsequent germination and must integrate these two stages with the environment. For this purpose Angiosperm seeds come up to desiccation and dormancy. The maintenance of a viable embryo in this condition necessitated the development of some remarkable mechanisms, including the accumulation of solutes such as sugars and proteins.

The seeds also have evolved protection against biotic stress. For this purpose they contain proteins with antifungal properties and molecules that discourage insects or other animals from eating them or feed on the seedlings. So the seeds have a highly successful adaptation that ensures the survival and dispersal of higher plants. Still, they are important for human life as a means of propagation of different crops and as a source of food, nourishment and textile fibres.

The recent interest in biology for the seed has led to a better understanding of the mechanisms behind the development of the seed; these constitute a prerequisite to new biotechnologies that complement the conventional outcross procedures.

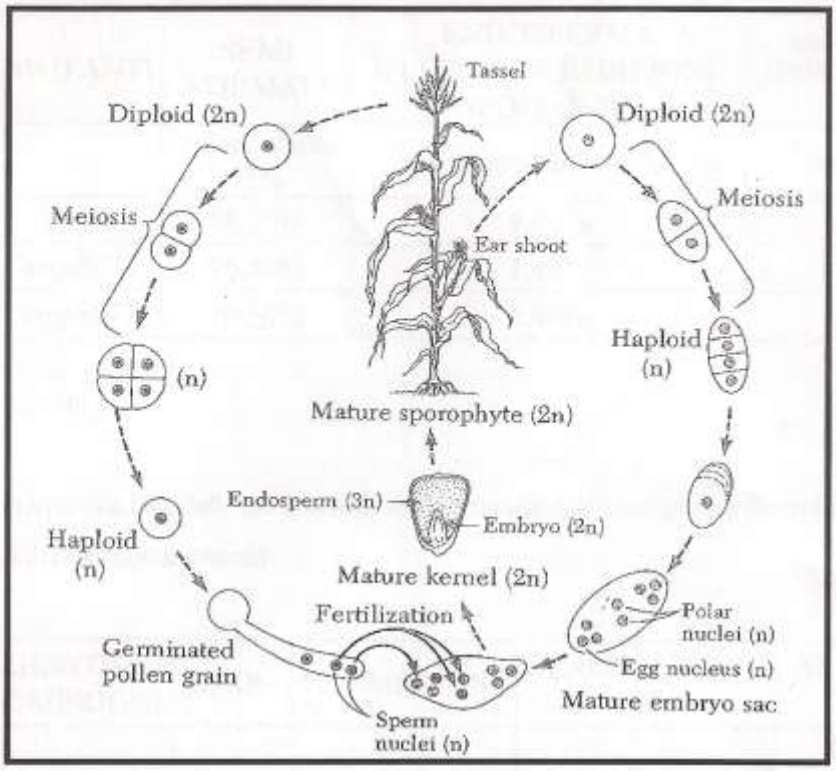

Figure1.

Main phases of the double fecundation in *Zea mays*. (from *A Feeling for the Organism. The life and Work of Barbara Mc Clintock. E*velyn Fox Keller, 1983).

1.1 Origin of embryo and endosperm

A century ago, Nawaschin (1898) in Russia and Guignard (1899) in France illustrated, independently, the origin of the seed by double fertilization event, which is something unique among living organisms. This process is achieved when the two sperm cells, genetically identical and existing in a single pollen tube, release their generative nuclei in the female gametophyte. A nucleus fuses with the egg cell and gives the diploid embryo whereas the second merges with polar nuclei of embryo-sac and gives rise to the triploid endosperm. The term "double fertilization" means that the endosperm originates from a sexual event however, the triploid nucleus is not part of an organism, but of a tissue with a well-defined development programme. The source, in evolutionary terms, of the endosperm is still controversial (Friedman 1998, 2001). Currently it is thought that the endosperm derives from a supernumerary embryo that has become a nutrient structure. Alternatively, it has been proposed that it represents the female gametophyte that differentiated tardively.

The zygote, one of two products by double fertilization, following numerous cell divisions and a process of morphogenesis, originates the embryo. During embryogenesis there are different morphologically distinguishable stages, corresponding to the acquisition and development of new functions. After multiple cycles of cell division embryo acquires a globular structure with radial symmetry that becomes bilateral and then undergoes an intense morphogenesis program leading to the formation of a primordial root and of a primordial bud at the two poles of the embryonic axis. At the end of this period the embryo begins a phase of ripeness related to an increase of abscisic acid content (ABA) and the acquisition of desiccation tolerance.

A widely shared vision maintains that embryogenesis proceeds through modules or domains, a concept originating from analysis of Arabidopsis mutants altered in embryonic development (Meyer *et al*., 1991). Alternatively, it appears not as an isolated event but as the first step in a continuous process of development interrupted by a retirement and resumed with germination (Kiaplan and Cooke, 1997).

1.2 the meristems of the shoot and root

The generative potential of the embryo is mediated by two meristematic regions the SAM (Shoot Apical Meristem) and the RAM (Root Apical Meristem), which are formed at opposite poles of the embryonic axis.

They represent the sites where the different organs are initiated and the paths of the stem and roots are established (Fletcher *et al.*, 2002). Contrary to what is observed in *Arabidopsis*, there are few mutants in maize which do not give rise to the formation of the SAM. The *sml* phenotype (*shoot meristem less*) reported by Pilu *et al.* (2002) is due to the interaction of two independent gene mutations.

Once developed, the SAM appears to be regulated by activators of transcription in the family gene knotted1 homebox (*kn1*) which was the first identified in plants homebox (Vollbrecht *et al*., 1991).

The dominant phenotype "knotted" consists of tissue masses on leaf surface which are formed by groups of cells, placed along the ribs, continuing division. By selecting for the loss of the dominant phenotype, Kerstetter *et al*. (1997) isolated recessive alleles of the gene caused a reduced ability to produce new cells meristematic.

A loss-of-function allele of the isolated gene *Kn1* revealed a new embryonic phenotype which gives rise to plants that arrested their growth at an early stage of development and was referred to as "limited shoot" (Vollbrecht *et al.*, 2000). Penetrance of this phenotype is dependant on the genetic background of this allele and can be explained by assuming that the loss of function of *kn1* is compensated by a duplicate locus or class 1 knox genes implicated in genetic redundancy in the control of the establishment and maintenance of SAM. Analysis of expression of kn1 in apical meristem shows that the level of kn1 transcripts decreases in the region where the first leaf primodium will be started. It is conceivable that kn1 can have a role in maintaining the area SAM morphogenetics (Vollbrecht *et al.*, 2000).

KN1 is a member of the KNOX proteins that are expressed in maize apical meristems and not in the leaves (Reiser *et al*., 2000). Their corresponding genes Introduction refer to the class 1 knotted like homebox (knox). They are expressed in the SAMs and not in the

beginning side organs and observation of this suggests a negative adjustment mechanism present in the meristem that occurs before initiation of lateral organs. Several genes have been identified in their ability to repress the expression of knox gene in leaf primordium i.e.: *rough sheath2* (*rs2*), *narrow sheath* (*ns*) and *leafbladeless* (*lbl*) (Reiser *et al.* .2000). It is believed that their action as repressors is exercised through separate pathways (Schneeberger *et al*., 1998).

In a new mutant of maize, called *semaphore1* (*sem1*) (Scanlon *et al*., 2002) its gene product is required for negative regulation of a subgroup of knox genes.

The regulation of KNOX is in normal cells that gives rise to leaf primordium while in *sem1* and *rs2* mutants, only in the last stages of the development of lateral leaf primordium an accumulation of the protein KNOX is observed. This suggests that SEMAPHORE is required to maintain the repression of transcription by KNOX during the final stages of the development of leaf primordium, while the initial regulation downstream of accumulation of KNOX in the young leaf primordium is controlled by separate gene functions (Scanlon *et al*., 2002).

In maize, the observation that both mutants *rs2* and *sem1* are correlated with a defective transport of auxin in the shoot, provides further evidence that there is a link between the ectopic expression of KNOX and the defective regulation of hormone concentrations in the plant's meristems (Hay *et al*., 2002).

The RAM generates new cells above the center to form the main body and under the center to form the radical cap. There is a group of cells that aren’t involved in the processes of division representing the quiescent center (QC) surrounded by mitotically active cells.

The QC appears as an "organizer" of the root architecture, as it inhibits the differentiation of surrounding cells and determines the cell differentiation thanks to the production of specific signals that allow a “cell to cell communication "(Ponce *et al*., 2000). An understanding of the organization of RAM could be reached through the analysis of mutants impaired in the root system. Unfortunately, this is difficult because the roots are not suited to phenotypic screening: they are greatly affected in their architecture by environmental changes. Also several characters of the root are controlled by multiple genes.

Despite these difficulties the application of specific screening systems has led to the isolation of several monogenic mutants with altered radical architecture.
They can be grouped into four classes based on the presence or absence of lateral roots on the main root and presence or absence of root hairs: *rt1, rth2, rth3* and *rth1* (Hochholdinger *et al*., 2004). In this way some genes specifically involved in the formation of roots were isolated and characterized at the molecular level.

1.3 the embryo maturation phase

Maturation begins when the embryo stops cell division and increasing the size of its cells is characterized by deposition of stock products and the acquisition of desiccation tolerance through which water is removed from mature seed. During this phase, there is an increase in the production of abscisic acid (ABA) which is involved in processes of accumulation of reserve protein in tolerance to desiccation and germination.
The ABA then goes back to low levels in the dry seed and embryo remains in a stage of quiescence.

1.4 specification and formation of endosperm cell domains

The endosperm of the maize seed is nucellar type. In this type of endosperm, very common in cereals, development begins with several divisions of triploid nucleus without citokinesis (Olsen 2001).
The primary endosperm nucleus is located in the micro pilar pole of the embryo sac and the first mitosis begins on a plane perpendicular to the longitudinal axis of the same.
At this stage the basal domains (calazal) and distal (micropilar) are already discernible and subsequent divisions occur simultaneously following a precise pattern.
The resulting eight nuclei are ordered in a single floor at the calazal pole of the endosperm. After this stage, central nuclei migrate to the pheriphery continuing to proliferate. The nuclei are regularly distributed in the cytoplasm surrounding the central portion of the cell, which is occupied by a large vacuole.

Depending on the genotype, 256-512 nuclei are produced as a result of continuous and synchronous divisions (Walbot, 1994; Olsen, 2004). After three days of pollination (3 DAP), cellularisation begins. This follows a precise pattern of divisions, in which the nuclei become cellularized and a new layer of nuclei is formed proceeding toward the cellularized middle of the endosperm (Walbot, 1994; Olsen, 2004). Cellularisation continues until the centripetal endosperm becomes completely cellularized.

This period, between 8 and 16 DAP, is the faster during growth of endosperm and the sequences of cellular divisions have been traced thanks to sectoral analyses. The *waxy* locus (*Wx*) controls the accumulation of amylose, and the *Ac* transposon excision in the *Wx* gene generates sectors that can be visualized with iodine.

Sectoral analysis shows that early divisions create the left and right halves of the endosperm while late divisions generate conical sectors (Mc Clintock, 1978).

Cell divisions cease in the central region of the endosperm after 12 DAP, while those in the subaleuronic region continue until 20 DAP. At this stage the nuclei of the central region begin their phase of endoreduplication. The endoreduplication extends from the base of the endosperm where transfer cells reside; the number of endocycles and the degree of endoreduplications depend on different genotypes (Larkins *et al.*, 2001). It is believed that the phenomenon of endoreduplication can be correlated to high levels of gene expression in a tissue requiring a gene activity and where there are severe limitations in terms of space and time.

The Authors Leiva-Neto *et al.* (2004) propose an alternative explanation suggesting that endoreduplication in endosperm of maize creates a reserve of nucleotides that will be used during embryogenesis and germination.

Today mutants in which the endoreduplication is suppressed have not yet been isolated.

dek mutants are often considered good candidates for studying the genes involved in mitotic cell cycle-endoreduplication. In this context, the study of 35 *dek* mutants showed a reduced level of endoreduplication in all cases except one (Kowles *et al.*, 1992). Their analysis will clarify the molecular defects in the mechanisms that control endoreduplication.

References

Fletcher JC. 2002. Coordination of cell proliferation and cell fate decisions in the angiosperm shoot apical meristem. *BioEssays* **24**: 27-37.

Friedman WE. 1998. The evolution of double fertilization and endosperm: an historical perspective**.** *Sexual Plant Reproduction* **11**: 6-16.

Friedman WE. 2001. Developmental and evolutionary hypotheses for the origin of double fertilization in endosperm. *Comptes Rendus de l'Académie des Sciences de Paris* **324**: 559-567.

Guignard L. **1899.** Sur les anthéroziodes et la double copulation sexuelle chez les végétaux angiospermes. *Comptes Rendus de l'Académie des Sciences de Paris* **128**: 864-871.

Hay A, Hardip K, Phillips A, Hedden P, Hake S, Tsiantis M. 2002. The gibberellin KNOTTED1-type homeobox function in plants with different body plans. *Current Biology* **12**: 1557-1565.

Hochholdinger F, Park WJ, Sauer M, Woll K. 2004. From weeds to crops: genetic analysis of root development in cereals *Trends in Plant Science* **9**: 42-48.

Kaplan DR, Cooke TJ. 1997. Fundamental concepts in the embryogenesis of dicotyledons: a morphological interpretation of embryo mutants. *The Plant Cell* **9**: 1903-1919.

Kerstetter RA, Laudencia-Chingcuanco D, Smith LG, Hake S. 1997. Loss-of-function mutations in the maize homeobox gene, *knotted-1,* are defective in shoot meristem maintenance. *Development* **124**: 3045-3054.

Kowles RV, McMullen MD, Yerk G, Phillips RL, Kraemer S, Srienc F. 1992. Endosperm mitotic activity and endoreduplication in maize affected by defective kernel mutations. *Genome* **35**: 68-77.

Larkins BA, Dilkes BP, Dante RA, Coelho CM, Woo Y-M, Liu Y. 2001. Investigating hows and whys of DNA endoreduplication. *Journal of Experimental Botany* **52**: 183-192.

Leiva-Neto JT, **Grafi G**, **Sabelli PA, Dante RA, Woo Y-M, Maddock S, Gordon-Kamm WJ, Larkins BA. 2004.** A dominant negative mutant of cyclin-dependent kinase a reduces endoreduplication but not cell size or gene expression in maize endosperm. *The Plant Cell* **16**: 1854-1869.

McClintock B. 1978. Development of the maize endosperm as revealed by clones. In: Subtelny S, Sussex I, eds. *The Clonal Basis of Development.* New York, Academic Press, 217-237.

Meyer U, Torres Ruiz RA, Berleth T, Misera S, Jurgens G. 1991. Mutations affecting body organization in the *Arabidopsis* embryo. *Nature* **353**: 402-407.

Nawaschin SG. 1898. Resultate einer Revision der Befruchtungsvogänge bei *Lilium martagon* und *Fritillaria tenulla*. *Bulletin de l'Académie des Sciences de Saint Petersbourg* **9**: 377-382.

Olsen OA. 2001. Endosperm development: cellularization and cell fate specification. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**: 233-267.

Olsen OA. 2004. Nuclear endosperm development in cereals and *Arabidopsis thaliana*. *The Plant Cell* **16**: S214-S227.

Pilu R, **Consonni G**, **Busti E**, **MacCabe AP**, **Giulini A**, **Dolfini S**, **Gavazzi G. 2002.** Mutations in two independent loci lead to suppression of the shoot apical meristem in maize. *Plant Physiology* **128**: 502-511.

Ponce G, Lujan R, Campos ME, Reyes A, Nieto-Sotelo J, Feldman LJ, Cassab GI. 2000. Three maize root-specific genes are not correctly expressed in regenerated caps in the absence of the quiescent center. *Planta* **211**: 23-33.

Reiser L, Sanchez-Baracaldo P, Hake S. 2000. Knots in the family tree: evolutionary relationships and functions of knox homeobox genes. *Plant Molecular Biol*ogy **42**: 151-166.

Scanlon MJ, Henderson DC, Bernstein B. 2002. SEMAPHORE1 functions during the regulation of ancestrally duplicated *knox* genes and polar auxin transport in maize. *Development* **129**: 2663-2673.

Schneeberger R, Tsiantis M, Freeling M, Langdale JA. 1998. The *rough sheath2* gene negatively regulates homeobox gene expression during maize leaf development. *Development* **125**: 2857-2865.

Vollbrecht E, Veit B, Sinha N, Hake S. 1991. The developmental gene *Knotted-1* is a member of a maize homeobox gene family. *Nature* **350**: 241-243.

Vollbrecht E, Reiser L, Hake S. 2000. Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, *knotted 1*. *Development* **127**: 3161-3172.

Walbot V. 1994. Overview of key steps in aleurone development. In: Freeling M, Walbot V eds. *The Maize Handbook*. New York: Springer-Verlag, 78-80.

Chapter 2

Interaction between different genes controlling endosperm development in maize

Interaction between different genes controlling endosperm development in maize.

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Abstract:

The symbol *emp* (*empty pericarp*) refers to the phenotype of a group of defective kernel mutants with drastic reduction in endosperm tissue production and embryo-lethals.

They can be subdivided into two major subgroups: one including those with a flat appearance of the kernel and smooth pericarp and another one with a wrinkled pericarp.

In all mutants, the analysis of longitudinal sections of mature seeds reveals absence of morphogenesis in the embryo proper, an observation that correlates with the failure of these mutants to germinate.

We report the results of a complementation test of nine of these monogenic mutants, isolated as independent events, either spontaneous in their origin or in stocks carrying an active transposon (*MuDr* or *Spm*). The chromosomal arm position of six out of nine mutants was successfully established by crosses with the entire set of B-A translocations.

The 9 *emp* mutants have been crossed *inter-se* in all pairwise combinations with the aim to establish their allelic relationship as well as their interaction in the homozygous double mutant genotypes recovered in the F_2 generation. These crosses identified those non-complementing (*i.e.* allelic) and those complementing (*i.e.* non-allelic) in the F_1 generation. Most results in the F_1 were concordant to those obtained in the F_2 generation with the exception of three cases where the F_1 results suggest allelism (i.e. one gene) whereas those in F_2 suggest presence of two genes. This unexpected observation seem to suggest an interaction between different *emp* mutants, attributable to a phenomenon that is often referred to as second site non-complementation (SSNC).

In addition evidence of specific epistatic interactions is observed when examining the F_2 progeny obtained by selfing F_1 parents heterozygous for two *emp* isolates with distinguishable phenotype (*i.e.* emp flat *vs* emp wrinkled).

In these cases, in fact, the expected 9 to 7 segregation can be subdivided into a 9:4:3, expected when one *emp* is epistatic over the other, or 9:6:1 when the two genes are duplicated and have a cumulative effect.

Keywords: *empty pericarp,* complementation, non-complementation, interaction, second site non-complementation

Introduction

The recent revival of interest in the study of the genetic control of seed development in cereals is due to the opportunity it offers to improve some important traits such as yield and quality. The maize seed consists of the embryo and the endosperm, the two products of double fertilization, and of a seed coat named pericarp, of maternal origin (Kiesselback, 1949). The embryo follows a developmental pattern characterized by a sequence of phases, the first one of histodifferentiation where the embryo proper is formed, followed by the acquisition of polarity and bilateral symmetry and morphogenesis, a maturation phase where reserve food accumulates, dehydration, and quiescence (Kiesselbach, 1949; Vernoud *et al*., 2005). The endosperm shows four developmental phases: syncytium formation, cellularization, differentiation, and cell death (Olsen, 2001). The differentiated endosperm consists of four major cell types or domains: the starchy endosperm, representing the central bulk; the single-cell aleurone layer covering the periphery of the endosperm; the embryo surrounding region, consisting of the cells lining the region where the embryo develops; and the basal endosperm transfer cells involved in the transport of nutrients. Each cell type has distinct morphology and function and is characterized by specific gene expression (Lopes and Larkins, 1993; Olsen, 2001; Bommert and Werr, 2001).

Starchy endosperm cells undergo PCD (Programmed Cell Death), a process that is initiated at approximately 16 d after pollination (DAP), whose pattern and timing of progression is well established and appears to be related to cell age (Buckner *et al*, 1998-200). A cell death process also occurs in the placenta-chalazal (P-C) cell layers in maternal pedicel tissues, as described by Kladnik *et al*., 2004. During embryogenesis the events of PCD occur in structures (scutellum and suspensor) that have only a transitory function and do not

contribute to the formation of an adult plant. The degeneration of the scutellum starts after 14 DAP (Giuliani *et al*., 2002). The suspensor follows the same fate, because its function – the transfer of nutrients from maternal tissues to the embryo- is confined to the early stages of embryogenesis. Other organs that undergo PCD in the embryo are the coleoptile and the root cap that exert their function in a short period of time.

Mutants affecting endosperm development represent an important tool for discovering the identities of the corresponding genes and elucidating their roles.

Several mutants with defects in the endosperm and in the embryo have been described.

Many of these mutants are well characterized but only a few have been cloned.

Those so far analyzed are:

1.Those altered in the composition of the starch in the endosperm but with a normal embryo. (for reviews, see Shannon and Gaewood, 1984; Boyer and Hannah, 1994; Nelson and Pan, 1995). They include *bt1, bt2, sh2* and *su1* which seriously affect kernel dry matter accumulation (i.e. starch deposition) and *ae1, du1, sh1* which produce more moderate phenotypes (Glover and Metz, 1987). In this group *mn1* and *rgf1* are also included characterized by a reduced activity of the cell wall invertase that converts saccharose into glucose and fructose in an irreversible manner (Cheng *et.al.* 1996; Byung Ho Kang *et al,* 2009; Maitz *et al* 2000).

2.Those with non vitreous endosperm that include the "high-lysine" mutants *o2*, *fl2*, and *o7*. They are characterized by soft, starchy endosperm, which makes them brittle and insect susceptible. All three have been cloned (Schmidt *et al*, 1987; Motto *et al*, 1988; Coleman *et al*, 1997; Miclaus *et al*, 2011).

3.Mutants altered in the accumulation of carotenoids in the endosperm. They are expressed both in the endosperm and in sporophytic tissues except *y1*, coding for phytoene synthase, an enzyme required in the first step of the carotenoid biosynthesis, whose expression is confined to the endosperm (Buckner *et al*, 1996).

The viviparous mutants are the result of a mutation in an early step of carotenoid biosynthesis; thus accounting for a block in carotenogenesis and vivipary a consequence of a block in ABA biosynthesis (Giraudat *et al*, 1994; Hable *et al*, 1998).

They are useful to analyze the final events of embryogenesis (Mc Carty, 1995; Finkelstein *et al.*, 2002; Durantini *et.al*, 2008).

4.Another group of mutants impaired in embryo development but not in endosperm development is that of the *embryo-specific* (*emb*). (Clark and Sheridan, 1991; Sheridan and Clark, 1993, Heckel *et al*., 1999; Elster *et al*., 2000; Consonni *et al*., 2003, Sosso *et al*, 2011).

These mutants, useful for the study of embryogenesis, cause a delay in embryonic development that appears morphologically abnormal while the endosperm is normal. The mutant seed usually shows a depression on the germinative face indicating the presence of a defective embryo.

Their histological analysis can determine at which stage the mutant is blocked in its development (J.K. Clark and W.F. Sheridan 1991).

However the definition of *emb* as mutants impaired in a specific manner in the embryo formaton is not appropriate, because the expression of the *emb* genes in tissues different from those present in the seed is difficult to notice due to their lethality.

This difficulty can be overcome by the clonal analysis of mutant tissues induced in the leaves of heterozygous*+/emb* seedlings with X rays, as reported for the *emp2* a lethal mutant of maize that causes the nearly total suppression of endosperm formation (Fu and Scanlon 2004).

Only 17 *emb* mutants have been characterized (Sheridan and Thorstenson, 1986; Heckel *et al.*, 1999; Elster *et al.*, 2000; Consonni *et al.*, 2003; Sosso *et al.*, 2011) and only three of them were cloned: *emb 8522*, *emb 8516* and *lem1* (Sosso *et al*, 2012, Magnard *et al*, 2004, Ma and Dooner 2004).

Mutants impairing the aleurone development are the *Dap* mutants (Gavazzi *et al*, 1997) *dek1* (Becraft and Asuncion-Crabb, 2000; Becraft *et al*., 2002; Lid *et al*., 2002; Wisniewski and Rogowsky, 2004) *cr4* (Becraft *et al*., 1996; Jin *et al*., 2000) *sal1* (Shen et al., 2003; Tian et al., 2007) *dil1* and *dil2* (Lid *et al* 2004) whereas *etched1* (*et1*) is pleiotropic affecting both the aleurone and the seedling (Da Costa e Silva *et al* 2004).

The largest group of seed mutants impairing both endosperm and embryo development are referred to as *dek* (*defective kernel*) mutants (Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980).

In these mutants both embryo and endosperm development is generally altered, but in most cases the developmental lesion has not been identified. The majority of these mutants grow into some form of a plant when germinated as mature seeds or cultured as immature embryos, while a minority lack this capacity. According to Sheridan and Neuffer (1981) they represent nutritional-type and developmental-type mutants respectively.

In particular we focused our attention on a subclass of the class of *dek* mutants referred to as *empty pericarp (emp)* (Fu *et al*., 2002; Fu and Scanlon, 2004). Only *emp2* and *emp4* have been characterized at the molecular level (Fu *et al*, 2002, Consonni *et al*, 2007). *emp2* encodes a protein with high similarity to HEAT SHOCK BINDING PROTEIN (HSP1), a negative regulator of the heat shock response, while *emp4* encodes a mitochondrion-targeted pentatricopeptide repeat protein necessary for seed development and plant growth.

Aim of the work:

In this paper we describe the isolation and characterization, of nine novel maize mutants referred to as *empty pericarp* (*emp*), with very reduced endosperm and loose pericarp.

The main purpose of this work is not only to establish which of these mutants are allelic to each other or identify different genes but also to study their interaction because in the literature there are few works regarding the interaction between *emp* genes (Avery and Wasserman 1992, Carlborg and Haley, 2004; Phillips, 2008; He, Quian, Wang, Li and Zhang 2010).

Materials and methods

Isolation of mutants

The nine *emp* mutants here analyzed, *emp4, emp*-8075, emp*-8077, emp*-8300, emp*-8376, emp*-8971, emp*-9106, emp*-9475* and *emp*-Dap3* where chosen out of a collection of mutants obtained by transpositional mutagenesis (Consonni *et al*.,1998) or spontaneous in their origin.

They were originally isolated in the F_2 of selfed F_1 plants derived by crossing a line maintained in our laboratory as inbred stock for at least four generations with a line carrying active *Mu* (*MuDR*) or *Spm* transposons.
They all behave as single gene recessive mutants in their original backgrounds and are propagated as heterozygotes, being lethal in the homozygous condition.

Allelism test of the emp mutants

The nine mutants under test were crossed *inter-se* in all pairwise combinations to assay their complementation pattern.
For each combination, pollen of a given *+/emp* plant, whose heterozygous condition was ascertained by selfing, was applied to the silks of plants representing the selfed progeny of a different *+/emp* isolate. When the lines to be crossed carry contrasting color genes (i.e. colored *vs* colorless or yellow *vs* white seeds) we made double pollinations to overcome the problem of the determination of the genetic constitution of the female parent, and to ensure the obtainment of double heterozygous individuals in the F_1 generation.To this aim, silks of the female parent, in a white stock, were split with the help of cardboard and one sector was selfed while the other was pollinated with the male parent in a colored or yellow stock.

B-A translocations in maize and chromosomal arm attribution of the emp mutants

The B-A translocations of maize provide an efficient method for locating recessive mutants to the proper chromosome arm. (Beckett, 1978).
B-A translocations are intherchanges that involve a supernumerary B-chromosome and members of the basic (A) set of chromosomes.
The B chromosome is widely distribuited in races of maize and is relatively inert and innocuous when low numbers are present (Randolph LF, 1941).
Translocations are obtained irradiating with x-rays mature pollen of plants that carry B chromosomes. Individuals carrying a B chromosome produce unbalanced gametes carrying 0-1-2 B chromosomes.

This phenomenon is due to non-disjunction at the second microspore mitosis occurring with a frequency of 90-100%, giving pollen grains with dissimilar sperms: one hyperploids with two B-A chromosomes and one hypoploid with none.

Fertilization of the egg-cell and central cell by the two sperms from such a pollen grain gives a kernel with non-corresponding embryo and endosperm genotypes; when the embryo receives two B-A chromosomes, the endosperm receives none and viceversa (Roman,1947).

The sperm carrying the extra B-A chromosome usually fertilizes the egg about 2/3 of the time (preferential fertilization Roman, 1948).

The mutant located in the hemizygous chromosomal segment, will manifest itself, allowing its attribution to a specific chromosomal arm.

Chromosomal arm attribution was established for these mutants: *emp4*, *emp*-8075*, *emp*-8077*, *emp*-8300*, *emp*-8971*, and *emp*-9475*; by crossing the progeny of outcrossed heterozygous *+/emp* plants with male parents including the entire set of B-A translocations covering about 90% of the genome. The source of these stocks is the Maize Cooperation Stock Center, Urbana, Ill. USA. (http://maizecoop.cropsci.uiuc.edu/)

Ears (5-10) from these crosses were scored for the presence of *emp* seeds with a frequency of 15-20% as evidence that the translocation is uncovering the mutant under test.

Light stereomicroscope

For light stereomicroscope (STEMI DRC Carl Zeiss), longitudinal sections of mature dry seeds of wild-type and *emp* mutant seeds at 60 DAP were obtained by placing the seeds on a wet, thin sponge and cutting them with a robust and sharp blade in combination with a hammer.

These sections were observed using the 1X magnification.

Photomicrographs were taken with a digital camera Casio Exilim-Z77 of 7.2 Megapixels.

Gametophytic selection

It was assayed by dividing F_2 ears segregating for a given *emp* into three sectors (apical, central, basal) of equal length and establishing the mutant distribution in each sector. Their distribution was assayed with the X^2 heterogeneity test.

Since the distance covered by the pollen tube to fertilize the basal egg cells is longer than that needed to reach the apical egg cells, there will be a significantly lower percentage of *emp/emp* kernels in the basal versus apical portion of F_2 ears obtained by selfing *+/emp* heterozygous parents if *emp* pollen tube growth is slower than that of wild-type siblings (Meinke 1982). If the mutant is not undergoing gametophytic selection, a random mutant distribution should be observed in all sectors, whilst a significant deviation from the random distribution is expected in case of gametophytic mutant gene expression during pollen development, pollen germination or pollen tube growth. For these three mutants analysis, data from two or more ears were combined.

Results

Genetic analysis and characterization of emp mutants

The *emp* mutants here analyzed, belong to the class of *dek* (*defective kernel*) mutants with the most severe reduction in endosperm development. They arise with a frequency of about one out of ten *dek*, as determined in a sample of *dek* mutants following chemical mutagenesis (Pilu *et al*, 2002).

They can be detected on a selfed *+/emp* ear as early as 10–12 DAP, on the basis of their reduced size and a pale, translucent appearance. Their endosperm has a soft and fluid consistency, whereas their embryo appears smaller than that of wild-type sibs and retarded in its morphogenetic development. Mature mutant seeds appear flattened and smaller than the wild-type sibs since they lack a well-developed endosperm. Hence the '*emp*' (*empty pericarp*) symbol applied to similar mutants in the literature. As to the embryo, the analysis of dissected mature seeds under low magnification microscopy indicates that the embryo proper is no longer recognizable. In its place, one can observe a scutellum-like structure in *emp4* mutant embryos and a less organized structure surrounded by a cavity in *emp*-8075* and in *emp*-8077*. This finding explains why these mutants are unable to germinate.

The origin of the nine mutants can be traced back to a cross between a line carrying active *Mutator* elements (*MuDR* or *Spm*) and the same genetic stock or spontaneous origin.

(See the table below)

Mutant isolated	Origin:
emp4	*Mutator (MuDR)*
emp-8075*	*Mutator (MuDR)*
emp-8077*	*Mutator (MuDR)*
emp-8300*	*Mutator (MuDR)*
emp-8376*	*Suppressor-mutator (Spm)*
emp-8971*	*Mutator (MuDR)*
emp-9106*	*Mutator (MuDR)*
emp-9475*	*Mutator (MuDR) or not determined?*
emp-Dap3*	*Spontaneous*

As reported in Table 1 they all behave as monogenic recessive mutants in their different genetic backgrounds. These values are obtained in the F_2 generation obtained following outcross of the mutants to different inbred lines.

Table 1. Segregation values of nine *emp* isolates. For each mutant the genetic background in which it was originally isolated is reported.

Mutant isolated	Genetic background	Mutant Phenotype	wt	*emp*	n	% of emp
emp4	A188	emp flat	121	47	168	**28**
emp-8075*	W64A	emp wrinkled	1920	591	2511	**23.5**
emp-8077*	ACR sc m-2	emp flat	1558	554	2112	**26.2**
emp-8300*	A188	emp wrinkled	396	125	521	**24**
emp-8376*	W64A	emp wrinkled	1159	393	1552	**25.3**
emp-8971*	B73	emp flat	695	219	914	**24**
	W23	emp wrinkled	977	310	1287	**24.1**
emp-9106*	A188	emp wrinkled	502	199	701	**28.4**
emp-9475*	Rsc m-2	emp wrinkled	245	84	329	**25.5**
	ACR sc m-2	emp wrinkled	885	266	1151	**23.1**
	B73	emp flat or wrinkled	6005	1794	7799	**23**
emp-Dap3*	ACR sc m-2	emp wrinkled	1253	414	1667	**24.8**

Table 2. Segregation values of nine *emp* isolates. For each mutant, the genetic background and the corresponding phenotype are reported.

Mutant isolated	Genetic background	Mutant Phenotype	wt	*emp*	n	% of emp
emp4	A188	*emp flat*	121	47	168	28
	ACR	*emp wrinkled*	913	291	1204	24.2
	H99	*emp dek*	528	185	713	25.9
emp-8075*	W64A	*emp wrinkled* or *emp-dek*	1920	591	2511	23.5
emp-8077*	B73	*emp dek*	543	148	691	21.4
	ACR sc m-2	*emp flat*	1558	554	2112	26.2
emp-8300*	W23	*emp dek*	606	185	791	23.4
	B73	*emp dek*	532	168	700	24
	A188	*emp wrinkled*	396	125	521	24
emp-8376*	W64A	*emp wrinkled*	1159	393	1552	25,3
	B73	*emp dek*	688	145	833	17.4*
	B73	*emp wrinkled*	190	84	274	30.7
	A636	*emp dek*	1110	343	1453	23.6
emp- 8971*	B73	*emp flat*	695	219	914	24
	W23	*emp wrinkled*	977	310	1287	24.1
	W23	*emp dek*	628	211	839	25.1
emp-9106*	A188	*emp wrinkled*	502	199	701	28.4
emp-9475*	Rsc m-2	*emp wrinkled*	245	84	329	25.5
	ACR sc m-2	*emp wrinkled*	885	266	1151	23.1
	Mo17	*emp dek*	1289	426	1715	24.8
	RAlex0	*emp dek*	572	214	786	27.2
	B73	*emp flat or wrinkled*	6005	1794	7799	23
emp-Dap3*	ACR sc m-2	*emp wrinkled*	1253	414	1667	24.8

*****segregation much less than 1/4

Gametophytic selection of emp mutants.

Table 3. Mutant segregation and distribution in three different sectors of selfed *+/emp* maize ears. P Probability, df degrees of freedom.

Mutant	N° of kernels	Segregation %	Distribution in different ear sector (%)			Heterogeneity X^2 test[a]	df	P
			Basal	Central	Apical			
emp-8971*	1139	25.81	23.76	27.59	25.33	1.503	2	0.50-0.70
emp-8077*	1990	26.33	26.72	26.15	26.04	0.098	2	0.70-0.90
emp-Dap3*	1450	25.10	23.59	25.00	23.46	0.887	2	0.50-0.70

Table 4. Mutant segregation and distribution in three different sectors of selfed F_2 maize ears from the cross *(+/emp*-8075*[c] x *+/emp*-8971*[c]). P Probability, df degrees of freedom. The symbol [c] indicate that the two parental F_1's plants have the verified heterozygous constitution using the double pollination technique.

NB: these two mutants are allelic.

N° of kernels	Segregation %	Distribution in different ear sector (%)			Heterogeneity X^2 test[a]	df	P
		Basal	Central	Apical			
1595	18.24	17.98	17.31	19.81	0.848	2	0.50-0.70

The transmission values of these three different *emp* mutant in F_2 ears, as well as the distribution in basal versus apical portion of the ear, are reported in Table 3. All the three mutants tested show the expected one-quarter segregation in the F_2 generation, and the heterogeneity X^2 test indicates that the mutant distribution in the three sectors is homogeneous. These results suggest that *emp* gene function is not required for male gametophyte development. The same conclusion applies to the double heterozygous F_2 ears of the two alleles *emp*-8075* and *emp**-8971 (Table 4) disclosing a significant shortage of mutant seeds over the expected one-quarter (X^2=38.821 P <0.01). However the heterogeneity X^2 test indicates that in these F_2 ears the distribuition of mutant kernels in the three sectors is homogeneous, thus indicating that the deviation over the expected distribuition is not due to male gametophytic selection.

Figure 1. Longitudinal sections of mature dry seeds scored under low magnification. From left to right: wt seed, *emp* flat of *emp*-8971* in B73 and *emp* wrinkled of *emp*-Dap3* in ACR

Wild type seeds show a well developed embryonic axis while in the *emp/emp* seeds, although the endosperm tissue is still present, the embryo proper is no longer recognizable and in its place a embryo cavity with necrotic margins is recognizable.

Results of the test of allelism in F_1 and in F_2/F_3

Mutant *emp inter se* crosses allow us to identify those complementing *vs* those non-complementing in the F_1 generation. (See Table 5).

Table 5. Results of the complementation test in the F_1 of the nine *emp* mutants isolated.

The + and – signs indicate complementation and non complementation respectively; signs in parenthesis refer to results that need further validation. Numbers inside the brackets, refer to the observed cases of + or – (in bold), over the total crosses made. For colors see the legend of Table 6.

↓ →	*emp4*	*emp*-8075*	*emp*-8077*	*emp*-8300*	*emp*-8376*	*emp*-8971*	*emp*-9106*	*emp*-Dap3*	*emp*-9475*
emp4	-	+ (**22**/22)	+ (**15**/15)	+ (**14**/14)	+ (**19**/19)	+ (**28**/28)	+ (**16**/16)	+ (**10**/10)	- (**13**/45)
emp-8075*		-	+ (**16**/16)	- (**11**/34)	(-) n.d.	(-) (**1**/9)	+ (**7**/8)	+ (**11**/11)	+ (**24**/33)
emp-8077*			-	+ (**8**/8)	+ (**8**/8)	+ (**12**/12)	- (**14**/28)	+ (**14**/14)	+ (**17**/17)
emp-8300*				-	+ (**8**/8)	- (**21**/34)	- (**9**/39)	+ (**3**/3)	+ (**17**/17)
emp-8376*					-	+ (**15**/15)	+ (**7**/7)	+ (**20**/20)	+ (**8**/8)
emp-8971*						-	+ (**19**/19)	+ (**49**/53)	- (**6**/36)
emp-9106*							-	+ (**6**/6)	+ (**10**/10)
emp-Dap3*								-	+ (**7**/7)
emp-9475*									-

If only **wild-type** seeds are observed in all the F_1 ears the two mutants are considered **not allelic**.

If some progeny ears yield **mutants in about one-quarter** of the seeds this is taken as evidence of **allelism.**

The F_2/F_3 progeny of these crosses should yield

only **3:1 segregation** in case of **allelism**

3:1 segregation and an additional class of ears with **9:7 segregation** if the two mutants **are not allelic**.

The results obtained in the F_1 and F_2/F_3 generation, are concordant in their conclusions in ten cases.

In the remaining three they show a contrasting result *i.e.* one gene as inferred from the lack of complementation observed in the F_1 and two genes based on the observation of a 9 to 7 segregation in F_2/F_3 expected when the F_1 plants are heterozygous for two *emp* mutants. (See Table 6)

Table 6. Validation of allelism and non-allelism of the F_1 results by analysis of the segregations in F_2/F_3.

Segregation values of 9 to 7 wild-type and mutant seeds on F_2/F_3 ears are taken as evidence of double mutant heterozygous constitution of F_1 ears.

Numbers in parenthesis refer to insufficient results that need further validation.

	Expected ratios (complementation)							
	1	2	1	1	4	4		
	Ears distribution:						Inferred number of genes from:	
Progeny of F_1 exhibiting non complementation (-)								
Cross made	**F_2**			**F_3**			**F_1**	**F_2/F_3**
	+/+	3:1	9:7	+/+	3:1	9:7		
emp 4 x emp*- 9475	27	59	10	4	24	19	1 gene	2 genes
emp*- 8971 x emp*- 9475	24	24	10	33	37	14	1 gene	2 genes
emp*- 8300 x emp*- 9106	24	30	5	9	18	7	1 gene	2 genes
emp*- 9106 x emp*- 8077	24	41	2*	11	35	6*	1 gene	(2 genes)
emp*- 8075 x emp*- 8300	22	38	1*	5	25	1*	1 gene	1 gene
emp*- 8971 x emp*- 8300	24	34	3*	3	14	0	1 gene	1 gene
emp*- 8971 x emp *-8075	4	7	0				1 gene	1 gene
emp*- 8075 x emp*- 8376	Not tested							
Progeny of F_1 exhibiting complementation (+)								
Cross made	**F_2**			**F_3**			**F_1**	**F_2/F_3**
	+/+	3:1	9:7	+/+	3:1	9:7		
emp*- 8077 x emp*- Dap3	3	3	1	3	24	13	2 genes	2 genes
emp*- 8971 x emp*- Dap3	18	25	5	6	12	9	2 genes	2 genes
emp*- 8075 x emp*- 9475	42	42	14	2	3	6	2 genes	2 genes
emp*- 8376 x emp*- Dap3	9	11	2	2	10	6	2 genes	2 genes
emp*- 8075 x emp*- 9106	16	10	1				(2 genes)	(2 genes)
emp*- 8300 x emp*- Dap3	5	9	6				(2 genes)	(2 genes)

* 9:7 with a segregation of about 35%

In yellow we have the cases where the F_1 results suggest allelism whereas those in F_2/F_3 suggest segregation of two genes. These conflicting results seem to suggest the occurrence of an interaction between different *emp* mutants, due to second site non-complementation. The cases in blue and green indicate one gene and two genes inference respectively.

Table 7. Comparison of the observed *vs* expected frequencies of F2 segregations. For each cross in the F_2 and F_3 generations total X^2 and P values are reported.

	Expected ratios (complementation)									
	1	**2**	**1**			**1**	**4**	**4**		
	Ears distribution:									
Progeny of F_1 exhibiting non complementation (-)										
Cross made	**F_2**					**F_3**				
	+/+	**3:1**	**9:7**	**x^2**	**P**	**+/+**	**3:1**	**9:7**	**x^2**	**P**
emp 4 x emp*- 9475	**27**	**59**	**10**	**11.06 S**	**<1%**	**4**	**24**	**19**	**0.92 NS**	**55.72%**
emp*- 8971 x emp*- 9475	**24**	**24**	**10**	**8.48 S**	**1.74%**	**33**	**37**	**14**	**76.61 S**	**<1%**
emp*- 8300 x emp*- 9106	**24**	**30**	**5**	**12.25 S**	**<1%**	**9**	**18**	**7**	**12.13 S**	**<1%**
emp*- 9106 x emp*- 8077	**24**	**41**	**2***	**17.81 S**	**<1%**	**11**	**35**	**6***	**23.51 S**	**<1%**
emp*- 8075 x emp*- 8300	**22**	**38**	**1***	**18.15 S**	**<1%**	**5**	**25**	**1***	**21.70 S**	**<1%**
emp*- 8971 x emp*- 8300	**24**	**34**	**3***	**15.26 S**	**<1%**	**3**	**14**	**0**	**13.71 S**	**<1%**
emp*- 8971 x emp *-8075	**4**	**7**	**0**	**3.73 NS**	**17.16%**					
emp*- 8075 x emp*- 8376	**Not tested**									
Progeny of F_1 exhibiting complementation (+)										
Cross made	**F_2**					**F_3**				
	+/+	**3:1**	**9:7**	**x^2**	**P**	**+/+**	**3:1**	**9:7**	**x^2**	**P**
emp*- 8077 x emp*- Dap3	**3**	**3**	**1**	**1.29NS**	**58.74%**	**3**	**24**	**13**	**3.93 NS**	**15.53%**
emp*- 8971 x emp*- Dap3	**18**	**25**	**5**	**7.13 S**	**2.95%**	**6**	**12**	**9**	**3.75 NS**	**17%**
emp*- 8075 x emp*- 9475	**42**	**42**	**14**	**18 S**	**<1%**	**2**	**3**	**6**	**1.48 NS**	**54.41%**
emp*- 8376 x emp*- Dap3	**9**	**11**	**2**	**4.45 NS**	**11.27%**	**2**	**10**	**6**	**1 NS**	**65.33%**
emp*- 8075 x emp*- 9106	**16**	**10**	**1**	**18.48 S**	**<1%**					
emp*- 8300 x emp*- Dap3	**5**	**9**	**6**	**0.30 NS**	**85.53%**					

The critical value of chi-square is 5.991 with P of 0.05 and two degrees of freedom.

In some cases the observed number of ears with the 9:7 segregation ratio is lower than the expected (see table).

When double *emp* heterozygotes are selfed, the distribution of F_2 or F_3 ears segregating 3:1 or 9:7 or not segregating is frequently showing a deficit in the number of 9:7 ears over the expected one, as shown in Table 6.

This deficit of ears segregating 9:7 is unexpected. However we noticed that frequently the mutant segregations in the F_2 or F_3 generation have intermediate values between the one expected for a single mutant (25%) and the one for two mutants (43.75%) as reported in Table 8 (see Discussion).

Table 8. Evidence of a % of mutants lower than the expected one of 7/16 (43.75%) in some cases of 9:7 segregation.

Cross made	n° of putative 9:7 ears in F_2/F_3	n° of 9:7 ears in F_2/F_3 with a segregation<7/16 (43.75%)	Average value of segregation of these 9:7
emp 4 x emp*- 9475	29	7	37.54%
emp*- 8971 x emp*- 9475	24	7	37.93%
emp*- 8300 x emp*- 9106	12	5	35.01%
emp*- 9106 x emp*- 8077	8	8	35.52%
emp*- 8077 x emp*- Dap3	14	2	38.91%
emp*- 8971 x emp*- Dap3	14	2	38.87%
emp*- 8075 x emp*- 9475	20	11	37.79%
emp*- 8376 x emp*- Dap3	8	0	/
emp*- 8075 x emp*- 9106	1	0	/
emp*- 8300 x emp*- Dap3	6	1	37.19%

Interaction among emp mutants

The recovery of two different emp phenotypes (emp flat vs emp wrinkled or emp wrinkled vs emp-dek), in some cases of 9:7 segregation is the evidence of an epistatic interaction between the two *emp* mutants.

In the case of the cross between *emp4* and *emp*-9475* the scoring of F_2 ears revealed a segregation ratio of 9:4:3 (See Table 9).

Table 9. Interaction between F_2 progeny of *emp4/+ +/emp*-9475* double heterozygous parents exhibiting non complementation in F_1.

Code n°	Genotype	% of *emp* seeds	Phenotype of the seeds	Segregation ratio	Observed	Expected	X^2 value	P
09.30 (12) ⊗	*emp4/emp*-9475*	41.5%	Wt	9	371	356.625	1.433 NS	55.5%
			emp wrinkled	4	153	158.5		
			emp flat	3	110	118.875		

Scoring the selfed progeny of the *emp4/+ +/emp*-9475* double mutant disclosed in some cases a segregation ratio of 9:4:3 (wt: emp wrinkled: emp flat) suggesting that *emp*-9475* conditioning a wrinkled phenotype, is epistatic over *emp4*, determining a flat phenotype.

Table 10. Interaction in the F_3 progeny of *emp*-8077/+ +/emp*-Dap3* double heterozygous parents exhibiting complementation in F_1

Code n°	Genotype	% of *emp* seeds	Phenotype of the seeds	Segregation ratio	Observed	Expected	X^2 value	P
09.39 (9) ⊗	*emp*-8077/emp*- Dap3*	45%	Wt	9	330	337.5	0.587 NS	74.73%
			emp wrinkled	6	234	225		
			emp flat	1	36	37.5		
09.39 (28) ⊗	*emp*-8077/emp*- Dap3*	47.84%	Wt	9	278	299.81	3.699 NS	24.24%
			emp wrinkled	6	220	199.88		
			emp flat	1	35	33.31		

Scoring the selfed progeny of the *+/emp*-8077 +/emp*-Dap3* double mutant disclosed a segregation ratio of 9:6:1 (wt: emp wrinkled: emp-flat).

Table 11. Interaction in the F_3 progeny of *emp*-8376/+ +/emp*-Dap3* double heterozygous parents exhibiting complementation in F_1

Code n°	Genotype	% of *emp* seeds	Phenotype of the seeds	Segregation ratio	Observed	Expected	X^2 value	P
09.51(10) ⊗	*emp*-8376/emp*-Dap3*	48.45%	Wt	9	289	317.8125	9.836 S	0.77%
			emp wrinkled	4	173	141.250		
			emp dek	3	103	105.938		
09.51 (9) ⊗	*emp*-8376/emp*-Dap3*	45.64%	Wt	9	306	317.25	8.612 S	1.49%
			emp wrinkled	4	169	141		
			emp dek	3	89	105.75		

Scoring the selfed progeny of the *+/emp*-8376 +/emp*-Dap3* double mutant heterozygous parents disclosed a segregation ratio close to 9:4:3 (wt: emp wrinkled: emp-dek) suggesting that *emp*-Dap3*, that conditions a wrinkled phenotype, is epistatic over *emp*-8376 (*in A636*)*, with a dek-emp phenotype.

Out of 70 dek-emp planted in the 2012 field, 15 germinated and gave stunted plants. Only one of these plants was successfully reproduced giving rise to a small ear homozygous for dek-emp, as expected in the 9:4:3 interpretation of the observed segregation.

Table 12. reports the results obtained by crossing each mutant with the entire set of B-A translocations

Mutant	Chromosomal arm
emp4	1 L bin 1.10
emp-8075*	3 S
emp-8077*	9 S
emp-8300*	**3 S** or 8 L or 10 L (needs further validation)
emp-8376*	not mapped yet
emp-8971*	**3 S** or10 L (needs further validation)
emp-9106*	not mapped yet
emp- 9475*	1 L bin 1.10 (associated to *emp4*)
emp-Dap3*	not mapped yet

The localization of *emp4* and *emp*-9475* on the same chromosomal arm is expected due their allelism observed in the F1 generation. The same result applies to *emp*-8300* and *emp*-8971* mutants allelic to each other, although the data collected need further validation.

Discussion

In spite of the many mutants impairing seed development so far isolated in maize, very few have been thoroughly analyzed in detail.

Here we present the results obtained with the study of a collection of nine *empty pericarp* mutants isolated as independent events.

In all these mutants, the analysis of longitudinal sections of mature seeds reveals absence of morphogenesis in the embryo proper, and observation that correlates with their failure to germinate.

To ascertain if these mutants are due to alleles of the same gene or to independent genes we compared the results of the F_1 obtained by crossing *inter-se* the different *emp* isolates with the F_2 segregations observed in the progeny of double heterozygous F1 parents.

Most results obtained in the F_1 are concordant with those obtained in the F_2 generation with the exception of three cases in which the F_1 results suggest allelism whereas those in F_2/F_3 are in accord with segregation of two genes.

Particular attention was given to the *emp4* and *emp*-9475* interaction: their relationship appears discordant in F_1 versus F_2/F_3; this result based on a large sample of observations could be explained by assuming that the products of the two genes interact functionally as proposed in the Second Site Non Complementation hypothesis (Hawley and Gilliland 2006).

This hypothesis assunes interaction between defective proteins in the double heterozygotes or that the mutant form of one protein sequesters the wild-type form of the other protein into an inactive complex. Both cases may lead to the appearance of mutant phenotype in the F_1 generation.

An alternative explanation is that these two mutants are allelic and a third *emp arising the novo segregates* in the F_2/F_3 progenies. An additional evidence of interaction between different *emp* isolates is the recovery of at least two different emp phenotypes in cases of 9:7 segregation.

However we should consider that the mutant interaction we are studying is performed in a non homogeneous genetic background.

In spite of this difficulty in two cases of 9 to 7 segregation we were able to identify two distinct emp phenotypes that fit the 9:4:3 segregation ratio suggesting the existence of an epistatic interaction between these *emp* mutants.

As previously mentioned the presence of a 9:7 segregation in the F_2/F_3 progeny is taken as evidence of two genes, while the 3:1 segregation indicates that only one gene is segregating.

However in many cases (see Table 7) while scoring F_2/F_3 progeny, we observed segregations with intermediate values between 25% expected for 1 gene and 43.75% expected for two genes.

In these cases 9:7 segregations, could be misclassified as 3:1, thus accounting for the observed deficit of 9:7 segregations.

Alternatively F_2 ears segregating for one gene could be misclassified as double mutant heterozygotes yielding progeny with only the 3:1 segregation.

We have also applied a test to assay presence of male gametophytic selection: against the mutant in the case of *emp*- 8971*, *emp*- 8077* and *emp*- Dap3*, all three behaving as single gene mutant with a good 3:1 segregation (see Table 1). In all these mutants no male gamethopytic selection was detected.

We also tested a progeny of F_1 with heteroallelic genotypes (*emp*-8075* and *emp*-8971*).

In this F_2 the segregation of mutant seeds is significantly less than 1/4.

Even in this case we didn't find male gametophytic selection, so the deficit cannot be explained with defects in male gametophyte development.

A possible explanation is that these *emp* genes are required for the development or functioning of the female gametophyte (embryo sacs) or are involved in the sterility of pollen such that fewer than the expected 50% of the functional gametophytes of *Emp/emp* plant carry the defective allele (Clark and Sheridan 1988).

This work will provide new knowledge about the two compartments of the seed, the endosperm and the embryo, and will help to clarify the interaction between these genes that are essential for the correct development of these major parts of the maize kernel.

References

Avery and Wasserman 1992 Ordering gene function: the interpretation of epistasis in regulatory hierarchies. *Trends in Genetics* **8**, 312-316.

Beckett JB. 1978. B-A translocations in maize. Use in locating genes by chromosome arms. *Journal of Heredity* **69**, 27-36.

Becraft PW, Stinard PS, Mc Carty D, 1996. CRINKLY4: A TNFR-like receptor kinase involved in maize epidermal differentiation. *Science* **273**: 1406-1409.

Becraft PW, Assuncion-Crabb Y.2000. Positional clues specify and maintain aleurone cell fate in endosperm development. Development 127: 4039-48.

Becraft PW, Li K, Dey N, Assuncion-Crabb YT. 2002 The maize *dek1* gene functions in embryonic pattern formation and in cell fate specification. *Development* **129**: 5217-5225.

Bommert P, Werr W. 2001. Gene expression patterns in the maize caryopsis: clues to decisions in embryo and endosperm development. *Gene* **271**, 131–142.

Boyer CD, Hannah LC. **1994.** Kernels mutants of corn. In *AR Hallauer, ed, Specialty Corns. CRC, Boca Raton, FL*, pp 1-28.

Buckner B., San Miguel P., Janick-Buckner D., Bennentzent J. 1996. The *y1* Gene of Maize Codes for Phytoene Synthase. *Genetics* **143**: 480-488.

Buckner B, Janick-Buckner D, Gray J, Johal GS. 1998. Cell death mechanisms in maize. *Trends in Plant Science* **3**, 218–223.

Buckner B, Johal GS, Janick-Buckner D. 2000. Cell death in maize. *Physiologia Plantarum* **108**: 231-239.

Carlborg and Haley 2004. Epistasis: too often neglected in complex trait studies? *Genetics* **5**, 618-625.

Cheng WH, Taliercio EW, Chourey PS. 1996. The *Miniature1* seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. *Plant Cell* **8**: 971–983.

Clark JK, Sheridan WF. 1991. Isolation and characterization of 51 embryo-specific mutations of maize. *The Plant Cell* **3**: 935-951.

Coleman C.E., Clore A.M., Ranch J.P., Higgins R., Lopes M.A. 1997. Expression of a mutant alpha-zein creates the floury2 phenotype in transgenic maize. *Proc. Natl. Acad. Sci. USA* **94**: 7094-7097.

Consonni G, Busti E, Dolfini S, Giulini AP, Landoni M, Maccabe AP, Pilu R, Gavazzi G. 1998. Genetic dissection of early embryogenesis in maize. *PIP Newsletter* **15**, 12–14.

Consonni G, Aspesi C, Barbante A, Dolfini S, Giuliani C, Giulini A, Hansen S, Brettschneider R. 2003. Analysis of four maize mutants arrested in early embryogenesis reveals an irregular pattern of cell division. *Sexual Plant Reproduction* **15**: 281-290.

Consonni G, Gutierrez-Marcos J.F., Dal Prà M, Giulini A, Costa LM, Gavazzi G, Cordelier S, Sellam O, Tatout C, Wyatt P, Perez P, Dickinson H.G. 2007. *empty pericarp4* encodes a mitochondrion-targeted pentatricopeptide repeat protein necessary for seed development and plant growth in maize. *The Plant Cell* **19**: 196-210.

Da Costa e Silva O, Larbiecke R, Garg P, Muller L, Wabmann M, Lavert P, Scanlon M, Hsia AP, Schinable PS, Krupinska K, Wienand U, 2004. The *Etched1* gene of *Zea mays* (L) encodes a zinc ribbon protein that belongs to the transcriptionally active chromosome (TAC) of plastids and is similar to the transcription factor TFIIS. *The Plant Journal* **38**: 923-939.

Durantini D, Giulini A, Malgioglio A, Pilu R, Tuberosa R, Sanguineti C, Gavazzi G. 2008. Vivipary as a tool to analyze late embryogenic events in maize. *Heredity* **101**: 465-470.

Elster R.; Bommert P.; Sheridan W.F.; Werr W.; 2000. Analysis of four embryo specific mutants in Zea mays reveals that incomplete radial organization of the proembryo interfers with subsequent development. *Development genes evolution* **210:** 300-331.

Finkelstein RR, Gampala SSL, Rock CD. 2002. Abscisic acid signalling in seeds and seedling. *The Plant Cell* **14** Suppl:S 15-45.

Fu S, Meeley R, Scanlon MJ. 2002. *empty pericarp2* encodes a negative regulator of the heat shock response and is required for maize embryogenesis. *The Plant Cell* **14**, 3119–3132.

Fu S, Scanlon MJ. 2004. Clonal analysis of EMPTY PERICARP2 reveals nonredundant functions of the duplicated HEAT SHOCK FACTOR BINDING PROTEINs during maize shoot development. *Genetics* **167**, 1381–1394.

Gavazzi G, Dolfini S, Allegra D, Castiglioni P, Todesco G, Hoxha M. 1997. *Dap* (*Defective aleurone pigmentation*) mutations affect maize aleurone development. *Molecular General Genetics* **256**: 223-230.

Giraudat, J; Parcy, F; Bertauche, N; Gosti, F; Leung, J; Morris, PC; Bouvier-Durand, M; Vartanian, N. 1994. Plant MolBiol **26**: 1557-1577.

Giuliani C, Consonni G, Gavazzi G, Colombo M, Dolfini S. 2002. Programmed cell death during embryogenesis in maize. *Annals of Botany* **90**: 287-292.

Glover DV, Mertz ET. 1987. Corn. *In* RA Olson, KJ Frey, eds, Nutritional Quality of Cereal Grains: Genetic and Agronomic Improvement. American Society of Agronomy, Madison, WI, pp183-336.

Hable WE, Oishi KK, Schumaker KS. 1998 Viviparous-5 encodes phytoenedesaturase, an enzyme essential for abscisic acid (ABA) accumulation and seed development in maize. *Mol Gen Genet*. **257**:167-76.

Hawley and Gilliland 2006. The second site non-complementation. *Genetics* **174**: 5-15.

Heckel T, Werner K, Sheridan WF, Dumas C, Rogowsky PM. 1999. Novel phenotypes and developmental arrest in early embryo specific mutants of maize. *Planta* **210**: 1-8.

He X, Quian W, Wang Z, Li Y, Zhang J. 2010. Prevalent positive epistasis in *Escherichia coli* and *Saccharomyces cerevisiae* metabolic networks. *Nature Genetics* **42**, 272-276.

Jin P, Guo T, Becraft PW (2000) The maize CR4 receptor-like kinase mediates a growth factor-like differentiation response. Genesis **27:** 104-116.

Kang Byung-Ho, Xiong Y, Williams D.S, Pozueta-Romero, Chourey P.S. 2009. *Miniature1* encoded cell wall invertase is essential for assembly and function of wall-in-growth in the maize endosperm transfer cell. *Plant Physiology* **151**, 1366-1376.

Kiesselbach TA. 1949. The structure and reproduction of corn. *Lincoln, NE: University of Nebraska Press.*

Kladnik A, Chamusco K, Dermastia M, Chourey P. 2004. Evidence of programmed cell death in post-phloem transport cells of the maternal pedicel tissue in developing caryopsis of maize. *Plant Physiology* **136**, 3572–3581.

Lid SE, Gruis D, Jung R, Lorentzen JA, Ananiev E, Chamberlin M, Niu X, Meeley R,Nichols S, Olsen O-A 2002. The *defective kernel 1* (*dek1*) gene required for aleurone cell development in the endosperm of maize grains encodes a membrane protein of the calpain gene superfamily. Proc Natl Acad Sci USA **99**: 5460-5465.

Lid SE, Ronald H, Trygve Krekling, Robert B, Meeley, Jerry Ranch AE, Hilde-Gunn Opsal-Ferstad, Odd-Arne Olsen 2004. The maize disorganized aleurone layer 1 and 2 (dil1, dil2) mutants lack control of the mitotic division plane in the aleurone layer of developing endosperm. *Planta* **218**: 370-378.

Lopes MA, Larkins BA. 1993. Endosperm origin, development, and function. *The Plant Cell* **5**, 1383–1399.

Ma Z, Dooner HK. 2004. A mutation in the nuclear-encoded plastid ribosomal protein S9 leads to early embryo lethality in maize. *The Plant Journal* **37**: 92–103.

Magnard JL, Heckel T, Massonneau A, Wisniewski JP, Cordelier S, Lassagne H, Perez P, Dumas C, Rogowsky PM. 2004. Morphogenesis of maize embryos requires ZmPRPL35–1 encoding a plastid ribosomal protein. *Plant Physiology* **134**: 649–663.

McCarty DR. 1995. Genetic control and integration of maturation and germination pathways in seed development. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**: 71-93.

Maitz M, Santandrea G, Zhang Z, Lal S, Hannah LC, Salamini F, Thompson RD. 2000. *rgf1* a mutation reducing grain filling in maize through effects on basal endosperm and pedicel development. *The Plant Journal* **23** (1), 29-42.

Meinke DW. 1982. Embryo-lethal mutants of *Arabidopsis thaliana*: evidence for gametophytic expression of the mutant genes. Theor Appl Genet **63**:381–386.

Miclaus M, Wu Y, Xu J.H, Dooner HK, Messing J. 2011. The maize high-lysine mutant *opaque 7* is defective in an acyl-CoA synthetase like protein. *Genetics* **189**: 1271-1280.

Motto M, Maddaloni M, Ponziani G, Brembilla M, Marotta R, Di Fonzo N, Soave C, Thompson R.D, Salamini F, 1988. Molecular cloning of the *o2-m5* allele of *Zea mays* using transposon marking. *Mol Gen. Genet.* 212, 488.

Nelson O, Pan D. 1995 Starch synthesis in maize endosperms. *Annu Rev Plant Physiol Plant Mo1 Biol* **46:** 475-496.

Neuffer MG, Sheridan WF. 1980. Defective kernel mutants of maize. I. Genetic and lethality studies. *Genetics* **95**, 929–944.

Neuffer MG, Sheridan F. 1981. A group of new mutants defective in kernel developent – the *dek* mutants. *Maize Genet. Coop. News Lett*. **55**: 29-30.

Olsen O-A. 2001. Endosperm development: cellularization and cell fate specification. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 233–267.

Phillips P. C. 2008. Epistasis – the essential role of gene interactions in the structure and evolution of genetic systems. *Genetics* **9**, 855-867.

Pilu R, Consonni G, Busti E, MacCabe AP, Giulini A, Dolfini S, Gavazzi G 2002. Mutations in two independent loci lead to suppression of the shoot apical meristem in maize. *Plant Physiol* **128**:502–511.

Randolph L.F. 1941. Genetic characteristics of the B chromosome in maize. *Genetics* **26**: 608-631.

Roman H. 1947. Mitotic nondisjunction in the case of interchanges involving the B-type chromosome in maize. *Genetics* **32**: 391-409.

Roman H. 1948. Directed fertilization in maize. *Proc. Nat. Acad. Sci.* **34**: 36-42.

Scanlon MJ, Stinard PS, James MG, Myers AM, Robertson DS. 1994. Genetic analysis of 63 mutations affecting maize kernel development isolated from Mutator stocks. *Genetics* **136**, 281–294.

Shannon JC, Garwood DL 1984. Genetics and physiology of starch development. *In* RL Whistler, JN Bemiller, EF Paschall, eds, Starch: Chemistry and Technology, Ed 2. Academic Press, New York, pp 25-86.

Schmidt, R. J., F. A. Burr, and B. Burr, 1987. Transposon tagging and molecular analysis of the maize regulatory locus *opaque-2*. *Science* **238**: 960–963.

Shen B, Li C, Min Z, Meeley RB, Tarczynski MC, Olsen OA. 2003. *sal1* determines the number of aleurone cell layers in maize endosperm and encodes a class E vacuolar sorting protein. *Proc Natl Acad Sci U S A* **100:** 6552-6557.

Sheridan WF, Clark JK. 1993. Mutational analysis of morphogenesis of the maize embryo. *The Plant Journal* **3**: 347–358.

Sheridan WF, Neuffer MG. 1980. Defective kernel mutants of maize. II. Morphological and embryo culture studies. *Genetics* **95**, 945–960.

Sheridan WF, Neuffer MG. 1981. Maize mutants altered in embryo development. *In: Alan R Liss, ed. Levels of genetic control in development. New York: Alan R. Liss. Inc*, 137–156.

Sheridan WF, Thorstenson YR. 1986. Developmental profiles of three embryo-lethal maize mutants lacking leaf primordia: *ptd*-1130, cp*-1418* and *bno*-747B. Developmental Genetics* **7**: 35–49.

Sosso D, Javelle M, Rogowsky P. 2011. Embryogenesis andearly grain development. In: JL Prioul, C Thévenot, T Molnar, editors,*Advances in maize* vol. **3.** London: Society for Experimental Biology pp. 163–188.

Sosso D, Canut M, Gendrot G, Dedieu A, Chambrier P, Barkan A, Consonni G, Rogowsky PM. 2012. PPR 8522 encodes a chloroplast-targeted pentatricopeptide repeat protein necessary for maize embryogenesis and vegetative development. *Journal of Experimental Botany* **63** (16), 5843-5857.

Tian Q, Olsen L, Sun B, Lid SE, Brown RC, Lemmon BE, Fosnes K, Gruis DF, Opsahl-Sorteberg HG, Otegui MS, Olsen OA. 2007. Subcellular localization and functional domain studies of DEFECTIVE KERNEL1 in maize and *Arabidopsis* suggest a model for aleurone cell fate specification involving CRINKLY4 and SUPERNUMERARY ALEURONE LAYER1. *Plant Cell* **19:** 3127-3145.

Vernoud V, Hajduch M, Khaled A-S, Depege N, Rogowsky P. 2005. Maize embryogenesis. *Maydica* **50**, 469–483.

Wisniewski JP, Rogowsky PM 2004. Vacuolar H+-translocating inorganic pyrophosphatase (Vpp1) marks partial aleurone cell fate in cereal endosperm development. *Plant Mol Biol* **56:** 325-337.

Chapter 3

Effects of the genetic background on the expression of empty pericarp mutants

Effects of the genetic background on the expression of *empty pericarp* mutants.

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Abstract

In this report evidence is presented of the existence of a cryptic genetic variation (CGV) in the genetic background of different inbred lines.

Such variation is monitored by changes in the phenotype of the *emp* mutants characterized by a strong, almost complete, suppression of the endosperm and a developmentally blocked embryo.

The unmasking of CGV by introducing the mutants in different genetic backgrounds is the result of interaction of the *emp* mutants with a modifier (m-ed) with no obvious phenotype of its own, present in the genetic background of the inbred lines where the *emp* mutants are transferred .

The presence of *emp* mutants is instrumental in disclosing presence of these modifiers.

Four cases of interaction between a specific *emp* and a modifier have been so far detected.

The specificity of the modifier, *i.e.* its capacity to affect a given *emp* or several ones has not yet been elucidated.

In one case the modifier behaves as dominant (*emp*-9475 M-ed*) and allows the recovery of germination even though in a low percentage (25%) and the resulting seedlings appear stunted with narrow leaves. In another case (*emp*-8971 M-ed*) the modifier is dominant but its enhancing effect on the endosperm tissues is not sufficient to resume the embryonic potential to germinate although a very low % of the seeds, with the improved endosperm, (8%) germinate into apparently normal seedlings.

For the *emp*-8300* partial recovery of the germination was observed in a low % (3%) but the nature of its modifier has not yet been elucidated.

In the last case the modifier of *emp*-8376* (*m-ed *8376*) behaves as recessive and surprisingly restores almost completely the germination giving rise to lethal seedlings with a pale green phenotype.

If these results are confirmed *emp* mutants could be used as a tool for the detection of genetic factors enhancing the amount of endosperm tissue in the maize kernel to exploit in breeding programs.

Keywords: *emp* mutants, embryo, improved endosperm, cryptic variability, modifier

Introduction

Cryptic genetic variation (CGV) is defined as genetic variation that does not contribute to the normal range of phenotypes observed in a population, but that is unmasked after environmental changes or the introduction of novel alleles.

A promising strategy for detecting CGV involves introgression of a mutant allele into a set of wild-type lines, and make use of changes in the mutant phenotype to monitor such criptic variation.

Naturally occurring variation can be employed as a gene discovery tool, by detecting interactions between a defined mutation and natural alleles in other genes from various genetic backgrounds. This variation only becomes apparent when it is combined with a mutation of interest. (Queitsch *et al*., 2002; Sangster *et al*., 2008).

Ironically, CGV is often observed and deliberately discarded as the "background effect" that can confound the early analysis of a mutation. However, recent characterization of diverse germplasm for species like maize (Flint-Garcia *et al*., 2005; Whitt and Buckler, 2003; Yu *et al*., 2008) and *Arabidopsis* (Alonso-Blanco *et al*., 2005; de Meaux and Koornneef *et al*., 2004; Keurentjes *et. al.*, 2008) suggests that we should re-examine genetic background effects.

Waddington (1952/53) suggested a mechanism to explain the maintenance of the hidden genetic variation and an alternative model for the evolutionary process know as "genetic assimilation". The model of genetic assimilation predicts that in presence of unusual environmental conditions or new mutations, phenotypes can be genetically "captured" by

the process of natural selection. Implicit in this evolutionary model is the existence of cryptic genetic variation for the trait unmasked following appropriate environmental stimuli, as well as buffering mechanism, referred to as "canalization" which helps to "store" the genetic variation.

Materials and methods

Isolation of mutants

The *emp* mutants analyzed, *emp*-8300, emp*-8376, emp*-8971* and *emp*-9475* where chosen from a collection of mutants obtained by transpositional mutagenesis (Consonni *et al*.,1998) or spontaneous in their origin.

They were originally isolated in the F_2 of selfed F_1 plants derived by crossing a line maintained in our laboratory by repeated selfing with a line carrying active *Mu* (*MuDR*) or *Spm* transposons.

They all behave as single gene recessive mutants in their original backgrounds and are propagated as heterozygotes, being lethal in the homozygous condition.

emp-8300* and *emp*-8971* are allelic while the two remaining mutants, not allelic to each other, identify two different genes.

Germination test

Mutant seeds were selected and surface sterilized for 20 min in a solution of 1:1 bleach : water then rinsed in water and sowed on a layer of three wet filter papers plexiglass containers. They were then covered with one sheet of filter paper and kept for three days in dark in a growth chamber at 24-25°C and then transferred to continuous light.

The seedlings, after a week, were transferred and mentained in pots in the growth chamber for a month before transplantation to the field.

Results

While scoring ears segregating for a single *emp* mutants, in different genetic backgrounds, we noticed that some mutant seeds exhibited a more pronounced endosperm tissue and occasionally an embryonic axis.

This phenomenon was observed for four mutants: *emp*-8300* in W23, *emp*-8376* in A636, *emp*-8971* in W23 and *emp*-9475* in Mo17.

As reported in Table 1, a low % of these seeds germinate, except *emp*-8376* that shows almost normal germinability.

Table 1. Germination (%) of "*modified emp*" mutants, as determined in the F_2 progeny. For each mutant, from 25 to 300 seeds were tested.

Genotype	*% of germination*	*Phenotype of the seedling*
+/emp- 8300* in W23 ⊗	*3%*	Small green seedling *(1)*
+/emp- 8971* in W23 ⊗	*8%*	Normal green seedling *(3)*
+/emp- 8376* in A636 ⊗	*93%*	Pale green lethal seedling with narrow leaves *(2)*
+/emp-9475* in Mo17 ⊗	*25%*	Stunted green seedling with narrow leaves *(4)*

Seedling phenotype:

(1) (2) (3) (4)

Figure 1: Morphology of *emp* caryopsis in different genetic backgrounds. From left to right: entire and longitudinal sections of mutant seeds of *+/emp*-8077* in ACR (a, f), *+/emp*-8376* in A636 (b, g), *+/emp*-8971* in W23 (c, h), *+/emp*-8300* in W23 (d, i) and *+/emp*-9475* in Mo17 (e, l). *emp*-8077* is here included as a reference phenotype.

Identification of endosperm modifiers

When plants heterozygous for these abnormal *emp* (designated in our lab with the provisional term of *dek-emp*) were crossed with plants carrying the original *emp*, referred as "*emp Ref*", the resulting progeny ears shows seeds of the two phenotypes in a 1:1 ratio, a result expected in the presence of a segregating modifier of *emp*, enhancing the amount of endosperm tissue in the seed.

The results obtained with these crosses are showed in the Table 2.

Table 2. Evidence of segregation of an endosperm modifier: two classes of mutant seeds are recovered in a 1:1 ratio in the F1 progeny of the crosses here reported.

Cross made	Total	Number of wt	Number of m	% of mutant seeds	*dek-emp*	*emp*
+/emp-8376 Ref* in B73 x *+/emp*-8376* segr for *dek-emp* in A636	256	200	56	21.87%	28	28
+/emp-8971 Ref* x *+/emp*-8971* segr *dek-emp* in W23	1093	844	249	22.78%	126	123

We then selected wild-type seeds from crosses reported in Table 2, selfed the resulting plants and scored the ears for presence of the two emp phenotypes expected in presence of a segregating modifier. The results obtained with *emp*-8971* are reported in Table 3.

Table 3. Identification of a dominant (M-*emp*) modifier of *emp*-8971*

Field code	**Total**	**m[(1)]**	**wt**	**dek-emp**	**emp**
11.20-2 ⊗	**434**	**89 (20.50%)**	**345**	**61 (68%)**	**28 (32%)**
11.20-3 ⊗	**656**	**140 (21.34%)**	**516**	**100 (71%)**	**40 (29%)**
Total values	**1090**	**229 (21%)**	**861**	**161 (70%)**	**68 (30%)**

(1) total number of emp (emp + dek-emp) mutants

The observed segregation (12 wt, 3 dek-emp and 1 emp) suggests that the paternal *+/emp*-8971* carries a dominant modifier (M-*emp*) of *+/emp*-8971*.

Table 5: Identification of a dominant (M-*emp*) modifier of *emp*-9475*

Field code	Total	m[(1)]	wt	dek-emp	emp
11.23 B-1 ⊗	**471**	**120 (25.47%)**	**351**	**90 (75%)**	**30 (25%)**
12.111(1) ⊗	**403**	**122 (30.27%)**	**281**	**92 (75%)**	**30 (25%)**
Total values	**874**	**242 (27.69%)**	**632**	**182 (75%)**	**60 (25%)**

(1) total number of emp (emp + dek-emp) mutants

In the case of *emp*-9475* the behavior of this modifier was ascertained by selfing plants of *+/emp*-9475* introduced in the Mo17 inbred line that disclosed a 3:1 segregation for *dek-emp*:*emp* seeds. Progeny ears revealed a segregation ratio of 12 wt, 3 dek-emp and 1 emp that indicate the dominant nature of this genetic factor.

The fact that the wt seeds from the ear that segregates for dek-emp gave this segregation could be explained by assuming that this ear is not really homozygous for the modifier.

Table 6: Identification of a recessive (m-*emp*) modifier of *emp*-8376*

Field code	Total	m[(1)]	wt	*emp*	*dek-emp*
11.21 (2) ⊗	**504**	**98 (19.44%)**	**406**	**61 (62%)**	**37 (38%)**
11.21 (3) ⊗	**549**	**150 (27.32%)**	**399**	**103 (69%)**	**47 (31%)**
11.21-6 ⊗	**460**	**111 (24.13%)**	**349**	**78 (70%)**	**33 (30%)**
Total values	**1513**	**359 (23.73%)**	**1154**	**242 (67%)**	**117 (33%)**

(1) total number of emp (emp + dek-emp) mutants

Among the *emp* mutants observed in the progeny of *+/emp*-8376 Ref* in B73 x *+/emp*-8376* segregating for *dek-emp* in A636, about 3/4 are *emp* in phenotype and 1/4 *dek-emp*.

This segregation (12 wt, 1 *dek-emp* and 3 *emp*) suggests that the paternal *+/emp*-8376* carries a recessive modifier (m-*emp*) of *+/emp*-8376*.

Conclusions

The segregation of *emp* and *dek-emp* observed after transferring the *emp* mutants from their native background to the one of an inbred line is expected in presence of the interaction of the *emp* mutant with a modifier, originally present in the inbred where the *emp* was introduced.

The effect of the modifier is that of enhancing the amount of endosperm tissue of the mutant. We still have to establish how specific is the interaction between *emp* mutants and the modifier.

Of the four modifiers so far detected two are dominant and one recessive in their action while the behavior of the last one is not yet established.

In one case (M-ed of *emp-*9475*) the modifier can partially rescue the germination capacity of the mutant embryos. The resulting seedlings appear stunted in their growth and with impaired morphogenesis.

The other dominant modifier (M-ed of *emp*-8971*) does not restore germination.

As to recessive modifier of *emp*-8376,* this is the only case where the embryo proper is clearly visible although it appears more irregular and enlarged when compared with the wt sibs and its scutellum exhibits an irregular shape. It's not clear, why the seedling from these dek-emp seeds is lethal with pale green, narrow leaves. A possible explanation of this seedling phenotype is that *emp*-8376* may have a general role in plastid function and/or development. In this view, mutant amyloplast development would cause disruptions in endosperm structure, whereas mutant chloroplast development would lead to discolored, striated or albino seedlings.

The observation of the cryptic variability of the endosperm/embryo interaction and the presence of dominant and recessive modifiers, needs to be further investigated, but if confirmed, *emp* mutants could be used as a tool for the detection of genetic factors enhancing the amount of endosperm in the maize kernel to exploit in breeding programs.

References

Alonso-Blanco, C., B. Mendez-Vigo, and M. Koornneef. 2005. From phenotypic to molecular polymorphisms involved in naturally occurring variation of plant development. Int.J. Dev.Biol. **49**:717-732.

Consonni G, Busti E, Dolfini S, Giulini AP, Landoni M, Maccabe AP, Pilu R, Gavazzi G. 1998. Genetic dissection of early embryogenesis in maize. PIP Newsletter **15**, 12–14.

de Meaux, J., and M. Koornneef 2004. The cause and consequences of natural variation: The genome era takes off! Curr. Opin. Plant. Biol. **11**: 99-102.

Flint-Garcia, S.A., A.C. Thuillet, J. Yu, G. Pressoir, S.M. Romero, S.E. Mitchell, J. Doebly, S. Kresovich, M.M. Goodman, and E.S. Buckler, **2005.** Maize association population: A high resolution platform for quantitative trait locus dissection. Plant J. **44**: 1054-1064.

Keurentjes, J.J., M. Koornneef and D. Vreugdenhil. 2008. Quantitative genetics in the age of omics. Curr. Opin. Plant. Biol. **11**: 123-128.

Koornneef, M., C. Alonso-Blanco, and D. Vreugdenhil 2004. Naturally occurring genetic variation in Arabidopsis thaliana. Annu. Rev. Plant Physiol. **125**: 156-159.

Queitsch, C., Sangster T.A. and S. Lindquist. 2002. Hsp 90 as a capacitor of phenotypic variation. Nature **417**: 618-624.

Sangster T.A., N.Salathia, H.N.Lee, E. Watanabe, K.Schellenberg, K. Morneau, H. Wang, S.Undurraga, C. Queitsch and S. Lindquist. 2008. HSP90 buffered genetic variation is common in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA **105**: 2969-2974.

Waddington, C.H. 1952. Selection for the basis of an acquired character. Nature **169**, 278.

Waddington, C.H. 1953. Genetic assimilation of an acquired character. Evolution **7**, 118-126.

Witt, S.R. and E.S. Buckler. 2003. Using natural allelic diversity to evaluate gene function. Methods Mol. Biol. **236**: 123-140.

Yu, J., J.B. Holland , M.D. Mc Mullen, and E.S. Buckler. 2008. Genetic design and statistical power of nested association mapping in maize. Genetics **178**: 539-551.

Chapter 4

Preliminary analysis of the role of brassinosteroids in ear development.

Preliminary analysis of the Role of brassinosteroids in the ear elongation and number of seeds: a study about the *lilliputian1-1* mutant of maize

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Abstract

Brassinosteroids (BRs) are a group of naturally occurring plant steroidal compounds with wide ranging biological activity. Their role in plant growth and development has been well-characterized in several plant species. However, very little is known about the role of brassinosteroids in maize. Because BRs control several important plant traits such as flowering time, plant architecture, leaf angle, plant height, seed yield and stress tolerance; we focused our attention on an extremely dwarf mutant of maize, called *lilliputian1-1*, that has this feature due to a defect in the BRs synthesis (Makarevitch *et. al.* 2012).

We compared the ear length, seed production and seed weight of plants heterozygous for mutant with the homozygous *Lil1-1/Lil1-1* sibs in two different genetic backgrounds (A188 and B73): the results obtained in this study, although preliminary, suggest that in maize BRs are involved in the control of ear elongation and number seed per ear. Heterozygous plants produce ears that are shorter with less seeds respect to the normal plants.

Further data are needed to confirm these results and it is also necessary to investigate the fact that these differences are due to male or female gametophytic selection.

The data collected will help to unravel the role of BRs in this important crop system and in it's productivity.

Keywords: brassinosteroids, *lil1-1* mutant, primary root, productivity, seed weight

Introduction

Manipulation of plant height and growth habits in order to improve agronomic production in crop plants is a practice that still has a great importance (Salas Fernandez *et al.*, 2009).

Gibberellins (GAs), a large group of cyclic diterpene compounds that promote stem elongation, and brassinosteroids (BRs), commonly occurring steroids hormones which regulate multiple aspects of plant growth and development, are two classes of hormones that alter plant architecture when aberrations occur in their biosynthesis or signaling pathways (Salas Fernandez *et al*., 2009; Clouse *et al*., 2011).

For example mutations in GA-related genes are responsible for the semidwarf phenotypes associated with the green revolution (Peng *et.al*., 1999).

Mutants deficient in brassinosteroid biosynthesis and signaling display various levels of dwarfism (Salas Fernandez *et al*., 2009). BR biosynthesis and signaling pathways are well established and several mutants from these pathways are characterized in *Arabidopsis*, pea, tomato, and rice (Fujioka *et al*., 2003, Hong *et al*., 2003, Hong *et al*., 2005., Tanabe *et al*., 2005). However, relatively little is known about the specific functional role of brassinosteroids in maize (Salas Fernandez *et al*., 2009).

With the aim to investigate the possible relationship that might exist between brassinosteroids and productivity –that manifest itself as weight and number of seeds carried by the ear-, we focused our attention on a unique monogenic, recessive mutant of maize, called *lilliputian1-1* (*lil1-1*), isolated by mutagenesis with the transposable element *Mutator*. This mutant exhibits a reproducible phenotype consisting of a large primary root, extremely reduced stature and crinkly leaves.

Histological analyses (Dolfini *et al*, 1999) revealed that this mutation is due to a disruption at the level of microtubule organization: mutant cells of the primary root have a prolonged mitotic activity and defects in the division plane alignment.

Experiments conducted by M. Galbiati in the year 1994 and M. Landoni in the year 1999 ruled out the link between the lesion in the *Lil1-1* gene and the pathway of gibberellins. Other tests made with applications of exogenous brassinolide on seeds grown on paper for two weeks, showed a partial response of this mutant to the administration of this hormone: the roots tips were curled and the primary root of the mutants on BR was thinner with

respect to that of the untreated mutants. The growth of the shoot did not show differences between control and treatment. Assuming therefore that this mutant has got some defects in the synthesis of BRs and because in the literature there were several works carried on rice, tomato and *Arabidopsis* that showed the role of BRs in the productivity, intended as number of seeds and weight of them, a new work started following the data present in the work of Wu *et al.* 2008.

Molecular analysis performed in our laboratory has shown that this gene encodes for the enzyme brassinosteroid C-6 oxidase that catalyzes the final steps of brassinosteroid biosynthesis.

Materials and methods

Mutant isolation and propagation

This mutant was isolated from the selfed progeny of a *Mutator* stock outcrossed to an unrelated stock (Gavazzi *et al.*, 1993). Mutant segregation was confirmed through two cycles of selfing and segregation ratios were established in the F_2 progenies obtained by outcrossing heterozygous mutant plants first to the inbred line W64A, a "non *Mutator*" line that reduces the elevate number of copies of the transposable elements, and then to the inbred lines A188 and B73.

Ears analyzed in this work were obtained from crosses between heterozygous plants to these inbred lines A188 and B73. The resulting F_1 seeds were planted and F_1 plants self-pollinated to obtained the F_2 families employed in each test.

B-A translocations in maize and chromosomal arm attribution of the mutant

The B-A translocations of maize provide an efficient method for locating recessive mutants to the proper chromosome arm. (Beckett, 1978).

B-A translocations are intherchanges that involve a supernumerary B-chromosome and members of the basic (A) set of chromosomes.

The B chromosome is widely distribuited in races of maize and is relatively inert and innocuous when low numbers are present (Randolph LF, 1941).

Translocations are obtained irradiating with x-rays mature pollen of plants that carry B chromosomes. Individuals carrying a B chromosome produce unbalanced gametes carrying 0-1-2 B chromosomes.

This phenomenon is due to non-disjunction at the second microspore mitosis occurring with a frequency of 90-100%, giving pollen grains with dissimilar sperms: one hyperploids with two B-A chromosomes and one hypoploid with none.

Fertilization of the egg-cell and central cell by the two sperms from such a pollen grain gives a kernel with non-corresponding embryo and endosperm genotypes; when the embryo receives two B-A chromosomes, the endosperm receives none and viceversa (Roman,1947).

The sperm carrying the extra B-A chromosome usually fertilizes the egg about 2/3 of the time (preferential fertilization Roman, 1948).

The mutant located in the hemizygous chromosomal segment, will manifest itself, allowing its attribution to a specific chromosomal arm.

Chromosomal arm attribution was established by crossing the progeny of outcrossed heterozygous *+/lil1-1* plants with male parents including the two set of B-A translocations (1La and 2Sa). The source of these stocks is the Maize Cooperation Stock Center, Urbana, Ill. USA. (http://maizecoop.cropsci.uiuc.edu/)

Ears (5-10) from these crosses were germinated to score the presence of mutant seedlings with a frequency of 5-10% as evidence that the translocation is uncovering the mutant under test.

Ear analysis

Ears were harvested and data were collected in this manner.

-The values of the length of the ears were obtained by marking the basis and the tip of the ear on a sheet of paper and measuring the distance between the two marks with a ruler.

-The ears were shelled and the total number of seeds was determined. The seeds were weighed on a normal scales.

-To genotype each ear, 20-40 kernels were germinated on filter paper and scored for *lil1-1* mutant segregation.

-In an additional test, from each ear 20 kernels were chosen, these seeds were mixed together in two bulks: one from the segregating population and one from the non-segregating population. Seeds, were then weighed individually on an analytical scale. Subsequently they were sowed into small pots in order to correlate the phenotype of the seedling to the weight of the seed that originated the seedling.

The data collected were analyzed with the statistical test of ANOVA (SPSS 18.0 for Windows).

Results

Genetic analysis of lil1-1 mutant

lil1-1 behaves as a monogenic recessive mutant (Gavazzi *et. al.*, 1993), and exhibits a reproducible phenotype consisting of a large primary root, crinkly leaves and extremely reduced stature (Figure1).

Figure 1 *Lil1-1* phenotype. A: Comparison between wt (left) and *lil1-1* mutant seedling (right) after 8 DAG (days after germination).

B: Mutant seedling at 8 DAG. Note the thick primary root, partially insensible to the gravity force.

C: A mutant plant at maturity. Note the extremely reduced stature and the crinkly leaves.

A B C

Molecular analysis performed in our laboratory has shown that the *lil1-1* gene sequence is localized on the long arm of chromosome 1. The crosses between plants heterozygous for *lil1-1* and plants of two genetic stocks carrying TB-A (1La and 2Sa) revealed that the mutant is localized on the long arm of chromosome 1 thus confirming the result obtained with the molecular tools.

Ears segregating for the *lil1-1* mutation were compared with wild-type ears for different traits. The analysis was performed on three different F_2 progenies. One was obtained from introgressing the mutation in the A188 line- C 574 (6) ⊗, C 574 (7)⊗, C 574-34⊗ e C 574-93 ⊗- and two were obtained after introgressing one- C 575-4 ⊗, C 575-18 ⊗, C575-24 ⊗, C 575-27 ⊗ C 575-30 ⊗, C 575 (1) ⊗, C 575-105 ⊗- or two times- C 675 (1) ⊗, C 675 (2) ⊗, C 675 (4) ⊗, C 675 (5) ⊗, C 675 (11) ⊗ C 675-8 ⊗, C 675-46 ⊗, C 676-4 ⊗, C 676-25 ⊗, C 676-28 ⊗, C 676-41 ⊗ e C676-56 ⊗- respectively the mutation in the B73 line.

Data collected from each experiment along with statistical analysis are reported in table 1, 2, 3. Data are also shown as percentage of mutant graph.

Table 1. Phenotype data from heterozygous and homozygous wild-type plants. Ears were from selfing F_1 *+/lil1-1* (4) and from F_1 *Lil1-1/Lil1-1* (4) plants obtained from introgressing the mutation in the A188 line.

Trait	Ears segregating for *lil1-1*	Ears not segregating for *lil1-1*
Average length of ears (cm)	15.3*	18.50*
Total number of kernels	1696*	2106*
Average number of kernels ear^{-1}	424*	527*
Average weight of 100 kernels (g)	28.60	28.20
Average weight of a single kernel (g)	0.281	0.280

Test: ANOVA UNIVARIATE (SPSS 18.0 for Windows)

*Statistically different at 5% level

Table 2. Phenotype data from heterozygous and homozygous wild-type plants. Ears were from selfing F_1 *+/lil1-1* (7) and from F_1 *Lil1-1/Lil1-1* (7) plants obtained from introgressing the mutation one time in the B73 line.

Trait	Ears segregating for *lil1-1*	Ears not segregating for *lil1-1*
Average length of ears (cm)	9.57*	11.86*
Total number of kernels	2061*	2736*
Average number of kernels ear^{-1}	294	391
Average weight of 100 kernels (g)	29.58	29.02
Average weight of a single kernel (g)	0.290	0.283

Test: ANOVA UNIVARIATE (SPSS 18.0 for Windows)

*Statistically different at 5% level

Table 3. Phenotype data from heterozygous and homozygous wild-type plants. Ears were from selfing F_1 *+/lil1-1* (12) and from F_1 *Lil1-1/Lil1-1* (12) plants obtained from introgressing the mutation two times in the B73 line.

Trait	Ears segregating for *lil1-1*	Ears not segregating for *lil1-1*
Average length of ears (cm)	nd	nd
Total number of kernels	5262*	5943*
Average number of kernels ear^{-1}	439	495
Total weight of the kernels (g)	1240.289	1401.369
Average weight of a single kernel (mg)	235.707	235.802
Average weight of a single kernel (mg) determined on an analytical scales	237.318	237.968
Average weight of 100 kernels (g)	23.571	23.580
Average weight of a single kernel (g)	0.236	0.236

Test: ANOVA UNIVARIATE (SPSS 18.0 for Windows)

*Statistically different at 5% level

nd no data

Ears developed by heterozygous plants are shorter and carry fewer seeds if compared to ears obtained from homozygous wild-type sibling plants. However the total kernels' weight of the two progenies (segregating and not-segregating ears) is not different

An additional test was performed on the three populations to ascertain if in the same segregating progeny the weight of mutant seeds is different from that of normal seeds.

The test consisted in weighing each single seed on an analytical scales and determining the seed phenotype through germination and seedling analysis. For each population a pool of 140, 160 and 240 seeds from ears obtained by selfing sibilings plants was used.

Table 4. Results of the germination test of segregating ears in the A188 inbred line.

Phenotype of the seedling	Average weight (mg)	N
lil	286.388	48
wt	284.731	109
not germinated	/	3

Test: ANOVA UNIVARIATE (SPSS 18.0 for Windows)

*Statistically different at 5% level

Table 5. Results of the germination test of segregating ears after introgressing one time the mutant in the B73 inbred line.

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Phenotype of the seedling	Average weight (mg)	N
lil	292.407	41
wt	295.929	98
not germinated	/	1

Test: ANOVA UNIVARIATE (SPSS 18.0 for Windows)

*Statistically different at 5% level

Table 6. Results of the germination test of segregating ears after introgressing two times the mutant in the B73 inbred line.

Phenotype of the seedling	Average weight (mg)	N
lil	245.836	58
wt	248.243	175
not germinated	/	7

Test: ANOVA UNIVARIATE (SPSS 18.0 for Windows)

*Statistically different at 5% level

The results presented in Table 4, Table 5, and Table 6, indicate that the weight of the mutant seeds is not significantly different from that of the normal sibs. This data may indicate that the mutation have no influence on the seed weight.

Conclusions

The results here presented indicate that the *lil1-1* mutation has some influence on maize ear development. The number of seeds differ significantly between the segregating and non segregating ears. Plants heterozygous for the *lil1* gene produces ears with fewer seeds than plants that do not have the recessive allele. In contrast ears produced by homozygous *Lil1-1/Lil1-1* plants were longer and set more seeds. These results suggest that the reduction of BRs have a negative influence on the length of the ear and seed set. BRs may influence ear elongation process or alternatively might have an effect on ovule and seed development. We will also investigate the presence of male gametophytic selection in the pollen of *+/lil1-1* plants by applying to ear sector the statistic test of heterogeneity. *lil1*-1 pollen tube growth might be slower than that of wild-type siblings. As consequence, since the distance covered by the pollen tube to fertilize the basal egg cells is longer than that needed to reach the apical egg cells, there might be a lower percentage of *lil1-1/lil1-1* kernels in the basal versus apical portion of F_2 ears obtained by selfing *+/lil1-1* heterozygous parents (Meinke 1982).

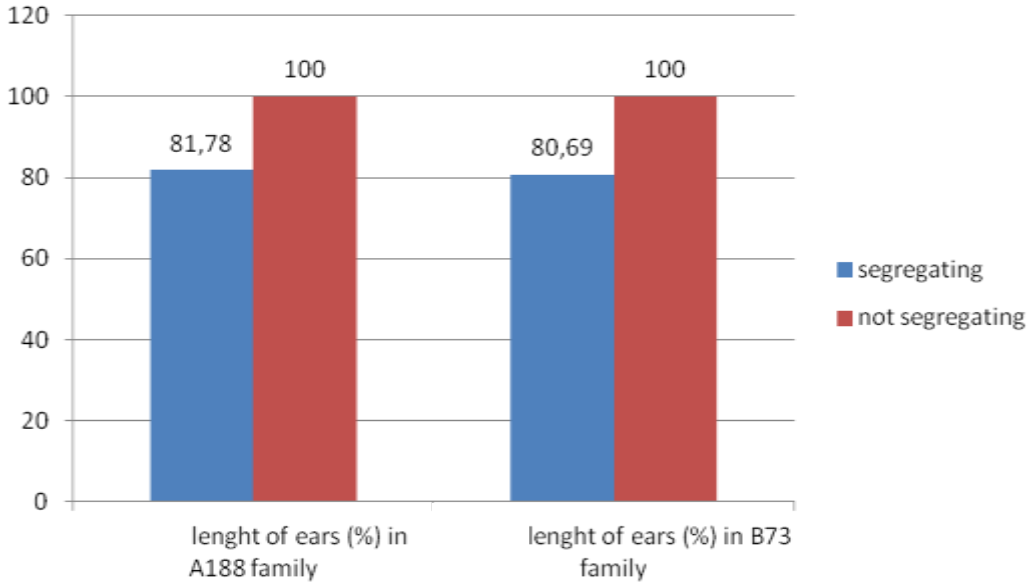
To better understand the effect of the mutation on a specific seed compartment (embryo or endosperm), we will also analyze progenies obtained by crossing plants heterozygous for the *lil1-1* gene and the TB-A 1La. These crosses with the TB-A generate two classes of seeds with embryo and endosperm that have discordant genotypes. The first class carries the hypoploid embryo (*lil1-1/*-) and hyperploids endosperm (*lil1-1/lil1-1*/+ /+) and the second class has the reciprocal combination: hyperploids embryo (*lil1-1/+ /+)* and hypoploid endosperm (*lil1-1/ lil1-1/* -).

Several works in rice (Sakamoto, T. *et al*. 2006, Wang, L. *et al.* 2007, Morinaka, Y. *et al.* 2006) show that slight decreases in BRs levels or in BR signalling have lead to significant increases in yield owing to changes in plant architecture. Another approach to increase yield might consist in controlling the BRs production during ear development.

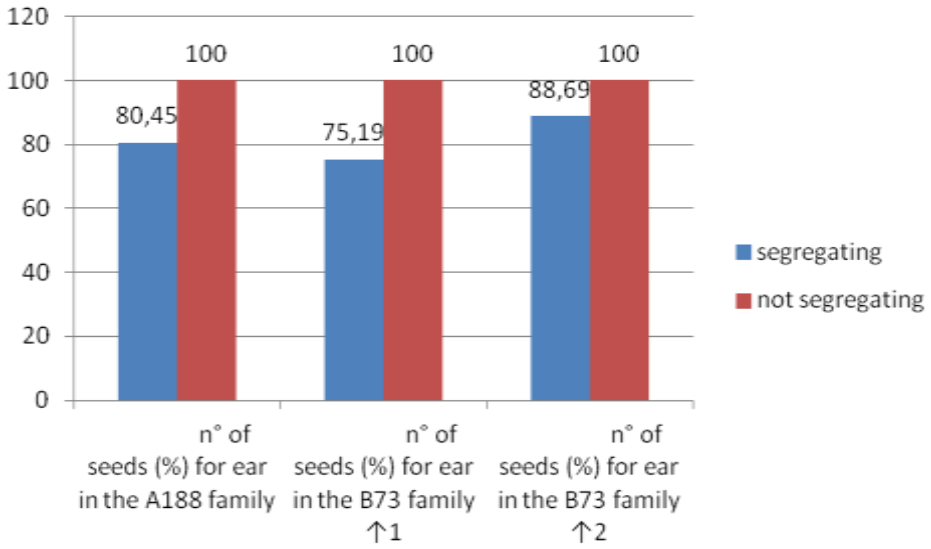
Supplemental materials

In the following graphs data are presented as percentage.

Graph 1. Ears segregating for *lil1-1* and wild-type ears from A188 and B73 genotypes are compared.



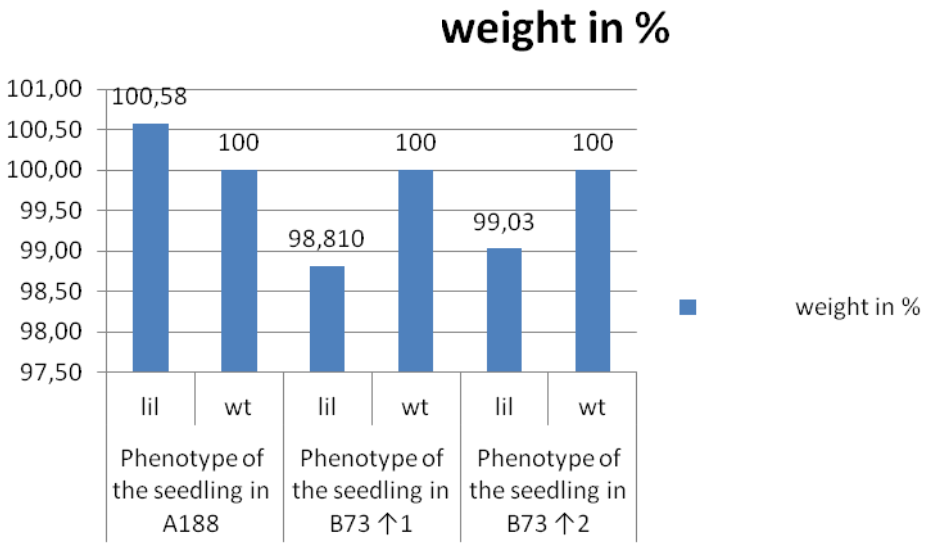
Graph 2. Percentage of seeds per ear in segregating and non-segregating population of A188 and B73 genetic background.



Plants heterozygous for the *lil1-1* gene produced ears with a reduction of 18.22% (A188 line) and of 19.31% (B73) in their elongation. A reduction of 19,55% is observed in the

A188 line for segregating ears. The difference is also present, although variable, in the two progenies of the B73 line, with a reduction in seed number of 24,81% and 11,31%.

Graph 3. shows the weight of the seeds from the segregating ears for the mutant in A188 and B73 lines.



As one can see no obvious differences were detected among the mutant seeds compared to the wt sibs suggesting that the mutation have no or very little influence on the weight of the seeds.

References

Beckett JB. 1978. B-A translocations in maize. Use in locating genes by chromosome arms. *Journal of Heredity* **69**, 27-36.

Clouse SD. 2011. Brassinosteroid signal transduction: from receptor kinase activation to transcriptional networks regulating plant development. *Plant Cell* **23**: 1219–1230.

Dolfini S, Landoni M, Consonni G, Rascio N, Dalla Vecchia F, Gavazzi G. 1999. The maize *lilliputian* mutation is responsible for disrupted morphogenesis and minute stature. *The Plant Journal* **17**: 11-17.

Fujioka S, Yokota T. 2003. Biosynthesis and metabolism of brassinosteroids. *Annu Rev Plant Biol* **54**: 137–164.

Galbiati M, 1994. Study of mutants affecting germination and first phases of development in the maize plant. PhD thesis.

Gavazzi, G., Dolfini, S., Galbiati, M., Helentjaris, T., Landoni, M., Pelucchi, N., and Todesco, G. 1993. Mutants affecting germination and early seedling development in maize. *Maydica* **38**, 265-274.

Hong Z, Ueguchi-Tanaka M, Umemura K, Uozu S, Fujioka S, *et al.* 2003. A rice brassinosteroid-deficient mutant, ebisu dwarf (d2), is caused by a loss of function of a new member of cytochrome P450. *Plant Cell* **15**: 2900–2910.

Hong Z, Ueguchi-Tanaka M, Fujioka S, Takatsuto S, Yoshida S, *et al*. 2005. The Rice brassinosteroid-deficient dwarf2 mutant, defective in the rice homolog of *Arabidopsis* DIMINUTO/DWARF1, is rescued by the endogenously accumulated alternative bioactive brassinosteroid, dolichosterone. *Plant Cell* **17**: 2243–2254.

Landoni M, Gavazzi G, Rascio N, Della Vecchia F, Consonni G, Dolfini S. 1999. A maize mutant with altered vascular pattern. *Annals of Botany The Plant Journal* **17**: 11-17.

Makarevitch I, Thompson A, Muehlbauer GJ, Springer NM. 2012. Brd1 gene in maize encodes for a Brassinosteroid C-6 Oxidase. PloS ONE 7(1): 1-9.

Meinke DW. 1982. Embryo-lethal mutants of *Arabidopsis thaliana*: evidence for gametophytic expression of the mutant genes. Theor Appl Genet **63**:381–386.

Morinaka, Y. *et al.* 2006. Morphological alteration caused by brassinosteroid insensitivity increases the biomass and grain production of rice. Plant Physiol. **141**, 924–931.

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, *et al*. 1999. 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400**: 256–261.

Randolph L.F. 1941. Genetic characteristics of the B chromosome in maize. *Genetics* **26**: 608-631.

Roman H. 1947. Mitotic nondisjunction in the case of interchanges involving the B-type chromosome in maize. *Genetics* **32**: 391-409.

Roman H. 1948. Directed fertilization in maize. *Proc. Nat. Acad. Sci.* **34**: 36-42.

Sakamoto, T. *et al*. 2006. Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. *Nat. Biotechnol*. **24**, 105–109.

Salas Fernandez MG, Becraft PW, Yin Y, Lubberstedt T. 2009. From dwarves to giants? Plant height manipulation for biomass yield. *Trends Plant Sci* **14**: 454–461.

Tanabe S, Ashikari M, Fujioka S, Takatsuto S, Yoshida S, *et al.* 2005. A novel cytochrome P450 is implicated in brassinosteroid biosynthesis via the characterization of a rice dwarf mutant, dwarf11, with reduced seed length. *Plant Cell* **17**: 776–790.

Wang, L. *et al.* 2007. Transgenic rice plants ectopically expressing AtBAK1 are semidwarfed and hypersensitive to 24-epibrassinolide. *J. Plant Physiol.* **164**, 655–664.

Wu C, *et al.* 2008. Brassinosteroids regulate grain filling in rice. *The Plant Cell.* **20**, 2130-2145.

Chapter 5

Maize variants susceptibily to Plodia interpunctella

Maize variants susceptibility to *Plodia interpunctella*

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Abstract

The behavior of the Indian meal moth *Plodia interpunctella* on maize genotypes differing in embryo development, both on whole and longitudinally sectioned kernels, was studied. In the test with whole kernels, damage was very low or absent, and only viviparous mutants were significantly attacked. However, 100% damage was observed in all genotypes on longitudinally sectioned kernels. In this test, mutant seeds lacking embryos were less damaged and showed the lowest mean number of adult insects. These results indicate that larval penetration is influenced by the embryo properties and first shows that the employment of genetic variants is a valuable approach to study insect behavior and an opportunity to highlight maize genotypes with characteristics that can minimize quality reduction caused by insect attacks.

Keywords: Indian Meal moth, maize embryo, maize seed mutants.

Introduction

Maize, with wheat and rice, is one of the most important crops for the world economy. Several insect pests can infest stored maize and research on the development of pests on different varieties has been carried out in order to find the most tolerant ones (Abdel-Rahman *et al*., 1968; Wiseman *et al.*, 1970; Dobie, 1974, 1977; Adesuyi, 1977; Falomo, 1981; Morah and Mbata, 1986; Mbata *et al*., 1988; Siwale *et al*., 2009).

Moths colonize the surface of cereals stored as a mass, causing heavier damage in warehouses than in silos. Moth larvae cause losses by feeding and by contaminating food with silk and frass. Silk net is produced by larvae on the surface of the cereal mass, favoring the development of moulds which can produce mycotoxins. Some authors have studied the development of moths on different varieties of maize (Bhattacharya et al., 1976; Rose and Behl, 1985; Mbata *et al.*, 1988). *Plodia interpunctella*, the Indian meal moth, is one of the most frequent species that can damage this cereal (Abdel-Rahman *et al*., 1968; Hockensmith *et al*., 1986; Mbata, 1987, 1990).

The maize seed comprises two major compartments: the embryo and the endosperm (Consonni *et al.*, 2005). The mature embryo consists of a well-differentiated axis, with root and shoot primordia and five or six internodes bearing a leaf at each node, surrounded by a single massive cotyledon, the scutellum. The differentiated endosperm consists of four major cell types or domains: the starchy endosperm, representing the central bulk; the single-cell aleurone layer at the periphery; the embryo-surrounding region, lining the cavity where the embryo develops; and the basal endosperm transfer cells involved in the transport of nutrients from the mother plant (Consonni *et al*. 2005). The endosperm is the main storage site of starch and proteins, whereas the embryo reserves mainly lipids.

The influence of genetic traits related to the maize caryopsis on susceptibility to insect attack has never been evaluated. A better knowledge of maize seed-insect interactions may allow the isolation, and subsequent introduction in commercial hybrids, of genetic variants limiting insect attack.

With this aim we propose in this study the analysis of the effect of maize lines differing in developmental and/or metabolic traits, as a tool to investigate the molecular basis of the

interaction between stored product insects and a specific seed compartment (Consonni *et al*. 2005). Particularly in this work we analyze the behavior of *P. interpunctella* on entire and longitudinally sectioned kernels of the RAlex0 line, characterized by high oil content in the embryo, and the B73 reference line. Two embryo-related recessive monogenic mutants were also tested: an *embryo-specific* mutant (*emb*-8908*), that causes an early block in embryo development, and the viviparous mutants (*vp2* and *vp5*), characterized by the precocious germination of the seed while it is still attacked to the ear (Giraudat *et al*., 1994; Hable *et al.*, 1998).

Materials and methods

Plant material

Four maize genetic stocks were used in this study. The inbred lines and the viviparous mutants were provided by the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu/), whereas the *emb*-8908* mutant was isolated in our laboratory in an active *Mutator* line.

Inbred lines were propagated via siblings mating. Mutants were backcrossed to the B73 line and segregating ears were obtained by selfing heterozygous plants. Homozygous mutants and related wild-type kernels used in the tests were obtained from F_2 segregating ears.

The seeds harvested were dried to about 12-13% moisture content and stored at room temperature.

The B73 line: the inbred line B73, developed at Iowa State University (Russell, 1972), exhibits high yield and is among the most used laboratory accessions and the main source of commercially important germplasm. Its genome sequence was released in 2009 (Schnable *et al*., 2009).

The RAlex0 genetic stock: also known as Alexander High Oil Synthetic, characterized by the presence of a larger embryo, is the result of a selection made at the University of Illinois for grain with high oil concentration (http://www.maizegdb.org, Gerdes *et al*., 1993).

The *emb*-8908* mutant: the *emb*-8908* recessive mutant was isolated in a genetic line carrying active *Mutator* transposable elements, which act as endogenous mutagens thus

allowing single gene specific mutant isolation (Walbot, 1992). The mutant was subsequently introgressed in the B73 inbred line. It belongs to the class of *embryo specific* (*emb*) mutants, that are characterized by impaired or arrested embryo development but normal endosperm (Clark and Sheridan, 1991). As shown in fig.1, emb*-8908 mutants are easily distinguishable from wild type seeds by the absence of the embryo axis and the presence of a scutellum with a reduced size. Seeds used in this work were obtained from ears with a segregation ratio of 3:1 for wild-type: emb phenotype kernels, obtained by selfing heterozygous *+/emb*-8908* plants. The mutant seeds' genotype is indicated as *emb*/*emb*, whereas wild-type seeds' genotype is indicated as *Emb*/-.

The *vp* mutants: maize *viviparous* (*vp*) mutants have been shown to affect either ABA biosynthesis or ABA signalling. *vp2* and *vp5* (Giraudat *et al*., 1994; Hable *et al*., 1998) are associated with reduced or suppressed carotenoid accumulation in both endosperm and vegetative tissues as a result of a mutational block in the early biosynthetic steps before the branching point separating ABA and carotenoid biosynthesis. Seeds were obtained from ears with a segregation ratio of 3:1 for wild-type: vp phenotype kernels; pools of vp2 and vp5 mutant kernels (*vp/vp*) and of relative wild-type controls (*Vp/-*) were used in each experiment.

Insect rearing and tests

Plodia interpunctella were reared on an artificial diet[1] in a thermostatic chamber at 26±1°C, 70±5% RH (Relative Humidity), and a photoperiod of 16:8 (light:dark). The tests were carried out by placing maize kernels in glass containers (diameter 35 mm, height 20 mm) with 20 newly emerged larvae. Such containers, closed with a net (120 mesh) to provide ventilation, were placed in an incubator at 26±1°C, 70±5% RH and 16 h of light alternating with 8 h of darkness. For each of the four maize genotypes, tests were carried out with 20 entire kernels and with 20 longitudinally sectioned kernels. Four replicates were carried out for each test.

[1]Ingredients of the rearing diet: 57 g bran, 61 g corn flour, 55 g wheat flour, 17 g wheat germ, 14 g dried yeast, 85 g glycerine, 67 g honey.

The number of emerged adults, the developmental period, and the percentage of damaged seeds were observed and data were analyzed using one-way ANOVA, Duncan's multiple range test and Student's t test (SPSS 19.0 per Windows).

Results

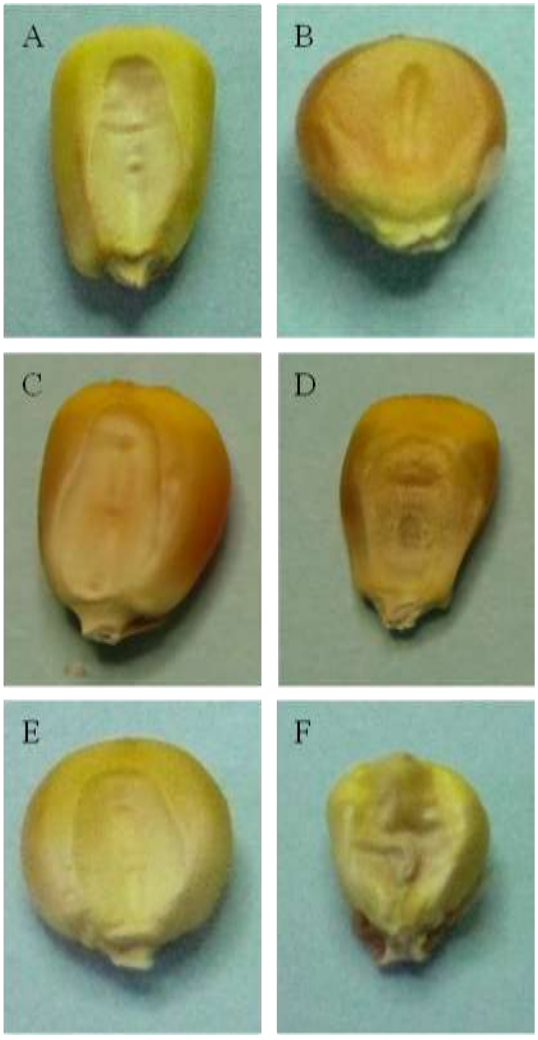

Fig. 1 Maize seed phenotypes. Representative seeds from: B73 inbred line (A); RAlex0 inbred line (B); Emb*-8908 wild-types (C) and emb*-8908 mutants (D) from a self-pollinated segregating ear; Vp5 wild-types (E) and vp5 mutants (F) from a self-pollinated segregating ear.

Tests with entire kernels

In Table 1 the results of tests carried out on the development of first instar larvae of *Plodia interpunctella* on entire kernels of the different genetic stocks of maize are reported. In all the genotypes tested no or only one adult developed among the four replicates, with the exception of the homozygous viviparous (*vp*/*vp*) mutants, which exhibited a mean of 7.5 adults with 55% of seed damage, while the respective control seeds (*Vp*/-) were undamaged. Even if the number of adults emerged is not significantly different from the other genotype, a higher percentage of damaged seeds was observed in B73, 15%, and in RAlex0, 7%. The mean developmental period of the larvae was between 48 and 53 days. On *Emb*/- and *Vp*/- larvae died without feeding, as seeds were undamaged.

Tab. 1 - Mean number (±S.D.) of emerged adults[1], mean development period (±S.D.) of *Plodia interpunctella* (Walker) and damaged seeds percentage observed on whole kernels of different maize genetic stocks.

Maize genetic stocks	Mean number of adults	Min-max	Mean development period	Min-max	Damaged seeds (%)
B73	0.7±0.5b	0-1	51.7±3.05	49-55	15
RAlex0	0.2±0.5b	0-1	48±0	-	7
Emb/-	0±0b	0-0	0±0	-	0
emb/*emb*	0.2±0.5b	0-1	51±0	-	2.5
Vp/-	0±0b	0-0	0±0	-	0
vp/*vp*	7.5±1.91a	5-9	52.7±7.49	42-68	55

[1]Observations were carried out on twenty newly emerged larvae for each replicate.
One-way Anova: Adults $F_{5,18}$=48.01 P<0.001; Development period $F_{3,31}$=0.157 n.s.
Means followed by different letters are significantly different according to Duncan's multiple range test.

Tests with longitudinally sectioned kernels

In Table 2 the results of tests carried out on 20 first instar larvae of *P. interpunctella* on longitudinally sectioned kernels of the different genetic stocks of maize are reported.

Tab. 2 - Mean number (±S.D.) of emerged adults[1], mean development period (±S.D.) of *Plodia interpunctella* (Walker) and damaged seeds percentage observed on longitudinally sectioned kernels of different maize genetic stocks.

Maize genetic stocks	Mean number of adults	Min-max	Insect developmental period (mean)	Min-max	Damaged seeds (%)
B73	12±3.65b	8-16	34.9±2.07d	33-39	100
RAlex0	15.2±2.5a	12-18	36.9±4.1bc	33-53	100
Emb/-	6.5±2.1c	4-9	36.6±2.31bcd	33-40	100
emb/*emb*	1.7±0.5d	1-2	44.3±5.32a	39-53	45
Vp/-	9.5±0.57bc	9-10	35.9±2.63cd	33-42	100
vp/*vp*	10±0.81b	9-11	38.5±2.98b	33-43	100

[1]Observations were carried out on twenty newly emerged larvae for each replicate
One-way Anova: Adults $F_{5,18}$=20.59 P<0.001; development period $F_{5,214}$=14.767 P<0.001
Means followed by different letters are significantly different according to Duncan's multiple range test.

Adults emerged in all the genotypes tested and 100% of damaged seeds were observed in all genotypes, except *emb*/*emb*.

In emb mutants the lowest percentage of damaged seeds, 45%, the lowest mean number of adults, only 1.75, and the longest mean developmental period, 44.3 days, were observed. Even the comparison between emb mutant seeds (*emb*/*emb*) and related wild-types (*Emb/-*) seeds, which exhibited a reduced number of moths in comparison with the other genotypes, showed a significant reduction, according to the Student's t test (t=4.437, df=3.345, P=0.01), in the mean number of moths produced with emb mutant seeds (1.7). The mean developmental period was significantly longer in emb mutants versus wild-type seeds (t=3.725, df=6.625, P=0.008). The highest number of emerged adults was observed on RAlex0 (15.2±2.5), followed by B73 (12±3.65), but the two lines were not significantly

different for this parameter if compared by Student's t test (t=1.469, df=5.306 P=0.199). Notably, the mean developmental period observed on B73 (34.9±2.07) was the shortest of all genotypes tested, and significantly differs from RAlex0 (t=3.426, df=89.987, P=0.001).

The results obtained in this test with sectioned vp mutants (*vp/vp*) did not confirm those obtained in the first test with entire kernels performed in this study. With sectioned kernels the mean number of adults emerged was not significantly different, according to the Student's t test (t=1, df=5.4, P=0.360), from the related controls, *Vp*/-, and the mean developmental period was significantly longer (t=4.172, df=75.58, P=0.0001). The mean number of adults emerged from vp seeds was also lower than that of the two inbred lines.

Discussion

In this study we have examined the behavior of *P. interpunctella* on entire and longitudinally sectioned maize kernels with different genotypes affecting embryo development. In most of the maize genotypes tested as entire kernels the mean number of attacked seeds was very low or seeds were not attached (*Emb*/- and *Vp*/-) and few adults of *Plodia interpunctella* emerged. On the contrary the same genotype tested as longitudinally sectioned kernels showed in most cases 100% of damaged kernels and a higher number of adults.

Several authors observed that the percentage of larval survival increased as the percentage of broken kernels augmented (Locatelli and Limonta, 1998; Kaliyan et al., 2005) and maize is mainly damaged in the germ area (Fraenkel and Blewett, 1945; Abdel- Rahman et al., 1968; Mbata, 1990). In this study the developmental time required from egg hatching to adult was from 48 to 52 days on entire kernels. However, unlike our results, Arbogast (2006) observed a shorter mean development period of 34.5 days (from egg to adult) on entire kernels of the maize Pioneer 3320 at 27°C, at 70 or 80 % RH, thus indicating that our lines were less favorable to *P. interpunctella* development than the commercial hybrid adopted in the Arbogast (2006) study.

Interestingly, in the tests with entire kernels, the only exception was represented by homozygous *vp/vp* mutant kernels that were significantly attacked by the insects, with 7.5 the mean number of emerged adults and 55 % of damaged seeds and where 37.5% of larvae

reached the adult stage. vp embryo are characterized by deficiency in ABA synthesis, that causes pre-germination of the mutant embryo while still attached to the ear. As consequence the pericarp, the external tegument of the seed, is less hard or fractured in the germ area, thus making the embryo more accessible, and allowing the insect to easily penetrate the kernel surface. The comparison between sectioned vp and wild-type sectioned kernel did not confirm a preference for the mutants, thus proving that the results observed in the entire kernels test were due to physical and not to nutritional properties of the embryos.

We may thus conclude that several factors influence the development of the pest, not only nutrients in the cereal but also the physical structure, such as hardness and the more or less smooth surface. Also Silhacek and Murphy (2006) observed that "nutrient availability depends upon the amounts of nutrients actually consumed and upon physical factors that restrict the ingestion of nutrients".

As to the other genotypes in this study, the observation that in longitudinally sectioned emb mutant seeds only few adults with a longer developmental period were observed, provides a proof of the requirement of this seed domain for *P. interpunctella* development. The emb mutant adopted in this study belongs to the class of *embryo specific* (*emb*) mutants, in which only the embryo but not the endosperm is affected (Clark and Sheridan, 1991); mutant seeds lack or exhibit a smaller scutellum and their embryo axis does not differentiate, whereas starchy endosperm seems normal both in volume and texture. As previously shown by Fraenkel and Blewett (1946), *P. interpunctella* can obtain all the nutrients it needs from wheat germ, but it is unable to use the starch in the endosperm. Our work may support the theory that *P. interpunctella* needs nutrients present in the germ, most probably liposoluble vitamins and proteins.

Another interesting result is related to the analysis of the RAlex0 inbred line, which is the result of a selection process aimed at increasing the oil concentration in the scutellum. It is conceivable, on the basis of the number of emerged adults, that RAlex0 nutritional properties have a positive effect on stimulating the speed of insects' development.

A preference for the B73 lines was also observed, as shown in the test with sectioned kernels. The comparison of B73 versus RAlex0 with Student's t test indicated that the two lines were not significantly different in terms of number of emerged adults; moreover in the

first line the mean developmental period was shorter. B73, which has been selected by breeders for its robustness and suitability in hybrid production, appears indeed not to be the ideal genotype for maize storage since it is a suitable substrate for *P. interpunctella* development. We observed, for instance, in the test with sectioned kernels that the wild-type controls of the two mutants used in the study, referred to as *Emb*/- and Vp/-, are more resistant to the infestation of *P. interpunctella*. Since both mutations have been introgressed in the B73 line, we may speculate that the introgression has brought some genetic factors that lead to a modification of the kernel properties.

Many mutants affecting embryo and endosperm development and metabolism are available in maize, and have been adopted for the isolation of genes involved in seed formation as well as the characterization of the molecular mechanisms implied. This study, although preliminary, shows that maize genetic variants constitute a valuable tool for the study of insect-seed interactions. Developmental mutants give the opportunity to explore the role of the seed components, i.e. embryo, endosperm and integuments, in determining insects attack. Similarly, mutants showing metabolic defects could be adopted to specifically investigate the role of molecules, such as oils, proteins, carbohydrates and secondary metabolites, in promoting or reducing insect attack.

This type of study will provide useful information for the selection of genotypes resistant to insect pests of stored products. If one takes into account that during its development *P. interpunctella* produces on the surface of the cereal a thick silk net that favors mold proliferation, we can conclude that the adoption of maize seeds more resistant to mechanical damage and insect attacks can also prevent mycotoxin accumulation thus enhancing both quality and safety of stored products.

References

Abdel-Rahman H.A., Hodson A.C., Christensen C.M., 1968. - Development of *Plodia interpunctella* (Hb.) (Lepidoptera: Phycitidae) on different varieties of corn at two levels of moisture. *J. Stored Prod. Res*., **4**: 127-133.

Adesuyi S.A. 1977. Relative resistance of some varieties of maize to attack by *Sitophilus zeamais* (Motsch.). Report of the Nigerian Stored Products Research Institute, 1976/1977, *Technical report no*. **7**: 79-82.

Arbogast R.T. 2006. A wild strain of *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) from farm-stored maize in South Carolina: development under different temperature, moisture, and dietary conditions. *Journal of Stored Products Research.* **43**:160-166.

Bhattacharya A.K., Chaudhary R.R.P., Rathore R.R.S. 1976. - Susceptibility of several varieties of soybean to *Ephestia cautella* (Walker) (Lepidoptera: Phycitidae). *Journal of Stored Product Researches*, **12**: 143-148.

Clark JK, Sheridan WF. 1991. Isolation and Characterization of 51 embryo-specific Mutations of Maize. *Plant Cell* **3**, 935-951.

Consonni G., Gavazzi G., Dolfini S. 2005. Genetic analysis as a tool to investigate the molecular mechanism underlying seed development in maize. *Annals of Botany,* **96**: 353-362.

Dobie P. 1974. The laboratory assessment of th einherent susceptibility of maize varieties to post harvest infestation by *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae). *Journal of Stored Products research* **10**: 183-197.

Dobie P. 1977. The contribution of the tropical stored products centre to the study of insect resistance in stored maize. *Tropical Stored Products Information* **34**: 7-16.

Falomo A.A. 1981. Post-harvest susceptibility of maize varieties to infestation by *Sitophilus zeamais* Motsch. Report of the Nigerian Stored Products Research Institute, 1981, Technical report no. **6**: 57-63.

Fraenkel G. and Blewett M. 1945. The dietetics of the caterpillars of three *Ephestia* species, E. *kuehniella*, *E. elutella*, and *E. cautella*, and of a closely related species, *Plodia interpunctella*. *J. Exp. Biol.* **22**: 162-171.

Fraenkel G. and Blewett M. 1946. Linoleic acid, vitamin E and other fat-soluble substances in the nutrition of certain insects, *Ephestia kuehniella*, *E. elutella*, and *E. cautella* and *Plodia interpunctella* (Lep.). *J. Exp. Biol.* **22**: 172-190.

Giraudat, J; Parcy, F; Bertauche, N; Gosti, F; Leung, J; Morris, PC; Bouvier-Durand, M; Vartanian, N. 1994. *Plant MolBiol* **26**: 1557-1577.

Gerdes, J.T., C.F. Behr, J.G. Coors, and W.F. Tracy. 1993. Compilation of North American maize breeding germplasm. Misc. Publ. CSSA, Madison, Wisconsin,USA

Goldman, I.L. *et al*. 1994. Molecular markers associated with maize kernel oil concentration in an Illinois High Protein X Illinois Low Protein cross. *Crop Sci*. **34**, 908–915.

Hable WE, Oishi KK, Schumaker KS. 1998. Viviparous-5 encodes phytoenedesaturase, an enzyme essential for abscisic acid (ABA) accumulation and seed development in maize .*Mol Gen Genet.* **257**:167-76.

Hockensmith P.E., Devine T.L., Legg D.E., Rodriguez J.G., 1986. Energy consumptions and food utilization of the Indian meal moth (Lepidoptera: Pyralidae) on different corn genotypes. *Journal of the Kansas Entomological Society*, **59 (4)**: 598-603.

Kaliyan N., Carrillo M.A., Morey R.V., Wilcke W.F., Cannon C.A., 2005 - Indian meal moth survivability in stored corn with different levels of broken kernels. *Great Lakes Entomologist*, **38 (3/4)**: 177-185.

Locatelli D.P., and Limonta L. 1998. Development of *Ephestia kuehniella* (Zell.), *Plodia interpunctella* (Hbn.) and *Corcyra cephalonica* (Staint.) (Lepidoptera: Pyralidae) on kernels and wholemeals of *Fagopyrum esculentum* Moench and *Triticum aestivum* L. *J. stored Prod. Res.* **34 (4)**: 269-276.

Mbata G.N., 1987. - Studies on the susceptibility of groundnut varieties to infestation by *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae). *J. Stored Prod. Res.* **23 (1)**: 57-63.

Mbata G.N., 1990. - Suitability of maize varieties for the oviposition and development of *Plodia interpunctella* (Hubner) (Lepidoptera, Pyralidae). *Tropical pest management*, **36 (2)**: 122-127.

Mbata G.N., Osuji F.N.C., Okere A.N., 1988. - Studies on the developmental biology of *Corcyra cephalonica* (Stainton) (Lepidoptera: Galleriidae) on 13 maize varieties. *Trop. Sci.*, **28**: 25-34.

Morah S.C., and Mbata G.N. 1986. An assessment of relative susceptibility of some maize varieties to post harvest infestation by the maize weevil *Sitophilus zeamais* (Motsch.). Report of the Nigerian Stored Products Research Institute, 1982, *Technical report no*. **5**: 63-68.

Rose H.S., Behl N.K., 1985. - Studies on the development of *Corcyra cephalonica* (Stainton) (Galleriinae: Pyralidae: Lepidoptera) on different varieties of maize. *Ann. Entomol.*, **3 (1)**: 25-28.

Russell W.A. 1972. Registration of B70 and B73 parental lines of maize. *Crop Science*, **12**, 721.

Schnable PS, Ware D, Fulton R, Stein J, Wei F, *et al.* 2009. The B73 maize genome: complexity, diversity and dynamics. *Science* **326**: 1112-1115.

Sedlacek J.D., Komaravalli S.R., Hanley A.M., Price B.D., Davis P.M. 2001. - Life history attributes of Indian meal moth (Lepidoptera: Pyralidae) and Angoumois grain moth (Lepidoptera: Gelechiidae) reared on transgenic corn kernels. *Journal of Economic Entomology*, **94 (2)**: 586-592.

Silhacek D. and Murphy C. 2006. A simple wheat germ diet for studying the nutrient requirements of the Indian meal moth, *Plodia interpunctella* (Hubner). J. Stored Prod. Res. **42 (4)**, 427-437.

Siwale J., Mbata K., McRobert J., and Lungu D. 2009. Comparative resistance of improved maize genotypes and landraces to maize weevil. *African Crop Science Journal* **17 (1)**: 17-24.

Wiseman R., McMillan R., and Widstrom N.W. 1970. Husk and kernel resistance among maize hybrids to an insect complex. *Journal of Economic Entomology* **63**: 1260-1262.

Chapter 6

Maize variants susceptibily to Rhyzopertha dominica

Maize variants susceptibility to *Rhizopertha dominica*

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Abstract The behavior of *Rhyzopertha dominica* on different maize genotypes affecting endosperm development, on whole and longitudinally sectioned kernels in competition was studied. The RAlex0 inbred line with high oil content in the embryo, the B73 reference line, two mutants altering endosperm development and a line that accumulates high quantity of anthocianins in the aleurone layer were analyzed.

In the first test with whole kernels in competition, the damage was very low or absent for most of the genotypes; only the seeds heterozygous and homozygous for the mutation *sugary1* were significantly attached, showing 30% and 18.75% of damage seeds respectively. As to the endosperm only the entire seeds homozygous for the *sugary1* mutation were significantly attached. A high % of damaged seeds was instead observed in all genotypes of longitudinally sectioned kernels. The test with longitudinally sectioned kernels in competition shows interesting results among the four replicates for each genotype tested. For example in the test with B73+RAlex0 inbred lines the highest % of damaged seeds was observed in the RAlex0 (45%). A similar result was obtained in the test with B73+Su1wt; Y1 wt+y1m and Col wt+cls m. These preliminary results indicate that larvae penetration is influenced not only by the embryo properties but even by the endosperm properties and shows that employment of maize genetic variants is a valuable approach to study insect behavior.

Keywords: development, capuchin of cereals, maize genetic stock, endosperm.

Maize, with wheat and rice, is one of the most important crop for the world economy; several stored products insects can infest it and researches on the development of pests on different maize varieties have been carried out in order to find the most tolerant ones (Osuji 1982).

Among insect pests, beetles colonize the surface of cereals mass, and they cause heavier damages in warehouses than in silos. Capuchin larvae cause losses by feeding from and by contaminating food with frasses that are produced by larvae on the surface of cereal mass, favouring the settlement of moulds that can produce mycotoxins. Some Authors have studied the development of this beetle on different varieties of maize (Osuji 1982).

The maize seed comprises two major compartments, the embryo and the endosperm (Consonni *et al*, 2005). The mature embryo consists of a well differentiated axis, with primordial root and shoot and five or six internodes bearing a leaf at each node, surrounded by a single massive cotyledon, the scutellum. The differentiated endosperm consists of four major cell types or domains: the starchy endosperm, representing the central bulk; the single-cell aleurone layer cover at the periphery; the embryo-surrounding region, lining the region where the embryo develops; and the basal endosperm transfer cells involved in the transport of nutrients from the mother plant (Olsen, 2001). The endosperm is the main storage site of starch and proteins, whereas the embryo reserves mainly lipids.

Maize genetic variants, showing different developmental and metabolic defects, which are instrumental for the isolation of genes involved in seed formation, are herein first proposed as a tool to investigate the molecular basis of the interaction between insects and a specific seed compartment.

In this work we analyzed the behavior of *R. dominica* on different maize genotypes affecting endosperm development, on whole and longitudinally sectioned kernels. In particular we adopted the RAlex0 line characterized by high oil content in the embryo and the B73 reference line. Three types of recessive monogenic mutants were also included: the *sugary1* mutant that is altered in the composition of the starch, the *yellow endosperm1* mutant that has got a low amount of carotenoids in the mutant endosperm but they are present in normal amount in the embryo and a line named ACR that is charachterized by the

presence of a single monogenic recessive mutation in one structural or regulatory gene of the biosintethic pathway of the anthocianins (Fig.1).

Materials and methods

Plant material:

Five maize genetic stocks were used in this study. The inbred lines, the *yellow endosperm1*, and the *sugary1* mutant were provided by the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu/).
The ACR line was already present in the maize genetic stocks of our laboratory.
Inbred lines were propagated via siblings mating. The *sugary1* mutant was backcrossed to the *a1-eap* line and segregating ears were obtained by selfing heterozygous plants while homozygous ears were obtained by planting and selfing mutant seeds. Homozygous mutants and relative wild-type kernels used in the tests were obtained from F_2 segregating ears.
The seeds harvested were dried to about 12-13% moisture content and stored at room temperature.

The B73 line: the inbred line B73, developed at Iowa State University (Russell, 1972), exhibits high yield and it is among the most used laboratory accessions and the main source of commercially important germplasm. Its genome sequence was released in 2009 (Schnable *et al*., 2009).

The RAlex0 genetic stock: also known as Alexander High Oil Synthetic, it is the result of a selection performed at the University of Illinois for grain with high oil concentration (http://www.maizegdb.org, Gerdes *et al*. 1993, Goldman *et al*. 1994). It is also characterized by the presence of a larger embryo.

The *sugary1* mutant: The *sugary1 (su1)* gene, located on the short arm of chromosome 4, is expressed in the kernel during starch biosynthesis and codes for the enzyme isoamylase-type starch debranching1, belonging to the family of α-amylase enzymes hydrolytic starch (Pan and Nelson, 1984). Loss of *su1* gene function results in the accumulation of sugars and the highly branched water-soluble polysaccharide phytoglycogen in the endosperm. The endosperm of mutant seeds is wrinkled and translucent when dry, sweet at the milk stage. Sugary kernels germinate poorly under unfavorable conditions and produce seedlings that are lighter green and weaker than normal. Gene variants of *su1* are used for the production of "sweet corn", whose immature ears are employed in the industry of canned food. Mutant seeds used in the test also have an accumulation of anthocyanin pigments in the region surrounding the embryonic axis; due to the characteristic expression of an allele of the *anthocyaninless1* gene, named *a1-eap* (*embryonic axis profile*).
For the tests seeds were obtained from ears with a segregation ratio of 3:1 for wt: su1 phenotype kernels and from selfed homozygous ears.

The *yellow endosperm1 mutant:*
Homozygous y1 seeds can be easily distinguished on segregating ears. They are characterized by a low content in carotenoids in the endosperm, and appear colorless or pale yellow. The gene *y1*, located on the long arm of chromosome 6, controls the production of the enzyme phytoene synthase (PSY), involved in first step of the biosynthesis of carotenoids in the endosperm (Buckner *et al*, 1996). During the development of the endosperm, gene transcription and accumulation of the product of the same gene are correlated with the accumulation of carotenoids. The presence of the recessive allele *y1* in homozygosity implies a reduced content of carotenoids in the endosperm.
Seeds used in the tests were obtained from ears with a segregation ratio of 3:1 for wt: y1 phenotype kernels.

The ACR line choosen for the tests is characterized by the strong accumulation of anthocyanin pigments, that are aromatic compounds of the flavonoids class, in the aleurone layer.

Many genes are involved in their biosinthetic pathway, including the structural genes *A1*, *A2*, *Bz1* and *Bz2*. The activity of this group of genes is controlled by some regulatory genes like R1 and C1 that are responsible for the accumulation of anthocyanins in seeds (Ludwig *et al*., 1990; Chandler *et al*., 1989; Paz Ares *et al* 1987; Cone *et al*. 1993). In the presence of functional alleles of these genes the phenotype of the seeds is colored, while mutations in a single regulator gene or in a structural gene cause the colorless seed phenotype.

Seeds used in the tests were obtained from ears with a segregation ratio of 9/16 for wt kernels and 7/16 for mutant kernels attributable to the segregation of two independent alleles.

Insect rearing and tests:

Rhizopertha dominica was reared on 200 g of wheat in a thermostatic chamber at 29±1°C, 70±5% RH and a photoperiod of 16:8 (light:dark). The tests were carried out by placing 20 maize kernels of one genotype and 20 maize kernels of the other genotype in glass containers (diameter 40 mm, height 20 mm) with 20 newly emerged larvae. Such containers closed with a net (120 mesh) to provide ventilation, were placed in an incubator at 29±1 °C, 70±5% RH and 16 h of light alternating with 8 h of darkness. For each of the five maize genotypes, tests were carried out with 40 whole kernels (20 of one genotype and 20 of another genotype) and with 40 longitudinally sectioned kernels (20 of one genotype and 20 of another genotype). Four replicates were carried out for each test.

Data were analyzed using one-way ANOVA and Duncan's multiple range test (SPSS 19.0 for Windows).

Results

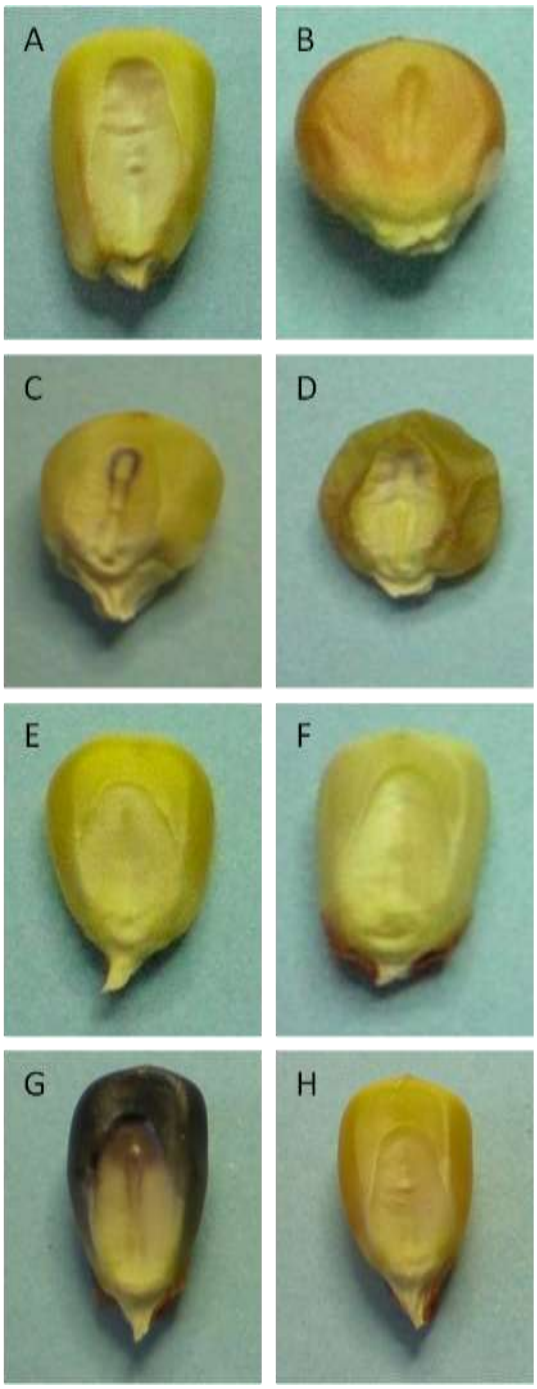

Fig. 1 Maize seed phenotypes. Representative seeds from: B73 inbred line (A); RAlex0 inbred line (B); Su1 wild-types (C) and su1 mutants (D) from a self-pollinated segregating ear; Y1 wild-types (E) and y1 mutants (F) from a self-pollinated segregating ear; ACR wild-types (G) and ACR mutants (H) from a self-pollinated segregating ear.

Note the ring of anthocyanins surrounding the embryonic axis in (C) and (D) seeds due to the introgression of the *su1/su1* mutant in a line homozygous for this trait referred as *eap* (*embryonic axis profile*).

Tests with entire kernels

In Table 1 the results of tests carried out on 20 first instar larvae of *Rhyzopertha dominica* on whole kernels in competition with the different genetic stocks of maize are reported.

Tab.1 Mean number (±S.D.) of emerged adults, mean development period (±S.D.) of *Rhizopertha dominica* F. and damaged seeds percentage observed on whole kernels of different maize genetic stocks.

Maize genetic stocks	Mean number of adults	Min-max	Mean development period	Min-max	Damaged seeds (%)
B73 + RAlex0	1.25±0.96b	1-2	49.8±23.21ab	33-84	6.25% for B73 0% for RAlex0
B73 + *Su1/-*	1±0.82b	1-2	39±3.46 b	36-42	0% for B73 5% for Su1/-
Su1/- + *su1*/*su1*	9.75±4.03a	5-14	63.5±16.82 a	32-86	30% for Su1/- 18.75% for su1/su1
Y1/- + *y1/y1*	4.75±2.63a	1-7	51.6±13.66 ab	33-76	12.5% for Y1/- 11.25% for y1/y1
Col/- + *cls/cls*	1.50±0.58b	1-2	50.3±11.31 ab	40-69	6.25% for Col/- 1.25% for cls/cls

One way Anova: Adults $F_{5,18}$=20.59 P<0.001; development period: $F_{4, 68}$=4,064; p≤0,005. Means followed by different letters are significantly different according to Duncan's multiple range test.

In all genotype tested very few adults developed among the four replicates with the exception of seeds in competition of these genotypes: *Su1/- + su1/su1* and *Y1/- + y1/y1*.
In these two samples a mean of 9.75 adults was observed in the test with Sugary1 and sugary1 seeds. In this test the Sugary1 seeds were much damaged with 30% of seed damage and only 18.75% of seed damage for the sugary1 seeds; while in the test with *Y1/- + y1/y1* a mean of 4.75 adults was observed with 12.5 % of seed damage for the Yellow endosperm1

seeds and nearly same percentage (11.25%) for the yellow endosperm 1 seeds.In the other tests a very low percentage of damaged seeds was observed.

Tests with longitudinally sectioned kernels.

In Table 2 the results of tests carried out on 20 first instar larvae of *Rhyzopertha dominica* on longitudinally sectioned kernels in competition of the different genetics stocks of maize are reported.

Tab.2 Mean number (±S.D.) of emerged adults, mean development period (±S.D.) of *Rhizopertha dominica* F. and damaged seeds percentage observed on longitudinally sectioned kernels of different maize genetic stocks.

Maize genetic stocks	Mean number of adults	Min-max	Insect developmental period	Min-max	Damaged seeds (%)
B73 + RAlex0	15.25±1.26a	14-17	44.09±9.95	30-68	31.25% for B73 45% for RAlex0
B73 + *Su1/-*	17±0,82b	16-18	42.13±9.27	30-67	26.25% for B73 58.75% for Su1/-
Su1/- + *su1*/*su1*	19.25±0.96c	18-20	41.48±8,78	30-67	47.5% for Su1/- 48.75% for su1/su1
Y1/- + *y1/y1*	17±1.15b	16-18	43.48±10.44	30-69	50% for Y1/- 35% for y1/y1
Col /- + *cls*/*cls*	15.75±0.96ab	15-17	41.75±8.95	30-65	43.75% for Col/- 35% for cls/cls

One way Anova: Adults $F_{5,18}$=20.59 P<0.001; development period: $F_{4, 68}$=4,064; p≤0,005. Means followed by different letters are significantly different according to Duncan's multiple range test.

Adults emerged in all genotypes tested and a high percentage of damaged seeds was observed in all genotypes. Also we observed a high number of adults in all the five tests and a shorter development period, from 30 to 69 days.

In each comparison very interesting results were obtained:

In the test with the two lines B73 and RAlex0 the seeds of RAlex0 were higly damaged: probably due to the fact that this genotype has got a larger embryo with a high oil concentration that has favoured the development of *Rhizopertha dominica*.

In the test in which the B73 seeds and the wt seeds derived from an ear segregating for the *sugary1* mutant were compared we observed a high % of damaged seeds in the *Su1/-* genotype while for the test that compared the wt seeds from an ear segregating for the *sugary1* mutant and the mutant sibs no significant difference was observed (47.5% vs 48.75%).

The last two tests (the first one with the Y1/-seeds vs y1/y1 seeds and the second one with the Col/-seeds vs cls/cls seeds) showed significant differences in the % of damaged seeds since there were more attacked the Y1 seeds (50%) and the Col seeds (43.75%) respect to one another.

Conclusions

In most of the maize genotypes tested in this study very few adults of *R. dominica* emerged from whole kernels and the development time required from egg to adult was from 39 to 63 days.

In particular the lowest number of adults was observed in the following tests: B73+RAlex0; B73+Su1/-, and Col/- + cls/cls.

Among our samples the only two exceptions were represented by the following tests: Su1/- + su1/su1 and Y1/- + y1/y1.

In particular the higher number of emerged adults in the test with the Sugary1 seeds with the sugary1 ones could be explained by the fact that these seeds represent a suitable substrate for *R.dominica* development. Mutant kernels have a glass and wrinkled appearance when dry and all the kernels of the *Su1/su1* ⊗ genotype have a ring of anthocyanins that surrounds the embryonic axis, this factor together with the altered composition of the starch (Pan and Nelson 1984) could be explained the preference of *R. dominica* for these seeds.

Here we presented the main results and conclusions for each test with the **whole kernels**:
As resulted in Table 1 in the test B73+RAlex0 only the B73 seeds were damaged even if in a very low percentage (6.25%) while none seed of RAlex0 was attacked: this data could be ascribable to the hardness of RAlex0 pericarp that represents a hard barrier for the larvae of *R dominica.*

In the test B73 + Su1/-, only the Sugary1 seeds were damaged (5%): a possible explanation to this fact is that these seeds, derived from an ear segregating for the mutant: it is possible to explain the preference of *R.dominica* for these seeds with the *su1* gene dose effect in the endosperm which manifests itself in 2/3 of the seeds wt with the heterozygous genotype. Some of these seeds have one dose of the gene *su1* in the endosperm that has no effect on the starch content, while another part has two doses of the recessive gene in the endosperm which reduces the amount of normal starch of about 29% when compared to *Su1/Su1* seeds (Singletary *et al.*, 1997). Another factor is the presence, in all the Su1 seeds, of a ring of anthocyanins surrounding the embryonic axis: even this factor could explain the preference of *R. dominica*'s larvae for these seeds.

The same explanation is good for the Su1/-+su1/su1 test in which the Su1/- seeds were more damaged with respect to the other (30%) vs (18.75%).

In the test with Y1/- + y1/y1 no differences in the attack between wt and mutant kernels derived from the selfed genotype *Y1/y1* and the low percentages (12.5% and 11.25%) are ascribable to the hardness of the pericarp of these seeds.

In the last test Col/- + cls/cls the larvae have preferred the coloured seeds that were damaged in a percentage of 6.25% while the colorless seeds were damaged only for the 1.25%. It's conceivable that the high concentration of the anthocyanin pigments in the aleurone layer had a more attractive effect on the young larvae of *R. dominica*.

A high number of adults and a shorter development period, from 30 to 69 days, were observed in the tests with longitudinally sectioned kernels.

Several authors observed that the percentage of larval survival increased as the percentage of broken kernels increased (Locatelli & Limonta, 1998; Kalyan *et al*, 2005, Osuji *et al*, 1982) and maize is mainly damaged in the germ area (Fraenkl & Blewett, 1945; Abdel-Rhaman *et al*., 1968).

Also in these tests larvae feed almost solely on the embryo with the only exception of homozygous *su1/su1* kernels. This data is ascribable to the endopsperm features of this mutant: the wrinklness of it's surface and it's tenderness probably have favoured the attack by the larvae.

Now the main results and conclusions are presented for each test with the **longitudinally sectioned kernels**:

As resulted in Table 2 in the test B73+RAlex0 both the two genotypes were higly damaged. In particular the RAlex0 seeds were badly damaged (45%): this result is due to the fact that the dissection of the seeds has brought the hard pericarp of this line rendering it's large embryo,with a high oil concentration, more accessible.

In the test B73 + Su1/-, the Sugary1 seeds were higly damaged (58.75%): a possible explanation to this fact is that these seeds, derived from an ear segregating for the mutant: it is possible to explain the preference of *R.dominica* for these seeds with the *su1* gene dose effect in the endosperm which manifests itself in 2/3 of the seeds wt with the heterozygous genotype. Some of these seeds have one dose of the gene *su1* in the endosperm that have no effect on the starch content, while another part has two doses of the recessive gene in the endosperm which reduces the amount of normal starch of about 29% when compared to *Su1/Su1* seeds (Singletary *et al*., 1997). Another factor is the presence, in all the Su1 seeds, of a ring of anthocyanins surrounding the embryonic axis: even this factor could explain the preference of *R. dominica*'s larvae for these seeds.

No significant differences were observed between longitudinally sectioned kernels in the test that compare the Su1/- seeds with the su1/su1 ones.

In the test with Y1/- + y1/y1 the high percentage of damaged seeds was observed in the Y1 seeds (50%): this data is due to the high content of carotenoids in the endoperm tissue that attracted the larvae. Plants can also contain carotenoids in nonphotosynthetic tissues, such as flowers and fruits. Here, their function has been attributed to making these tissues more

attractive to animals, thereby promoting pollination and seed dispersal. (Goodwin, 1971; Buckner et.al, 1996).

Even the last test Col/- + cls/cls the larvae have preferred the coloured seeds that were damaged in a percentage of 43.75% while the colorless seeds were damaged only for the 35%. It's conceivable that the high concentration of the anthocyanin pigments in the aleurone layer have a more attractive effect on the young larvae of *R. dominica*.

As was observed in the tests conducted on *Plodia interpunctella*, even for *Rhizopertha dominica* the more appetible region of the seed was the embryo.

The non-admission of young larvae in the whole kernel is presumably due to the hardness of the pericarp (Osuji, 1982). This Author has used kernels previously kept in thermostated cells to 27 ° C and RH of 67% for two weeks: this preliminary treatment has increased the percentage of moisture content of the kernels favouring the entry of larvae in the seeds, since the low concentration of moisture and the integrity of the pericarp are an obstacle for the trophic activity of the larvae.

References

Abdel-Rahman H.A., Hodson A.C., Christensen C.M., 1968. - Development of *Plodia interpunctella* (Hb.) (Lepidoptera: Phycitidae) on different varieties of corn at two levels of moisture. *J. Stored Prod. Res*., **4**: 127-133.

Buckner B., San Miguel P., Janick-Buckner D., Bennentzent J. 1996. The *y1* Gene of Maize Codes for Phytoene Synthase. *Genetics* **143**: 480-488.

Chandler V.L., Radicella J.P., Robbins T.P., Chen J., Turks D. 1989. Two regulatory genes of the maize anthocyanin pathway are homologous: Isolation of B using R genomic sequences. *Plant Cell* **1**: 1175-1183.

Cone K.C., Cocciolone S.M., Burr F.A., Burr B. 1993. Maize anthocyanin regulatory gene *pl* is a duplicate of c1 that functions in the plants. *Plant Cell* **5**: 1795-1800.

Consonni G., Gavazzi G., Dolfini S., 2005. - Genetic analysis as a tool to investigate the molecular mechanism underlying seed development in maize. *Annals of Botany*, **96**: 353-362.

Fraenkel G. and Blewett M. 1945. The dietetics of the caterpillars of three *Ephestia* species, E. *kuehniella*, *E. elutella*, and *E. cautella*, and of a closely related species, *Plodia interpunctella*. *J. Exp. Biol*. **22**: 162-171.

Fraenkel G. and Blewett M. 1946. Linoleic acid, vitamin E and other fat-soluble substances in the nutrition of certain insects, *Ephestia kuehniella*, *E. elutella*, and *E. cautella* and *Plodia interpunctella* (Lep.). *J. Exp. Biol*. **22**: 172-190.

Gerdes, J.T., C.F. Behr, J.G. Coors, and W.F. Tracy. 1993. Compilation of North American maize breeding germplasm. Misc. Publ. CSSA, Madison, Wisconsin,USA.

Goldman, I.L. *et al.* 1994. Molecular markers associated with maize kernel oil concentration in an Illinois High Protein X Illinois Low Protein cross. *Crop Sci.* **34**, 908–915.

Goodwin, TW. 1971. Biosynthesis, pp. 577-636 in *Carotenoids*, edited by 0. ISLERB. irkhauser Verlag, Basel.

Kaliyan N., Carrillo M.A., Morey R.V., Wilcke W.F., Cannon C.A., 2005. - Indian meal moth survivability in stored corn with different levels of broken kernels. *Great Lakes Entomologist*, **38 (3/4)**: 177-185.

Locatelli D.P., and Limonta L. 1998. Development of *Ephestia kuehniella* (Zell.), *Plodia interpunctella* (Hbn.) and *Corcyra cephalonica* (Staint.) (Lepidoptera: Pyralidae) on kernels and wholemeals of *Fagopyrum esculentum* Moench and *Triticum aestivum* L. *J. stored Prod. Res*. **34 (4)**: 269-276.

Ludwig S.R., Wessler S.R. 1990. Maize R gene family: Tissue specific helix loop helix proteins. *Plant Cell* **62**: 850-851.

Olsen O-A. 2001. Endosperm development: cellularization and cell fate specification. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 233–267.

Paz-Ares, J., Ghosal D., Wienand U., Peterson P.A., and Saedler, H., 1987. The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. *Embo Journal*. **6**:3553-3558.

Osuji F.N.C. 1982. Development of the lesser grain borer, *Rhyzopertha dominica*, in maize kernels as affected by site of larval entry. *Entomologia Experimentalis et Applicata* **31**: 392.

Pan D., Nelson O.E. 1984. A debranching enzyme deficiency in endosperm of the 7 Sugary mutants of maize. *Plant Physiology.* **74**: 324-328.

Russell W.A. 1972. Registration of B70 and B73 parental lines of maize. *Crop Science*, **12**, 721.

Schnable PS, Ware D, Fulton R, Stein J, Wei F, *et al.* 2009. The B73 maize genome: complexity, diversity and dynamics. *Science* **326**: 1112-1115.

Singletary G.W., Banisadr R., Keeling P.L. 1997. Influence of gene dosage on Carbohydrate Synthesis and Enzymatic Activities in Endosperm of Starch-Deficient Mutants of Maize. *Plant Physiol* **113**: 293-304.

Chapter 7

General discussion

Chapter 7 General discussion

The division of Angiosperms comprises all the cultivated plants that have some importance from an economic point of view, such as cereals, legumes, vegetables, ornamental plants and the majority of the woody plants.

Due to the central role of the Angiosperms in the food chain, the understanding of their development and of the genetic mechanisms that determine their development is still of a great interest.

The zygote of higher plants derived from the fusion of a male gamete with a female gamete and is the beginning of the embryogenetic process controlled by a specific genetic programme.

The completion of the embryogenesis requires a balance between morphogenesis, cellular differentiation, cellular divisions, growth, metabolism and maturation.

What happens to the embryonic level-histogenesis and organogenesis, meristematic activity-represents an iterative process that takes place throughout the lifecycle of the plant. Unravel the mechanisms underpinning of embryogenesis opens doors to the understanding of the processes that occur in the post phase of germination. It is likely that some genes are their only function at the level of embryogenesis, others will express both in the embryonic and post-embryonic phases; others belong to families, multi-genetic illnesses whose individual elements are characterized by expression profiles different spatial and temporal, and are activated in different stages of the life cycle of the plant.

Mutant phenotypes analysis suggests the existence of pathways that regulate the histogenesis and organogenesis overlapping and redundant during the different stages of development.

In this context the study of the mutants altered in the embryogenetic processess takes therefore a twofold purpose: on the one hand, to identify specific processes and characteristics of the development of the embryo and related genes, on the other hand to help to understand the processes that are started during embryogenesis but which are not distinctive of this period because they are almost unchanged during the life of the plant. One of the most direct in this second case is represented by the formation of organs at the apical meristem, a phenomenon that distinguishes the plant system in all stages of its life cycle and

that, presumably, shares the same structure in both the embriogenetic processes that post – phase germination.

All the works in this thesis focus on the kernel of maize that is the core of the new plant generation and the main source of food and other materials both for human and animal consumption.

The choice of maize is due to it's importance for man. If one considers only maize, rice and wheat, they represent almost the 65% of the calories produced on the earth and utilized directly and indirectly, by the consumption of animal product and maize is the species with the highest level of productivity. Maize seeds are also considered as a "bioreactor" for the production of compounds that have a great value.

The maize seed comprises the two compartiments, the embryo and the endosperm, both originated from the double fertilization event.

The mature embryo is surrounded by an organ of big dimensions, referred as scutellum, and comprises a well developed embryonic axis, whilst the endosperm, that occupied almost the entire seed comprises the amylaceous central region in which the stored products are accumulated, mainly complex carbohydrates, proteins and lipids.

The economic and nutritional value is due to the high content of starch, that represents almost the 75% in weight of the mature caryopsis, but also to the complement of reserve proteins, especially zeins (10%) and oils (4.6%).

Due to the central role and importance of the seed, in this thesis several works were conducted in order to elucidate better the roles exerted by the genes involved in the development of seed, embryo and endosperm formation.

About the *emp* (*empty pericarp*) mutants of maize, they represent a useful tool to elucidate the role of these genes in embryo-endosperm development: the study conducted about the interaction of these genes in all the pairwise combinations allowed us to identify an epistatic interaction between these mutants.

The complementation test not only answers the central question "are the two mutations due to alleles of the same gene or to different genes?" but also provides the opportunity to study how and in that manner these genes interact with one another.

Even if we encountered many difficulties during this work, mainly due to the extreme similarities between the phenotypes of these mutants (it's quite impossible to classify these *emp* only on the bases of their phenotype because it is always the same with only slightly differences in the wrinkles of the pericarp), the different lines of maize employed and the lethality of these mutants when in the homozygous condition were identified; we were able to individuate some cases of 9:7 segregation in which the recovery of at least two different phenotypes of mutant seeds (wrinkled vs flattened and wrinkled vs dek-emp) in a 9:4:3 or 9:6:1 ratio is the evidence of an epistatic interaction between the two genes.

We noticed another fact during the study of the interactions between these mutants: one of these (*emp*-8376*) in the A636 background germinates very well but seems to be lethal because during the years we tried to grow mutant seedlings in the field but nonetheless it was able to reach the reproductive maturity. But when this mutant was crossed to *emp*-Dap3* in the ACR background and the resulting F_1 generation was conducted in F_2/ F_3 and the dek-emp from an ear that segregated 9:4:3 for wt, emp-wrinkled and dek-emp were sown directly in the field, among the few survivors, that were small and chlorothic, one of them reached the reproductive maturity giving a small ear homozygous for these dek-emp seeds.

It's thus conceivable that there is not only the interaction between the *emp* genes but also with the genes present in the backgrounds: this fact could explain the survival of the mutant (probably *emp*-8376*) in the field: it is the result of a positive interaction not only with the *emp*-Dap3* but also with the two different genetic backgrounds: in this case they were the A636 line and the ACR line.

The fact that the collection of these novel mutants of the seed in maize is in different genetic backgrounds on the one hand is a factor of "disturb" in the classification of the phenotypes of single *emp* mutants but on the other hand allow us the identification of genetic factors that are able to improve the endosperm of some of these mutants in some genetic backgrounds.

The identification of these genetic factors, provisionally designated with the term "modifiers" allowed us to clarify their behavior: dominant or recessive.

To do so, first appropriate crosses were made between plants heterozygous for the *emp* "Ref" and plants heterozygous for these abnormal *emp*: the resulting progeny ears show the segregation of the two *emp* phenotypes in a 1:1 ratio that indicate the presence of a segregating genetic factor. Wild-type seeds from the same ears were selected, planted and selfed in order to ensure the nature of this modifier.

In the F_2 progeny we noticed the segregation ratio of the two mutant phenotypes near to 12:3:1.

In the case of *emp*-8971* in W23 we could identify a dominant modifier because the seeds with the improved endosperm are 3/4 whereas those with the reference phenotype are in 1/4. The same result was observed for *emp*-9475* in Mo17.

Instead for *emp*-8376* in A636 the modifier of the endosperm tissue is recessive because in the 12:3:1 ratio the seeds with the reference phenotype were 3/4 whereas those with the improved endosperm were only 1/4.

One important point remains to be elucidated: if these genetic factors present in these inbred lines are specific or not for a given *emp* mutant.

We noticed that the presence of improved endosperm is not sufficient to promote the germination of impaired embryo.

About the work on the *lilliputian1-1* of maize although the data are preliminary we can notice that plants heterozygous for the mutant produce ears that are shorter and set less seeds with respect to the dominant wild-type sibs.

These data suggest that a strong reduction in brassinosteroid byosinthesis causes a reduction in the productivity.

On the other hand a slight reduction in the byosinthesis of brassinosteroids has a positive effect on the increase of productivity.

On the last two works conducted on *Plodia interpunctella* and *Rhyzopertha dominica*, we were able to identify the genotypes that were more resistant and more susceptible to the attack on whole and longitudinally sectioned kernels.

Tests carried out on *P. interpunctella* demonstrate that on the whole kernels larvae have encountered severe difficulties to break the hard pericarp of the kernels with the exception of the homozygous seeds for the two mutations: *vp2* and *vp5*. This result is due to the pregermination of the mutant kernels that make the pericarp less hard and the embryo more accessible.

On the other hand in the tests conducted on longitudinally sectioned kernels all the genotypes employed were attacked with the exception of homozygous kernels for the *emb*-8908* mutation that were damaged in the endosperm area due to the absence of the embryonic axis. These results indicate the preference of *P.interpunctella* for the embryo that contains more nutritive substances with respect to the endosperm.

Very similar results were obtained in tests with *R. dominica*: on the whole kernels a high mortality of the young larvae was observed due to the hardness of the pericarp, while on longitudinally sectioned kernels more larvae reached the adult stage and even in this case the embryo was the part of the seed preferred respect with to the endosperm.

Attacks on the endosperm were detected only on the whole homozygous kernels for the *su1* mutation and, partially, on sectioned kernels of the two lines B73 and RAlex0.

In conclusion all these works provide a good example of how one can understand several aspects regarding the seed development in maize with the employement of several techniques such as the complementation test, the use of B-A translocations, the use of insect pests, and many more.

Summary

One of the most interesting aspects, that is still poorly charachterized from the genetic and molecular points of view, of the development of the higher plants is the formation of the seed.

A better knoweledge of the steps of development is achieved with the isolation, in model species, of mutants impaired in the formation of the two components of the seed: the embryo and the endosperm.

In this thesis several works are summarized regarding the study of different classes of mutants in *Zea mays* of different origin affecting either the seed or the plant development.

In **Chapter 2** of this thesis we provide the state of the art about the collection of nine emp (*empty pericarp*) of maize isolated as independent events. These mutants are embryo lethal at maturity and drastically reduced in their endosperm size. In this chapter we present the results obtained with the complementation test performed in order to establish which of these mutants are allelic and which are not allelic and to study the forms of interactions in the cases of 9:7 segregation. In this way we were able to recover two different epistatic forms of interaction that fit the segregation values of 9:4:3 and 9:6:1 in which we can hypothesise that one *emp* gene is epistatic respect to one another. However the data need to be increased to demonstrate that the hypothesis postulated is correct.

In **Chapter 3** we present the study of the different expression of *emp* mutants in certain genetic backgrounds with the discovery of a new improved endosperm phenotype referred to with the provisional term of dek-emp that in some cases allow the recovery of germination, although in a low percentage, in the mutant seeds.

With the appropriate crosses between plants that carry the *emp Ref* mutation with plants that have the same mutant with more endosperm tissue, we were able to identify first the presence of a genetic factor called “modifier” of the endosperm tissue and then in the F_2 generation the nature of this modifier.

In this chapter the results of these crosses were discussed and hypothesis act to explain why some *emp* mutants in some genetic backgrounds have an improved endosperm were also formulated.

Summary

In **Chapter 4** we present the preliminary results of a work regarding the role of brassinosteroids in the productivity of a unique mutant of maize referred to as *lilliputian1-1 (lil1-1)*. As showed in the work of Makarevitch *et. al.* 2012 this gene encodes for a Brassinosteroid C-6 Oxidase. We analyzed the data collected about the length of the ears and the average weight of kernels of plants +/+ and plants heterozygous for the *lil1-1* gene in two different genetic backgrounds: A188 and B73.

The results indicate that the plants heterozygous for the *lil1-1* gene displayed ears that are significantly shorther in length and set a lowest number of seeds when compared with the ears obtained from plants that haven't got the recessive allele. This preliminary data is in accordance with other data from the literature in Arabidopsis and rice that showed an increased level of productivity when the plants have got a slightly low level of BRs in their tissues.

Further work is needed to elucidate the role of this gene during the male and female gametophyte development.

In the last two chapters (**Chapter 5** and **Chapter 6**) there are two works about the susceptibility of different maize genetic variants to the attack of two different insect pests: *Plodia interpunctella* a Lepidoptera Pyralide and *Rizopertha dominica* a Coleoptera Bostrichidae.

In the work with *P. interpunctella* the behavior of this pest was tested on four maize stocks with significant differences in the embryo. The stocks were: B73 reference line, RAlex0 line two mutants pooled together: *vp2* and *vp5* and another mutant: *emb*-8908*. Tests were carried out on whole and longitudinally sectioned kernels and the results obtained were discussed.

In the other work with *R. dominica*, we tested five maize genetic stocks in competition (in a couple) with significant differences in the endosperm. The stocks were B73 reference line, RAlex0 line, ACR line that accumulate anthocyanins in the aleurone layer and two mutants of the endosperm: *sugary1* and *yellow endosperm1*. Tests were carried out as those on *Plodia interpunctella* and the results were discussed in the relative chapter.

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