Phenotypic and molecular evaluation of maternal effects for a number of agronomic traits in onion

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STUDY AND EVALUATION OF THE POSSIBLE MATERNAL EFFECTS ON THE FINAL PHENOTYPE IN THE ONION (*Allium cepa L.*)

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ABSTRACT

Onion (*Allium cepa L.*) is one of the most important vegetable crops in the world, ranking third only after potato and tomato for total yield and acreage. Although its importance, onion can be considered an “orphan crop” as it has received little attention from researchers compared to the other monocot species like rice, maize and wheat. Plant pathology and molecular information, useful for improving the existing breeding programs in the species, are lagging behind major commercial crops, mainly because the onion genome is too complex to sequence or characterize. A. cepa L. (2n=16) is a biannual allogamous plant, with a very large nuclear genome (16.5 Gbp per 1C, approximately 6X larger than rice and maize), characterized by a high presence of repeated sequences and one of the lowest percentage of GC content (32%) known for any angiosperm. In fact, the onion genome has not yet been sequenced because of its very large size, a high percentage of repeated sequence and a life cycle (biannual plant) unfavourable to scientific studies. For these reasons, at present just a limited number of EST (about 20.000 EST) are available in public database (ncbi, the gene index project, TIGR, or in related specie like Garlic: GarlicESTdb). A strong maternal influence has also been observed in the species, but few studies are nowadays available to clarify the molecular mechanisms underlying this effect.

It was already observed in model plant that parental imprinting influence the progeny's phenotype during their development as seed size, germination timing, leaf production, timing of flowering and reproductive success. Moreover it has been suggested that maternal influence can increase the individual adaptation to the environment where its mother lived, but the frequent modification and fragmentation of the natural environment by man (anthropization), the probability for a seed to germinate and develop in the same environmental conditions as its mother is always lower, thus reducing the fitness of the plants. These development difficulties encountered by the plant in unusual environmental conditions could be possible and related to the global marketing, where it’is normal to have a seed production in a place and the plant production in another one. In this case, the maternal influence can be a disadvantage for all crops, since it could adverse the plant performances in a different environment. Consequently, it becomes very important to understand the molecular process which regulates this aspect strongly present in onion, in order to minimize any negative effect and improve crop performance and quality. The main objectives of this PhD research were to study the maternal effects on a number of agronomic traits in onion with two different strategies: an epigenetic analysis of genome and analysis of possible genetic basis of tolerance-resistance of onion to *Fusarium oxysporum* f. sp. *cepae*, a common soil fungus causing of an important disease that affect the normal field production and the storage of the bulb kept in stock. Epigenetic analysis will be focused on the methylation status of specific genes involved in two important metabolic pathways for this crop, photoperiod and vernalization, in order to understand the heritability, penetrance and interaction with environment of candidate genes.

We took advantage from the novel techniques and the coordinated efforts of international research groups of recent years, which enabled to sequence many plant nuclear genomes and to share all the information with whole community scientists. This made it possible to apply synteny based research and to study quickly but deeply plant genomes not yet sequenced using new bioinformatics methods.

The results obtained in this PhD project allow saying, with reasonable certainty, that maternal effects influences multiple agronomic traits in onion. It been shown, in pathological test and field trials, how the plants of the hybrids generated by crossing the parental DH lines are phenotypically closer to the maternal lines. In particular, in the tests of resistance/tolerance to the pathogenic fungus Fusarium, has been observed as hybrids and their reciprocal respond differently to infection of the pathogen. These data were also confirmed by the data collected in the field, consequently it prove how the gene expression in onion is influenced by some molecular mechanism which promotes the mother's chromosomes

The results of molecular analysis have instead allowed seeing how environmental conditions directly influence the molecular aspects such as epigenetic modifications in the genome

Key words

Allium cepa L., *Fusarium oxysporum* f. sp. *cepae* (FOC), Maternal inheritance, Developmental traits, Candidate Genes

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CHAPTER 1

General Introduction

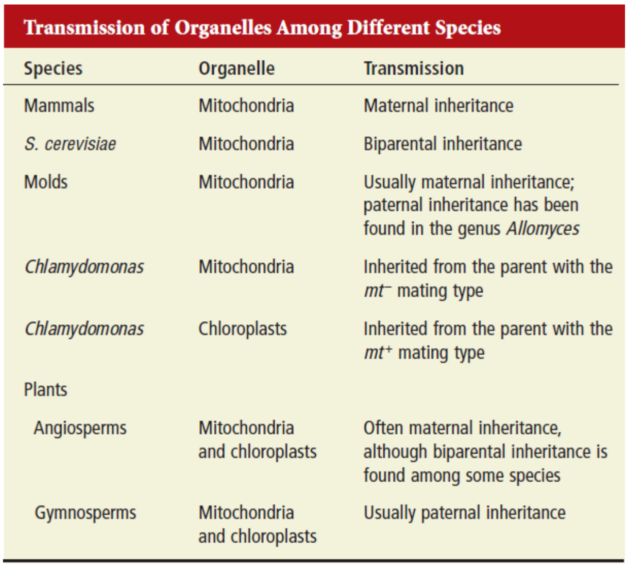
1.1 Epigenetics in Plants

1.1.1 Maternal inheritance

In most eukaryotes, and then also in plants, in addition to the transmission of genetic information through the nuclear chromosomes from the two parents, additional molecular mechanisms are present and they promote preferential transmission to the progeny of the genetic heritage and experience of one of the two parents. In fact, one of the main characteristics of this particular genetic inheritance is that does not display a Mendelian pattern, showing an evident deviation from this notorious genetic law. The main advantage of this alternative molecular mechanism allows the offspring to have a phenotypic plasticity that increase their adaptation to a specific environment. In plants, that are sessile organisms, the most frequent parent imprinting is of maternal origin this is because species characterized by limited seed dispersal area increase the fitness of their offspring generations if latter inherit good phenotypic traits from mother (Galloway; 2005). In conclusion, for a better phenotypic adaptation of progeny to local environment the maternal genetic pool and its biochemical modifications, fixed during her life experience, are used as a source of molecular tools to increase chances of survival by offspring. Regarding maternal influence, there are mainly two extra nuclear source of genetic information: the maternal inheritance and maternal effects.

In the first case, the mechanism known from more time, the inheritance of genetic information occur through the transmission of the genome of cytoplasmic organelle (plastid and mitochondria). In this case several phenotypic variations could be due to the expression of extra nuclear DNA present in the ovum during fertilization. It is because during the union of gametes the ovum retains its mitochondria. In fact, the female gamete is often bigger than male gamete because contains a big quantity of cytoplasm with all organelles. This simple observation allows understanding how citoplasmic organelles are not allocated in equal way in daughter cells during in meiosis and therefore do not segregate into gametes.

The maternal inheritance is a universal way to transmit the genetic information from mother in most sexual organisms. The traits influenced by this system are many, for example from sugar production through the enzyme RUBISCO, of which the large subunits are encoded from genes that lies in the chloroplast genome, the ATP molecule produced in the matrix of mitochondria with the respiratory chain.



Transmission of Organelles Among Different Species

Species	Organelle	Transmission
Mammals	Mitochondria	Maternal inheritance
S. cerevisiae	Mitochondria	Biparental inheritance
Molds	Mitochondria	Usually maternal inheritance; paternal inheritance has been found in the genus *Allomyces*
Chlamydomonas	Mitochondria	Inherited from the parent with the *mt*⁻ mating type
Chlamydomonas	Chloroplasts	Inherited from the parent with the *mt*⁺ mating type
Plants		
Angiosperms	Mitochondria and chloroplasts	Often maternal inheritance, although biparental inheritance is found among some species
Gymnosperms	Mitochondria and chloroplasts	Usually paternal inheritance

Table 1.1. Description of the inheritance patterns of mitochondria and chloroplasts in several selected species (Brooker RJ, 2012)

Chloroplasts and mitochondria are considered ancestral bacterial which have been incorporated within the plant cell during the evolution (Deusch, O. et al., 2008. - Gray MW et al., 1999 - Lang BF et al., 1999 - Karlin S et al., 1999). In particular, chloroplasts are considered endosymbiotic descendants of cyanobacteria-like prokaryotes (Deusch, O. et al., 2008), while it is assumed that mitochondria genome are discended from (eu)bacterial (Gray MW et al., 1999).

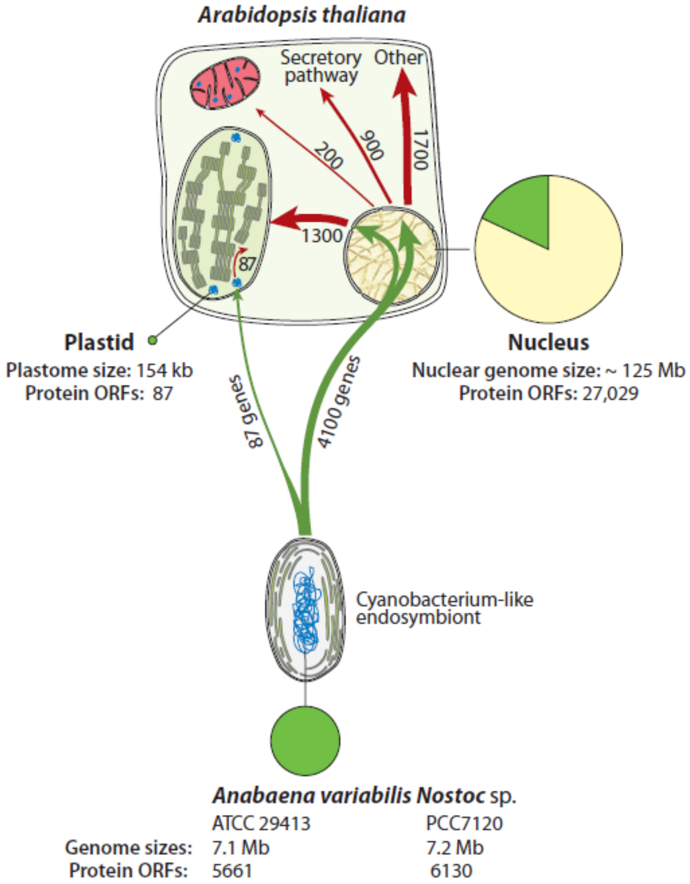


Fig. 1. 1

Diagram illustrating the hypothesis on the origin of chloroplasts in plants.

Fate of cyanobacterial genes and the intracellular targeting of their products in the flowering plant *Arabidopsis thaliana*. Chloroplasts such as those in *Arabidopsis* are descended via primary endosymbiosis from a cyanobacterium-like endosymbiont. On the basis of genome-wide sequence comparisons, the ancestral endosymbiont was most probably similar to contemporary filamentous, heterocyst-forming (nitrogen-fixing) cyanobacteria (Kleine et al., 2009a)

The beneficial effects of these mutual interactions between prokaryotic and eukariotic cell has been important for both organisms: from one hand the eukaryote provided protection and nutrients to the prokaryote, and in return, the prokaryotic endosymbiont provided additional energy to its eukaryotic host through its respiratory cellular machinery (Palmer J. D., 2000). During the evolutionary process, the genomes of these organelles have decreased dimension in consequence to transmission of many genes to nuclear genome. In fact, it was estimated that 10 % of all plant proteins are mitochondrial and 14 % chloroplastic (Emanuelsson et al. 2000).

Although evolution has led to the transfer of many genes from the organelles to the cell nucleus, this does not mean that it is a quick and easy process. In fact, the transfer and integration of a organelle gene in the nucleus to be successful important genetic and molecular events must occur, for example at the beginning of the gene must be inserted a promoter for a proper nuclear transcription, must be add a poly-A tail to protect the mRNA from degradation, must be add a signal sequence to allow to address the final protein to a correct cellular apparatus or compartment. Finally, a specific molecular communication among organelles and nucleus to synchronize their interaction must be established through specific signalling molecules that convey information to the nucleus. (Kleine T. et al., 2009 – Inaba T., 2010). For example, in the case of chloroplast at least four pathways which generate signals have been discovered: redox change, tetrapyrrole synthesis, plastid gene expression and protein import defects. In the figures below it is shown a simple representation of the pathways just listed above between chloroplast and nucleus (Kleine T. et al., 2009):

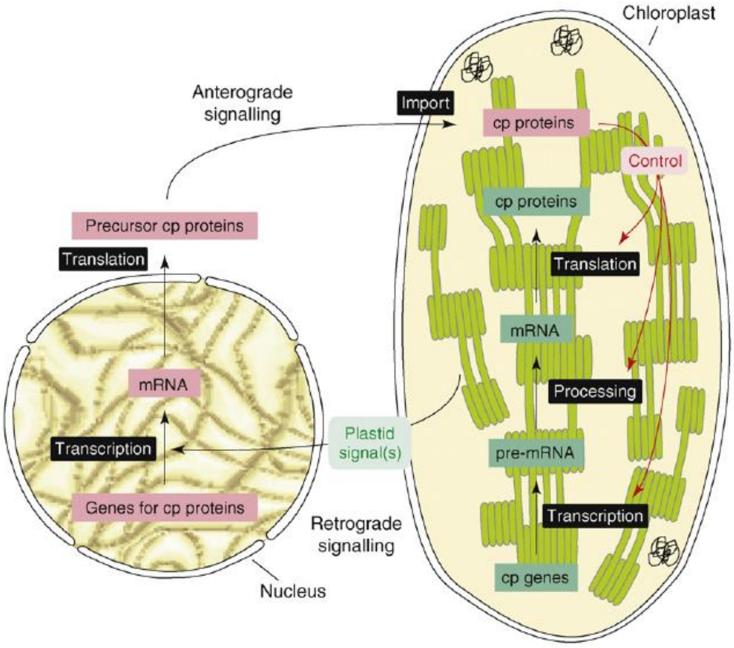


Fig. 1. 2
Concept of anterograde and retrograde signalling
Modulation of the level of transcripts of nuclear genes for chloroplast (cp) proteins controls the abundance of many cp proteins. The post-translational import of proteins into chloroplasts is facilitated by the Tic–Toc complex. (Kleine *et al*., 2009b).

Chloroplast genomes have a greater number of genes compared to those of mitochondria and its dimension range from 100,000 to 200,000 bp in length, consequently the first are larger than of those of mitochondria (Brooker R. J., 2012 - Book). Below is showed a map of tobacco chloroplast genome as an example.

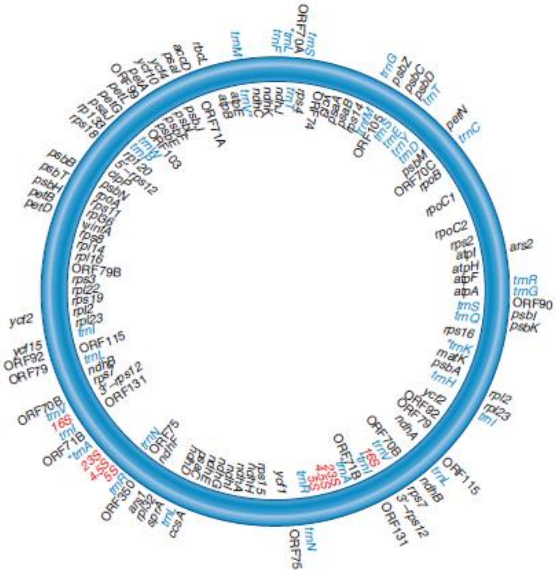


Fig. 1. 3
A genetic map of tobacco chloroplast DNA (cpDNA).
This diagram illustrates the locations of many genes along the circular chloroplast chromosome. The gene names shown in blue encode transfer RNAs. The genes that encode ribosomal RNA are shown in red. The remaining genes shown in black encode polypeptides that function within the chloroplast. The genes designated ORF (open reading frame) encode polypeptides with unknown functions. (Brooker RJ, 2012)

An evident example of maternal inheritance, namely the uniparental transmission of maternal cytoplasmic organelles, is given by the “Four o’clock” plant (Fig. 1.4). Carl Correns in 1909, in an experiment with this plant, observed that the leaf color was inherited by offspring with a strong deviation of Mendel ratio. After a careful analysis of all combinations obtained with its plant-mating design, he observed that the heritability of the leaf phenotype could be explained only with maternal inheritance.

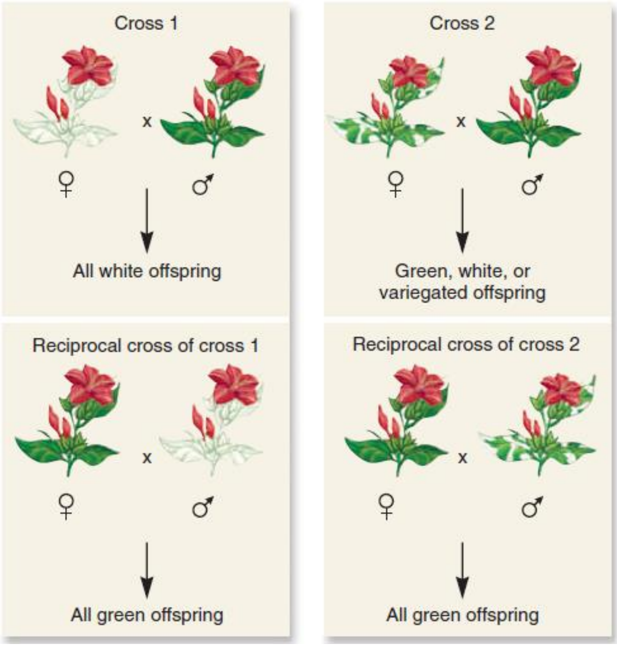


Fig. 1.4
Maternal inheritance in the four-o’clock plant, Mirabilis jalapa.
The reciprocal crosses of four-o’clock plants by Carl Correns consisted of a pair of crosses between white-leaved and green-leaved plants, and a pair of crosses between variegated-leaved and green-leaved plants. (Brooker RJ, 2012)

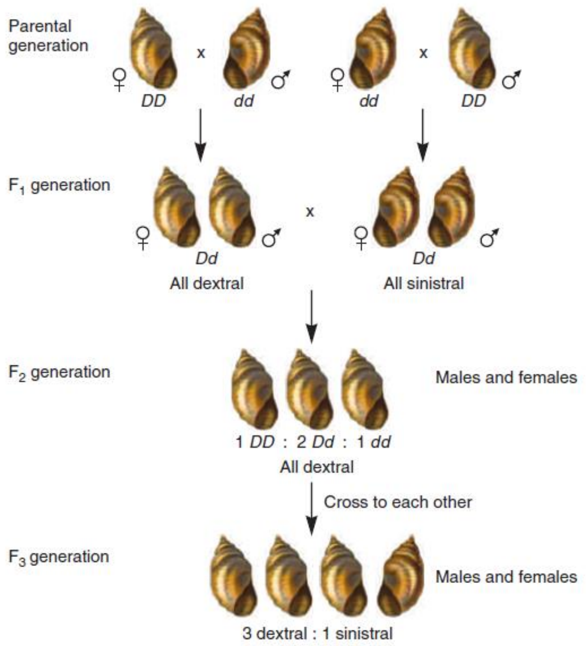
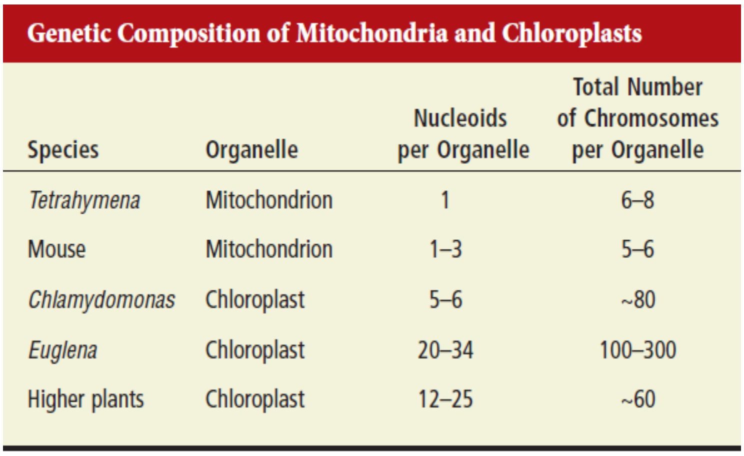


Fig. 1.5
Experiment showing the inheritance pattern of snail coiling.
In this experiment, *D* (dextral) is dominant to *d* (sinistral). The genotype of the mother determines the phenotype of the offspring. This phenomenon is known as the maternal effect. In this case, a *DD* or *Dd* mother produces dextral offspring, and a *dd* mother produces sinistral offspring. The genotypes of the father and offspring do not affect the offspring's phenotype. (Brooker RJ, 2012)

In the mitochondrion, there are just 40 proteins transcribed from its genome compared to several thousand arising from the nucleus of the plant cell (Emanuelsson et al. 2000). The process which led to the transfer of organellar genes in the nucleus is still taking place, as in the legume family with the subunit 2 of cytochrome oxidase (Adams et al. 1999). An interesting example of negative effect in a plant arising from a mutation in a gene of mitochondria genome is the phenomenon known as cytoplasmic male sterility, or cms. The plant with this genetic disorder in the organelle isn't able to produce viable pollen. The male sterility it is always maternally inherited, consequently does not display a Mendelian segregation ratio even if this handicap can be overcome if it is present a restorer gene in the nucleus which will be able to complement the original function of the mitochondria's gene (Zabala et al., 1997)

Another characteristic of these organelles is that they have more copies of their genome (nucleoid) in each organelle and they are present in high number of copies in a plant cell, as shown in the table below (Brooker R. J., 2012 - Book).



Genetic Composition of Mitochondria and Chloroplasts

Species	Organelle	Nucleoids per Organelle	Total Number of Chromosomes per Organelle
Tetrahymena	Mitochondrion	1	6–8
Mouse	Mitochondrion	1–3	5–6
Chlamydomonas	Chloroplast	5–6	~80
Euglena	Chloroplast	20–34	100–300
Higher plants	Chloroplast	12–25	~60

Table 1.2.
Description of the genetic composition of mitochondria and chloroplasts for a few selected species (Brooker RJ, 2012)

1.1.2 Maternal effects

Maternal effects are the result of the second most important mechanism to imprint the final phenotype of the offspring in uniparental way. It is important to note that, in contrast to what we have described previously, in this case the uniparental influence on progeny phenotype doesn't occur with an unequal segregation of some compartment cell and its genetic patrimony but through of alteration of gene expression with an epigenetic modification of DNA of specific sequences of nuclear chromosomes. This alternative molecular mechanism produces a segregation which is not in accordance with Mendel's laws. In fact, a variation in the hereditability of methyl groups during the mitosis and meiosis can occur. In case of mitosis, transient marks cannot be passed to a sister cell while stable one is mitotically inherited. In case of meiosis, marks inherited could be lost after several generations because erased for unknown reasons or because promoted by specific environmental conditions. These mechanisms are still unclear, and further investigations are required to understand laws that govern the hereditability and the effects on gene transcription across generations (Jablonka E., and Raz G., 2009 - Kumar et al., 2013). In conclusion, even if the final phenotype of a plant is mainly due to genetic differences, it is clear that there is an additional minor component of hereditability influenced by epigenetic modifications. Finally, the methylation of DNA can be considered a quick way of adaptation to environmental modification for a plant, as it was observed recently by Gonzalez et al. (2013). Given the characteristics of the molecular mechanism of the epigenetic marks just described, it is evident that these DNA modifications can increase the phenotypic variations in a population without any changes into its pool gene sequences (Galloway, 2005 – Schmitz and Ecker, 2012. - Schmitz et al., 2013).

In recent year increasing attention has been dedicated to the study of epigenetic; consequently also plants have been subject to this type of molecular analysis. Epigenetic studies are at the first steps in plants, and limited to a few model plants, like Maize and Arabidopsis (Kermicle J.L., 1970 - McWhirter KS, Brink RA, 1963 – Grossniklaus et al., 1998). Subsequently, epigenetic information is lacking or limited for horticultural crops, such as onion, even if it is evident that many genes and traits are under the control of this molecular mechanism.

Epigenetic studies how covalent modifications of DNA may cause physical variations in chromatin structure altering the normal expression of several genes. The final effect of this alteration in an organism cold be observable in a possible deviation from the average parameters of the final phenotype of the population (Galloway, 2004 – Schmitz and Ecker, 2012. - Schmitz et al., 2013).

Epigenetic changes can be perceived as an alternative way to control the gene expression, amplifying the phenotypic variation, normally due to variation in DNA sequences. Usually, a modification in the phenotype is a consequence of one or more modifications in the DNA sequence (SNPs, in-dels, deletions, etc), Instead epigenetic marks could only modify the transcription of a chromosome region, compromising the interactions between genes and proteins, like transcription factors. In other words, changes in the phenotype do not correspond to modifications in the primary sequence of the genome.

Interestingly, these modifications can be inherited by the next generation with a specific parental imprinting (Jablonka E., and Raz G., 2009 - Kumar et al., 2013).

1.1.3 Type of modifications

The covalent modifications that can occur in the sequence and structure of DNA are the methylation of cytosine, where a methyl group is added at position 5 of the nucleotide (5-methylcytosine; 5mC) by a DNA methyltransferase , and methylation of a aminoacid in the tail of histone. In this case, the aminoacid involved in this modification is lysine 27 which undergoes to modification by a Polycomb Group complex (PcG). Regarding the cytosine, only the nucleotides which lies in specific sequences, CG, CHG and CHH (where H can be C, A or T), can be modified (Shen, X. et al., 2008 - Di Croce L. and Helin K., 2013).

Recently, another type of epigenetic mark was discovered also in plant (Tahiliani et al., 2009 - Piccolo and Fisher, 2014.), where cytosine is modified with a methyl group associated with a hydroxyl group (5hmC). Even if in plants a few is known regarding this mark, from research performed in mammalian it seems that modifications are associated with enhancer and gene bodies (Stroud et al., 2011).

1.1.4 Molecular pathways

Different molecular mechanisms are responsible for DNA modifications and involve different enzyme pathways at different stage of the plant life cycle.

Addition of a methyl group to the cytosine can happen by three different pathways. The first pathway consists in a series of modification through several DNA methyltransferases. In plant have been identified several enzymes that covalently attach methyl group to nucleobases of nucleic acids, among these only three have been well characterized: DRM2, MET1 and CMT3 (Köhler et al., 2012).

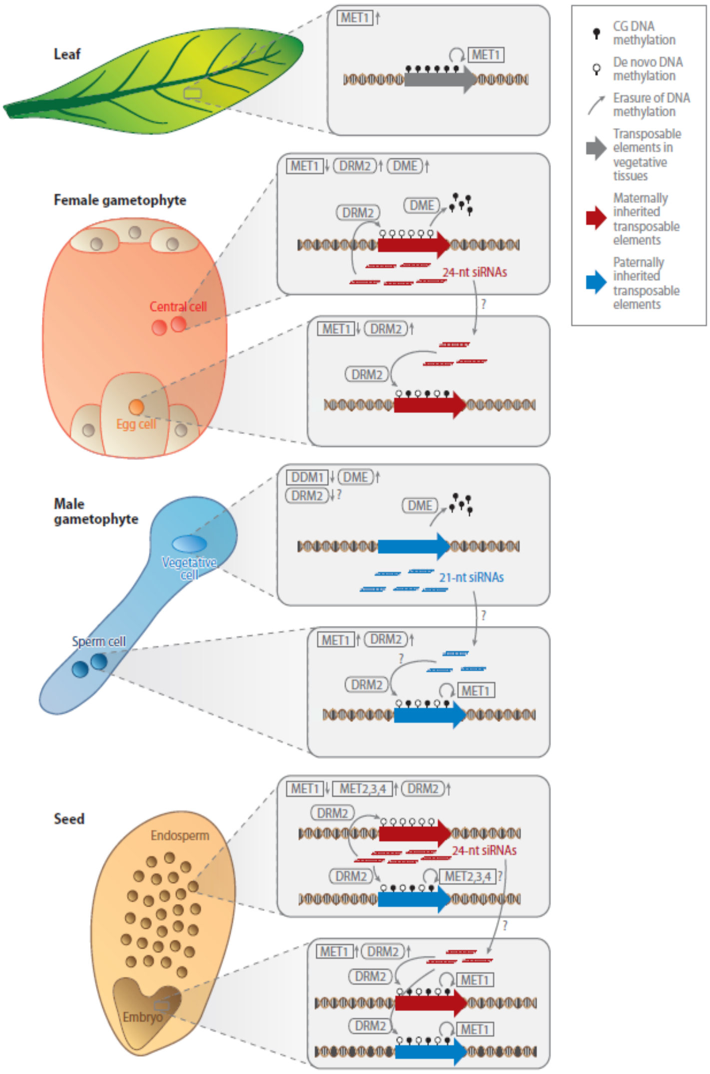


Fig. 1.6

Model depicting chromatin modifications in gametic and nongametic cells underlying imprinted gene expression.

CG DNA methylation (closed black circles) of transposable elements (solid arrows) in vegetative tissues is maintained by MET1 (processes denoted by looping arrow). In the central cell of the female gametophyte, MET1 is not active and CG methylation is removed by DME, causing transposable element transcription and generation of 24-nucleotide (nt) small interfering RNAs (siRNAs) that guide de novo DNA methylation by DRM2 at non-CG DNA residues (open circles). These siRNAs might migrate to the egg cell, where they guide de novo methylation by DRM2 at CG and non-CG residues. In the vegetative cell of the male gametophyte, CG methylation is removed by DME, causing generation of 21-nt siRNAs that might migrate to sperm cells guiding de novo methylation by DRM2 at CG and non-CG residues. In the endosperm, transcription of hypomethylated maternal alleles (denoted in red; for simplicity, only one allele is shown) generates 24-nt siRNAs that guide de novo methylation by DRM2 on non-CG residues. DRM2 is unlikely to target CG residues in the endosperm, for reasons that remain to be investigated. CG methylation on paternal alleles might be maintained by the MET1 homologs MET2, MET3, or MET4. The 24-nt siRNAs might migrate from the endosperm to the embryo, where they guide de novo methylation by DRM2.

(Köhler et al., 2012)

Moreover, these methyltransferases can be grouped in two classes, the first class is composed by enzymes that create new methylation marks on the DNA (DRM2), while the second class encloses enzymes which add methyl group in the symmetric nucleobases of new strand of DNA after the DNA replication (DRM2, MET1 and CMT3) (Cao and Jacobsen, 2002).

The second molecular pathway utilizes double-strand RNA (dsRNA). Specifically, these molecules belong to the “RNA silencing” system. This mechanism is characterized by two different steps. The first step consists in a “Post Transcriptional Gene Silencing” (PTGS) and occurs in the cytoplasm. During this phase a dsRNA or assRNA (single-strand RNA) cause the resetting of gene expression through the disruption of specific mRNA restraining the translation process. dsRNA or ssRNA are processed by DICER enzyme in small RNAs of 20-24 nt in length (microRNAs – miRNAS).These small ribonucleic acids drive a protein complex in the cytoplasm to specific mRNA through base-pairing, so the molecule target will cleavage in small part and the translation will be inhibited. In second phased sRNA causes the resetting of gene expression driving the methylation of specific DNA sequences by DNA methyltransferases, This process is known as “RNA-directed DNA methylation” (RdDM), or “RNAi” in mammals. (Le Trionnaire and Twell, 2010)

The PTGS is a very powerful system because allows a quickly adaptation of the plant in response to environmental modification. This system works in two different ways. The first pathway, known as ESTABLISHMENT, generates epigenetic marks in new positions in the genome, The second pathway, known as MAINTENANCE, is promoted and driven by an addition of a methyl group to a cytosine in a genome of a different cell during cell division or between generations. The sequence target of “RNA-directed DNA methylation” (RdDM) is the cytosine in all sequence context, that is CG, CHG and CHH (where H can be C, A or T), while the main sites target are the Transponable Elements (TE) and tandem repeats. This process will affect not only the transcription of TEs but also the transcription of those genes that lie around transposons, by modifying genes expression and the consequent phenotype of a plant. Examples of these methylation effects is the FWA gene, involved in flowering time in Arabidopsis (Fujimoto et al., 2011) or the paramutagenic allele at b1 locus in maize (Hollick, 2012). RdDM is considered a defense system which acts through the methylation and restriction of the expression of DNA elements. These element share the ability to move across the genome, protecting the genome from recombination damages, chromosome structure alterations, and mutagenic RNA viruses,. The proteic mechanism involved in this system is complex and act in both nucleus and cytoplasm. Moreover, for different pathways (establishment or maintenance) are recruited different DNA methyltransferases.

Polycomb Group complex (PcG) is a specific proteins complex that participates to the modification of DNA structure, modifying with a methyl group the lysine 27 placed in the tail protein complex of nucleosome. This change maintains DNA tightly wrapped around the histone, preventing the interaction between transcriptions factors and DNA. PcGs are also important for maintaining imprinted gene expression and these two mechanisms can act in synergy at the same locus or independently. RDdM and PcG are common across mammalian genomes, where the key processes of the genomic imprinting are always epigenetic phenomenon and histone modification, altering the normal gene expression (Scott and Spielman, 2006; Arnaud and Feil, 2006; Kohler and Makarevich, 2006).

Recent research on Arabidopsis thaliana, Vigna unguiculata and Campanula americana showed that many maternal traits of final phenotype are inherited by the offspring (Gehring et al., 2011; Manggoel and Uguru, 2012; Galloway, 2005). The frequency of these events is higher in plants than in animals, because in plants DNA methyltransferase are active during gametogenesis and embryogenesis, and that patterns of DNA methylation can persist from parents to progeny and do not need to be reset. (Bond and Baulcombe, 2014)

1.1.5 Timing of the modifications

Epigenetic variations are extremely interesting because these modifications are influenced by several factors like age, environment and pathogen interaction. Even if different epigenetic variations occur during the lifetime of an organism, not all the changes are inherited by the next generation.

Usually, DNA modifications occur in the early stage of a lifecycle, like gametogenesis and embryogenesis through the mechanisms described above. Specifically, molecular pathways that involve small RNAs are particularly common. In fact, many seed traits are influenced by genes imprinted with epigenetic marks.

In this paragraph, are briefly described the female gametophyte traits and the fertilization process in order to better understand the effects of these external modifications of DNA.

Female gametophyte contains three antipodal cell and a central cell (product of two polar nuclei), all within the same embryo sac. After a double fertilization, a diploid embryo (containing a set of maternal chromosomes and a set of paternal chromosomes) and a triploid endosperm (composed of 2 sets of maternal chromosmes and one set of paternal chromosomes) are formed. These parts of seed are covered by a seed coat (TESTA), a tissue exclusively derived from maternal tissue (external teguments of the ovule). Numerous studies have shown that small RNA control part of methylation machinery trough the Post Transcriptional Gene Silencing system and its main targets are Transposable Elements (TEs) and tandem repeats. One of the most important was the identification of specific sRNAs produced in a cell and then translocated in another cells, also for long distance. This feature is specific for class of sRNA and has been named trans-acting RNA (taRNA). Many research teams agree that during the gametogenesis, these molecules play an important role because allow the silencing of transponable elements in both female and male gametes. It was observed that during female gametogenesis the nucleus of synergid cells are characterized by a large decondensed area of chromatin with a very poor methylation of H3K9 and just some epigenetic marks at H3K27 (Leroy et al., 2007). Moreover, Slotkin et al. (2009) reported a wide decreasing of epigenetic marks at TEs, due to the action of a specific enzyme, such as the DNA glycosylase DEMETER (DME). The vegetative cells, also namely companion cells, of the male gametophyte undergo to the same process. Subsequently, taRNAs produced in the companion cells will move to egg or sperm cells where they will promote the methylation of specific target sequences in the DNA (TEs and tandem repeats).

Recently Agonaute effector proteins (AGO), specific of this gametogenesis phase of plant life cycle, have been discovered (Nonomura et al., 2007; Lippman al., 2003). In particular, in Arabidopsis, molecular analyses have demonstrated that many siRNAs were associated with AGO9, an AGONAUTE protein highly expressed in the ovule. The sequences of these siRNA have revealed, that they derived from TE and satellite repeats (Olmedo-Monfil et al., 2010), while in the male gametes it was observed that many retrotransposon elements were upregulated because hypomethylated whereas other TEs were ipermethylated and consequently silenced (Borges and Martienssen, 2013; Chen et al., 2010). Similarly in maize, AGO104 an homolog of Arabidoposis AGO9, has a pivotal role in the methylation of cytosine in the DNA sequences CHG and CHH (Singh et al., 2011).

The process, described above, is repeated after a double fertilization. The important role of endosperm, a triploid tissue, is to nourish the embryo and to produce siRNA and taRNAs arising from TEs. These will move into the embryo in order to silencing homolog sequences by methylation of cytosines through RdDM mechanism. A recent finding is that the sRNAs, produced by RNA polymerase IV, and arising from transposon in the endosperm, derive from maternal lineage, Consequently a parental epigenetic profile will be generated in the embryo genome, besides to modify indirectly the expression of genes integrated near to transponable elements targeted (Mosher et al., 2009).

In fact, it was demonstrated that TE regions are hypomethylated in the endosperm, while the same sequences are hypermethylated in the embryo (Hsieh et al., 2009; Gehring et al., 2009). On the base of these recent findings, it is possible to hypothesize a new role for the transponable elements, from simple parasitic elements to fundamental elements for the regulation of genes and chromatine remodeling. Moreover, it is interesting to note that sRNAs can modify the gene expression and final phenotype across generations.

In Arabidopsis, it has been demonstrated that several maternal genes (MEA, FWA and FIS2) are preferentially expressed, whereas the paternal alleles are silenced by DNA methylation (Kinoshita et al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000; Jullien et al., 2006). These data have been confirmed also in rice and maize, where endosperm is hypomethylated when compared to the embryo and to vegetative tissues (Gehring et al., 2009; Zemach et al., 2010; Lauria et al., 2004). This can be considered as an imposition in gene expression in a non-equivalent way on parental genomes or parental gametes, before or just after the double fertilization. Consequently, embryo and endosperm are epigenetically distinct, but they maintain a similar genetic background. In conclusion, even if the composition of a seed is characterized by three different genetic structures, the seed development is dependent on maternal control. Moreover, endosperm has a fundamental role during the seed development. Finally, in several model plants, like Arabidopsis thaliana, has been demonstrated that maternal effects influences many aspects during a life cycle of a plant, such as a seed size, germination timing, flowering time and ontology in general (Donohue, 2009).

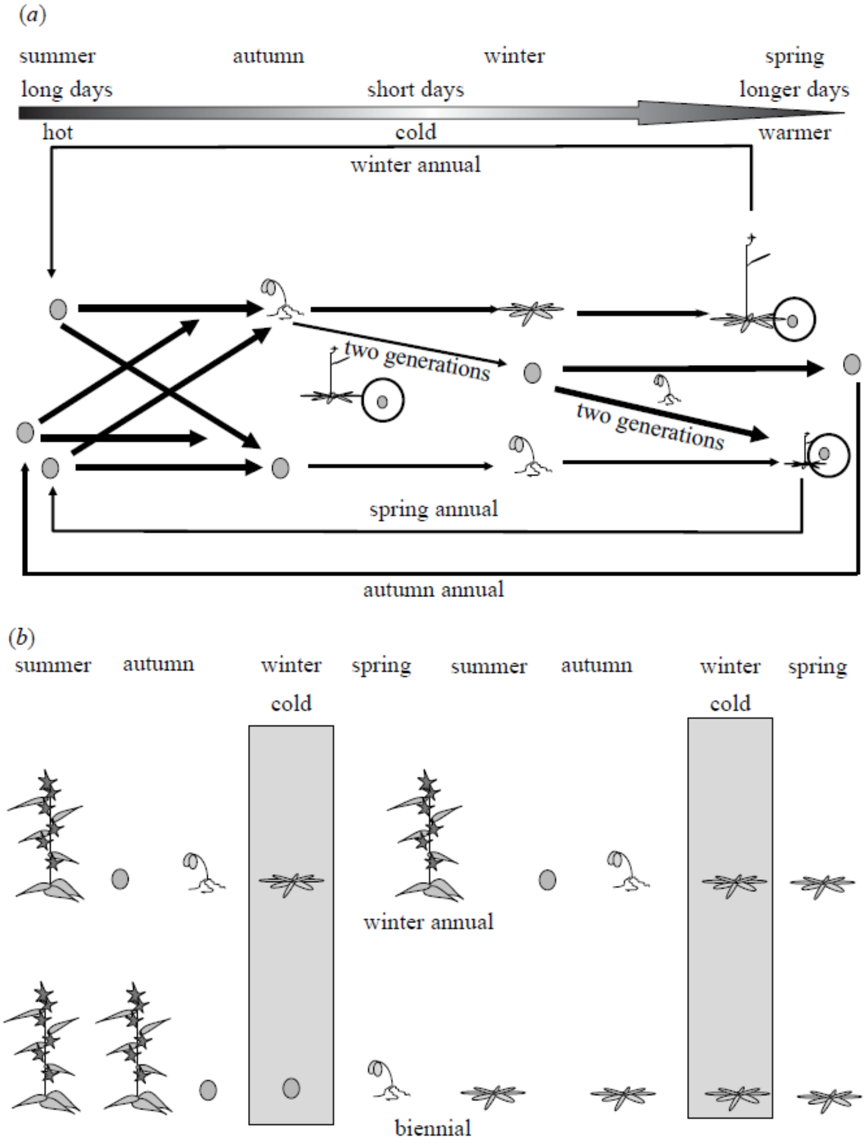


Fig. 1.7
Comparison between different plant life cycles.
(a) Life cycles of annual plants. Bold lines indicate branch points determined by the germination behaviour of seeds (circled) that matured during different seasons. The pathway from flower to seed represents the maternal effects on germination, and this path also completes the life cycle. The life cycle that is expressed depends on which path the seeds follow, which can be a function of when the seeds were matured and dispersed. (b) Life cycles of biennial plants. Germination timing determines whether the seeds or rosettes experience cold (shaded). This, in turn, determines whether the plants flower the following season and express an annual life cycle as opposed to a biennial life cycle. (Donhue K., 2009)

Donohue, 2009

Currently, there are several theories that attempt to explain this phenomenon. Kinship theory of imprinting (Haig, 2000) theorizes that there is a parental conflict between maternally and paternally genomes inherited by offspring over maternal resource allocation. Consequently, imprint the gene expression by DNA methylation contrasts this conflict and give an evolutionarily advantage to the progeny. Instead, the maternal-offspring coadaptation theory of imprinting, assert that in species where the mother take cares for the offspring, the expression of maternal alleles are favored because they increase the adaptive integration of maternal and offspring genomes (Wolf and Hager, 2006).

1.1.6 Gene cluster

Interestingly, several mini clusters of imprinted genes were reported in Arabidopsis. These kinds of gene clusters are called imprinting control regions (ICRs), and usually were observed in mammalians.

1.1.7 Expression of imprinted alleles

Interestingly, in two recent publications (Gehring et al., 2011; Gregg et al., 2010) it was reported that genes identified as imprinted exhibit a partial rather than a complete imprinting, both in plant and mammalians. In fact, both alleles of maternal and paternal parents are expressed at different rate. In particular are Paternally Expressed imprinted Genes (PEGs) that show a diffused “partial imprinting”, while for Maternally Expressed imprinted Genes (MEGs) this event is relatively rare. These studies also showed that PEGs encode for potential regulators of the epigenome, like 5-methylcytosine binding gene, histone deacetylase interacting protein, SRA (SET- and RING-ASSOCIATED) domains domain-containing histone lysine methyltransferases and the RNA polymerase PolIV. In addition, there is a subgroup of putative transcription factors and DNA binding proteins that can interact with these genetic elements. In case of MEGs, the groups of proteins identified are several zinc finger and leucine zipper genes, transcription factors and finally genes involved in cell wall modification, like pectin esterases.

It was also observed that there is a correlation between the imprinted genes that encode for regulatory proteins and hypomethylated genomic regions. MEGs transcriptional factors are also in correlation with DMRs.

1.2 Epigenetic effects on plant phenotypes

1.2.1 Flowering time

The flowering time specify the period when a plant produce flowers. To set up the right moment to have transition from vegetative to reproductive stage, plants have several important pathways and many genes which interact with the environment. Normally plants respond to changes in the day length (photoperiod) and cold temperature (vernalization), to avoid producing reproductive organs in unfavorable conditions. It is really interesting to notice how plants show a large variability in flowering time tuning their genetic network to the environment where they live, for example plants flowers during spring season in temperate regions to avoid freezing temperatures and when day length is increasing. Conversely, other plant species flower during cooler seasons in tropical regions to avoid hot temperature of summer. Clearly, in the examples listed above, it is evident how plants must have organs and molecular mechanisms that allow perceiving light and temperature to have a plastic adaptation to environment in order to increase the probability of success in the reproduction. Genetic research in model plants allowed identifying molecular pathways and many genes controlling seasonal flowering-responses. (Greenup et al., 2009)

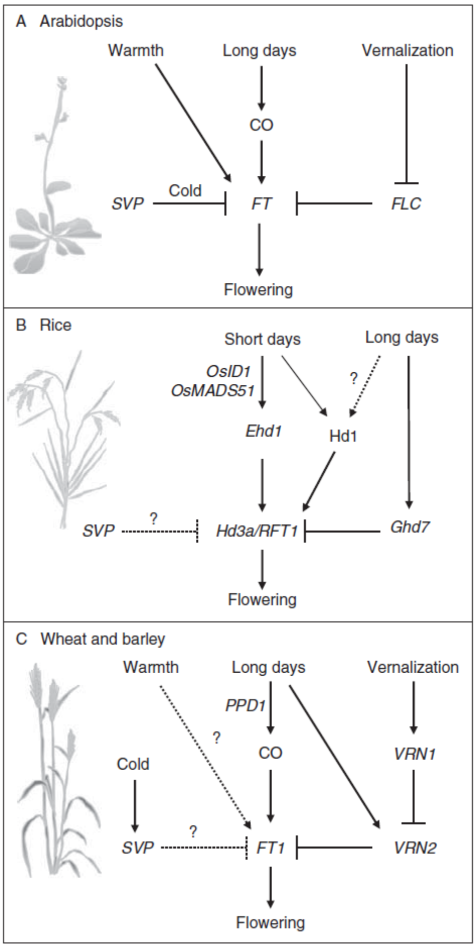


Fig. 1.8
A comparison of the molecular pathways regulating flowering time in (A) arabidopsis, (B) rice and (C) temperate cereals.
Vernalization and long days promote flowering in arabidopsis and temperate cereals wheat and barley (A, C), whereas short days promote flowering in rice (B). The role of *FT* in mediating the day-length response is conserved. The role of the CO protein might also be conserved. In rice a pathway involving *OsMADS51* and *Ehd1* promotes expression of *Hd3a* (*FT-like*) in short days, while Hd1 weakly activates *Hd3a* (dotted line). In long days *Ghd7* represses *Hd3a*. In arabidopsis and the temperate cereals vernalization is required for the long-day flowering response, but this response has evolved independently in these plants. In arabidopsis, *FLC* represses *FT* to block the day-length response until *FLC* is itself repressed by vernalization. In temperate cereals, *VRN2*, which is distantly related to *Ghd7*, blocks expression of *FT1* in long days. *VRN1* is induced by vernalization to repress *VRN2* and to allow long-day induction of *FT1. PPD1* is required for long-day induction of *FT1*. Warm conditions also promote expression of *FT* in arabidopsis, while *SVP* represses *FT* at lower temperatures. *FT-like* genes might have similar roles in regulating thermo-sensitive flowering responses in cereals. *SVP-like* genes are induced by low-temperatures in barley, but it is not known whether these genes inhibit expression of *FT1* at low temperatures in cereals. (Greenup et al., 2009)

Flowering time is one of most important agronomical trait modified by man in many worlds' major crop species because, even if they had a specific place of origin, they are cultivated in many different environments. Wild species have been domesticated with breeding activities through manipulation of flowering time.

Different flowering pathways are present in plant, like gibberellin, photoperiod, autonomous, and vernalization pathways, because this assure a properly flowering time in different environment. Interesting, in addition to photoperiod and vernalization pathways which react to light and temperature, gibberellin and autonomous pathways respond to internal cues (Putterill et al., 2004; Simpson, 2004). In any case, several functional differences are present in genes belonging to pathways among plants of different species and/or clades. In fact, it was observed with comparative analysis that many genes have an identical role but are characterized by a different molecular action: for example, the CONSTANS gene promote the transition from vegetative to reproductive growth in long day conditions in Arabidopsis, while its hortologue in rice (Hd1) represses the same transition in long but promote it in short day conditions. Moreover, it was identified some specific genes in a plant species, showing that new functions have been created during the evolution (Higgins et al., 2010).

The key genes of the photoperiod pathway, which perceive day length variation, are GIGANTEA, CONSTANS, and FLOWERING LOCUS T. In Arabidopsis, the product of first gene interact with FKF1 protein to form a complex after their concentration increase in presence of light, showing that these proteins are regulated in circadian way. This protein complex interacts and promotes the CONSTANS in long day condition (Sawa et al., 2011). The **CONSTANS (CO)** gene transcribe for a protein characterized by two B-boxes (a class of zinc-finger domain) and a CCT domain ("CONSTANS, CO-like, and TOC1" domain is a highly conserved basic module at the C terminus of plant proteins often involved in light signal transduction). Its expression is not constant during the day, in fact only in late afternoon is registered its maximum transcription while it is repressed by CDF1 at the morning. Other to be regulated the gene also its protein is regulated, showing that the maximum peak of CO takes place in the late afternoon, that in winter is already dark, the protein is quickly degraded. Conversely, when its maximum expression occurs with light (summer season) the Co is stabilized by PHYA and CRY proteins and can activate **Flowering Locus T (FT)** (Higgins et al., 2010). In long day condition the FT gene is expressed in the leaves and this protein will move to the apex to interact with FD protein in order to promote the expression of genes involved in floral development, such as the MADS box gene **APETALLA 1 (AP1)** (Greenup et al., 2009)

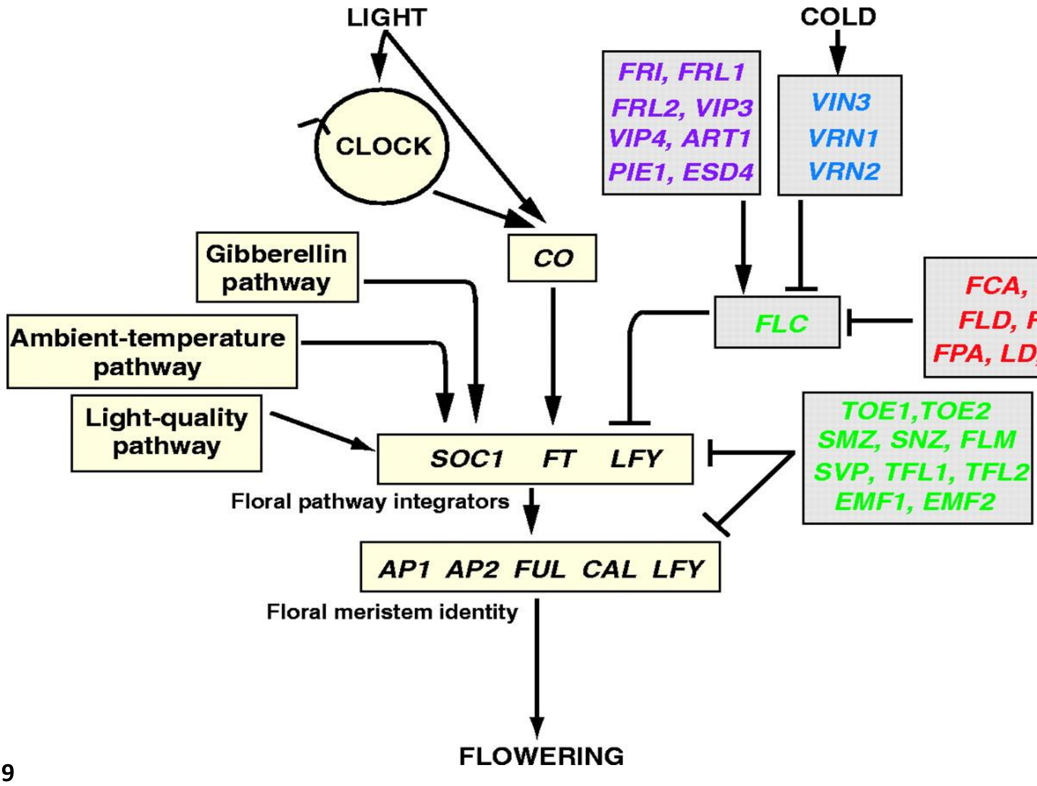


Fig. 1.9

Pathways controlling flowering-time in Arabidopsis.

The flowering-time pathways control the expression of the floral pathway integrators SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), FT and LEAFY (LFY). These genes encode proteins that activate the floral meristem identity (FMI) genes APETALA1 (AP1), APETALA2 (AP2), FRUITFULL (FUL), CAULIFLOWER (CAL) and LFY, which convert the vegetative meristem to a floral fate. Recent expression data has indicated that FUL may also act as a floral integrator (Schmid et al., 2004). The photoperiod, gibberellin, light-quality and ambient-temperature pathways activate floral pathway integrators. The CONSTANS (CO) transcription factor functions in the photoperiod pathway; long-day photoperiods promote flowering by circadian clock (CLOCK) dependent and independent mechanisms, which control the activity of CO. Activation of flowering is antagonised by the floral repressors encoded by (shown in green) FLOWERING LOCUS C (FLC), FLOWERING LOCUS M (FLM), TERMINAL FLOWER1 (TFL1), TERMINAL FLOWER2 (TFL2), SHORT VEGETATIVE PHASE (SVP), TARGET OF EAT1 (TOE1), TARGET OF EAT2 (TOE2), SCHNARCHZAPFEN (SNZ), SCHLAFMUTZE (SMZ) and EMBRYONIC FLOWER1/2 (EMF1, EMF2). TFL1 may also be downstream of CO, as it is induced after CO activation (Simon et al., 1996). FLC expression is controlled by a number of different pathways. The genes shown in purple, FRIGIDA (FRI), FRIGIDA-LIKE1 (FRL1), FRIGIDA-LIKE2 (FRL2), PHOTOPERIOD INSENSITIVE EARLY FLOWERING1 (PIE1), AERIAL ROSETTE1 (ART1), EARLY UNDER SHORT DAYS4 (ESD4), VERNALIZATION INDEPENDENCE3 (VIP3) and VERNALIZATION INDEPENDENCE4 (VIP4), encode proteins that promote FLC expression and delay flowering. FLC expression is downregulated in response to prolonged cold by proteins encoded by the genes (shown in blue) VERNALIZATION INSENSITIVE3 (VIN3), VERNALIZATION1 (VRN1) and VERNALIZATION2 (VRN2), and also by proteins encoded by the genes of the autonomous pathway (red): FCA, FY, LUMINIDEPENDENS (LD), FLOWERING LOCUS D (FLD), FVE, FLOWERING LOCUS K (FLK) and FPA. (Henderson & Dean, 2004)

In certain ecotypes of Arabidopsis, the floral inhibitor Flowering Locus C (FLC) represses the transition from vegetative to reproductive stage until plant undergo to a prolonged cold period, called vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999). FLC is a MADS box gene that transcribe for a transcription factor which repress the expression of FT by binding to a region inside FT gene (Michaels and Amasino, 1999; Sheldon et al., 1999; Michaels et al., 2005; Helliwell et al., 2006). This interact prevent the transition from vegetative to floral stage. It is important to note that FLC inhibit also SUPPRESSOR OF OVER EXPRESSION OF CONSTANS 1 (SOC1) that is another floral promoter. SOC1, as FLC, is a MADS box gene that act downstream of CO (Hepworth et al., 2002; Michaels et al., 2005; Helliwell et al., 2006). The main enhancer of FLC is FRIGIDA (FRI), which promote an over expression of FLC (Koornneef et al., 1994; Lee et al., 1994; Johanson et al., 2000). After a prolonged cold period (vernalization), expression of FLC in a plant decrease (Michaels and Amasino, 1999; Sheldon et al., 1999) allowing the expression of genes downstream listed before. Consequently, after winter period when cold is replaced by a mild temperature coupled with an increase of day length, FT and SOC1 are transcribed and will promote flowering formation. The repression of FLC during winter period is carried out by a Polycomb Repression Complex 2 (PRC2) (Schubert et al., 2006; Wood et al., 2006; De Lucia et al., 2008), methylating the lysine 27 residue of the histone 3 tail (H3K27me3) (Schubert et al., 2006; Sung et al., 2006; Greb et al., 2007; Finnegan and Dennis, 2007; De Lucia et al., 2008). PRC2 can target and repress FLC thanks to anther protein transcribed by VERNALISATION INSENSITIVE 3 (VIN3), which encode for a cold-induced Plant HomeoDomain (PHD). Genes that transcribe for a HomeoDomain (HD) are called HOMEOBOX, and their products are protein involved in regulation of development process. The HD are capable to recognize and bind specific DNA sequences regulating the expression of gene target (Sung and Amasino, 2004; Wood et al., 2006; De Lucia et al., 2008). Finally, also all genes belonging to Autonomous pathway (LD, FCA, FY, FPA, FVE, FLD, FLK and FLD)) repress FLC expression (Simpson, 2004; Marquardt et al., 2006).

1.2.2 MADS-box genes

During brief description of the main pathways, it has been reported several time genes belonging to **MADS box family**. Members of this family are transcription factors that play an important role in every development process in plants so not surprisingly it was observed that many genes have conserved modular sequences and function across the flowering plants (Smaczziak et al., 2012). MADS domain transcription factors are almost ubiquitous throughout all clades. In fact, genes with these characteristic sequences were found in plant (Yanofsky et al., 1990; Schwarz-Sommer et al., 1990), in human (Norman et al., 1988) and in yeast (Passmore et al., 1988). The combination of the first letter of all genes discovered in these researches gave the name to this family gene: MADS-box. Structural analysis showed that a MADS-box gene is a result of combination of modular domains that at the central and N-terminal parts interacting with specific DNA sequences, while the C-terminal part is involved in interaction with other proteins (normally another MADS protein) to form a protein complex (Pellegrini et al., 1995; Huang et al., 2000). These genes, besides to be involved in floral organ specification, play a role also in morphogenesis of almost all organs, like embryo and gametophyte development and it explains why this family is very large in plants compared to animals and fungi.

This interesting gene family can be divided into two class of proteins: type I is a heterogeneous group where any any gene function were still assigned; it can be subdivided in other three subclass Mα, Mβ and Mγ. The proteins of the type II class are characterized by a modular domain structure, called MIKC structure. As it is possible to see in the picture below, a MADS-domain is located in the N-terminal region of protein and it is important for the interaction with the DNA sequence target; continuing in direction of C-terminal there are two domains called Intervening (I) and Keratine (K) involved in dimerization and higher-order complex formation. The final part of the MADS protein is formed by high variable domain (C-terminal domain), both in sequence and in length, which may have roles in protein complex formation and transcriptional regulation (Kaufmann et al., 2005).

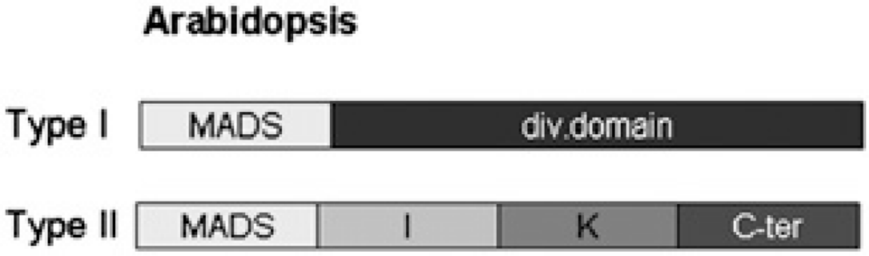


Fig. 1.10
Domain structure of MADS-domain proteins.
Schematic Representation of Type I and Type II MADS Box Transcription Factors.
C-ter, the divergent C region; I, intervening region; K, keratin K domain; M, MADS box domain. (Masiero et al., 2011)

The type II proteins are further subdivided in two groups based on differences in their domain structure: MIKC^{C} and MIKC* (Henschel et al., 2002). Proteins belonging to second group (MIKC*) have an altered protein domain structure, while the first group (MIKC^{C}) is formed by two group depending of their ancestral origin.

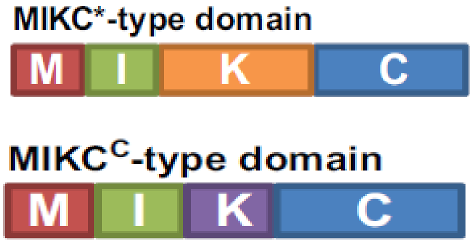
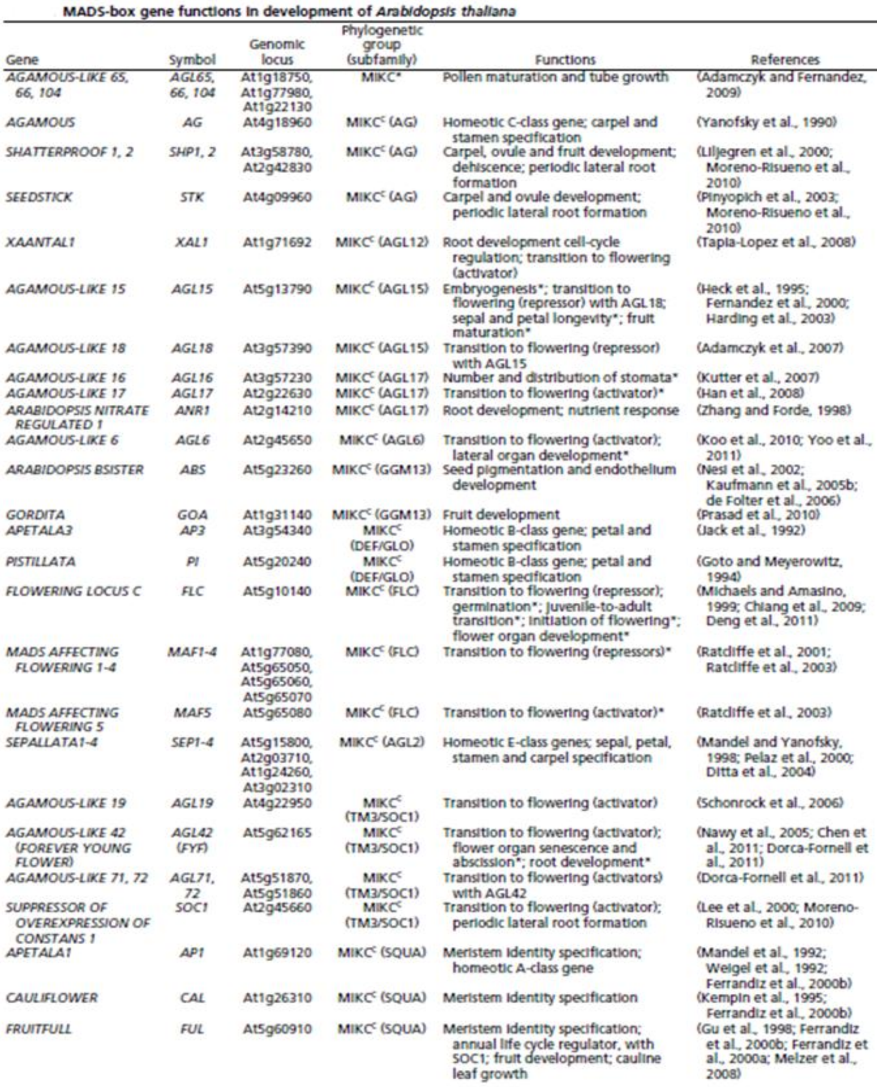


Fig. 1.11
Domain structure of MADS-domain proteins.
(Smaczniack et al., 2012)

Members of MIKC^{C} group can be involved in a specific development process or play more roles in different processes, for example genes of AGAMOUS (AG), DEFICIENS (DEF) and GLOBOSA (GLO) families are involved in the specification of stamens and carpels of flowers, while genes of SQUAMOSA (SQUA) and SEPALLATA (SEP or AGL2) families have a role in floral meristem. Similarly genes TOMATO MADS-BOX 3 (TM3), FLOWERING LOCUS C (FLC) and SOLANUM TUBEROSUM MADS-BOX 11 (STMADS11) act in floral transition, while proteins of families AGL12 and AGL17 are involved also in root development.



MADS-box gene functions in development of *Arabidopsis thaliana*

Gene	Symbol	Genomic locus	Phylogenetic group (subfamily)	Functions	References
AGAMOUS-LIKE 65, 66, 104	*AGL65, 66, 104*	At1g18750, At1g77980, At1g22130	MIKC*	Pollen maturation and tube growth	(Adamczyk and Fernandez, 2009)
AGAMOUS	*AG*	At4g18960	MIKC^{C} (AG)	Homeotic C-class gene; carpel and stamen specification	(Yanofsky et al., 1990)
SHATTERPROOF 1, 2	*SHP1, 2*	At3g58780, At2g42830	MIKC^{C} (AG)	Carpel, ovule and fruit development; dehiscence; periodic lateral root formation	(Liljegren et al., 2000; Moreno-Risueno et al., 2010)
SEEDSTICK	*STK*	At4g09960	MIKC^{C} (AG)	Carpel and ovule development; periodic lateral root formation	(Pinyopich et al., 2003; Moreno-Risueno et al., 2010)
XAANTAL1	*XAL1*	At1g71692	MIKC^{C} (AGL12)	Root development cell-cycle regulation; transition to flowering (activator)	(Tapia-Lopez et al., 2008)
AGAMOUS-LIKE 15	*AGL15*	At5g13790	MIKC^{C} (AGL15)	Embryogenesis*; transition to flowering (repressor) with AGL18; sepal and petal longevity*; fruit maturation*	(Heck et al., 1995; Fernandez et al., 2000; Harding et al., 2003)
AGAMOUS-LIKE 18	*AGL18*	At3g57390	MIKC^{C} (AGL15)	Transition to flowering (repressor) with AGL15	(Adamczyk et al., 2007)
AGAMOUS-LIKE 16	*AGL16*	At3g57230	MIKC^{C} (AGL17)	Number and distribution of stomata*	(Kutter et al., 2007)
AGAMOUS-LIKE 17	*AGL17*	At2g22630	MIKC^{C} (AGL17)	Transition to flowering (activator)*	(Han et al., 2008)
ARABIDOPSIS NITRATE REGULATED 1	*ANR1*	At2g14210	MIKC^{C} (AGL17)	Root development; nutrient response	(Zhang and Forde, 1998)
AGAMOUS-LIKE 6	*AGL6*	At2g45650	MIKC^{C} (AGL6)	Transition to flowering (activator); lateral organ development*	(Koo et al., 2010; Yoo et al., 2011)
ARABIDOPSIS BSISTER	*ABS*	At5g23260	MIKC^{C} (GGM13)	Seed pigmentation and endothelium development	(Nesi et al., 2002; Kaufmann et al., 2005b; de Folter et al., 2006)
GORDITA	*GOA*	At1g31140	MIKC^{C} (GGM13)	Fruit development	(Prasad et al., 2010)
APETALA3	*AP3*	At3g54340	MIKC^{C} (DEF/GLO)	Homeotic B-class gene; petal and stamen specification	(Jack et al., 1992)
PISTILLATA	*PI*	At5g20240	MIKC^{C} (DEF/GLO)	Homeotic B-class gene; petal and stamen specification	(Goto and Meyerowitz, 1994)
FLOWERING LOCUS C	*FLC*	At5g10140	MIKC^{C} (FLC)	Transition to flowering (repressor); germination*; juvenile-to-adult transition*; initiation of flowering*; flower organ development*	(Michaels and Amasino, 1999; Chiang et al., 2009; Deng et al., 2011)
MADS AFFECTING FLOWERING 1-4	*MAF1-4*	At1g77080, At5g65050, At5g65060, At5g65070	MIKC^{C} (FLC)	Transition to flowering (repressors)*	(Ratcliffe et al., 2001; Ratcliffe et al., 2003)
MADS AFFECTING FLOWERING 5	*MAF5*	At5g65080	MIKC^{C} (FLC)	Transition to flowering (activator)*	(Ratcliffe et al., 2003)
SEPALLATA1-4	*SEP1-4*	At5g15800, At2g03710, At1g24260, At3g02310	MIKC^{C} (AGL2)	Homeotic E-class genes; sepal, petal, stamen and carpel specification	(Mandel and Yanofsky, 1998; Pelaz et al., 2000; Ditta et al., 2004)
AGAMOUS-LIKE 19	*AGL19*	At4g22950	MIKC^{C} (TM3/SOC1)	Transition to flowering (activator)	(Schonrock et al., 2006)
AGAMOUS-LIKE 42 (FOREVER YOUNG FLOWER)	*AGL42 (FYF)*	At5g62165	MIKC^{C} (TM3/SOC1)	Transition to flowering (activator); flower organ senescence and abscission*; root development*	(Nawy et al., 2005; Chen et al., 2011; Dorca-Fornell et al., 2011)
AGAMOUS-LIKE 71, 72	*AGL71, 72*	At5g51870, At5g51860	MIKC^{C} (TM3/SOC1)	Transition to flowering (activators) with AGL42	(Dorca-Fornell et al., 2011)
SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1	*SOC1*	At2g45660	MIKC^{C} (TM3/SOC1)	Transition to flowering (activator); periodic lateral root formation	(Lee et al., 2000; Moreno-Risueno et al., 2010)
APETALA1	*AP1*	At1g69120	MIKC^{C} (SQUA)	Meristem identity specification; homeotic A-class gene	(Mandel et al., 1992; Weigel et al., 1992; Ferrandiz et al., 2000b)
CAULIFLOWER	*CAL*	At1g26310	MIKC^{C} (SQUA)	Meristem identity specification	(Kempin et al., 1995; Ferrandiz et al., 2000b)
FRUITFULL	*FUL*	At5g60910	MIKC^{C} (SQUA)	Meristem identity specification; annual life cycle regulator, with SOC1; fruit development; cauline leaf growth	(Gu et al., 1998; Ferrandiz et al., 2000b; Ferrandiz et al., 2000a; Melzer et al., 2008)

Table 1.3.
List of MADS-box genes and their functions in A. thaliana (Smaczniack et al., 2012)

It was observed in FLC members, which act as floral repressors, a natural variation in the flowering time that implies a gene variation in the different ecotypes of Arabidopsis and after a vernalization period, as already reported above, its expression is silenced through an epigenetic modification of the proteins associated with these DNA regions (histones).

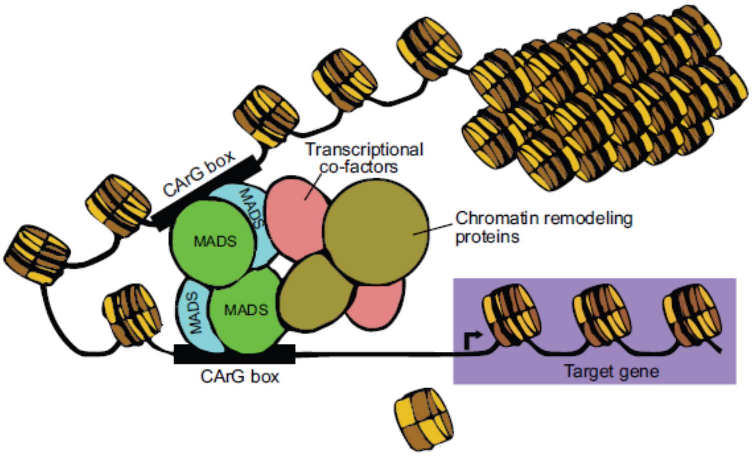
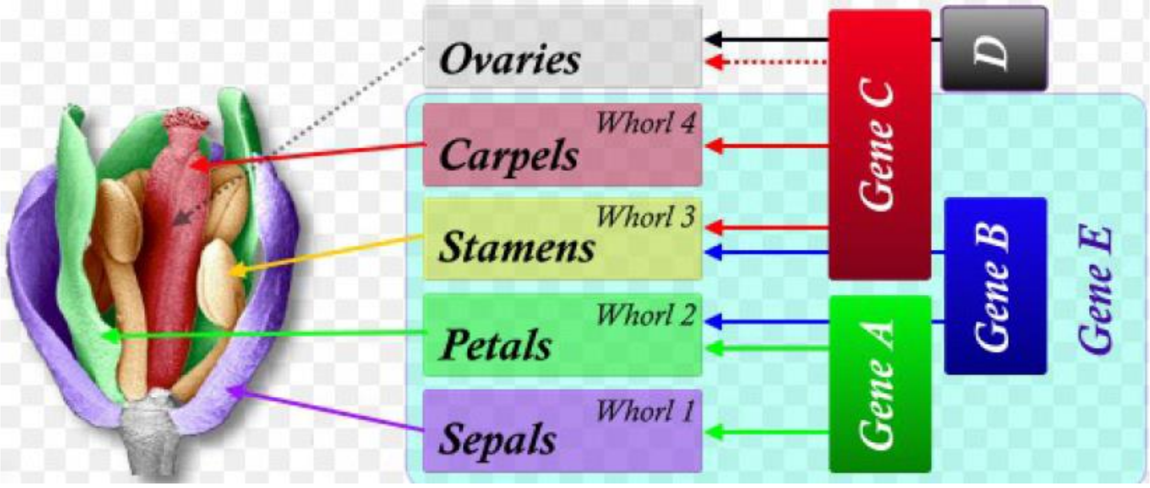
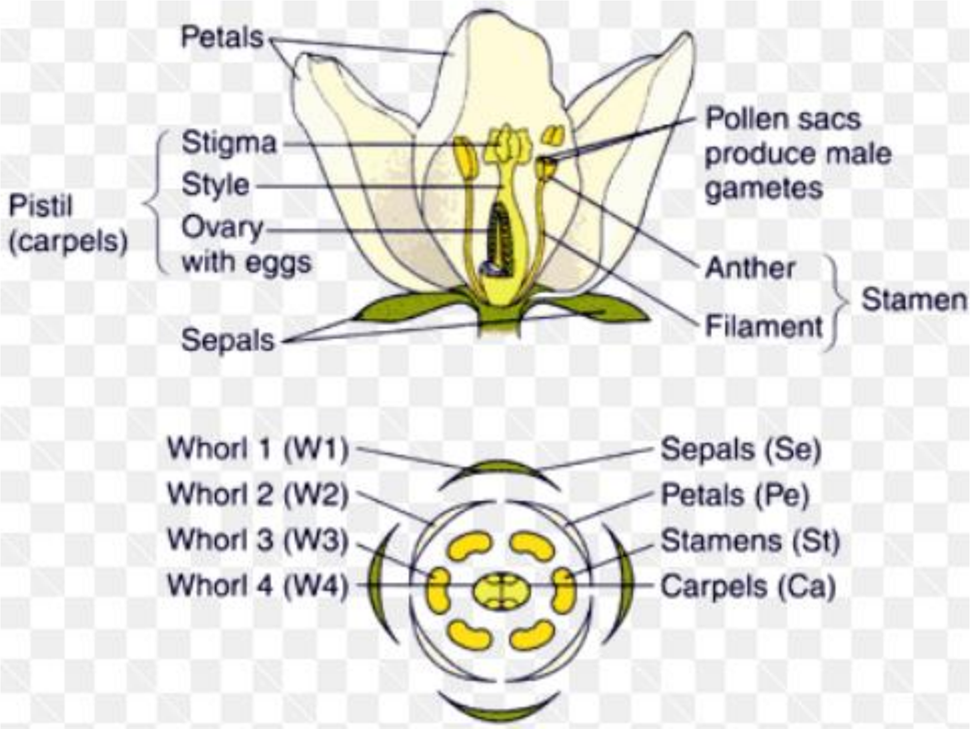


Fig. 1.12
Model for the action of MADS-domain protein complexes.
Shown is a model of MADS-domain protein complex formation and a hypothesized mechanism of regulatory action. In this model, MADS domain proteins (green and blue) form quaternary complexes according to the 'floral quartet' model and interact with two DNA binding sites (CArG boxes; black) in close proximity, resulting in DNA looping. Subsequently, MADS-domain proteins recruit transcriptional co-factors (pink), which mediate transcriptional regulation and may influence target gene specificity, as well as chromatin remodeling proteins (brown), which relax the chromatin structure at the target gene transcription start site allowing for the initiation of transcription. Depending on the selection of transcriptional co-factors and chromatin remodeling factors, the complex may also play a role as a transcriptional repressor. (Smaczniack et al., 2012)

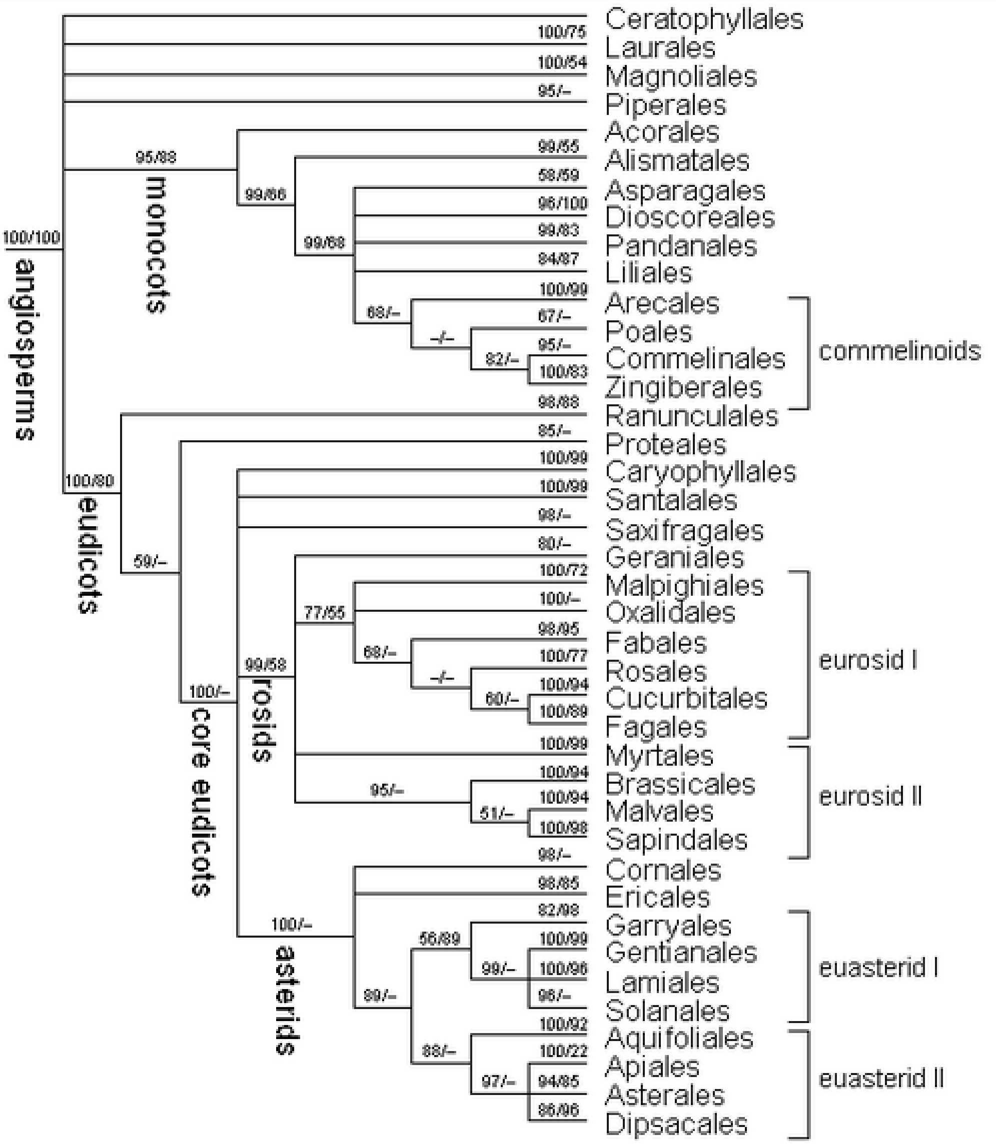
Regarding floral development it has been noted that morphology and number of reproductive organs are highly different according to different reproductive strategies, while the basic types of the same organs are largely conserved (Soltis et al., 2002). In fact, a similar genetic and molecular pathway involved in floral development is present in plants, At the beginning, after the identification of some homeotic floral mutants in Arabidopsis and Antirrhinum, it was elaborated the ABC model that explain how the combination of products of three class of homeotic genes (A, B, and C) is the basis of organ identities of flower (sepals, petals, stamens and carpels) present in the four concentric whorls of flower. The genes of the class A, [APETALA 1 (AP1) and APETALA 2 (AP2) in Arabidopsis and SQUAMOSA in Antirrhinum], just specify the identity of sepal, while those belonging to class B, [APETALA3 (AP3) and PISTILLATA (PI) in Arabidopsis and DEFICIENS (DEF) and GLOBOSA (GLO) in Antirrhinum] specify the identity of petals. When class B and class C genes act together produce the identity of stamens in the third whorl of flower.



Finally the class C gene, where it is identified just one gene AGAMOUS (AG), specify for the identity of carpels in the fourth whorl.



Genes of all three class are MADS box of the MIKC type characterized by a conserved structural organization of 56 amino-acids in the N-termial region (M) (Purugganan et al. 1995). The only one exception is AP2 that belong to the Ethylene-responsive element binding protein (EREBP) family an homeobox (Shore and Sharrocks 1995). In recent years, the previous ABC model has extended with two new classes: D and E (Theissen 2001). In the eudicot model plant Arabidopsis, the class D genes [SEEDSTICK (STK), SHATTERPROOF1, and 2 (SHP1 and SHP2)] control the ovary development in coordination with AG (class C), while the class E genes [SEPALLATA genes (SEP1, SEP2, SEP3, and SEP4)] are involved in the specification of all organs in all whorls of the flower, so sepals, petals, stamens and carpels (Pelaz et al., 2000, 2001b). Moreover, SEPALLATA (SEP) play a global role, in combination with B-, C- and D-class MADS-box genes, promoting plant development through many aspects such as floral meristem determination, organ identity, fruit maturation, seed formation and plant architecture. Phylogenetic analysis has revealed that genes of SEP subfamily are present in distant angiosperm lineages, therefore both in monocots and eudicots (Zahn et al., 2005). In Arabidopsis, an eudicot plant, are present four members of the SEP gene family (SEP1, SEP2, SEP3 and SEP4) as a result of gene duplication (Zahn et al., 2005), while many SEP-like genes have been identified in monocots. For example, at least eight and five different SEP-like genes have been identified in Maize and rice respectively (Becker & Theissen, 2003; Zahn et al., 2005; Cui et al., 2010). However, recent findings of SEP-like genes in monocots were mainly restricted within the Poaceae.



A frequent molecular mechanism used by plants to control expression of their MADS box genes is epigenetic modifications of their sequences. An important example of this genetic alteration is that given by the FLC gene that switches from active to repress form after a long period of cold (vernalization) that promote the amino acid methylation in the tail of histones associated with this gene.

1.2.3 Biotic and abiotic stress

Biotic and abiotic stress can induce epigenetic modifications in genes involved in interaction with the pathogen, and subsequently transgenerational inheritance in plants. Surprising it was registered a loss of methyl group from a gene sequence. In the case of interaction between Pseudomonas syringae and Arabidopsis it was reported a hypomethylation in the region around the defense gene and its transmission to offspring (Pavet et al, 2006). A similar resistance was observed when a mutation affects the RdDM pathway. From these results, it is possible to infer that genes involved in defense pathways can be silenced by epigenetic modifications through a sRNAs mechanism-dependent. The possible explanation for this unusual silencing could be that the plant is repressing the expression of a metabolically expensive pathway, when is not needed (low infection pressure). Conversely, the pathway can be activated under pathogen pressure, allowing a rapid response of the plant to infection, and transfer to next generation the activated gene. (Bond and Baulcombe, 2013)

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CHAPTER 2

Objectives and outline of the thesis

2.1 Objectives and outline of the thesis

The overall aim of the thesis is to unravel the genomic bases with a multidisciplinary approach of maternal effects on the offspring phenotype in onion

This has been done through three specific objectives: a) creation of specific plant material using Double Haploid (DH) lines, b) molecular analysis on plant genome in order to study global epigenetic modifications in different environment conditions and c) phenotypic analysis of plant through a physiopathological test with *Fusarium oxysporum* f. sp. *cepa*.

In Chapter 3, it is described the use of two populations derived from cross of the three DH lines, that differ mainly for their photoperiod characteristic and for other agronomical traits, to study variation in methylation profile during summer and winter seasons.

In Chapter 4, it was observed the different behavior of parental DH lines and F1, RF1 during some pathological tests.

CHAPTER 3

Maternal influence in onion developmental traits: flowering time, maturity and bulb features

3.1 Maternal influence in onion developmental traits: flowering time, maturity and bulb features

3.1.1 Taxonomy and diffusion of the crop

Onion (*Allium cepa L.*) is one of the most important commercial vegetable crops. The species belongs to the family of Liliaceae (also called Alliaceae), which also includes other vegetables such as shallots, leeks, garlic and asparagus. Thanks to the many properties contained in this horticultural plant, it is widely cultivated and used not only for food preparation but also for therapeutic purposes. Although archaeological evidences of consumption of onion as fresh vegetable date back to the Bronze Age (ca. 5.000 BC), it is still unclear whether the cultivation of this plant is actually so old or if its spreading happened only later (about 2000 years later).

The center of origin and domestication of onion is still under debate. Many archaeologists, botanists, and food historians believe onions originated in central Asia, while other researchers suggest onions were first grown in Iran and West Pakistan. However, the plant assumed great importance especially as vegetable for direct human consumption during the Middle Age, and was later introduced in the American continent after its discovery by Cristoforo Colombo in 1492. At present, onion is a “universal” crop, consumed in large quantities throughout the world.

A huge phenotypic variation exists among and within onion varieties and local landraces present in several germplasm collections around the world, which allow plant differentiation by shape, size and color. The main phenotypic variability can, in fact, be ascribed to bulb color. Of particular interest is the distinction between external bulb color and internal bulb color. The former refers to the "outer skin" of the bulb and ranges from white to pale yellow or golden yellow, or red, through the countless shades of purple; the latter concerns with the color of the bulb flesh that, in turn, can range from white or shaded in purple, through a yellowish hue (Kim et al., 2009; Khar et al., 2008; Kim et al., 2005). Another major distinction within the onion germplasm concerns the adaptation of the plant to the agro-ecological conditions of the crop in relation to latitude and thus photoperiod. Accordingly, 4 basic groups of cultivars/genotypes can be observed: 1) very long day onions (extra-long day), typical of Northern Europe (Great Britain, Scandinavia, Poland, Northern Germany, the Benelux countries); 2) long day onions (long day) typical of Central Europe, Northern Asia; 3) day-length intermediate onions (medium day) cultivated in the United States, Southern Europe, Central Asia, China, Oceania; and 4) short-day onions (short day) Africa, Central and South America, South Asia (Magruder and Allard, 1937; Brewster, 1977; Steer, 1980, de Ruiter, 1986; Lancaster et al., 1996).

It is estimated that around the world, over 3.6 million hectares (ca. 9 Mio acres) of onions are grown annually for the production of about 75 million tonnes of bulbs (source, FAOSTAT 2012). However, yield trends are in constant growth with an expected production for 2050 of 87 million tonnes. About 170 countries cultivate onions for domestic use and about eight percent of the global production is traded internationally. According to bulb color classification, the world market/hectarage of onion cultivation is dominated by 75% of yellow varieties (gold), followed by red (15%), white (6%) and pink (4%).

Major worldwide producers are China (20.5 Mio t), India (13.4 Mio t), United States (3.3 Mio t), Egypt (2.2 Mio t), Iran and Turkey (1.9 Mio t) that together represent 57% of the world total yield (source, FAOSTAT 2012). Table 3.1 and Table 3.2 summarize the total harvested hectarage, bulb yield and production realized in Europe and Italy, respectively.

Table 3.1: Total harvested area, yield and production scored by onion crop in Europe during years 2010-2012. (source: faostat.fao.org)

EUROPE

Major producers of onion	**Onions, dry**		
	Area Harvested (Ha)	Yield (Hg/Ha)	Production (tonnes)
Europe - 2010	374.927	231.297	8.671.951
Europe - 2011	400.056	260.682	10.428.740
Europe - 2012	384.653	254.200	9.777.864
Russia - 2010	88.000	174.580	1.536.300
Russia - 2011	95.500	222.276	2.122.740
Russia - 2012	92.100	225.930	2.080.814
Romania - 2010	33.766	109.324	369.142
Romania - 2011	33.108	119.097	394.305
Romania - 2012	33.038	104.528	345.340
Netherland - 2010	28.866	451.050	1.302.000
Netherland - 2011	29.842	516.353	1.540.900
Netherland - 2012	27.235	496.787	1.353.000
Poland - 2010	23.705	243.823	577.983
Poland - 2011	26.629	254.218	676.956
Poland - 2012	25.072	256.130	642.169
Spain - 2010	22.478	491.650	1.105.131
Spain - 2011	24.526	533.120	1.307.531
Spain - 2012	22.800	520.658	1.187.100
Italy - 2010	12.603	302.194	380.855
Italy - 2011	13.004	318.204	413.793
Italy - 2012	10.749	313.936	337.450

Table 3.2: Total harvested area, yield and production scored by onion crop in Italy during years 2010-2013. (source: agri.istat.it)

ITALY

Major producers of onion	Onions, dry		
	Area Harvested (Ha)	Yield (Hg/Ha)	Production (tonnes)
ITALY 2010	12.603	302,194	3.808.550
ITALY 2011	13.004	318,205	4.137.935
ITALY 2012	10.749	313,936	3.374.496
ITALY 2013	9.509	280,413	2.666.449
Emilia-Romagna 2010	3.203	383,077	1.226.995
Emilia-Romagna 2011	3.482	428,623	1.492.465
Emilia-Romagna 2012	3.104	392,445	1.218.150
Emilia-Romagna 2013	2.636	314,829	829.890
Sicilia – 2010	1.700	167,124	284.110
Sicilia – 2011	1.635	169,743	277.530
Sicilia – 2012	1.593	164,033	261.305
Sicilia – 2013	1.600	185,781	297.250
Puglia – 2010	1.145	232,808	266.565
Puglia – 2011	1.150	227,004	261.055
Puglia – 2012	1.155	216,970	250.600
Puglia – 2013	1.435	235,610	338.100
Campania – 2010	1.285	280,124	359.959
Campania – 2011	1.289	291,613	375.889
Campania – 2012	1.290	308,797	398.348
Campania – 2013	1.125	293,987	330.735
Veneto – 2010	1.399	299,412	418.878
Veneto – 2011	1.493	304,521	454.650
Veneto – 2012	1.368	288,223	394.289
Veneto – 2013	1.021	303,036	309.400
Piemonte – 2010	1.585	400,440	634.698
Piemonte – 2011	1.585	400,440	634.698
Piemonte – 2012	1.588	400,253	635.602
Piemonte – 2013	----	----	----

3.1.2 Morphology and biology of the onion plant

Onion is a monocot biennial herbaceous plant grown as an annual crop. Here follows a brief summary of the main morphology traits of the plant together with the key biological aspects of onion development.

<u>Root system</u>: bundle fibrous roots protrude from the underside of the bulb and extend for a short and superficial way into the soil to a depth of 35 cm.

Bulb: The edible part of the plant is formed by the accumulation of reserve substances in the basal portion of leaf (petiole) becoming tunics fleshy and succulent. Consequently, bulb size is directly related to the number of leaves. The thin external part appears papery, varying in color (white, yellow, coppery yellow, red, purple) and has a protective function. The final part of leaf petiole ends up in a kind of bottleneck at the top of the bulb called "neck" or "pseudostem" because it is the crossing point between the bulb and the leaf blades.

Leaves: Typically 20-to-70 cm long, leaves are fleshy, glaucous green covered with wax, emerge alternate and opposite, and are inserted concentrically (younger inside) around the central apex of the pseudo-stem. They show a tubular sheath and a basal erect lamina, cylindrical, slightly enlarged at the center, closed to the tip apex, full inside during the early stages of the development and then empty for the rest of the development. The production of new leaves is highly dependent on the temperature: growth rates are linearly increasing between 6 and 20 °C, consistently elevated between 20 and 27 °C and decreasing linearly between 27 and 30 °C.

Stem: Located below-ground the stem is strongly flattened due to shortened internodes, which form a sort of disc varying in width from 1 to 2 cm. In the central part of the disc is present the vegetative apex from which originate the leaves. Near the main apex, 2-to-4 other meristems are dormant during the first year of the plant life and can develop flowering stems in the second year of development.

Umbel: Floral structures form in the 2nd year starting from the central part of the bulb. A single bulb can produce more than one flowering scape composed by a fistular fusiform stalk, up to 1.5 m long, ending with a flower-head that takes the form of a globular umbel (5-10 cm in diameter). The inflorescence is initially enclosed by 2-4 bracts cartilaginous (spathe) that subsequently open releasing many (usually 200-600, up to 2.000) simple flowers, hermaphrodite, carried by long peduncles 15-40 mm. Each individual flower is made up of 6 tepals whitish or greenish-yellow, six stamens and three bilocular carpels. Blooming, does not occur at the same time but on different days, begins in late May-early June in the main stalk and continue for about 20-30 days on secondary scapes. The species is strongly proterandrous releasing its pollen several days before the stylus of the same plant is receptive. This means that the species is predominantly allogamous and the pollination is done by of insect.

Fruit: is a triloculare capsule with 2-3 (rarely up to 6) seeds in each lodge. The seeds are irregularly shaped, angular, flattened, black in color and weigh 2.5-5 mg.

3.1.3 Main developmental stages

Germination: The stages of germination and emergence have a duration ranging from 8 to 20 days after sowing depending on weather conditions and the time of sowing. The cotyledon after germination appears as a "loop or hook" above the ground surface. Given the sensitivity of the cotyledon and its poor penetration ability, it is evident how the emergency phase is very critical, therefore, the soil must not have absolutely the surface crust. Even if the minimum temperature for germination is slightly above zero, at low temperatures this phase is very slow (at 5 ° C takes about a month), the temperature optimum is found in a wide range with the average time of germination that, in any way, are rarely less than a week. It is during the emergency phase that the onion is particularly sensitive to frost although you can find a high variability among cultivars.

Vegetative stage: seedling, growing out of the ground, can bring outside the black seed tegument. The cotyledon assumes a typical horizontal position to the ground and it is called "flag stage". After the appearance of the second and third true leaf, cotyledon progressively loses the reserve substances and finally dries. Meanwhile, the seminal root elongates rapidly but with the formation of the first leaves degenerates and is replaced by a system composed of sorted adventitious roots, unbranched, short enough but numerous.

After the appearance of the fourth leaf, the plant neck begins to increase its thickness while the first leaf begins to wilt. During the formation of the fifth, sixth and seventh leaf, the first leaf dried completely, while the second leaf begin to show an early senescence.

The onion has different requirements towards the light, so that each variety need of different environmental conditions (light and temperature) to begin the bulbification process:

- Short day: they require a period of 10-12 hours of light per day (early varieties);
- Intermediate day: they require a period of 12-14 hours of light per day (mid-early variety);
- Long day: they require a period of 14-16 hours of light per day (late varieties or very late).

When, the daylength is appropriate to plant, the bulbification begin all reserve substances are translocated in the basal part of the leaves to form the bulb.

During this phase, the second and third leaf dry up while the leaves are formed from the eighth to the thirteenth and the plant reaches its maximum height (usually the longest leaf is the seventh or eighth and the subsequent ones gradually shorter) .

When bulb is already formed, the internal youngest leaves are unable to emergence outside and stay inside the bulb as fleshy scales, increasing bulb size. The beginning of bulbification can be identified when the ratio between the maximum diameter of the bulb and the minimum diameter of the neck is ≥ 2. The growing phase of the bulb last about 6-8 weeks while, at the same time, the gradual desiccation of the 4th-6th leaf edges and tips of the youngest leaves proceed. The leaves begin to fold under its own weight. During this phase can appear 1-2 new short leaf blades and protective outer bulb tunics begin to form.

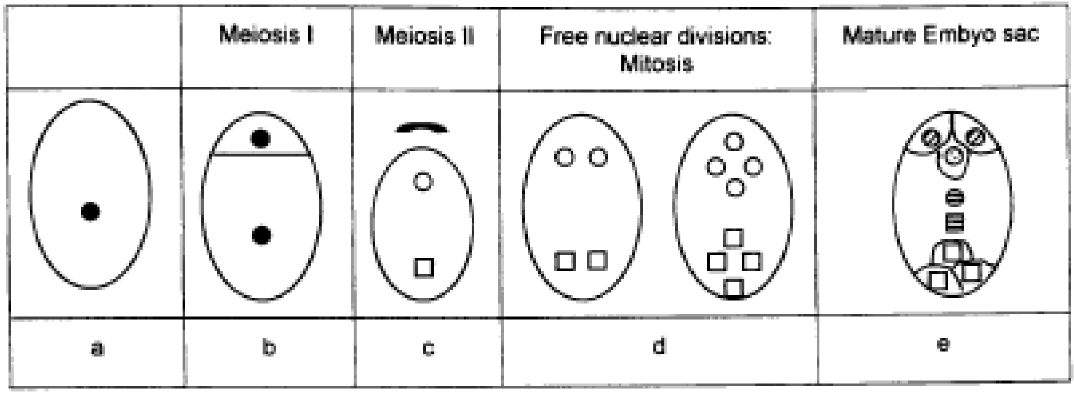
The neck begins to empty as the new leaf blades break off their growing, besides that leaf tissues lose their turgidity causing the collapse of the vegetative apparatus. In the final phase of the plant cycle the outer protective tunics of bulb begin to dry together with a complete leaf senescence. It was observed that the bulb during this stage, despite not having more photosynthetic activity, can further increase its weight and size due to translocation of the substances accumulated in the leaves.

Finally, the onion prefers medium-textured soils (sandy soils) but it also able to adapts to clay provided that fields are cool, deep, rich in organic matter with good water availability. It 'recommended a change long (every 4-5 years). It prefers soils with pH values between 6 and 7.

Reproductive stage: Onion flower induction is caused by low temperatures after a juvenile phase of development from seed. In the first year from seed, onion plants will produce a bulb and enter a dormancy period. Once dormancy period is completed, the apical vegetative meristem will differentiate into a reproductive meristem and an axillary vegetative meristem. Both meristems will begin to develop and elongate. The reproductive meristem will form the flowering inflorescence in the second year after seeding. Onion plants will form a seedstalk in the first year if they reach a critical size and then receive thermo induction. A reduction in plant size lowers the bolting percentage of a bolting-susceptible cultivar. The sensitivity to low temperatures increases with plant size (Cramer 2003).

Megasporogenesys: the genus *Allium* differs from other important horticultural crops, for the gametophyte development of the ovules. Normally, in the Angiosperm over 70% of the megasporogenesis occurs with a monosporic pattern (also knew as Polygonum type), where after two meiosis (1 and 2), the megasporocyte produces four megaspores (haploid cells). Three megaspores will degenerate, while the fourth will produce all set of nucleus present inside the ovule. Polygonum pattern is the typical morphogenesis pathway for many plant families like Brassicaceae (e.g., Arabidopsis, Capsella, Brassica), Gramineae (e.g., maize, rice, wheat), Malvaceae (e.g., cotton), Leguminoseae (e.g., beans, soybean), and Solanaceae (e.g., pepper, tobacco, tomato, potato, petunia) (Yadergari and Drews, 2004). In the Alliaceae, and therefore in onion, the molecular and tissue morphogenesis pathway in the megasporogenesis follows a bisporic pattern (also knew as Alisma type). In this process, after the first meiosis, one nucleus degenerates while the second will produce two nuclei which in turn will produce all set of nucleus present inside the ovule after two mitosis (Fig 3.1).

Fig 3.1. Female gametophyte (Bisporic megagametogenesis) development in *Allium*



●	Somatic megaspore nucleus or derivative
○ □	Germinal megaspore nucleus
⌒	Degenerating nucleus
⊘	Synergid nucleus
⊖ ▤	Polar nucleus
⊙	Egg nucleus

This difference in the tissue morphogenesis process is very important, because one of the two polar nuclei is derived from the four micropylar nuclei, while the second polar nucleus comes from chalazal region. Moreover, the two polar nulei are genetically different and consequently the endopserm and the embryo will differentiate for genetic inheritance by mother (Haig, 1990; Mangum, PhD thesis 2001).

3.1.4Genomics in onion

Despite its importance, onion can be considered an “orphan crop”, as it has received little attention from researchers compared to other monocot species, such as rice, maize and wheat. Due to the complexity of its genome, slow progress has been made to characterize and acquire sequencing data, in order to improve onion breeding programs. At approximately 16.4 giga (billion) bases per 1C, the onion nuclear genome is one of the largest nuclear genomes among all diploid and is over six times more than maize or humans (Arumuganathan and Earle 1991). Onion is diploid with 16 chromosomes and there is no evidence of a recent polyploidization event.

Biochemical analyses have provided insights about the structure of the nuclear genome of onion. The GC content of onion DNA is 32%, the lowest known among angiosperms (Kirk et al. 1970). Although gene-rich islands exist in some grass species with larger genome sizes (Keller and Feuillet 2000), Cot reassociation kinetics revealed that the onion genome consists of middle-repetitive sequences occurring in short-period interspersions among single-copy regions (Stack and Comings 1979). Fluorescent in-situ hybridizations (FISH) of random genomic fragments cloned into bacterial artificial chromosomes (BACs) supported significant amounts of repetitive DNAs in the onion genome; Suzuki et al. (2001) reported that 80% of random BACs carried common repetitive DNAs and hybridized to entire chromosomes, 15% hybridized to centromeric or telomeric regions, and only 5% of BACs hybridized to specific regions on chromosomes. These results suggest that much of the onion genome is composed of repetitive elements; however unique sequences exist at specific regions of the genome.

As large-scale DNA sequencing technologies become more efficient and cheaper, the genomic DNAs of more and more plants are being sequenced, assembled, and annotated. Using next-generation sequencing technologies and large computational abilities, sequencing, assembly, and annotation of large genomes has become almost routine. Paralleling these newer technologies, the declining cost of computational power and growing availability of on-demand cloud-based computing is making assembly and annotation more efficient. Standardized web-based resources allow sharing and browsing of integrated data from annotated genomes. As a result, large-scale and cost-effective sequencing, assembly, and annotation of onion DNA are becoming feasible. Recently, a "white paper" stating an international plan for sequencing and annotation of the whole onion genome has been presented and summarizing preliminary results of 1) sequencing of random genomic fragments, 2) transcriptome sequencing, and 3) reduced-representation sequencing of genomic regions (http://alliumgenetics.org/cmsmadesimple/).

3.1.5 Genetic resources

The availability of genetic resource is of pivotal importance for any commercial crop. Regarding this aspect Italy is one the most important Countries with the highest wealth of germplasm of onion because there is a widespread tradition among farmers to maintain and reproduce the many cultivars and local populations. These local varieties are extremely important for the genetic improvement and onion breeding activities. Ancient genetic resources allow preserving many useful traits that may be lost over time in consequence of the increasingly massive use of a few commercial hybrids with high performance. The characteristics of the product required by modern markets, such as improved yield, high storage ability, improved skin retention and a reduced number of growing centers have favored the spread of a few commercial hybrids that have allowed a standardization of many aspects along the entire production chain. A direct effect of the drastic reduction of the marketing of local open pollinated population has a high risk of erosion of old genetic resources. This situation has prompted the urgent need for conservation and enhancement of local ecotypes in recent years, through characterization, collection, preservation and regeneration of seed and vegetable clones (Astley, 1991).

The magnitude of genetic variability present in the genotypes provides the possibility of improving proportionally a crop. In a recent publication, Kik (2008) reported that a total of 9000 accessions of *Allium* are present worldwide, in particular the number of onion accessions are 7000. Moreover, the genebanks present in the world hold 12,740 onion accessions (Kik, 2008). Below is reported a short list of the major seed banks:

Country	Seed bank	web site
USA	**USDA - United States Department of Agriculture**	http://www.ars.usda.gov/Aboutus/docs.htm?docid=6248
Europe	**eurisco**	http://eurisco.ecpgr.org/home_page.html
Netherland	**CGN – Center for Genetic Netherland**	http://www.wageningenur.nl/en/Expertise-Services/Statutory-research-tasks/Centre-for-Genetic-Resources-the-Netherlands-1/Centre-for-Genetic-Resources-the-Netherlands-1/Expertise-areas/Plant-Genetic-Resources/CGN-crop-collections/Onion-and-leek.htm
United Kindom	**Warwick Crop Centre » Genetic Resources Unit**	http://www2.warwick.ac.uk/fac/sci/lifesci/wcc/gru/

3.1.6 *In silico* resources

There are not many useful resources for onion research on internet, but recently has created two new interesting website:

- “AlliumGenetics” coordinated by J. McCallum of the Institute of Plant and Food Research located in New Zealand. This website groups all molecular information about onion, as well as providing many useful links for this crop.
- RoBuST: This database has been developed to initiate a platform for collecting and organizing genomic information useful for Root and bulb vegetables (RBV) researchers. At present the RoBuST contains genomics data for 294 Alliaceae and 816 Apiaceae plant species (Bashi et al., 2010)

Data Base	web site
AlliumGenetics.org	http://alliumgenetics.org/cmsmadesimple/
RoBuST	http://robust.genome.com/
ncbi	http://www.ncbi.nlm.nih.gov/genome?term=txid4679[orgn]
Onion gene index	http://compbio.dfci.harvard.edu/tgi/plant.html
GarlicESTdb	http://garlicdb.kribb.re.kr/loginout.jsp

Nonetheless, a limited number of ESTs (about 20.000 EST) are available in public database.

3.1.7 Laboratory resources

Onion tissue culture: The breeding activities for genetic improvement of the onion are a very slow process because this species is a biannual plant and this feature results in a selective process very slowly. In fact, during the first year, parental lines are selected for desired characteristics (in the field, during storage and in processing), then bulbs are harvested and transported in a warehouse for storage. In the second year, during the generative growth, there is seed production. The final consequence is that it takes 17-20 years of hard work to get a new commercial variety: 8-12 years to develop parental lines, least 3 others years in the field to test the performance of the hybrids obtained, in these 15 they follow at least 2 years for validation in the main growing areas in the world, plus the same for their commercial introduction.

In order to accelerate the onion breeding program to obtain isogenic lines it has been successfully developed the technique of production of double haploids through in vitro culture of flowers collected before anthesis (gynogenesis – Fig 3.2 e 3.3) (Campion et al., 1992). Production of homozygous lines by gynogenesis is more advantageous than the traditional method of inbreeding because it allows:

- Time saving: gaining 2 years compared to compared to the time required to obtain an inbred line S3
- Maximum homozygosity: Double Haploids plants are 100% homozygotes compared to plants S3, which are 87,5% (Campion et al., 1995, Bohanec et al., 1995)
- opportunity to quickly obtain homozygous mantainer lines to be used for the production of male sterile lines

Finally, even if not all plant material responds in the same way to hormone stimulation during gynogenesis process, the production of haploid embryos is an average of 3-4%, making this technique very interesting for its use in a breeding program (Geoffriau et al., 1997).

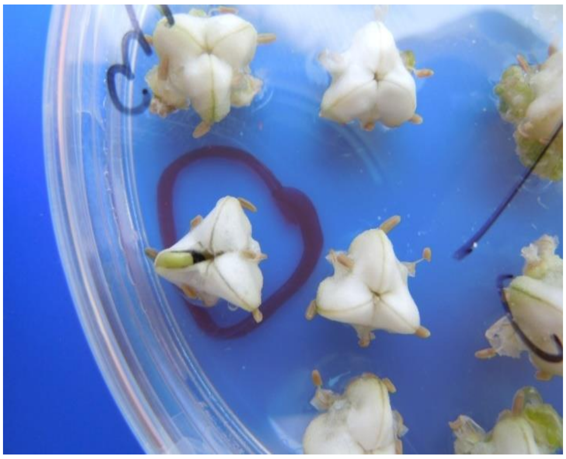


Fig 3.2.
Haploid embryo sprouting out from a carpel of *Allium cepae*

Fig 3.3.
Double Haploid (DH) plant of the *Allium cepae* in final stage of in vitro culture

3.1.8 Goals

Despite a strong maternal influence has been observed in onion, no studies are currently available to clarify the molecular mechanisms in the species, even though the importance of this aspect in a breeding program. In fact, the evident phenotypic differences between F1 and RF1 (reciprocal cross) in onion, allow understanding how this parental imprinting doesn't segregate in accordance with Mendel's Laws and how this inheritance is influenced by alternative molecular mechanisms. It is of crucial importance for a breeder because to predict the final phenotype of following generation during the selection activities is important to know which direction has to choose for an effective breeding program. The main goal of this chapter is to study changing in the methylation profile of the genome and some candidate genes in onion when environmental conditions change and deduce its implication on global physiology and performance of a plant.

3.2 Strategy

The research strategy followed during the PhD has been to produce and use Double Haploid (DH) plants in order to create, per each couple of parental lines, identical hybrids which differ only for their maternal origin. The final result of this mating strategy was to obtain plants with identical genome but differ in cytoplasm (Fig. 3.4). This plant material allow us studying the possible influence on the final phenotype by each parental, and the maternal effect in particular. Moreover, this approach eliminate the numerous allelic variations certainly present in an allogamous specie, like onion, to avoid errors in the interpretation of data

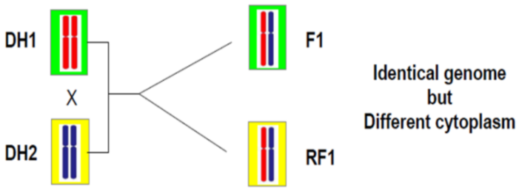


Fig 3.4.
Double Haploid (DH) parental lines crossed to obtain special hybrid and its reciprocal to use for analysis.

To be sure to study a true F1/RF1, and avoid choosing plant self-pollinated, it were used onion cultivars with different dry skin color as parental lines in order to analyse DNA samples coming from plant with a different aspect when compared to original phenotype

Moreover, it was used laboratory techniques and sequence of candidate genes (hortologues) to identify molecular mechanisms that are thought to be involved in the epigenetic regulation of relevant onion traits like phenology, development, and resistance to important diseases. This could in fact explain the deep phenotypic difference observed between plants belonging to reciprocal hybrids.

Finally, it was plan to analyze the general methylation status of DNA of onion plants (parent lines, F1 and F2) by MSAP in order to obtain a methylation profile of each lines and generation.

3.2.1 Plant material

The origin of Double Haploid (DH) plant material used for this project were several local Open Pollinated (OP) populations and 1 commercial hybrid. In the table below is reported several data about plant material:

Breeding code	GS	DSCol	Typology	MT	Storage
2P05	**OP**	YO	LDO	late	medium-long
SpR	**OP**	YO	LDO	late	long
BRal	**F1**	RO	LDO	early-medium	medium-long
BP	**OP**	WO	SDO	extra early	poor
Tr	**OP**	RO	SDO	medium	poor

Note: GS = genetic structure, DSCol = Dry Skin Color, MT = Maturity Time, DH lines = Double Haploid Lines

Table 3.3.
Description of some agronomical traits of the material used for molecular analysis

3.2.2 Tissue culture

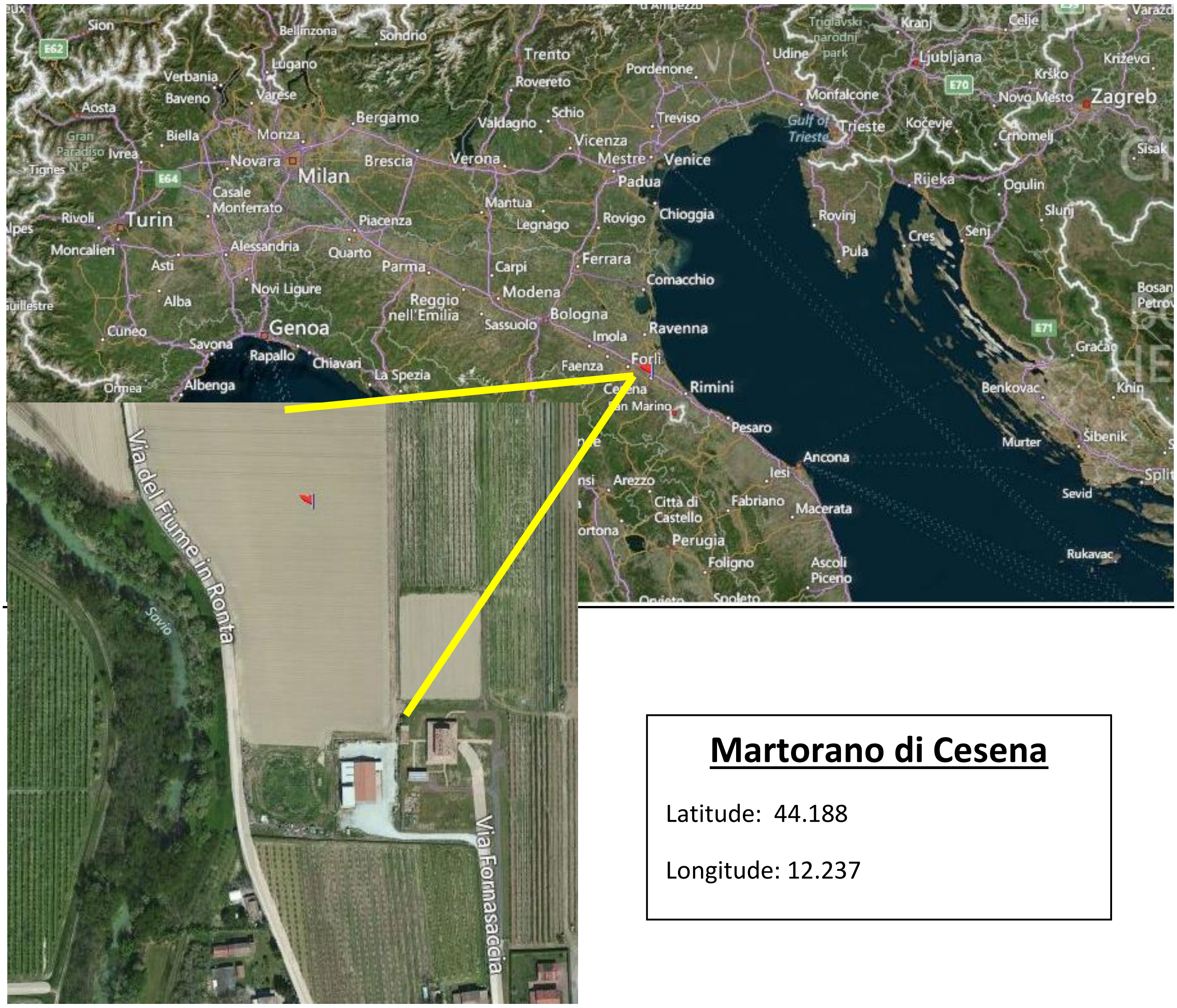
The laboratory activities aimed at obtaining haploid embryos with the technique of the gynogenesis, were conducted by placing in culture onion flowers before anthesis on specific substrates. The collecting of the flowers from the onion umbel occurred during the first days of June. after sterilized externally flowers with a solution of 50% sodium hypochlorite, has been cut the peduncle at the base of the calyx working in a sterile environment under a laminar flow hood. Flowers were placed on the first substrate and after 1 month these have been transferred on second substrate to induce the embryo formation (Jakse et al., 1996). The embryos, after about a month, began to escape from the ovary were taken and placed on a substrate and agar supplemented with colchicine. As this alkaloid is photolabile, embryos partially immersed in the substrate containing this molecule, were placed in the dark for 2 days embryos, after about a month, began to escape from the ovary were taken and placed on a substrate and agar supplemented with colchicine. As this alkaloid is photolabile, embryos partially immersed in the substrate containing this molecule, were placed in the dark for 2 days to induce the duplication of chromosomes through the inhibition of the formation of the mitotic spindle. The embryos, survived to treatment with colchicine, were placed on a growing substrate and have begun to develop producing real plants. Finally, the small Double Haploid plants have been, after having reduced in size roots and leaves, cut lengthwise in order to multiply on a monthly basis. All the steps were carried out in a controlled environment (growth chamber) where light and temperature have had a constant rate throughout the period of in vitro culture. The average rate of production of embryos was around 3%, as reported in the literature, although it has been observed a considerable variability in the response to the growing techniques among the different materials used

3.3 Field activities

3.3.1 Trial sites

All field activities during the period of the doctorate were carried out in the municipality of Cesena, near the village of Martorano di Cesena (FC). Soils of the fields where there were made the experimental activities were of clay typology.

Fig 3.5. Map and field position of the place where field trials have been made



Martorano di Cesena

Latitude: 44.188

Longitude: 12.237

2011

The first step consisted in transferring the DH plants obtained with gynogenesis from vitro conditions to small pot with peat and placed in environment at stable condition inside a growing room. After a relative short period of acclimatation the DH plants were transfered in greenhouse for a further acclimatization before being transplanted in the open field in order to produce seed of each single line. Meanwhile, DH bulbs coming from plants obtained with gynogenesis in 2010 were covered in April by metal structure and net to assure an environmental isolation in order to avoid any contamination by insect. Subsequently, several launches of flies were done in May-June in each cage in order to assure pollination for the desired crosses (see table below) and or for the simple reproduction of seed of each line.

At the end of July was the seed crop produced inside the isolators, clean, cataloged and stored in a refrigerated chamber. Finally, in mid-August, were collected bulbs of plants and stored in boxes and placed in a shady place at room temperature. Healthy DH bulbs were transplanted again in the field in mid October 2011 for the future seed production.

2012

During last winter (Oct 2011 - Feb 2012), bulbs of 34 DH lines were transplanted in open field in order to set up 59 different combinations. We preferred to grow a large number of plants and combinations because it is quite complicate the synchronization of flowering time among different onion typology, in addition to the fact that DH plants are notoriously less vigorous and consequently are less the plants that will survive during the winter.

Fig 3.6: Panoramic picture of a field during the placement of cages before anthesis

Fig 3.7 Detail of a cage with flies during anthesis period

Crosses made in 2012									
Onion Typology		Crosses	RO				YO		WO
			WO	YO	RO	PO	YO	WO	WO
SDO x SDO	cages	12	11	1					
SDO x LDO	cages	27	4	11	6	5		1	
LDO x LDO	cages	18	3	5	1		8		1
Total	cages	57	17	13	7	5	8	1	1

Tab 3.4: Summary of total number and different types of crosses set up in 2011-2012. SDO: Short Day Onion; LDO: Long Day Onion; RO: Red Onion; WO: White Onion, YO: Yellow Onion; PO: Pink Onion

2013

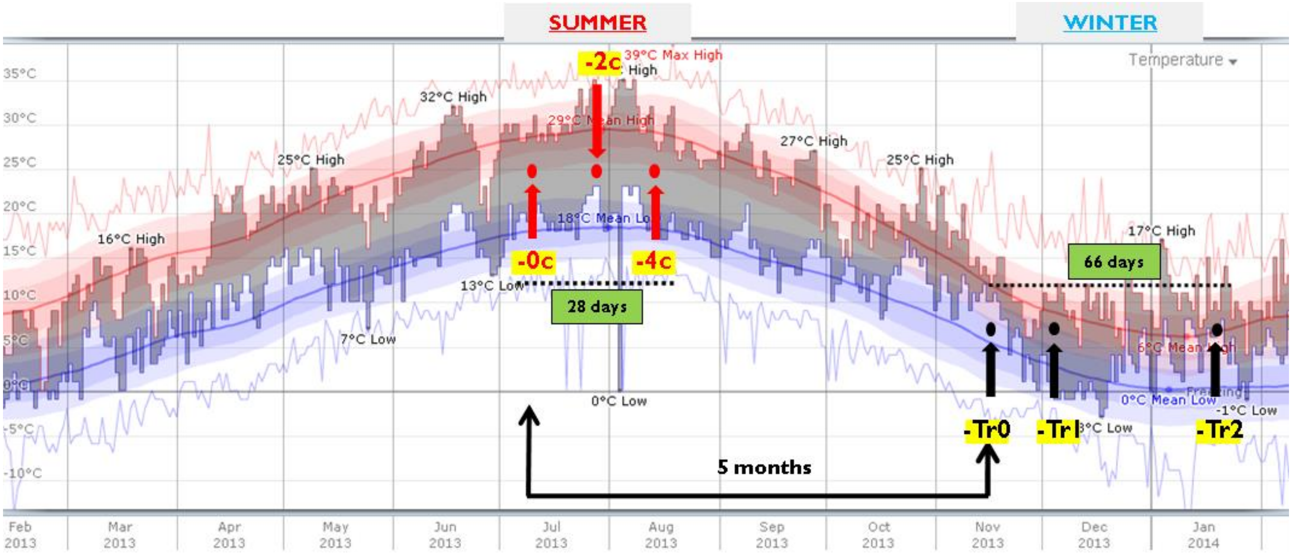
We chose two prospective crosses among four Haploid Double lines, and we set up appropriate field trials to study the phenotype of all F1 plants and their reciprocal (RF1) for several important agronomic traits of epigeal part, such growth rate, leaf erectness, leaf waxiness, leaf cruncking, blister, beginning of bulbification and day length sensitivity. Same type of phenotypization has been conducted for the bulb, such number of dry skin, dry skin retention, dry skin color, bulb firmness, ring thickness and number of centers. At the beginning the plants were seeded 0n 15 march 2013 in a seed tray and placed in a green house, then transplanted in open field at the end of April (25 April 2013).

Fig 3.8. Plots of onion in field trial

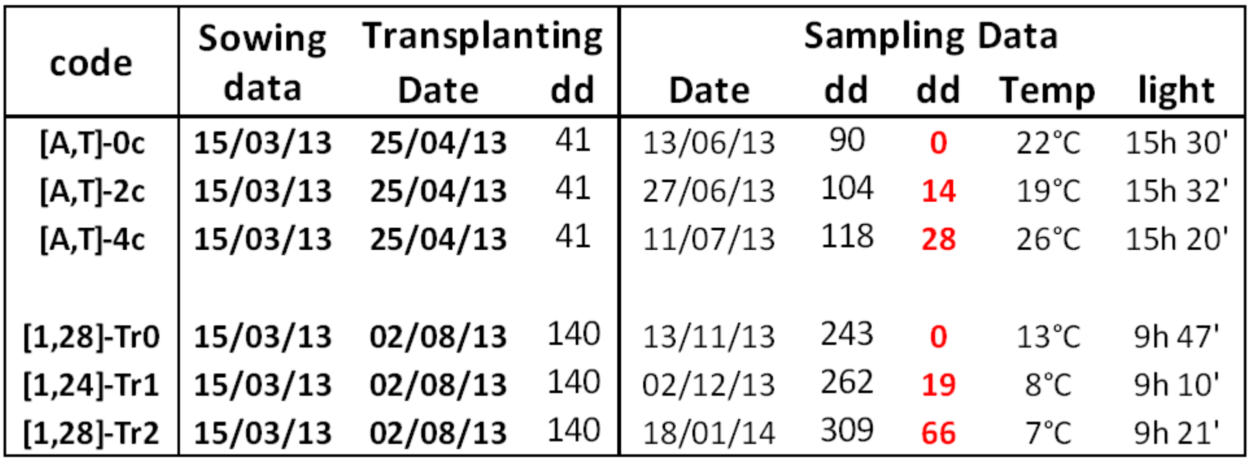
3.3.2 Growth conditions

Growing conditions which have been subjected the plants used for the analysis of methylation were seasonal ones, that is the samples of the leaves were carried out in summer and in winter. In the picture and table below it is possible to see data and seasonal temperatures in the area where the plants are grown studied.

Fig.3.9 Annual trend of temperatures in Cesena area which have been set up field trial



Tab 3.5 .Some data about field trial 2013



code	Sowing data	Transplanting		Sampling Data				
		Date	dd	Date	dd	dd	Temp	light
[A,T]-0c	15/03/13	25/04/13	41	13/06/13	90	0	22°C	15h 30'
[A,T]-2c	15/03/13	25/04/13	41	27/06/13	104	14	19°C	15h 32'
[A,T]-4c	15/03/13	25/04/13	41	11/07/13	118	28	26°C	15h 20'
[1,28]-Tr0	15/03/13	02/08/13	140	13/11/13	243	0	13°C	9h 47'
[1,24]-Tr1	15/03/13	02/08/13	140	02/12/13	262	19	8°C	9h 10'
[1,28]-Tr2	15/03/13	02/08/13	140	18/01/14	309	66	7°C	9h 21'

3.4 Laboratory activities

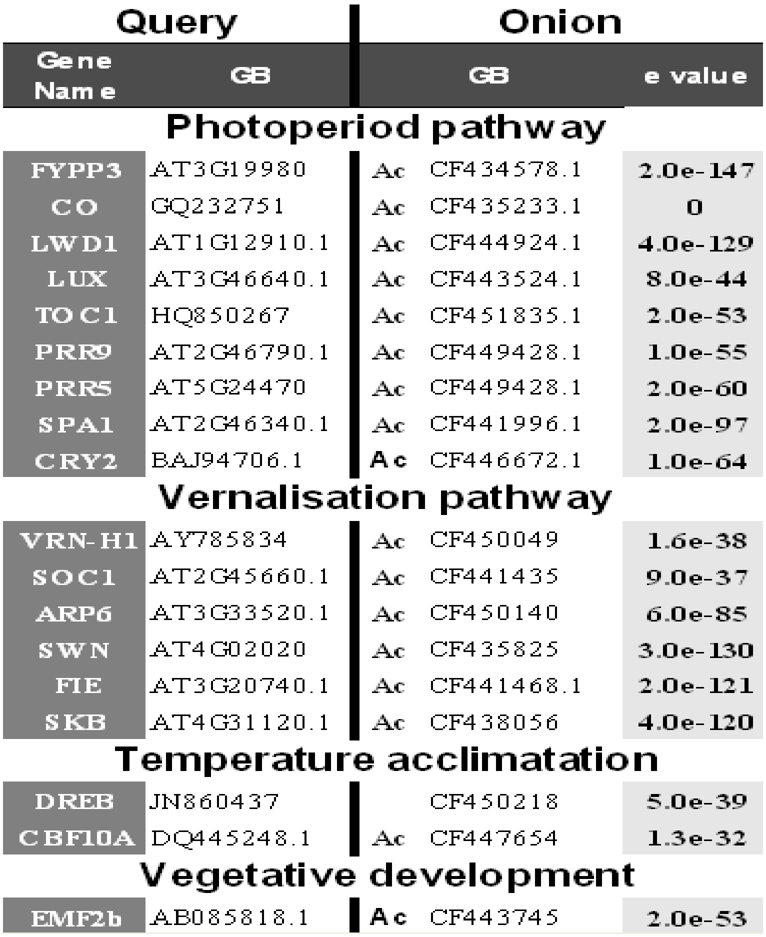
3.4.1 DNA extraction and purification

The DNA extraction from 500 mg of leaf tissue was performed following the CTAB protocol reported on the Maniatis manual, while DNA purification was done following the instructions provided in the commercial kit "NucleoSpin Extract II”, marketed by Macherey-Nagel, after to have excised with a scalpel a gel slice containing the DNA fragment amplified by PCR.

3.4.2 Re-sequencing of candidate genes

To identify EST of putative ortholog in *Allium cepa* (Ac) involved in photoperiod and vernalization pathways, we used 63 sequences (30 genes involved in photoperiod and 33 genes belonging to vernalization pathways) of Arabidopsis thailiana (At) and Hordeum vulgare (Hv) as a query sequence to perform BLAST and BlastX analysis.

The EST sequences were amplified by PCR with specific primers for each target sequence using primers designed with software Primer3 (http://bioinfo.ut.ee/primer3/)



Query		Onion		
Gene Name	GB		GB	e value
Photoperiod pathway				
FYPP3	AT3G19980	Ac	CF434578.1	2.0e-147
CO	GQ232751	Ac	CF435233.1	0
LWD1	AT1G12910.1	Ac	CF444924.1	4.0e-129
LUX	AT3G46640.1	Ac	CF443524.1	8.0e-44
TOC1	HQ850267	Ac	CF451835.1	2.0e-53
PRR9	AT2G46790.1	Ac	CF449428.1	1.0e-55
PRR5	AT5G24470	Ac	CF449428.1	2.0e-60
SPA1	AT2G46340.1	Ac	CF441996.1	2.0e-97
CRY2	BAJ94706.1	Ac	CF446672.1	1.0e-64
Vernalisation pathway				
VRN-H1	AY785834	Ac	CF450049	1.6e-38
SOC1	AT2G45660.1	Ac	CF441435	9.0e-37
ARP6	AT3G33520.1	Ac	CF450140	6.0e-85
SWN	AT4G02020	Ac	CF435825	3.0e-130
FIE	AT3G20740.1	Ac	CF441468.1	2.0e-121
SKB	AT4G31120.1	Ac	CF438056	4.0e-120
Temperature acclimatation				
DREB	JN860437		CF450218	5.0e-39
CBF10A	DQ445248.1	Ac	CF447654	1.3e-32
Vegetative development				
EMF2b	AB085818.1	Ac	CF443745	2.0e-53

Tab 3.6 .
List of EST identified in onion with BLAST analysis

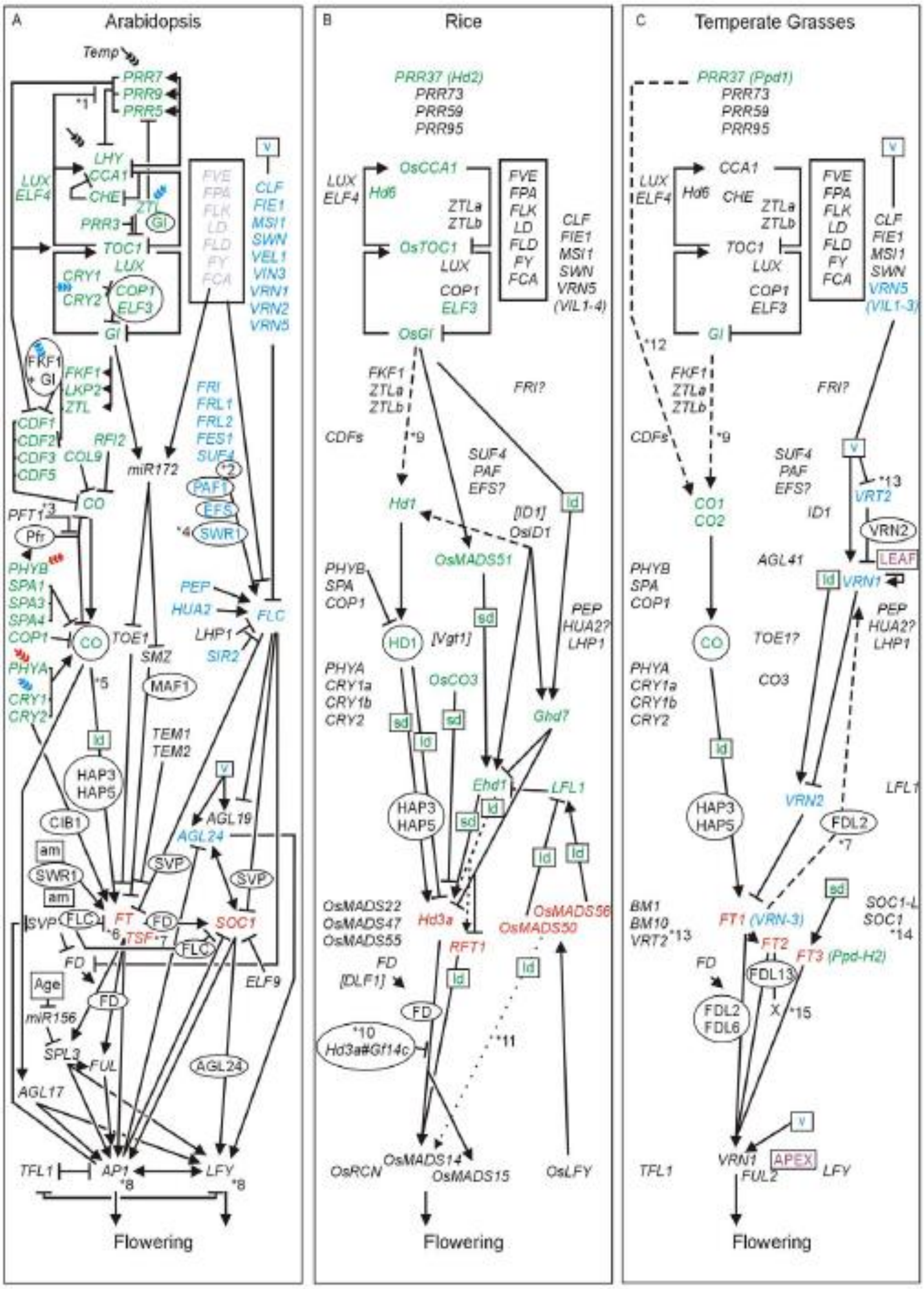


Fig 3.10.

Genetic pathways controlling flowering in Arabidopsis, rice and temperate grasses. Arrows show promoting effects, T-bars show repressing effects. Environmental cues are shown as lower case letters in square boxes; v is extended cold (vernalization); ld is long days; sd is short days; am is ambient (non-vernalizing) temperature. Genes are shown in italics and proteins in non-italics in ovals. Proteins are clearly involved at all stages but we make this distinction only in cases where separate controls are known to exist for transcription and protein function or stability. indicates inhibition of protein function.

Box A. In Arabidopsis genes assigned to pathways are shown in colour (photoperiod pathway in green, vernalization pathway in blue, autonomous pathway in light blue). Flowering pathway integrators are shown in red. Triple headed arrows indicate activation by red or blue light. **Box B**. Rice genes identified as homologues of those in Arabidopsis are shown in black. Ambiguous homology is indicated with a question mark. Genes with published roles in photoperiod regulated flowering are shown in green and orthologues of pathway integrators in red. **Box C**. In the temperate grasses box Brachypodium genes identified as homologues of those of Arabidopsis or rice are shown in black. The VRN2 and CO3 genes from barley and wheat are also included. Genes with published roles in photoperiod or vernalization regulated flowering in barley or wheat are shown in green or blue, respectively, and orthologues of pathway integrators in red. (Higgins et al., 2010)

3.4.3 Methylation-sensitive amplification polymorphism (MSAP)

The genomic DNA samples collected in the different periods of the year (see tab 6) were subjected to methylation analysis by the technique Methylation-sensitive amplification polymorphism (MSAP) a method is based on the principle of the amplified length fragment polymorphism (AFLP) technique. It was followed the protocol reported by Reyna-Lopez and co-workers (1997).

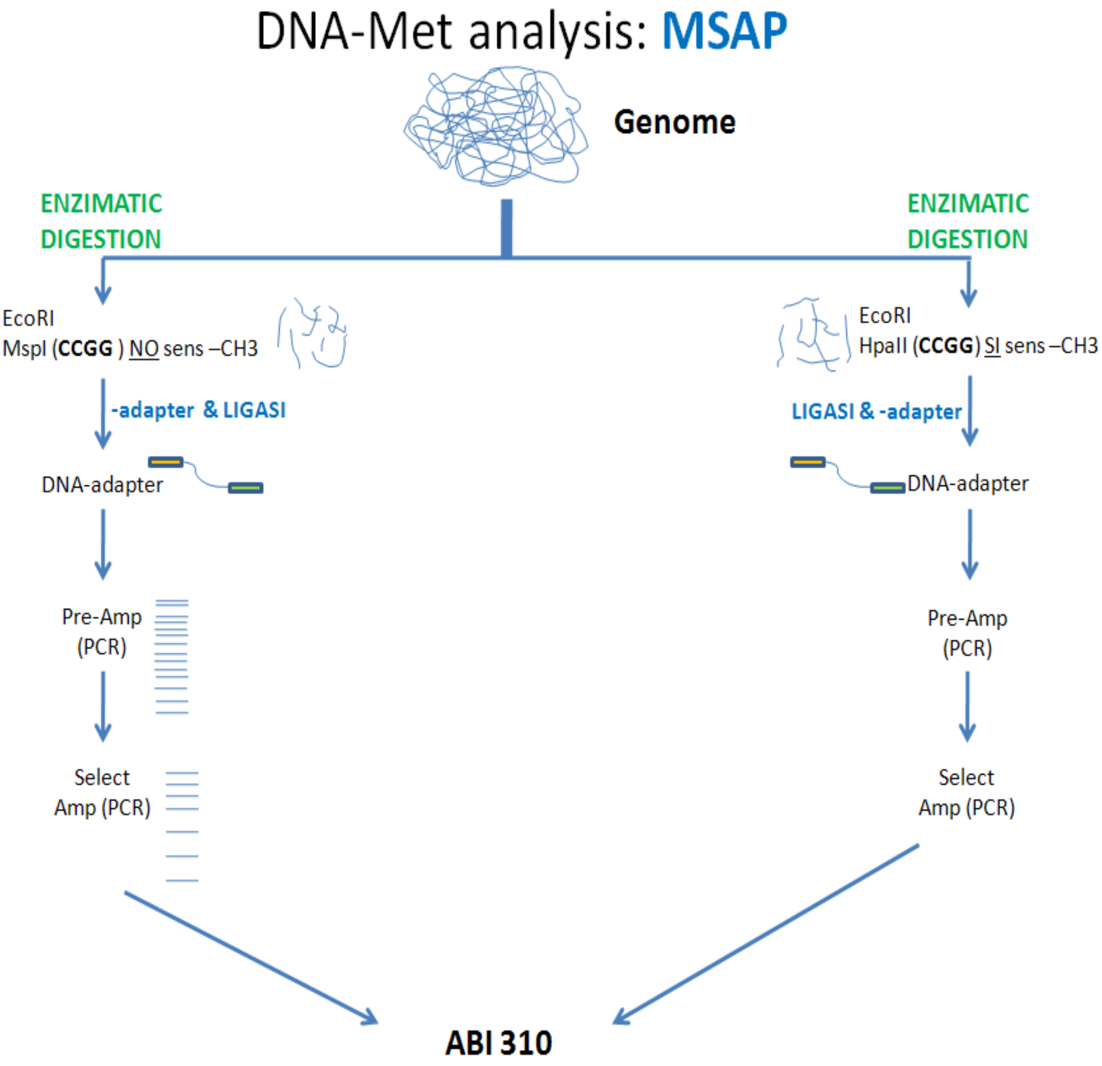


Fig.3.11 Flowchart showing the steps of the laboratory technique MSAP

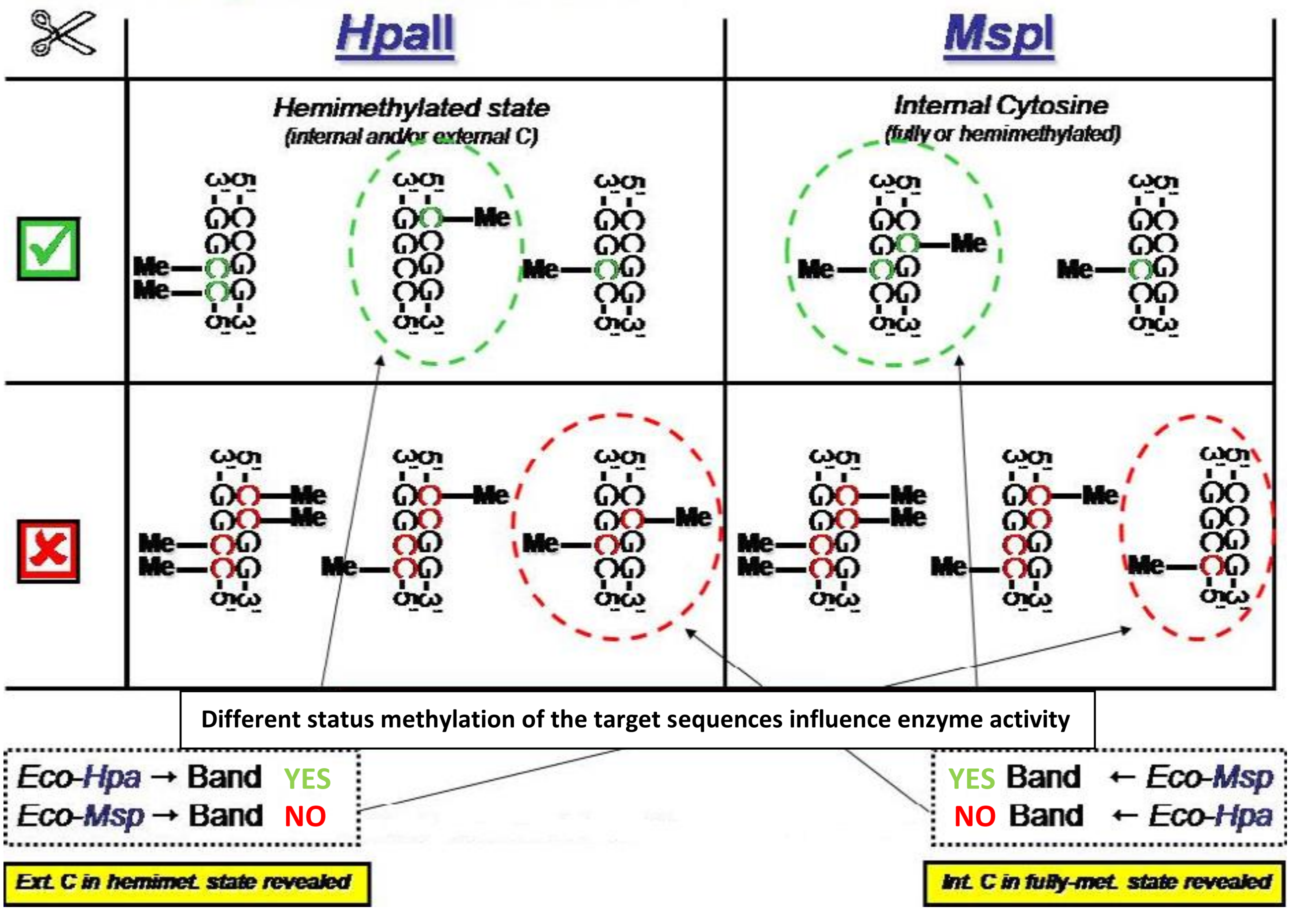
The pattern of DNA methylation typically occurs in regions rich in GC bases (the called "CpG island") are heritable, and correlate with tissue-specific gene expression. It was chosen to evaluate the methylation status of the sites 'GGCC' of total genomic DNA extracted from young leaves following the CTAB protocol of Stein et al. (2001).

The phenotypes in the study were analyzed with the technique Methylation-sensitive amplification polymorphism assay (MSAP), by modifying the protocol used by Portis et al. (2004).

MSAP is a technique based on the use of AFLP isoschizomers HpaII and MspI as "frequent cutter" (which differ in sensitivity to methylation in their recognition sequence on the genomic DNA), while the enzyme "rare-cutter" is the same EcoRI compared to other standard AFLP protocols.

The two combinations of restriction enzymes used were: EcoRI / HpaII, EcoRI / MspI. The isoschizomers HpaII and MspI both recognize the tetranucleotide sequence 5'-CCGG-3 ', but their activity is influenced by the state of methylation of cytosine residues external or internal: HpaII is active only on the sequences 5'-CCGG-3' hemymethylated , while MspI cuts the sequences 5'-CmCGG-3 'which is completely hemimethylated, but not the sequences 5'-mCCGG-3'.

Fig.3.12 schematic representation of all possible methylation in the target sequences recognized by restriction enzymes MspI/HpaII and the effect on their action



The samples were digested with the two enzymes using different combinations of: 125 ng µl-1 of genomic DNA, buffer (10x) "Tango ™" (Fermentas), EcoRI (10U µl-1), MspI or HpaII (10U-µl 1) in a final reaction volume of 25 l. The incubation took place at 37 ° C for 4 h, and subsequently at 70 ° C for 15 min. For the digested fragments from both combinations were ligated adapters for EcoRI and MspI or HpaII (Table …), incubating at 20 ° C for 2 h a reaction mixture consisting of: 24 µl of digested genomic DNA previously, 5 l buffer T4 ligase, 1 ĩl EcoRI adapter (2 mM), 1 ĩl adapter MspI or HpaII (20 mM), 1 ĩl T4 DNA ligase (5 U µl-1), leading to a final volume of 50 l with water. The ligation product was diluted 1:10 v / v in 0.1x TE (TrisHCl-EDTA, pH 8.0).

The pre-amplification was performed using a reaction mixture consisting of: 5 l of ligated DNA and diluted, 5 l of buffer (5x), 1.5 µl MgCl2 (25 mM), 0.6 µl dNTPs (10 mM), 1 ĩl primer "E0" (10 mM), 1 ĩl primer "HM0" (10 mM), 0.2 µl Taq DNA polymerase (5 U µl-1; Fermentas), leading to a final volume of 25 l with water. The PCR reaction used is composed of 35 cycles at 94 ° C for 30 sec, 56 ° C for 1 min and 72 ° C for 1 min, followed by 72 ° C for 10 min. The final product was then diluted 1:20 v / v in 0.1x TE. The selective PCR was performed by carrying out 10 cycles of 94 ° C for 1 min, 1 min of "annealing", and 72 ° C for 1 min and 30 sec. The phase of "annealing" is started at 65 ° C and was reduced by 1 ° C for each cycle up to 56 ° C, and maintained for the next 23 cycles at this temperature.

At the end of the cycle 33, the temperature was maintained at 72 ° C for 10 min. The reaction mixture was composed of: 5 l of the pre-amplified DNA, 4 µl of buffer (5x), 1.2 µl MgCl2 (25 mM), 0.4 µl dNTPs (10 mM), 0.2 µl DNA taq polymerase (5 U µl-1; Fermentas). For staining technique with "Silver staining" were added 0.6 µl of selective primers (10 mM), while for the fluorescent labeling was added 1 ml of selective primers "E" (5 mM) and 1 ml of selective primers fluorescence labeled "HM" (1 mM). The final volume of both reactions was 25 l, with addition of water. The amplification products obtained with primers marked in fluorescence, mixed at the molecular weight standard GS-500 Rox, were separated with the instrument AB 3130 Genetic Analyzer XL, and analyzed with the GeneMapper software (AB). All profiles obtained are running anyway were subjected to visual inspection.

Finally, some of the amplicons obtained by selective PCR were controlled by agar gel before to analyze their profile with analyzer.

Adapters/Primers	Sequences
EcoRI adattatore 1	5’-CTCGTAGACTGCGTACC-3’
EcoRI adattatore 2	5’-AATTGGTACGCAGTCTAC-3’
E0*	5’-GACTGCGTACCAATTCA-3’
E1	5’-GACTGCGTACCAATTCAAC-3’
E2	5’-GACTGCGTACCAATTCACG-3’
E3	5’-GACTGCGTACCAATTCACT-3’
E4	5’-GACTGCGTACCAATTCAGT-3’
*HpaII/Msp*I adattatore 1	5’-GATCATGAGTCCTGCT-3’
*HpaII/Msp*I adattatore 2	5’-CGAGCAGGACTCATGA-3’
HM0*	5’-ATCATGAGTCCTGCTCGGT-3’
HM1	5’-ATCATGAGTCCTGCTCGGTAA-3’
HM2	5’-ATCATGAGTCCTGCTCGGTCC-3’
HM3	5’-ATCATGAGTCCTGCTCGGTTC-3’
HM4**	5’-ATCATGAGTCCTGCTCGGTAG-3’
HM5**	5’-ATCATGAGTCCTGCTCGGTCA-3’

Tab 3.7
Adapters and primer combinations used for pre- and selective MSAP amplification in *Allium cepae*. Each “E” primer was used in combination with all the other “HM” primers.

3.5 Results

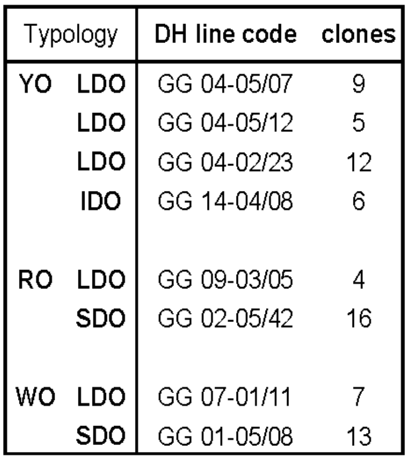
3.5.1 Tissue culture

The laboratory activities carried out to produce haploid embryos using the technique of gynogenesis started in the second half of the month of May harvesting onion umbels when some of their flowers were beginning to open up. After removing the flowers already opened, the umbels were sterilized by soaking for 20 min in a solution of 1.5% sodium hypochlorite.

Following the procedure already reported in materials and methods, it has began the collection of the embryos sprouting from ovaries in mid-August. The sprout timing of the embryos was not uniform and lasted several weeks as a function of genotype and for the different period in which they were placed on the substrate.

In the following months, after duplication of chromosomes by immersing the embryos in an agar substrate with colchicine for 2 days, diploid plants were propagated in vitro to increase their number and avoid losing genetic resource during various stages of acclimatization and transplantation.

In the table below are reported number of plants obtained from each DH lines.



Typology		DH line code	clones
YO	**LDO**	GG 04-05/07	9
	LDO	GG 04-05/12	5
	LDO	GG 04-02/23	12
	IDO	GG 14-04/08	6
RO	**LDO**	GG 09-03/05	4
	SDO	GG 02-05/42	16
WO	**LDO**	GG 07-01/11	7
	SDO	GG 01-05/08	13

Tab 3.8: DH lines and number of clones obtained with gynogenesis in 2011.

LDO = Long Day Onion, ISO = Intermediate Day Onion, SDO = Short Day Onion

3.5.2 Field activities

Field activities of the first year consisted in the transplanting of DH plants around the middle of March, obtained with the technique of gynogenesis in the year 2010, subsequent harvesting of bulbs of the plants survived and their storage in a dry environment. The following year, it was set up bulb transplanting and cages on the same plants in order to produce F1/RF1 seed. As expected, the number of crosses in which it was possible to harvest seeds from both parental lines was low: 25 on 57 (44%). Moreover the quantity of seed harvested was often poor. However the seed harvested it will allow us to set up future field trial, molecular analysis and pathology test on the parental and their F1 and RF1 (Reciprocal Hybrid Cross) offspring.

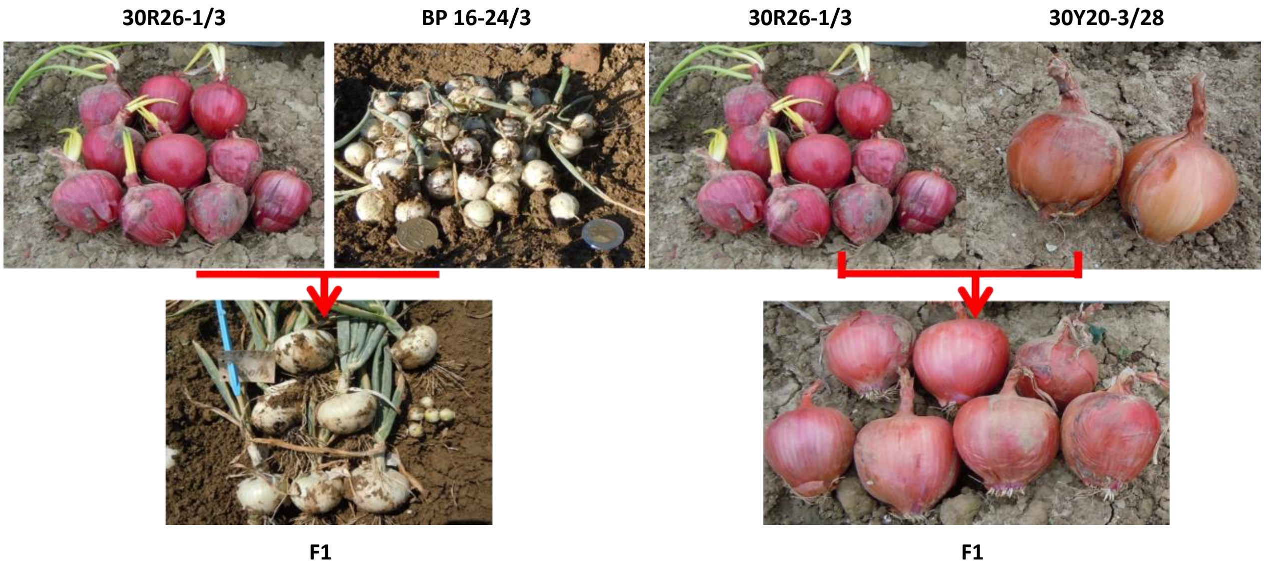
Crosses made in 2012									
Onion Typology		Crosses	RO				YO		WO
			WO	YO	RO	PO	YO	WO	WO
SDO x SDO	cages	12	11	1					
	seeds	**11**	**10**	**1**					
SDO x LDO	cages	27	4	11	6	5		1	
	seeds	**10**	**1**	**4**	**5**	**0**		**0**	
LDO x LDO	cages	18	3	5	1		8		1
	seeds	**4**	**0**	**2**	**0**		**2**		**0**
Total	cages	57	17	13	7	5	8	1	1
	seeds	**25**	**11**	**7**	**5**	**0**	**2**	**0**	**0**

Tab 3.9
Summary of total number and different types of crosses set up in 2011-2012, with an indication of those which have produced seeds (see numbers in bold). SDO: Short Day Onion; LDO: Long Day Onion; RO: Red Onion; WO: White Onion, YO: Yellow Onion; PO: Pink Onion

After choosing the seed of F1 and their reciprocal (RF1) taking in account the amount of seed available, dry skin color and their day length attitude of resulting progeny were sown in a seed tray and then transplanted in the open field to the middle of March. The aim of this field activity was to phenotype all components of each family: parental lines, F1 and RF1.

Below are reported several pictures and phenotipic traits (Table 3.10) of the two families that have been chosen

Fig.3.13 Some pictures of onion bulbs of parental lies and their hybrids



SOME PLANT TRAITS

LINES		Typology	Anthesis	B.Color	Intensity	B. Shape	B. Dimension	neck	B. Firmness	n°	thickness	retention	thickness	outside color	internal color	n°of centers	class	value	25/10/2013	10/12/2013
Range value = [1,5]				BULB						Dry Skins			RINGS				MT		sprout	
30R26-1/3	DH	LDO	late	R	3	bo	60-80	2	2,5	2	2	2	2	r	w	1	med	3	---	50
30R26-1/3 x 30Y20-3/28	F1	LDO	----	R	2	r-bo	70-90	2	2,5	2	2,5	2	2	p	w	1-2	late	4	---	25
30Y20-3/28 x 30R26-1/3	RF1	LDO	----	R	1,5	r-f	60-80	2,5	3	2	3	3	2	p	w	1	late	4,5	---	---
30Y20-3/28	DH	LDO	late	Y	3	r	50-70	2,5	3	2	3,5	3,5	2	Y	Y	1	late	5	---	---
30R26-1/3	DH	LDO	late	R	3	bo	60-80	2	2,5	2	2	2	2	r	w	1			---	50
30R26-1/3 x BP 16-24/3	F1	IDO?	----	W	3	bo-f	60-80	2,5	2,5	2	2		3	w	w	2	med	2	20	75
BP 16-24/3 x 30R26-1/3	RF1	IDO?	----	W	3,5	f-bo	40-60	3	2,5	2	2,5		3,5	w	w	2	early	1,5	40	90
BP 16-24/3	DH	SDO	early	W	4	f	5-10	3	2	2	2,5	2,5	3,5	w	w	3	ex. early	0,5	80	100

Tab 3.10
Phenotypic data of the parental lines and the hybrids obtained from their crossing collected during field trial.
SDO: Short Day Onion; LDO: Long Day Onion; RO: Red Onion; WO: White Onion, YO: Yellow Onion; PO: Pink Onion

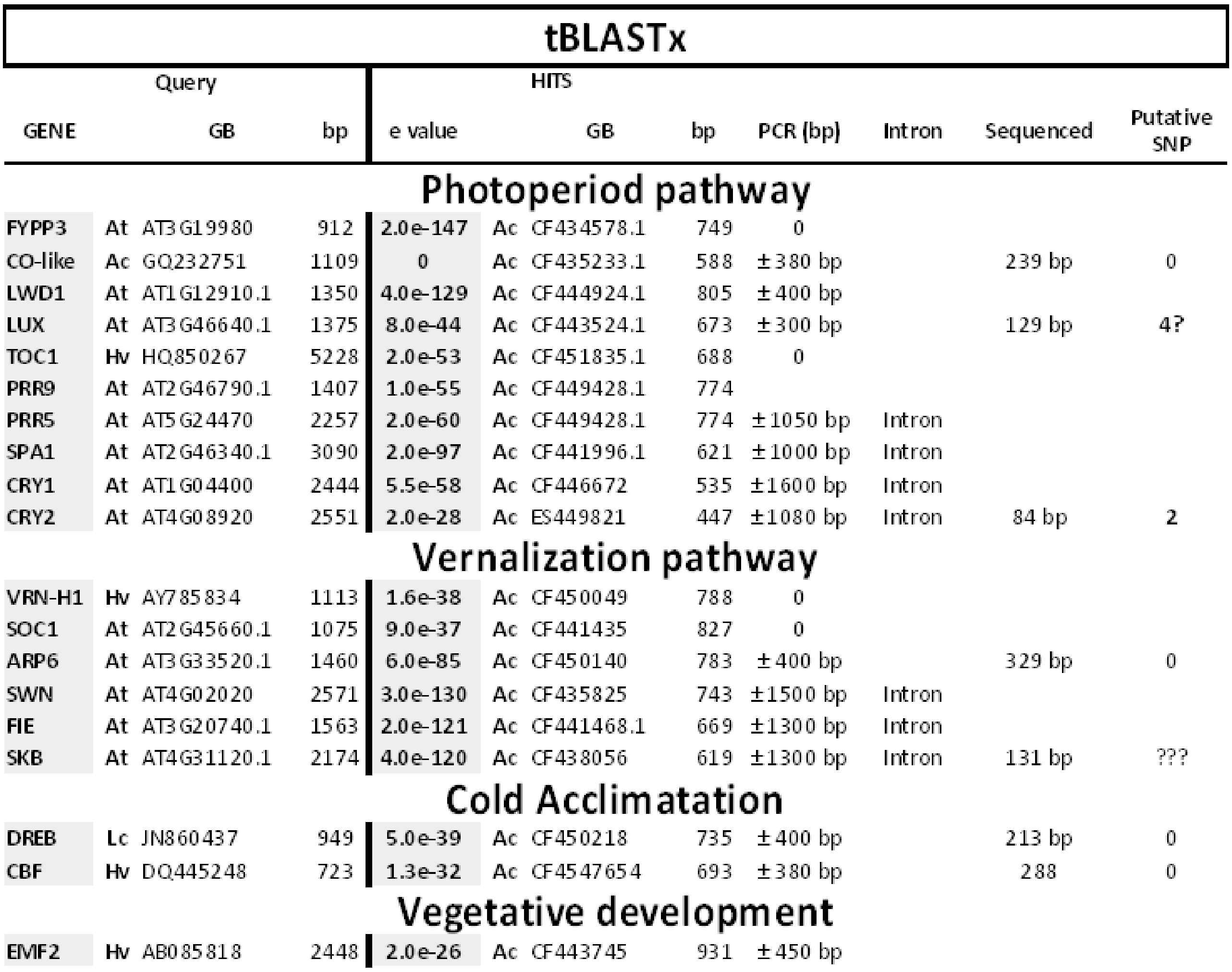
3.5.3 Laboratory activities

i. DNA extraction and purification

ii. Re-sequencing of candidate genes

As expected, given the poor number of onion sequences (just 20.000 EST) deposited in databases, many query sequences have no match or a very poor E-value. Consequently, we have chosen the sequence with the best alignment and with E-value ranging from e-37 to E-147. At the end, we identified 13 putative orthologs of onion: 7 EST homologous to genes involved in photoperiod and 6 EST homologous to genes involved vernalization pathways. The tBLASTx results are reported in the Table below and the ESTs with the best E-value were amplified by PCR in different onion genotypes (Table 3.11).

Tab 3.11
Putative ortholog ESTs of onion identified with the analysis tBLASTx



tBLASTx											
Query				HITS							
GENE		GB	bp	e value		GB	bp	PCR (bp)	Intron	Sequenced	Putative SNP
Photoperiod pathway											
FYPP3	At	AT3G19980	912	2.0e-147	Ac	CF434578.1	749	0			
CO-like	Ac	GQ232751	1109	0	Ac	CF435233.1	588	±380 bp		239 bp	0
LWD1	At	AT1G12910.1	1350	4.0e-129	Ac	CF444924.1	805	±400 bp			
LUX	At	AT3G46640.1	1375	8.0e-44	Ac	CF443524.1	673	±300 bp		129 bp	4?
TOC1	Hv	HQ850267	5228	2.0e-53	Ac	CF451835.1	688	0			
PRR9	At	AT2G46790.1	1407	1.0e-55	Ac	CF449428.1	774				
PRR5	At	AT5G24470	2257	2.0e-60	Ac	CF449428.1	774	±1050 bp	Intron		
SPA1	At	AT2G46340.1	3090	2.0e-97	Ac	CF441996.1	621	±1000 bp	Intron		
CRY1	At	AT1G04400	2444	5.5e-58	Ac	CF446672	535	±1600 bp	Intron		
CRY2	At	AT4G08920	2551	2.0e-28	Ac	ES449821	447	±1080 bp	Intron	84 bp	2
Vernalization pathway											
VRN-H1	Hv	AY785834	1113	1.6e-38	Ac	CF450049	788	0			
SOC1	At	AT2G45660.1	1075	9.0e-37	Ac	CF441435	827	0			
ARP6	At	AT3G33520.1	1460	6.0e-85	Ac	CF450140	783	±400 bp		329 bp	0
SWN	At	AT4G02020	2571	3.0e-130	Ac	CF435825	743	±1500 bp	Intron		
FIE	At	AT3G20740.1	1563	2.0e-121	Ac	CF441468.1	669	±1300 bp	Intron		
SKB	At	AT4G31120.1	2174	4.0e-120	Ac	CF438056	619	±1300 bp	Intron	131 bp	???
Cold Acclimatation											
DREB	Lc	JN860437	949	5.0e-39	Ac	CF450218	735	±400 bp		213 bp	0
CBF	Hv	DQ445248	723	1.3e-32	Ac	CF4547654	693	±380 bp		288	0
Vegetative development											
EMF2	Hv	AB085818	2448	2.0e-26	Ac	CF443745	931	±450 bp			

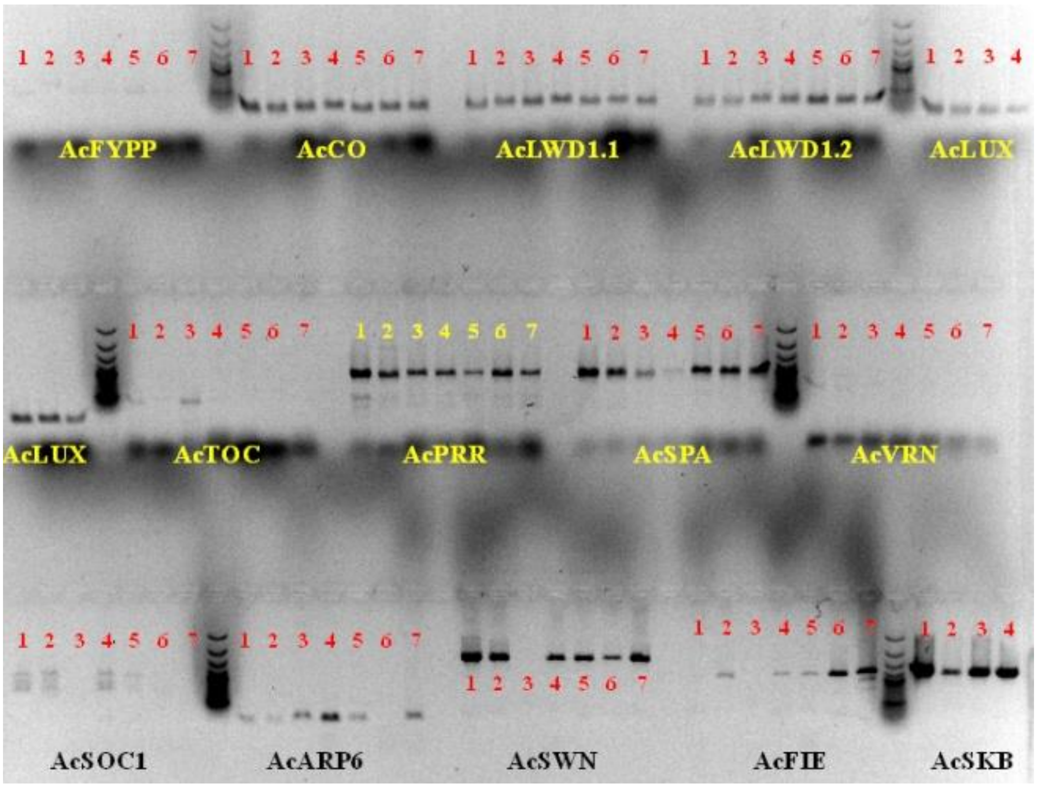
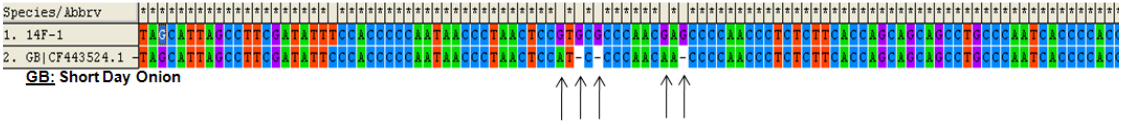


Fig 3.14. PCR products of putative ortologues genes of onion identified by tBLASTx analysis.

It has been attempted by sequencing all PCR products, and the result has been lower than expected as it was obtained just 7 out of 14 sequences analyzed. In 2 of the 7 were identified some SNPs that characterize different onion typologies, in particular these SNPs seem able to distinguish between the short-day from those long day onions (see fig 3.15. Furthermore, one of the two SNP identified in the sequence of the ortholog gene "cryptochrome 2" appears to induce the amino acid substitution in the sequence of the resulting protein, probably causing a conformational and functional modification. This SNP also lies in a sequence of the cutting enzyme HpyAv and can be used for a possible application as CAPS marker for a breeding program (see Fig 3.16)

Fig 3.15. Putative SNPs in two target genes identified with sequencing analysis in Long Day Onion variety comparing their sequence to that present in the database .

Ac LUX= Transcription factor component of a protein complex (Evening Complex) composed of EARLY FLOWERING 3 (ELF3) and EARLY FLOWERING 4 (ELF4) that directly regulates plant growth



Ac Cryptochrome 2 = blue light photoreceptor activity, chromatin organization, chromatin remodeling, positive regulation of flower development

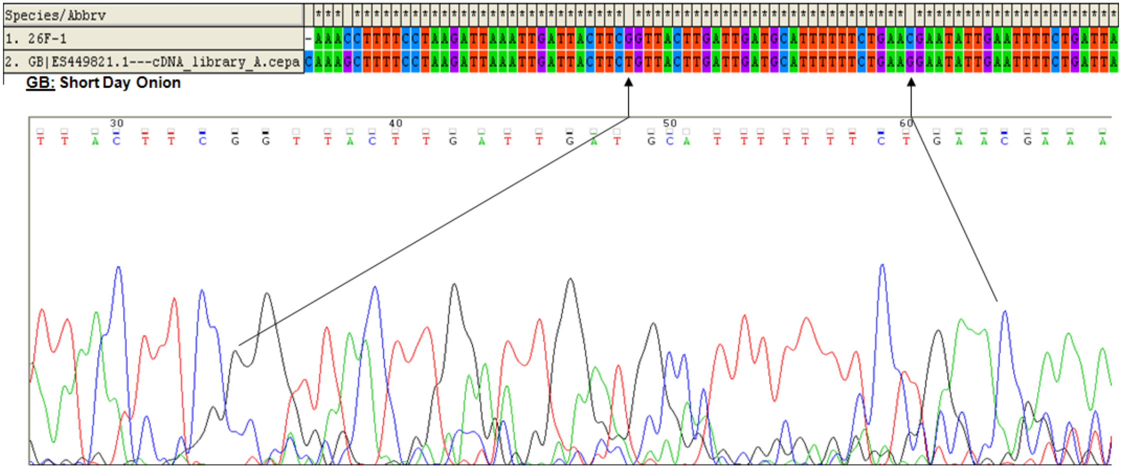
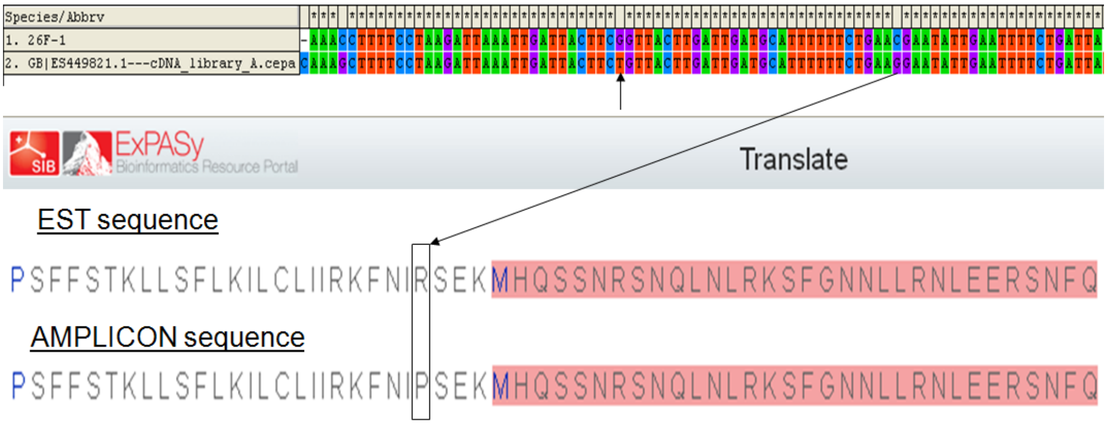


Fig 3.16. Translation analysis of the AcCRY2 nucleotide sequence and identification of the likely effects arising from the SNPs identified by sequencing analysis.

Cryptochrome 2 - Translation analysis



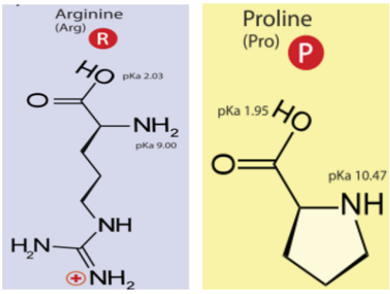
EST sequence

PSFFSTKLLSFLKILCLIIRKFNIRSEKMHQSSNRSNQLNLRKSFGNNLLRNLEERSNFQ

AMPLICON sequence

PSFFSTKLLSFLKILCLIIRKFNIPSEKMHQSSNRSNQLNLRKSFGNNLLRNLEERSNFQ

Amino Acid structures



Endonuclease/Restriction Analysis

HpyAV: Cleavage site (🟩) + Target sequence

TTGAAAATTACTTCTTTCCTCGAGGTTTCTCAACAGATTATTCCC
AAAGCTTTTCCTAAGATTAAGTTGATTACTTC**T**GTTACTTGATTGA
TGCATT🟩TTTTCTGAA**G**GAATATTGAATTTTCT🟥GATTATGAGGCA
TAAAATTTTGAGAAAGCTCAGCAGTTTCGTGGAAAAAAAGCTTG
G

AcuI: Target sequence + Cleavage site (🟥)

iii. MSAP – Agar gel

It was analyzed the PCR products after each step by agarose gel and in more detail the selective amplification products by sequencer.

From preliminary analysis of the electropherograms obtained with all pre-amplification products was possible to observe a difference in the macroscopic level among band profiles in DNA samples collected in summer and winter (Fig. 3.17 and 3.18).

Tab 3.12
Some data regarding the leaves sampling and the environmental conditions

code	Sowing data	Transplanting		Sampling Data				
		Date	dd	Date	dd	dd	Temp	light
[A,T]-0c	**15/03/13**	**25/04/13**	41	13/06/13	90	**0**	22°C	15h 30'
[A,T]-2c	**15/03/13**	**25/04/13**	41	27/06/13	104	**14**	19°C	15h 32'
[A,T]-4c	**15/03/13**	**25/04/13**	41	11/07/13	118	**28**	26°C	15h 20'
[1,28]-Tr0	**15/03/13**	**02/08/13**	140	13/11/13	243	**0**	13°C	9h 47'
[1,24]-Tr1	**15/03/13**	**02/08/13**	140	02/12/13	262	**19**	8°C	9h 10'
[1,28]-Tr2	**15/03/13**	**02/08/13**	140	18/01/14	309	**66**	7°C	9h 21'

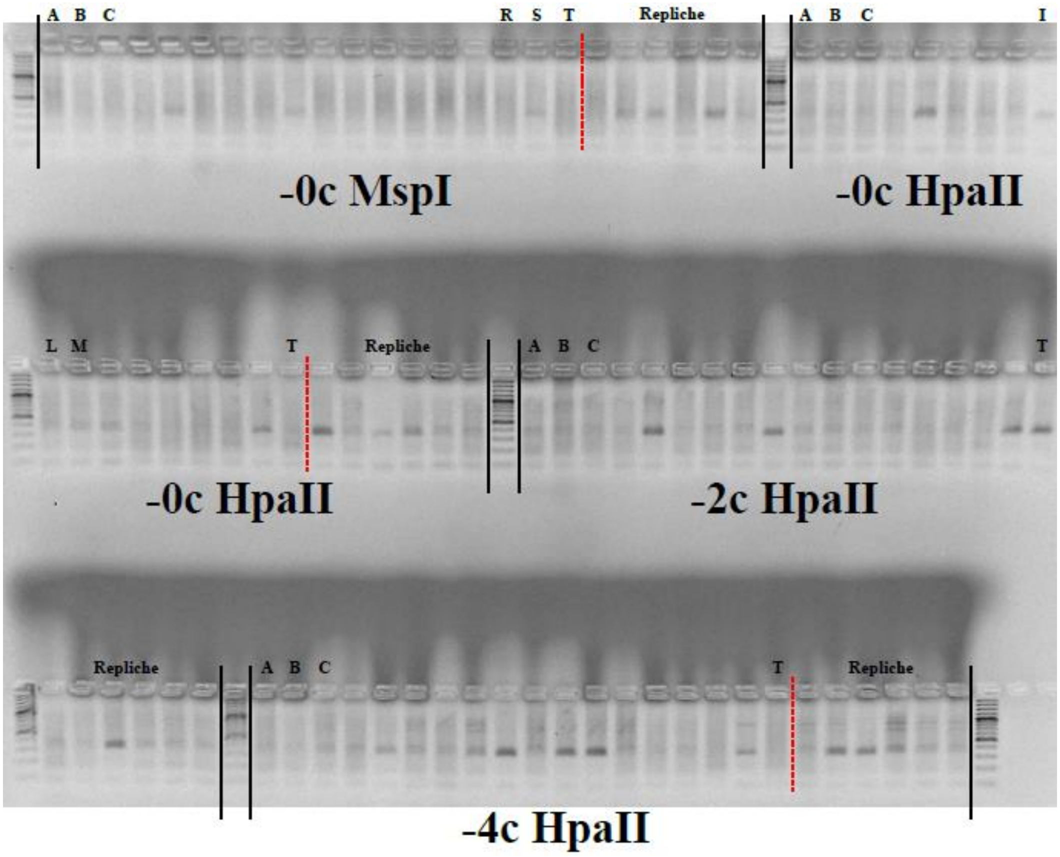


Fig. 3.17. Agar gel 2% Electropherogram of Pre-Amp PCR amplification from all DNA of plants grown only in summer conditions. **MspI-0c**: sample profiles obtained with MspI enzyme; **HpaII-0c/2c/4c** sample profiles obtained with HpaII

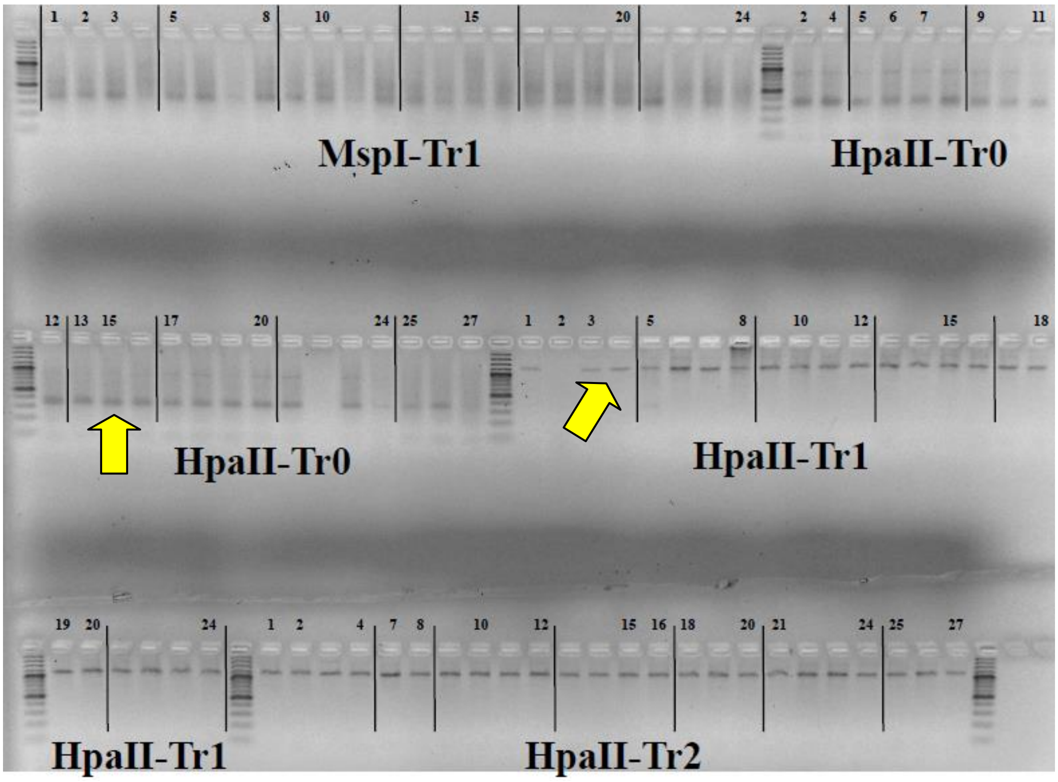


Fig 3.18. Agar gel 2% Electropherogram of Pre-Amp PCR amplification from all DNA of plants grown only in winter conditions. **MspI-Tr1**: sample profiles obtained with MspI enzyme; **HpaII-Tro/1/2:** sample profiles obtained with HpaII

In particular, must to note a clear modification in the methylation profile of genomes extracted from leaf tissues collected in second sampling (02/02/13). It is clear how the methylated sites are increased preventing the enzyme HpaII to cut its target sequences producing only DNA fragments with high molecular weight.

Same results were obtained with the SELECTIVE amplifications. Analyzing some electropherograms, stained with ethidium bromide, it was observed more evident and numerous methylation differences then observed with PRE-AMP amplifications. Even in this case, it is seen another methylation profile in winter samples.

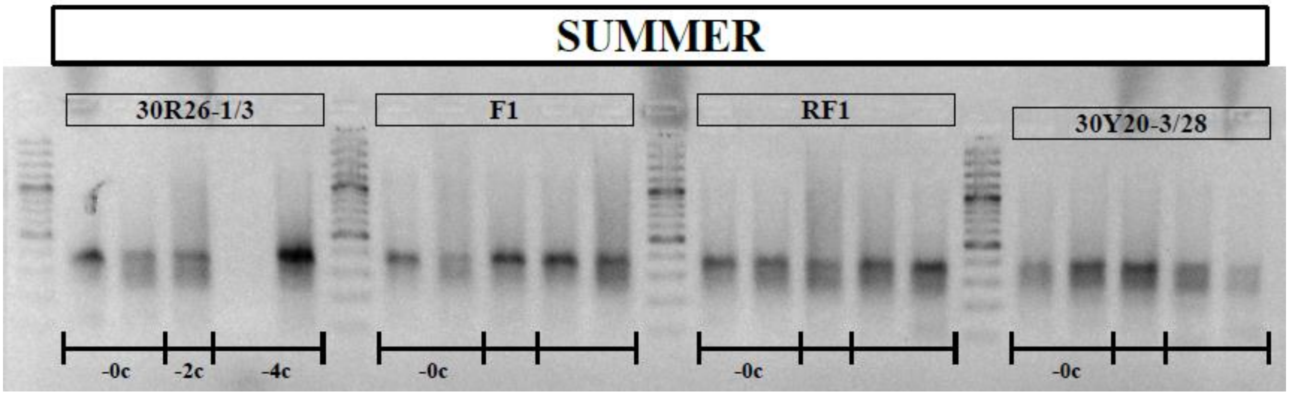


Fig 3.19. Agar gel 2% - Electropherogram of selective amplification obtained with one primer combination from some DNA of plants grown only in summer conditions. It is possible to compare the methylation profiles of parental lines (30R26-1/3 and 30Y20-3/28) and their F1, RF1

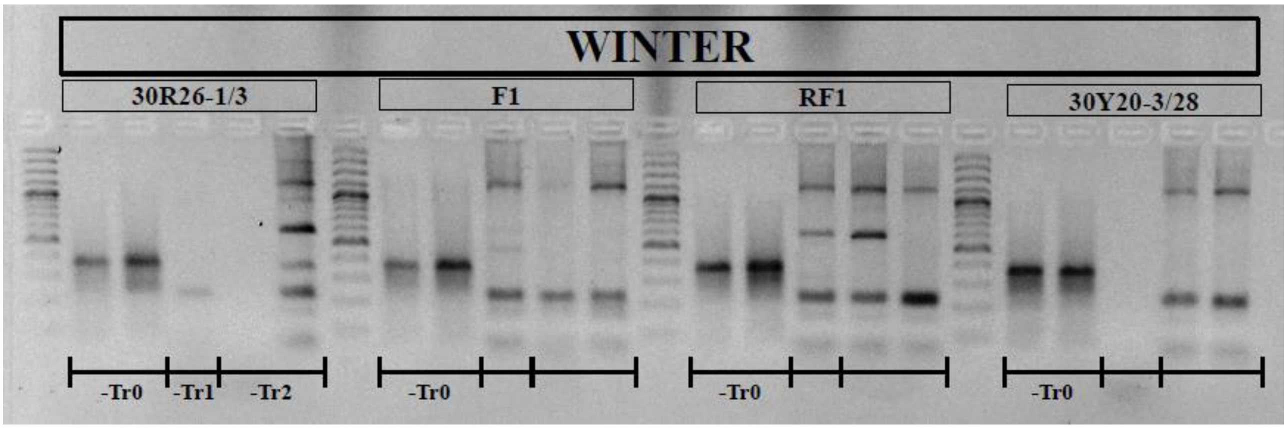


Fig 3.20. Agar gel 2% - Electropherogram of selective amplification obtained with one primer combination from some DNA of plants grown only in winterr conditions. It is possible to compare the methylation profiles of parental lines (30R26-1/3 and 30Y20-3/28) and their F1, RF1

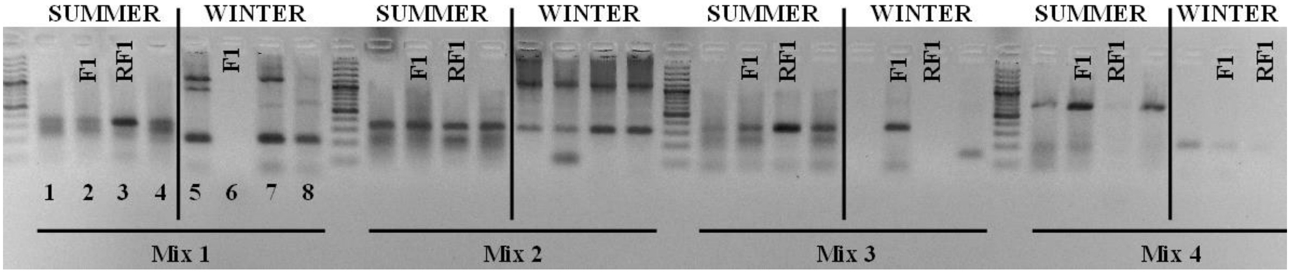


Fig 3.21 Agar gel 2% - Electropherogram of Selective amplification by PCR from all DNA of plants grown in summer and winter conditions. It is possible to compare the methylation profiles, obtained with different primer combinations (Mix 1/2/3/4), belonging to parental lines (30R26-1/3 and 30Y20-3/28) and their F1 and RF1.

These different methylation pattern at the genomic level seems most likely due to a change in climatic trend occurred between the first (13/11/13; –Tr0) and the second (02/12/13; -Tr1) sampling. In fact, if it looks at the daily temperatures of the period (see Fig 3.22 and table 3.13), we can observe that in the last 10 days of the month of November, there was a bad turn in temperatures (highlighted with a black boundary) which, until then, had remained at above the seasonal average.

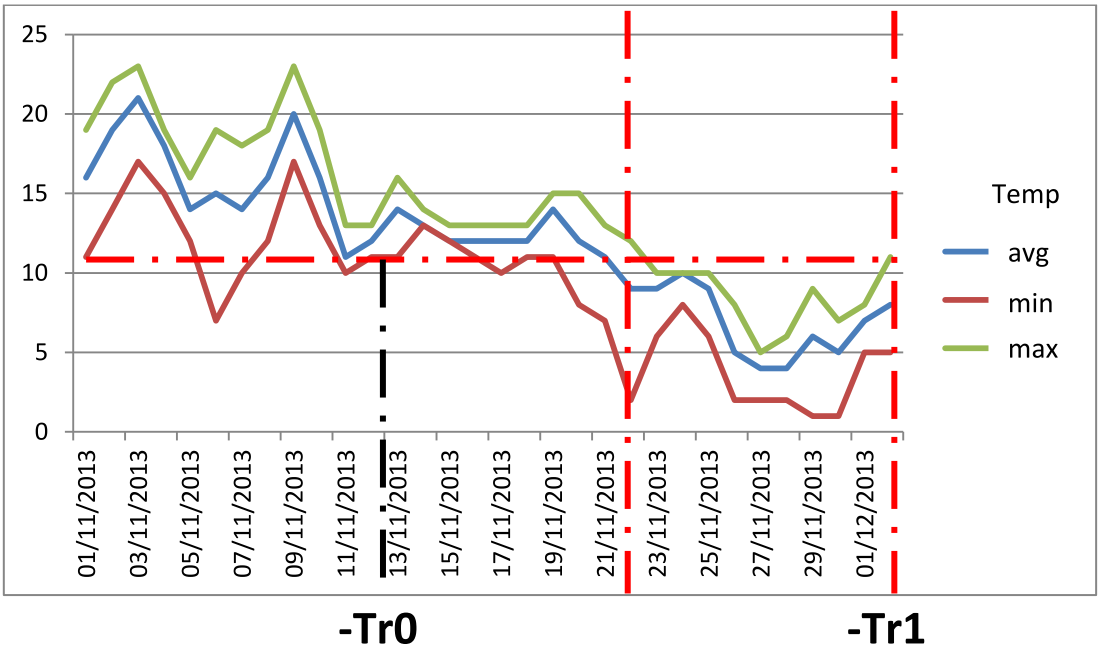


Fig 3.22 Detail of trend temperature between first two winter samples. It is possible to notice a sudden drop in temperature starting from 22 November

PLACE	DATE	Average T °C	Min T °C	Max T °C
Cesena	September	22,00	15,71	25,14
Cesena	October	17,42	13,55	19,97
Cesena	November	12,17	9,10	14,17
	Nov [01-21]	14,48	11,57	16,57
	Nov [22-31]	6,78	3,33	8,56
Cesena	Dicember	6,97	1,52	10,71
Cesena	January	6,45	3,18	9,73

Tab 3.13
Average monthly temperatures

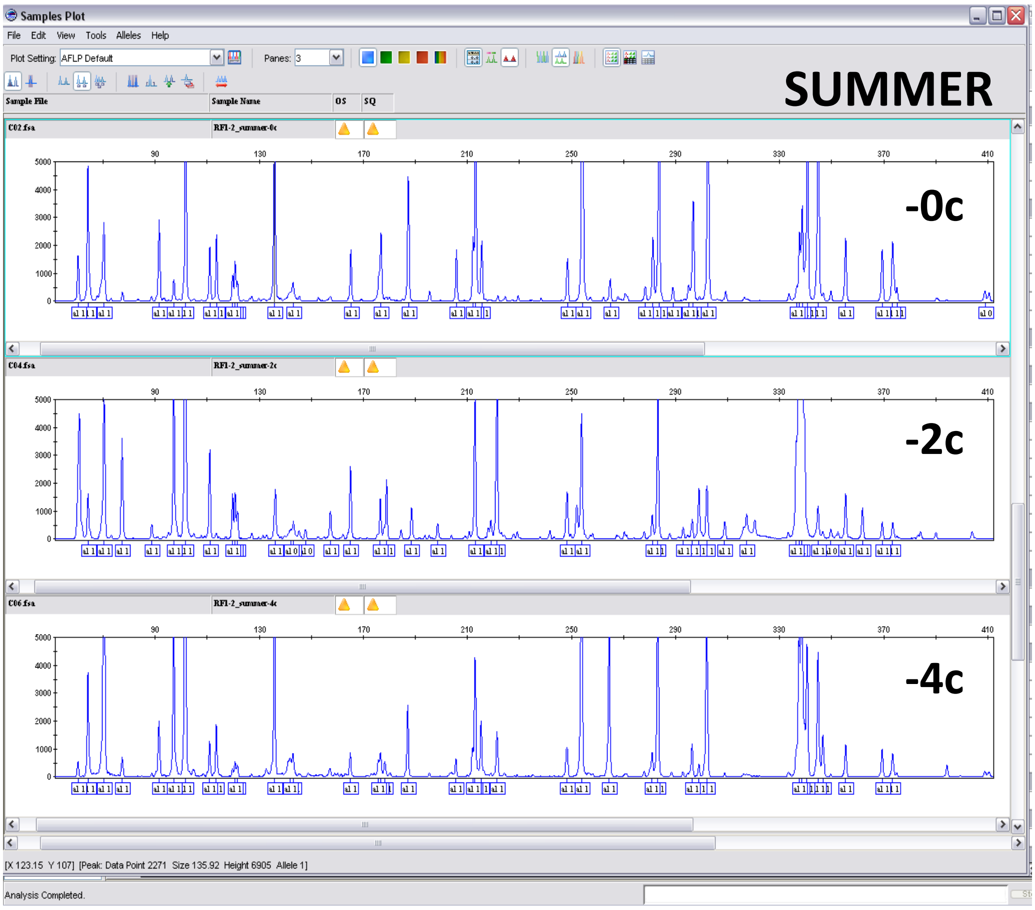
iv. MSAP – Sequencer

The use of the genetic analyzer allows the separation of the PCR products with a resolution extremely higher when compared to the profiles of the bands observed with the agarose gel (Fig. 3.19, 3.20, 3.21). In total, 20 selective fluorescently-labeled primers combinations were used (Table 3.7) and separated via capillary electrophoresis.

As an example, we report here the results of primer combination E1HM1, called mix1, while the other are still underway. As reported in Figure 3.18 a dramatic change in MSAP profiles can be observed, leading us to the conclusion that a sudden and significant increase in CpG islands methylation occurred at many chromosomal regions in plants starting from the second winter sampling (-Tr1).

Although this analytical technique can analyze DNA fragments with dimensions in the range of 50 to 500 bp, after a preliminary study of the results generated with the Mix1, it was identified 26 fragments that have changed their methylation status at the end of November (-Tr1). This result strengthens and extends what has been already macroscopically observed with agarose gel electrophoresis (Fig.3.20, 3.21), where a few bands had generated a modest polymorphism that had already allowed us to formulate a hypothesis. Furthermore, it must be stressed that some of the polymorphic products observed in the agarose gel (see Bands at ca. 600bp and 1,200 bp) have not been revealed with the Sequencer.

Below are two screenshots produced by the sequencer where the evolution of the methylation profile generated from the same plants can be observed. Changes, for each season, are probably depending on variation of the weather conditions during the season. The most related environmental parameter seems to be low temperature as during summer the profiles remain very similar among genotypes, whereas during the cold season many peaks disappear, especially starting from the end of November (Fig. 3.24).



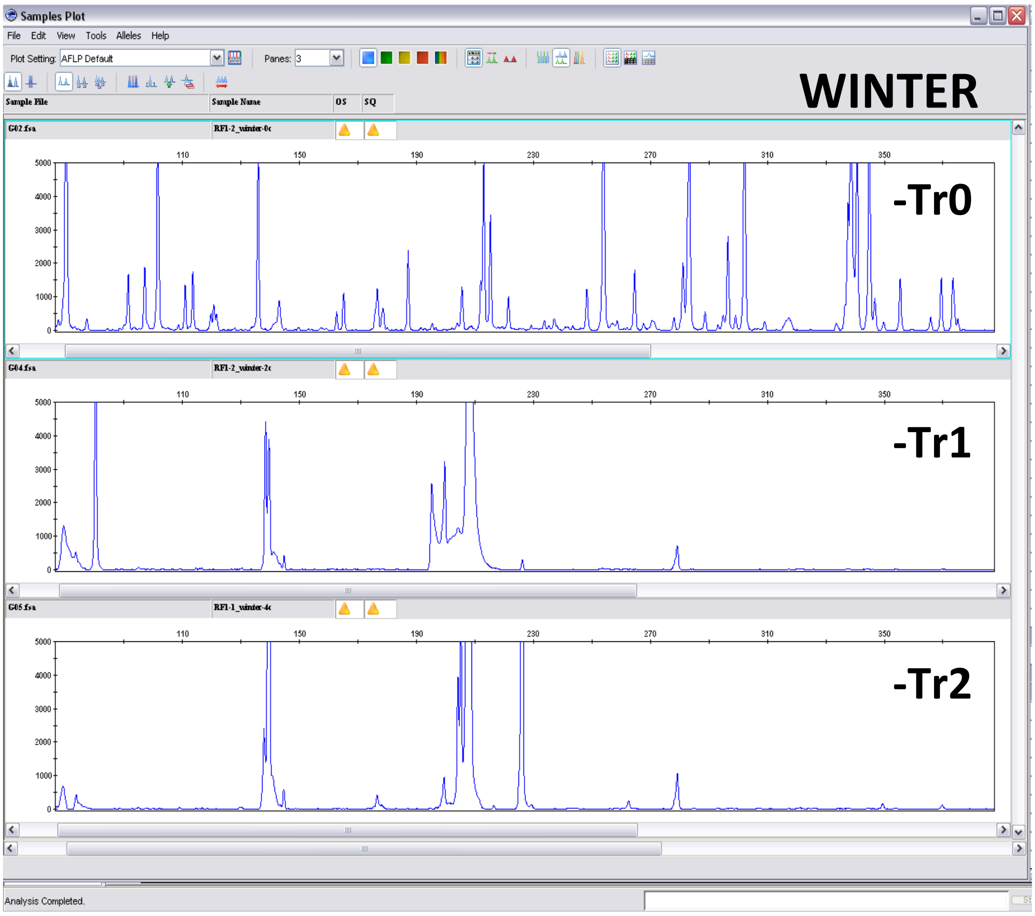
Plot Setting: AFLP Default

Panes: 3

[X 123.15 Y 107] [Peak: Data Point 2271 Size 135.92 Height 6905 Allele 1]

Analysis Completed.

Fig 3.23. Image of peaks produced by sequencer instrument during the analysis of fragment DNAs generated by plants sampled in summer



Samples Plot

Plot Setting: AFLP Default

Panes: 3

Sample File	Sample Name	OS	SQ
G02.fsa	RF1-2_winter-0	⚠	⚠
G04.fsa	RF1-2_winter-2	⚠	⚠
G05.fsa	RF1-1_winter-4	⚠	⚠

Analysis Completed.

Fig 3.24. Image of peaks produced by sequencer instrument during the analysis of fragment DNAs generated by plants sampled in winter

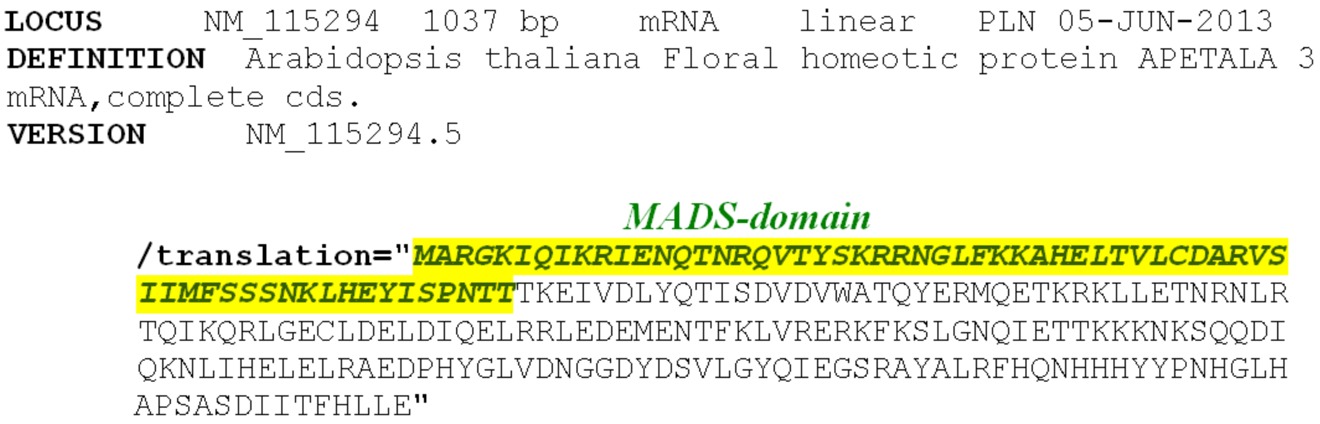
The *ad hoc* developed plant material (DH lines, F1 and RF1 progeny) revealed his usefulness also to obtain some indication about the parental imprinting effect. In particular, some peaks of the MSAP profiling that were present only in one of the two DH parental lines were constantly inherited by either the F1 or the RF1 genotypes. Further analysis of the MSAP data are underway to verify the hypothesis of a constant effect from one of the two parents.

v. MSAL

In addition to analyzing the changes in epigenetic markers at the genomic level in general, it was also attempted to restrict the analysis to specific genes by modifying the protocol MSAP so as to amplify specific target sequences.

As one of the most important classes of genes involved in the development of almost all tissues in plants are those that belong to the MADS-box genes, it was decided to analyze possible modifications of epigenetic marks in the regions close to onion ESTs of putative orthologous genes chosen for their e-value with a BLASTX analysis.

The first step of the BLAST analysis has been to use a MADS-box region of the gene apetala 3 of Arabidopsis (accession NM_115294.5) which allowed the identification of 4 ESTs in onion.



```
LOCUS       NM_115294   1037 bp    mRNA    linear   PLN 05-JUN-2013
DEFINITION  Arabidopsis thaliana Floral homeotic protein APETALA 3
mRNA,complete cds.
VERSION     NM_115294.5

                         MADS-domain
     /translation="MARGKIQIKRIENQTNRQVTYSKRRNGLFKKAHELTVLCDARVS
     IIMFSSSNKLHEYISPNTTTKEIVDLYQTISDVDVWATQYERMQETKRKLLETNRNLR
     TQIKQRLGECLDELDIQELRRLEDEMENTFKLVRERKFKSLGNQIETTKKKNKSQQDI
     QKNLIHELELRAEDPHYGLVDNGGDYDSVLGYQIEGSRAYALRFHQNHHHYYPNHGLH
     APSASDIITFHLLE"
```

Fig 3.25. MADS-box amino-acid sequence (yellow highlighted) belonging to Arabidopsis used as query in BLASTx analysis to find MADS-box sequences in onion ESTs.

Subsequently, each individual AcEST was compared with the protein sequences of all flowering plants deposited in the database with BLASTX. In the table below are reported the onion ESTs with the best alignments (Table 3.14)

Tab 3.14 - BLAST RESEARCH for MADS-box genes in Onion

Query				RESULTS										
	EST	**GB**	**bp**	**eTool**	**Organisms**								**e value**	**Ident**
At		**NM_115294.5**		tBLASTn	A. cepa						**CF441435.1**	EST	4E-024	68%
	It was used the MADS-box region of NM_115294.5 as a query for BLAST research										**CF450049.1**	EST	8E-024	61%
											CF449085.1	EST	1E-022	64%
											CF436727.1	EST	2E-022	56%
	EST	**GB**	**bp**	**eTool**	**Organisms**	**Species**	**Clade**		**Order**	**Gene/Prot characteristics**	**GenBank**		**e value**	**Ident**
Ac	EST677780	**CF441435.1**	827	BLASTx	Flowering Plants	A. cepa	Monocots	Liliopsida	Asparagales	***C-class MADS-box protein***	**AGV31153.1**	Prot	7E-166	99%
				BLASTx	Flowering Plants	A. cepa	Monocots	Liliopsida	Asparagales	***MADS-box transcription factor***	**AGC31682.1**	Prot	5E-164	99%
				BLASTx	Flowering Plants	Asparagus	Monocots	Liliopsida	Asparagales	MADS-box transcription factor	**BAD18011.1**	Prot	1E-133	84%
	AcMADS 1			BLASTx	Flowering Plants	Hosta plant.	Monocots	Liliopsida	Asparagales	agamous MADS-box transcr. Fact.	**ACB70410.1**	Prot	4E-129	83%
				BLASTx	Flowering Plants	Cr. sativus	Monocots	Liliopsida	Asparagales	agamous MADS-box transcr. Fact.	**AAS67611.1**	Prot	5E-129	80%
				BLASTx	Flowering Plants	Hosta plant.	Monocots	Liliopsida	Asparagales	agamous-like protein 2	**ADK35760.1**	Prot	3E-128	82%
				BLASTx	Flowering Plants	Cr. sativus	Monocots	Liliopsida	Asparagales	agamous MADS-box transcr. Fact.	**AAS67610.1**	Prot	3E-128	80%
Ac	EST686394	**CF450049.1**	788	BLASTx	Flowering Plants	Asparagus	Monocots	Liliopsida	Asparagales	MADS-box transcription factor	**ABC70710.1**	Prot	9E-156	88%
				BLASTx	Flowering Plants	Asparagus	Monocots	Liliopsida	Asparagales	MADS-box transcription factor	**ABC70706.1**	Prot	2E-154	89%
				BLASTx	Flowering Plants	Asparagus	Monocots	Liliopsida	Asparagales	MADS-box transcription factor	**AAQ83834.1**	Prot	7E-154	88%
	AcMADS 2			BLASTx	Flowering Plants	A. tequilana	Monocots	Liliopsida	Asparagales	MADS box protein 1	**AEX92976.1**	Prot	2E-152	89%
				BLASTx	Flowering Plants	Cr. sativus	Monocots	Liliopsida	Asparagales	SEPALLATA3-like MADS-box prot	**ACB69510.1**	Prot	6E-148	86%
Ac	EST673072	**CF436727.1**	765	BLASTx	Flowering Plants	Alstr. ligtu	Monocots	Liliopsida	Asparagales	MADS-box transcription factor	**BAM34481.1**	Prot	1E-102	70%
				BLASTx	Flowering Plants	Aco. americanus	Monocots	Liliopsida	Acorales	AGL2 (MADS-box gene)	**AAX15922.1**	Prot	6E-102	69%
	AcMADS 3			BLASTx	Flowering Plants	A. tequilana	Monocots	Liliopsida	Asparagales	MADS box protein 2	**AEX92975.1**	Prot	2E-101	69%
				BLASTx	Flowering Plants	Platanus acerifolia	Eudicots			Sepallata 1-like protein	**ADR83588.1**	Prot	3E-099	68%
Ac	EST685430	**CF449085.1**	717	BLASTx	Flowering Plants	A. cepa	Monocots	Liliopsida	Asparagales	***SOC1-like protein, partial***	**AGO81844.1**	Prot	3E-054	98%
				BLASTx	Flowering Plants	D. nemorosa	Eudicots		Brassicales	MADS-box protein	**AAP20425.1**	Prot	4E-041	72%
	AcMADS 4			BLASTx	Flowering Plants	Th. cacao	Eudicots		Malvales	AGAMOUS-like 20 isoform 3	**EOX96137.1**	Prot	5E-041	73%
				BLASTx	Flowering Plants	Th. cacao	Eudicots		Malvales	AGAMOUS-like 20 isoform 4	**EOX96138.1**	Prot	9E-041	73%
				BLASTx	Flowering Plants	Th. cacao	Eudicots		Malvales	AGAMOUS-like 20 isoform 5	**EOX96139.1**	Prot	1E-040	72%

Using the software Primer3 (http://bioinfo.ut.ee/primer3/) it was designed primers for each sequence. Subsequently it was evaluated their specificity and ability to amplification by PCR. In the gel below (Fig 3.26) there are the PCR products obtained for 3 out of 4 identified EST.

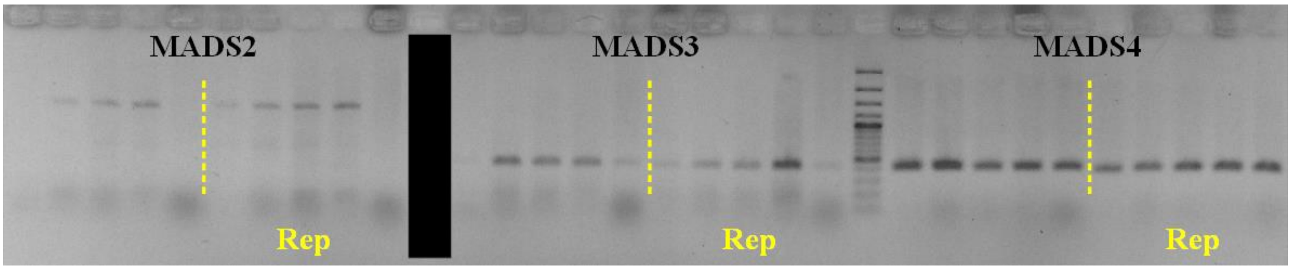


Fig 3.26. PCR products of putative ortologues MADS-box genes of onion identified by tBLASTx analysis.
Rep: sample replicated to have a greater amount of DNA to be purified and that could be used in a future sequencing analysis

EST	size
AcMADS 1	0 bp
AcMADS 2	1500 bp
AcMADS 3	400 bp
AcMADS 4	450 bp

Tab 3.14
Size of PCR products relative to AcMADS

The next step was to selectively amplify the regions adjacent to the EST sequence by modifying the technique used previously MSAP. In the figure below is shown schematically the procedure for this type of analysis. Not knowing the entire sequence of the candidate gene, but only the part corresponding to ESTs deposited in the data bank, it was used a primer combination: in the pcr solution it was put one specific primer to the known EST sequence and one of the two oligonucleotides used to make the “adapter HpaII / MspI”, in particular “HpaII / MspI” adapter 1 to amplify of the sequence contained between the two primers.

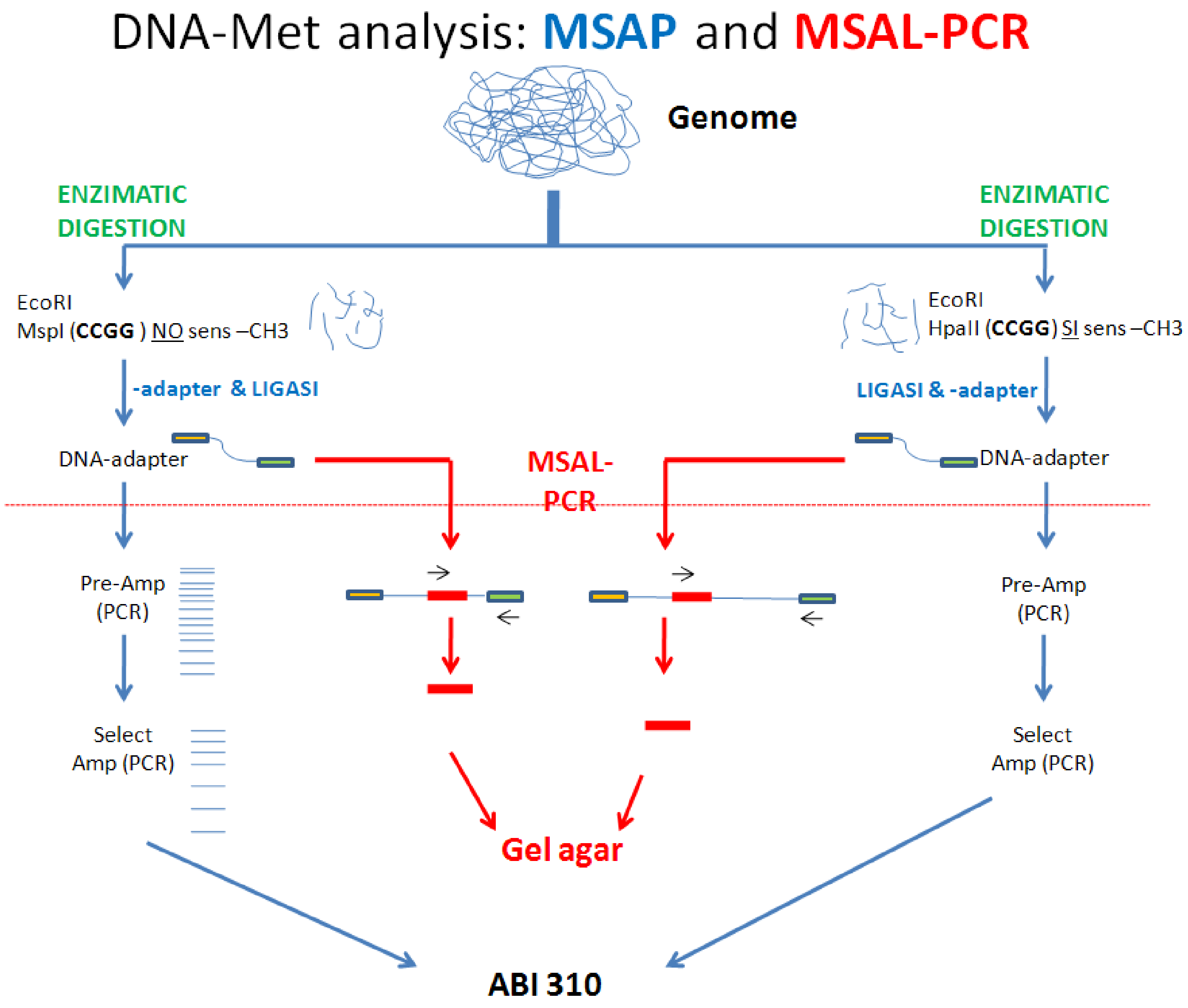


Fig.3.27 Flowchart showing the steps of the laboratory technique MSAL

What makes interesting this variant of the method MSAP is the ability to analyze any changes in the methylation profile that can occur both within the sequence known and in its proximity, exploiting the presence and the position of the adapters to the ends of the fragments. Different cleavage sites produce size polymorphisms of fragments amplified by PCR and that can be evaluated with a gel.

The onion EST (AcMADS3) is the most like ortholog of the MADS-box transcription factor gene *Alstroemeria ligtu subsp. Ligtu*, which would seem to transcribe proteins belonging to "E-class" of the MADS-box family involved in the development of all floral organs.

The restriction analysis with the software NEBcutter v2.0 (http://tools.neb.com/NEBcutter2/) of AcMADS3 did not reveal any sequence recognized by the enzymes HpaII / Maspi excluding a possible cut in the EST sequence.

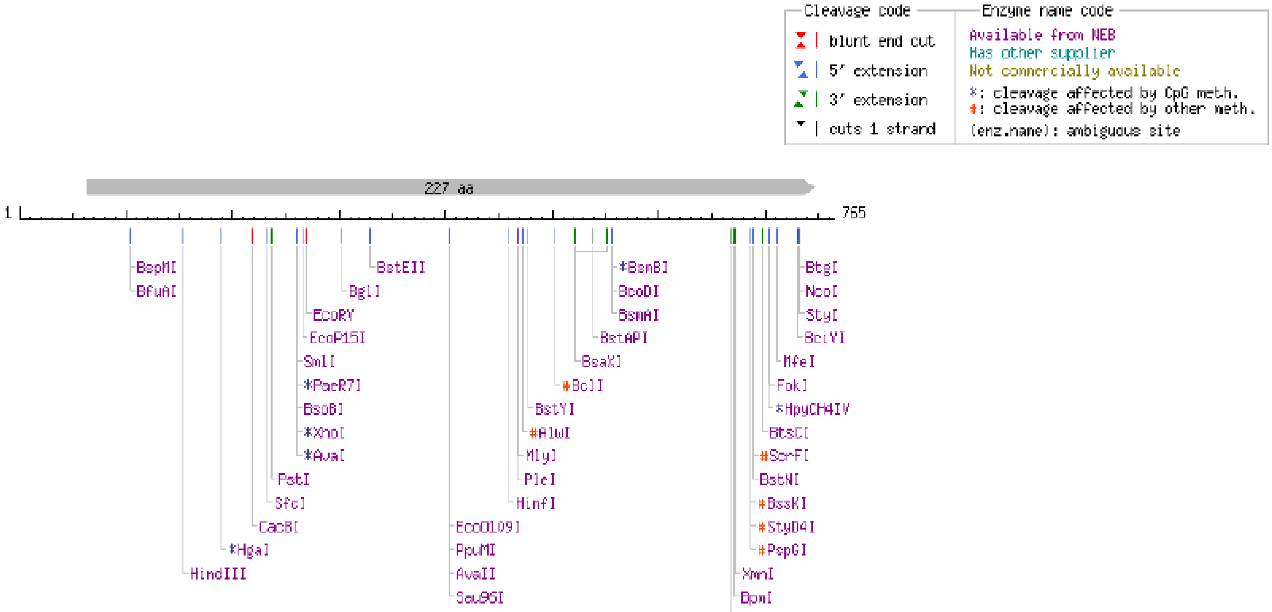


Fig.3.28 Result of restriction analysis for AcMADS3 obtained with NEBcutter v2.0

This information has allowed hypothesizing the production of fragments longer than that resulting from the same EST by PCR.

The screening of PCR products of the pre-amp derived from the DNA of all samples taken during the year, allowed uncovering a clear polymorphism between summer and winter samples. As a result of the pre-selective amplification in winter samples appear a second fragment that seems to be slightly bigger than 500 bp (Fig. 3.29), and in any case are bigger than the band obtained with the specific PCR (Fig. 3.26). The second band just described is added to that already present about 250 bp in all samples and sampling.

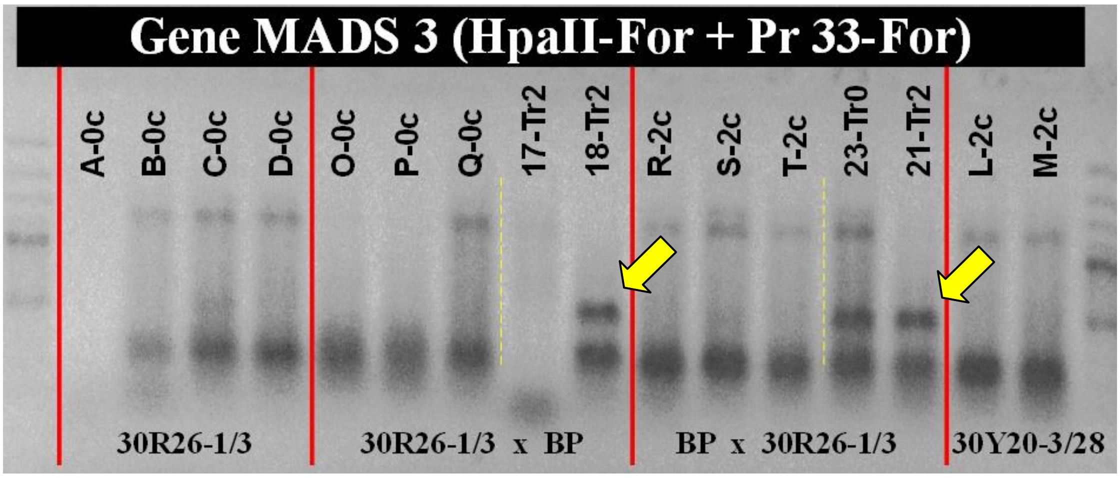


Fig.3.29 Polymorphismbetween summer and winter samples

This specific result is summarized in the table below

Sampling Data													
MADS-box 3		SUMMER						WINTER					
450 bp		-0c		-2c		-4c		-Tr0		-Tr1		-Tr2	
		MspI	HpaII	MspI	HpaII	MspI	HpaII	MspI	HpaII	MspI	HpaII	MspI	HpaII
30R26-1/3	P1	+	-	+	-	+	-	+	+	+	+	+	+
30R26-1/3 x 30Y20-3/28	F1	+	-	+	-	+	-	+	+	+	+	+	+
30Y20-3/28 x 30R26-1/3	RF1	+	-	+	-	+	-	+	+	+	+	+	+
30Y20-3/28	P2	+	-	+	-	+	-	+	+	+	+	+	+
30R26-1/3	P1	+	-	+	-	+	-	+	+	+	+	+	+
30R26-1/3 x BP 16-24/3	F1	+	-	+	-	+	-	+	+	+	+	+	-
BP 16-24/3 x 30R26-1/3	RF1	+	-	+	-	+	-	+	+	+	+	+	-
BP 16-24/3	P3	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Tab 3.15
Polymorphism (-/+) produced by a different methylation status of the onion EST "MADS-box 3" during
The two seasons (summer/winter) in which they are occurred leaves sampling

3.6 Discussion

The research strategy used for this project is found to be appropriate for the study of epigenetic variation of the genome and its phenotypic effect in onion. The goal of creating hybrids (F1), and their reciprocal (RF1), with Double Haploid (DH) lines has allowed obtaining plants F1/RF1 with a genome identical even if having a different maternal origin. The research strategy used for this project is appropriate for the study of epigenetic variation of the genome and its phenotypic effect in onion. The goal of creating hybrids (F1) and reciprocal hybrids (RF1) has allowed us to obtain plants with a genome identical but with different maternal origin respect to the DH lines from which they were derived.

The results of the phenotyping annotation in the field of different plant families, composed of the two parental lines and their crosses (F1/RF1), have revealed a difference for some agronomic traits between F1 and its RF1. In particular, it has been ascertained how many of the characteristics observed in the progeny are close to those of the mother (stockseed), this confirms what has already been observed in other plant species where it has been demonstrated a specific maternal effect (Lauria et al., 2013; Raissig et al., 2013; Cendan et al., 2011; Waters et al., 2011; Elwell et al., 2011; Riginos et al., 2007; Galloway et al., 2009; Galloway, 2004).

The molecular strategy, followed for the study of epigenetic modifications in the genome of the plants through the analysis of leaf samples collected for a long period (several months), has proven effective because it allowed to observe a direct influence of the external environment on the metabolism through epigenetic variations in the genome.

MSAP and MSAL tests have demonstrated their ability to analyze the nuclear genome in general and particular, through the identification of new methylation status of the target sequences, as there is a direct correlation to the plants and their environment. The analysis of epigenetic marks at specific times allowed us to photograph the exact moment in which there was an epigenetic upheaval in the genome of onion plants at the same time lowering the outside temperature. Even in this case were confirmed the observations that have been made on other species of plants

(Suter and Widmer, 2012; Galloway et al., 2009; Galloway, 2004)

The appearance of the second band at the cold season allows assuming that alteration in the methylation status in this gene region could be inferred that gene expression, in this specific case, could be in response to the environment.

It could be possible to speculate that in onion the mother lineage has a strong influence in the gene expression of the embryo because in the endosperm is present larger pool of TEs, retrotransposons, retroviruses and repeated sequences compared to paternal line. The number of these sequences with a specific epigenetic profile inherited from the mother could be bigger when compared to monosporic pattern, thanks to a larger genetic background represented by two genetically different polar nuclei which promote the gene silencing through the RdDM pathway. The “genetic memory” inherited by father lineage consequently could be largely silenced thanks to epigenetic modifications of its gene pool

These observations, taken together, are extremely valuable not only to adopt an effective breeding program in onion, but also to carefully analyze the microclimates of the places where the reproduction is made of the seed, in order to avoid an unsuitable environment to play the cultivar. In fact, it might even speculate that the reproduction of a commercial hybrid by crossing two parental lines in an environment not suited to the characteristics of the maternal line may result in changes to the phenotype of the offspring produced.

A last comment could be dedicated to the SNPs identified in the EST of putative ortologue gene CRYPTO 2 that, even if confirmed on a larger number of accessions and cultivars, could be used in a future research project to assess whether different allelic variants can influence flowering time and maturity time even in onion. In fact, it is known that in Arabidopsis thaliana the protein produced by the gene CRPTOCHROME stabilizes the protein COSTANS promoting the transition from vegetative to reproductive stage.

3.7 Reference

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CHAPTER 4

Maternal influence in onion: plant-pathogen interactions

4.1 Maternal influence in onion: plant-pathogen interactions

Introduction

4.1.1 Main diseases of onion

Diseases affecting onions and related crops are many and the causal agents of these injuries are fungi, bacteria or viruses. The onion, as other *Allioideae*, could be affected by serious plant diseases of parasitic nature. Onion is characterized by poor deference molecular traits which make it sensible to many pathogenic microorganisms. It is useful to list also the disorders caused by adverse weather, nutritional imbalances, air pollutants, herbicides, other chemical compounds and soil conditions. Despite different origins and causes of the physiological alterations, several diseases and/or disorder can be present at the same time.

4.1.2 Non infectious diseases

The main non-infectious diseases in onion are: bulb splitting, freeze damage, greening, herbicide injury, leaf variegation (chimera), nutrient disorders, storm damage, sunscald, Thrips damage and translucent scale.

The following is a brief description of the various non-infectious diseases.

i. BULB SPLITTING

The bulb affected by this physiological disorder shows a secondary growth of new growing points at the basal plate level causing the breakage of the bulb.

Small bulbs protruding from a split basal plate.


Small bulbs protruding from split basal plates.

Onion plants affected by bulb splitting show several small bulbs protruding from the split basal plate. The causal agent of this problem is just physiological, even if it was observed the presence of bulb mites of the genus *Rhizoglyphus* (see photo below) with frequency on the plant affects of this physiological disorder. Nonetheless, there are still no scientific evidences of their involvement in this specific agronomical problem. What seems to increase the development of this problem is a wrong irrigation management combined with an incorrect preparation of the seedbed. In particular, an alternation of abundance of water (over-irrigation) followed by a dry period, fosters the development of a not uniform growing of the different tissues of the plant. To minimize this problem attention must be paid to seedbed preparation, with application of a uniform timing and distribution irrigation and avoiding to use high doses of nitrogen fertilizer.

ii. FREEZE DAMAGE

This alteration can be noticed after a prolonged cold period below freezing point, which can occur in the field or during storage. Main symptoms are translucent tissue: in general the outside scales of bulb lose the cellular integrity. Often damaged scales show a grayish yellow color while inner scale, not damaged by cold since they are protected from the external ones, have a normal aspect. Even if a part of bulb is intact, it cannot be marketable. The conditions that promote this problem are low temperature, in particular when it drops below -2°C.


Longitudinal-section of a bulb showing water-soaking of freeze-damaged tissue.

Moreover, repeatedly changing in the temperature, from frozen to defrost situation, increase the probability that bulbs are affected by this problem. The damages caused by low temperature are minor when bulbs are under the ground compared to bulb placed on the soil surface. The appropriate agronomic practice to limit this cold injury is to harvest the bulb in the correct period, avoiding leaving them in the ground. It should be mentioned that onion is characterized by a great variation for tolerance to freezing temperature: this because the development of cold injuries are inversely related to the amount of dry matter present in the bulb, therefore a high percentage of dry matter lowers the probability of cold injuries.

Cross-section of a bulb showing water-soaking of freeze-damaged tissue.

iii. GREENING

This phenomenon is observed when in the outer scales of bulbs chlorophyll is produced.

Normally, the main case where this disorder is observed is when bulbs are left on the soil after harvesting for a prolonged period, allowing the sun light to promote the development of chlorophyll. Also, an excessive or late season nitrogen applications can delay maturity of the crop and increase the appearance of greening. To avoid this physiological problem it is necessary not supplying plants a late and excessive nitrogen fertilization. In addition it’s possible to leave bulbs dug on the ground in order to dry them maximum 5-7 days (curing).

Green outer bulb scales.

iv. HERBICIDE INJURY

Herbicides are chemical compounds used to control and eliminate unwanted weeds in agriculture. Many kinds of herbicides exist, from those that mimic natural plant hormones (for example the auxin 2,4-D) to those

Leaf tissue yellowing caused by glyphosate.

that contrast the growth of plants. For this reason they may be chosen for their selective action against specific plants in order to leave the desired crop relatively unharmed. In any case, a wrong dosage or a wrong application timing of herbicide at the unfavorable stage of plant growth could cause several problems. For example, a use of a wrong product could cause chlorotic or necrotic spots while, in some other cases, will produce leaf curling. Moreover, the drift problem due to wind during spray application at the boundary between two fields with different crops should be considered. To avoid or limit these kinds of injuries attention should be paid to the choice of the chemical and its dosage, together with the weather conditions during treatments.

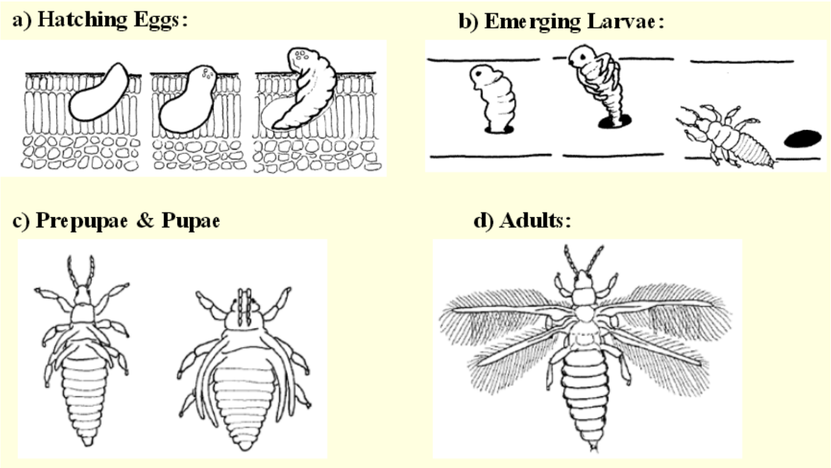
Leaf lesions and leaf curling caused by bromoxynil and oxyfluorfen.

v. THRIPS DAMAGE

Thrips are small insects widely present worldwide and feed on a large variety of plants sucking up the contents with their piercing-sucking buccal apparatus. Even if they have wings, they are not good flyers, nevertheless they can reach distant places when transported by wind. The speed of their life cycle is linked to environmental conditions, including the temperature and nutrient quality of their food sources, so during hot and dry summers can exponentially increase in population size and form large swarms. It should be noticed that a thrip can reach adult stage from egg within two weeks.

The effects of their presence and food habits are particularly negative for plants with commercial value, because they cause a reduced quality of harvested products. In fact, besides causing direct damage to the plant for the simple act of feeding, they are also vectors for plant diseases, such as viruses. For this reason they are considered pest and strictly controlled with insecticides by farmers, in order to protect

their crops. They breed and protect themselves in adverse weather conditions (cold and rainy days) in the leaves at the pseudostem region of onion plant, while they are active during the warm hours of the day.



Symptoms revealing their presence in an onion field are small dark green spots on the onion leaf, which turn silver or white color with time. Leaves can collapse during water stress conditions, if this tissue is strongly affected by thrips bites and, in any case, plant will produce a small bulb because the photosynthetic apparatus is damaged, thus causing a reduced translocation of molecules produced by photosynthesis in the bulb.

Thrips live in bulbs, while larvae or pupae are present in leaf litter, soil or alternative hosts, as during wintertime. It is important to control thrip populations with different strategy managements, in order to avoid direct and indirect damages on the onion crop: moreover, weeds should be controlled, since they can be a source of thrips and diseases. Sprinklers for an overhead irrigation system is suggested, together with plowing deeply to bury plant debris and larvae/pupae of thrips, spray insecticides several times for a chemical control (even if it is better with low frequency to avoid developing a resistant population of insects).

4.1.3 Infectious diseases

a. Viral diseases

vi. IRIS YELLOW SPOT VIRUS

This important virus is transmitted by thrips, consequently its distribution is related to its vectors. The localization of the virus in diseased plants is around the lesions produced by thrips while feeding and it appears as an elongated and chlorotic area that can turn in white color, enlarging its surface.

Figure 5. Lodging of onion flower scapes infected with IYSV. (Photo by L.J. du Toit)

When more patches are present in a plant, they tend to join (coalesce), causing the collapsing of the diseased tissue. This aspect can compromise seed productions because of lodging of onion flower scapes.

It is possible to control indirectly this disease controlling thrips (which is its vector) and weeds (possible reservoir plants of virus) populations. Moreover, for the same reasons, all onion residues should be removed from the field, since they could offer an overwintering place for thrips. Finally, onion varieties have a different susceptibility to this insect; in particular, it was observed that cultivar characterized by a low level of wax on leaves combined to a

Figure 3. Iris yellow spot lesions: (A) leaf lesions on an onion bulb crop, (B) close-up of severe leaf lesions, and (C) lesions on the flower scapes of an onion-seed crop. Photo source: (A) G.Q. Pelter, WSU Extension; (B) and (C) L.J. du Toit, WSU Department of Plant Pathology.

light yellow-green color are less attractive than plants with a strong presence of wax and with a dark green color (Molenaar ND 1984; Oliveira AP de, Castellane PD 1996).

vii. ONION YELLOW YELLOW DWARF VIRUS

This potyvirus cause severe foliar symptoms like flattening, crinkling, twisting and bending over of the leaf. Plant infected in a juvenile stage remaind dwarfed and produce small bulbs, while during the reproductive stage its umbel is characterized by few and small flowers.

As many viruses, onion yellow dwarf virus is 104noculums104a by vector from plant to plant by the green peach aphid, *Myzus persicae*, and other aphids in a non persistent manner. It is possible to control this pathogen controlling its insect vectors, and using virus-free seeds and bulbs. Finally, it could be tried to reduce the diffusion of the virus roughing out infected plants.

b. Prokaryotic diseases

viii. BACTERIAL BLIGHT OF LEEK

Pseudomonas syringae pv. *Porri* causes the formation of elongated water soaked lesions of dark-green color in the terminal part of leaf, which turns orange to brown with surrounding chlorosis after several days. Epigeal parts of a plant could be severely compromised, when a lesion extends into sheath causing leaf curls, splits and wilts. In fact, infected plants are misshapen and undersized. The environmental conditions promoting this disease are warm temperature and high humidity, so after the winter period where the bacteria remain latent in a plant, seeds and plant debris, they back to grow and produce symptoms in a host during the favorable season. To limit the problem, only clean seeds should be used, an excessive irrigation during the growing season should be avoided and all infected plants, together

Elongate orange to brown lesions with surrounding chlorosis.

with plant debris, should be removed in order to decrease 105noculums in the field. Finally, the pH of soil might be lowered with appropriate fertilizers, in order to hamper bacteria development.

ix. BACTERIAL SOFT ROT


Soft rot developing late in the season in two bulbs.

The causal agents of this disease are *Dickeya chrysanthemi* and/or *Pectobacterium carotovorum* ssp. *Carotovorum*, which affect mainly mature bulbs. Both bacteria cause rot of scales but with *D. chrysanthemi* scales appear water-soaked and pale yellow, while bleached gray to white when infected with *P. c.* ssp. *Carotovorum*. During the disease evolution, healthy scales become soft and sticky while and in the course of time bulb collapse. In fact, if bulb is squeeze, foul-smelling thick liquid can escape from neck.

As often happens, also in this case the main source of these bacteria are plant debris present in the soil, while the weather conditions suitable to its spread are high humidity and temperature and can be disseminated in the environment by insect, irrigation system and splashing water. They are able to enter in their host only through wounds. Not surprisingly, this disease is particularly prevalent during rainy seasons.

However these bacteria produce damages more frequently in storage, where they can cause the bacterial soft rot even at lower temperatures. Because these *Enterobacteriales* might be water saprophytes, frequent irrigation should be avoided. Moreover, care should be taken during the bulb harvesting to avoid any kind of bulb damage and store bulbs in a ventilated room to dry the environment and avoid moisture condensation.

x. SOUR SKIN

The main symptoms can be observed starting from stage of 2-3 leaves, because changed color turning in a light brown and can be easily removed from rest of the plant. Moreover, a watery rot develops in the pseudostem region and proceed in the neck direction. Finally, *Burkholderia cepacia*, the bacterial causal agent, will infect the outer bulb scales. Scales affected by bacterium have a slimy pale yellow to light brown. What makes the Sour Skin disease particular is an acrid and vinegar-like odor due to secondary invaders, like yeast.

The favorable conditions promoting this disease are high humidity in the soil, normally present in rainy season or period, or when there is an overhead irrigation. Infection begins through a wound present in a plant, or when the pathogen enters inside the bulb with water (rain or irrigation) through the neck, because basal part of the leaves (axil region) is not so close to rest of plant. The weather conditions favorable for the development of the Sour Skin disease are high humidity and temperature. To limit development and diffusion of this bacterium should be better use furrow irrigation, like drip systems, instead of overhead irrigation, like sprinkler systems, in order to avoid that water enter inside the bulbs through the necks. Leaf wounds before the harvesting of the bulbs should be avoided and storage is best in ventilated and cool rooms.


Sour skin of onion

Sour skin of onion caused by *Burkholderia cepacia*. Soft rot symptoms are observed on onion bulbs collected from a field near Walla Walla, WA.

c. Oomycetes and fungi

xi. DOWNY MILDEW

It is a fungal disease caused by *Peronospora destructor* and is favored by a high content of humidity of the atmosphere. Consequently, it is frequent to observe severe infections in rainy season periods and/or in environment with high humidity. In particular, the conditions for disease development of downy mildew are that the infected tissues have been covered with a water film for at least 4 hour with a relative low 106noculums106a (10-13°C). Usually the infection and sporulation occur during the night, since the absence of the sun promotes the presence of moisture. The *Peronospora* grows internally and continues to produce spores as long as weather remains cool and wet. The incubation period ranges from 9 to 16 days, at the end of which the conidia appear on the surface of the leaves. Normally, the infection will appear in small patches in the field and then, if not properly controlled, rapidly spreads to all plants. First symptoms appear on well-developed leaves with a flat, distinct, discolored area of elongated shape and with variable dimension. These alterations are characterized by white and/or gray-greenish color and in presence of high humidity they produce a mold fluff, which is the conidia during the sporulation period, with brownish-purple color. Conversely, agamic fructification occurs within the tissues of the host plant. These lesions progressively enlarge, and later a browning necrosis appears. Finally the infected leaves collapse and often they are invaded by other fungi, like *Stemphylium* or *Alternaria* species. Same physiological alteration can appear on seed stalk compromising the seed production and causing a high economic loss.

The control strategies to limit the spread of the disease and/or its effects make use exclusively of chemical compound (fungicide) through a regular spray program based on climatic conditions. The control strategies with chemical compounds are connected with several specific problem, due to two plant features of onion: leaves erectness and high content in wax on leave surface. These last aspects complicate the efficacy of treatment in that the aqueous solution containing the fungicide tends to slide quickly on leaves, leaving the plant tissue without protection. New chemical compounds characterized by a high resistance to water washout are appearing on the market to be more effective against this pathogen.

xii. BOTRYTIS

It is another genus of fungi infecting onions. Normally, in our rural environments, *B. cinerea* and *B. squamosa* are present, while *B. allii* is rare. The first of list, *B. cinerea*, is the causal agent of a disease known as **Botrytis Brown Stain**. It is characterized by producing of brown patches on the outer scales and neck of bulb during storage period. Generally, it is not able to infecting healthy leaf tissue, but when it happens, spores germinate on leaf surface producing superficial flecking. The environmental conditions promoting the disease development are a relative high moisture percentage combined with a temperature of approximately 21°C. During storage period, browning patch may decrease because the environmental conditions are not favorable for development of this fungus.

As many fungi, *B. cinrea* may survive as sclerotia, which are thick and dark cells with poor quantity of water which can live for long time in environment extremely unfavorable, in vegetable debris. An easy system to control the 108noculums source of this pathogen is to destroy any residual part of plant present in field before plowing a field to avoid burying vegetable debris. During the vegetative phase a fungicide spray program should be enough to control its development.

The casual agent of **Botrytis Leaf Blight** is *B. squamosa* and, conversely to *B. cinerea*, its main target is leaves. The main symptoms visible on infected plants are small white macules surrounded by a greenish halo, and often in the center of the patch is present a small brownish area which complicates the distinction from other injuries produced by insects, chemical compound and mechanical damage. As sclerotia the pathogen can live in the soil during the winter, while as fungus can over-winter in infected plant material. When the weather situation turns in favorable conditions, and it is moist and temperature increases, fungus and sclerotia produce fungal spores, that are dispersed in the environment and disease will spread rapidly. To control *B. squamosa* all plant debris must be destroyed to reduce the sources of 108noculums and a good preventive fungicide spray program must be applied too. Finally, good cultural practices should be followed, such as crop rotation, in order to decrease sclerotia in the soil.

Botrytis allii is responsible of **neck rot** and its distribution is worldwide. It is a bulb disease, consequently is rare to see its symptoms during the vegetative stage of the crop. The part of bulb invaded from this fungus is the neck, even if it can enter in another part of the plant through wounds. The infected tissues turn into a brown color and are water-soaked, prior to become soft and spongy. In some cases, a gray mycelium can grow on outer scales around the neck. Also this pathogen is favored by high humidity rate with moderate temperature: in fact. With prolonged wet conditions, *B. alli* produces spores from plant debris or sclerotia present in the soil. Subsequently wind will spread spores to other plants, which will be infected if they have some wounds or cuts. Same bad conditions listed for the field, high humidity and temperature, promote the development of neck rot in storage too. Transplanted plants followed by rainy period can increase the susceptibility to this pathogen. There are many practices that can reduce the presence of this pathogen in a growing area: for instance for a good management of this disease, it is important to avoid excessive much irrigation, use specific varieties adapted to the growing area to avoid harvest bulbs too late. For the same reason, fertilizers should be avoided since they promote the growing stage and cause a late maturity. Spray fungicide treatments at the right time can mitigate disease symptoms during the storage, destroy all plant debris combined with crop rotation it is necessary. Finally, deep plow fields is suggest, burying deeply all sclerotia.

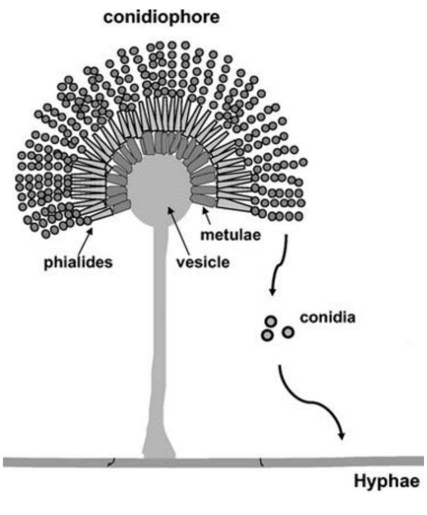
d. Fusariosis of onion

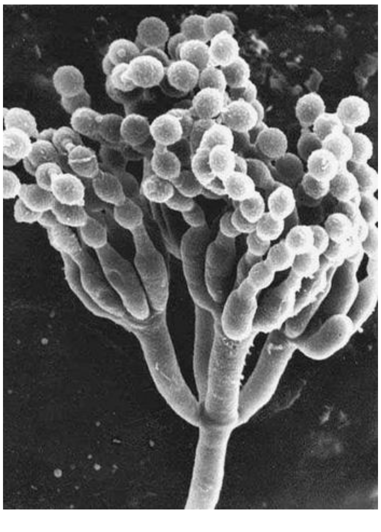
xiii. FUSARIUM OXYSPORUM

The isolation and characterization of *Fusarium oxysporum* dates back to 1824 by the German botanist DFL von Schjetendahl. *Fusarium oxysporum* is the species belonging to the genus more widespread in nature, with a distribution that ranges from the Arctic (Kommedahl et al., 1988), tropical and desert areas (Mandeel et al., 1995), soils virgins of the cultivated areas
(McMullen and Stack, 1983).

In addition, it is present in many different habitats. This species is characterized by a wide variety of biotypes with different characteristics: all are saprophytes, many are endophytes (are able to invade extensively some plant tissues without inducing any specific symptom), and some are plant pathogens of vegetable crops such as cucumber, tomato, onion, but also cereals and ornamental plants and spontaneous (Leslie and Summerell, 2006).

Many *F. oxysporum* strains appear to be host specific and this characteristic has been used to classify them in different special forms (*formae 109noculum*) and physiological races, depending on the specialization and the degree of aggressiveness that they have developed against particular plant species and cultivars (Snyder and Hansen , 1940, Armstrong and Armstrong, 1981). To date, more than 100 *formae 109noculum* have been described (Booth, 1971, Leslie and Summerell, 2006). The complex genetic relationships among isolates belonging to different pathotypes are not





yet clear. Moreover, it is not possible to distinguish strains on the basis of morphological and genetic basis or based on vegetative compatibility (Nogales Moncada et al., 2009).

The high intraspecific variability, as well as physiologically and genetically, is also evident in the morphology and pigmentation of the colonies, which may take a very different aspect depending on the culture medium used. The genus *Fusarium* encloses filamentous fungi and belongs to the *Ascomycota* phylum, *Ascomycetes* class, *Hypocreales* order. Over twenty species belong to this genus; some of these are known to be saprophytes and others to be important plant pathogens (deHoog et al.2000). Among the pathogens, *Fusarium oxysporum* is the cause of diseases and economic losses on numerous crops. Generally, *Fusarium* produces three different types of spore (macroconidia, microconidia and chlamydospores), that differ for shape, dimension and function.

All conidia are placed at the ends of elongated structures called phialides, which are the terminal part of coniodiophore. Macroconidia cells, the most important because they define the genus, are characterized by having a sickle-shaped form with some internal septa (3-5) while microconidia, smaller in size, have a shape from oval to ellipsoidal and could be aseptate or to be 1 septate. Finally, chlamydospores are resistant structures characterized by a thick cell envelope, which encompass protoplasm with high lipid content.

Interestingly, this large genus is formed by a large number of species and many of which are harmless and saprobes. In the taxonomical classification of *F. oxysporum*, the main problem was to differentiate the similar strains with analogue morphological traits belonging to same genus. After several attempts, plant pathologists decided to separate them for their ability to interact with host in a specific manner. This approach allowed obtaining many "*formae 110noculum*", even if not always this is host specific, because they have a broad host range. Recently, application of molecular techniques has improved and better defined the classification of these species.

However, the main problem for the taxonomic classification of *F. oxysporum*, was to differentiate among strains with analogue morphological traits belonging to same species. After long debating, plant pathologists agreed to classify them according their ability to interact with a specific host. Despite the name, *forma specialis* do not always corresponds to a specific plant host, and multiple hosts have been reported for different *Fusarium oxysporum*.

All isolates of *F. oxysporum* species are able to survive for long periods as saprophyte on organic material in the soil and in the rhizosphere of many plant hosts (Garrett, 1970). *F. oxysporum* is one of the most important species for plant pathology and for its economic impact in many commercial crops. The various special forms are in fact responsible for vascular disease (Fusarium wilt) and parenchymal rot in plants (O'Donnell et al., 2004).

Plant host range includes tomato (Jones et al. , 1991) , lettuce (Pasquali et al. , 2003) , melon (Wechter et al. , 1995) , banana (Nel et al. , 2006) , as well as various ornamental plants such as carnation (Cercone , 2005), daisy, lisianthus, gerbera, *Hebe* spp. , water lily and dimorfoteca (Gullino and Garibaldi, 2007).

Under the name of tracheomycosis we have diseases characterized by the prevailing location of the fungal pathogen in the plant vascular system (Frasconi , 2006). The typical symptoms of the disease include stunting, wilting, chlorosis, vascular discoloration, necrosis and death of the plant. These events can occur simultaneously or in succession, and prevail over each other depending on the host, pathotype, and environmental conditions. *F. oxysporum* may affect plants both in protected cultivation and in open field, at any stage and in any vegetative 111noculums111al stage, although early infections cause the most significant economic damage .

Fusarium basal rot of onion, caused by *Fusarium oxysporum* f. sp. *cepae* (FOC), is one of the most important soilborne disease of onion.

Infection and symptoms – *Fusarium oxysporum* f. sp. cepae, overwinters as spores or mycelia on crop residue. Optimal temperature for growth and infection ranges from 25 to 30°C, Fusarium can infect onion at all stages of its life cycle. It can bea primary or secondary invader and it can cause damping-off of seedling, root rot in a plant and basal rot of bulbs.

Fusarium during onion infection, when acting as primary invader, breaks down the cell wall of the onion root with its pectolitic enzymes to invade the apoplast of this tissue. Subsequently, hyphae penetrate the cells to invade the vascular system of the roots to reach the stem plate area. Once in the stem plate, if the conditions are favorable, infection will spread to the rest of bulb, causing changing of the color of the scale, followed by softening and putrefaction of the bulb.

During its vegetative stage *Fusarium oxysporum* can colonize the plant producing leaf symptoms, such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system and stunting. The mode of action of this pathogen is to colonize the vascular ways of the plant occluding them overtime. The occlusion blocks the proper transport of water and nutrients from the roots to the leaves, the host will die for lack of nourishment.

Distribution and spread – The geographical distribution of this pathogen is worldwide. *Fusarium oxysporum* f. sp. *cepae* and its negative effects have been reported in many locations of the world: from Arizona (Marlatt, 1958), where it was observed basal rot in onion bulbs, to Japan (Takakuwa et al., 1977) and Israel (Joffe et al., 1972) where several isolates were detected. The symptoms of *Fusarium* were detected also in Zambia (Naik and Burden, 1981), Italy (Fantino and Schiavi, 1987), Turkey (Koycu and Özer, 1997), Finland (Tahvonen, 1981), Nigeria (Wilcox and Balogh, 1998). Moreover, Brayford (1996), described *Fusarium* also in Egypt, Ethiopia, South Africa, India, Philippines, Greece, Hungary, USA, Brazil and Australia.
Usually, the dissemination of the pathogen occurs through the spores present in the seed of diseased plant, in agricultural equipments and contaminated tissues.

Alternative hosts – As mentioned above, many *formae 112noculum* of *Fusarium oxysporum* are not host specific. In fact, *Fusarium oxysporum* f. sp. *cepae* is able to infect a large spectrum of plants, like *A. oschaninii* (shallots), *A. sativum* (garlic), *A. fistolosum* (Japanese bunching onion) and *A. chinense* (rakkyo). Other also different crops can be infected by *Fusarium oxysporum* f. sp. *cepae*, like *Zea mays* (Corn), *Triticum aestivum* (wheat), *Oryza sativa* (rice), *Glycine max* (soybean), *Cucumis sativus* (cucumber), *Pisum sativum* (pea) and *Cucurbita maxima* (squash) (Cramer, 2000).

Moreover, it was observed that when *Fusarium* infects a host, symptoms are not always displayed and detectable. Other studies confirmed that *Fusarium oxysporum* acts as a primary pathogen on a crop and a secondary pathogen on others.

Interestingly, there are common weeds, worldwide spread in agricultural areas, which are symptomless when hosting *Fusarium oxysporum* f. sp. *cepae*. Different studies were carried out in USA by Abawi and Lorbeer (1972) and in South Africa by Holz and Knox-Davies (1976). In both research studies, the fungus was isolated from wild healthy plants of *Oxalis* spp. For this reason it is fundamental to control the weed populations in onion fields, considering also that this crop is a poor competitor for water and nutrients.

Control strategies – None of the approaches used for the control of this onion pathogen has been fully effective, so it is important to limit or avoid any contact between onions and *Fusarium oxysporum*. Several options are available for growers to manage the disease. In particular, they may opt among conventional chemical control, biological control, soil solarization, breeding programs and integrated management practices.

Chemical control – The use of chemical fungicide is effective against the soil-borne and seed-borne pathogens. In addition, chemicals can control population of soil insects, improving the germination, prevent the spread of other plant diseases.

Recently, it is common practice to apply chemical treatments to outer surface of onion seeds in order to protect seedling at the first stage of life and to prevent the development of soil-borne diseases. Usually, the active ingredients used for these treatments are a mixture of benomyl (benzimidazole) and mancozeb (a complex of Zinc and Manganese salts fungicides), or thiram (dimethyl dithiocarbamate). Treatments can be applied to seed, and, in several countries also to small bulbs (set onions). Treatments are carried out dipping bulbs in the antifungal solution for 15 min. This treatment increases yields by over 50% compared to the untreated bulbs. Unfortunately, negative effects on the beneficial interactions between mycorrhizal fungi and plants have been reported.

Biological control – Overtime, the massive use of chemical products in modern agriculture caused many environmental problems and risks for human health. Consequently, new approaches and agronomic practices have been evaluated. Among the different organic strategy two are particularly successful to control *Fusarium* infection: use of antagonistic organisms and soil solarization.

According to Baker and Cook definition (1974) biological control is promoting the reduction in natural way of the 114noculums or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man. In the last decades, several antagonistic interactions among *Fusarium* and other organisms have been studied. Kawamoto and Lorbeer (1976) observed that the bacterium *Burkholderia cepacia* (casual agent of "sour skin" in onion bulbs) protects onion seedlings from damage by *F. oxysporum*. As well, a research conducted in Iran, demonstrated that *Bacillus* species are antagonist to F*. oxisporum* on onion (Tehrani and Riseh, 2004). Other well known antagonists of *F. oxysporum* are *Trichoderma* spp,. In particular *Trichoderma virens*, suppress the growing of *F. oxisporum*. Furthermore, *Fusarium* is not a good competitor compared to natural soil microbiota (Abawi and Lorbeer, 1972).

Soil solarization, is performed during the summer. Plowed field, cleaned from plant debris, is covered with a polyethylene film for several weeks in the in order to increase the temperature of the soil and kill the soil borne pathogens. Thanks to this simple practice, the temperature can increase up to 50-60°C (Hartz et al, 1989), killing pathogens. For example, this technique significantly reduced *F. oxisporum* populationin Israel (Katan et al, 1980). In other experimental trials some *F. oxisporum* strains showed a tolerance to high temperature (50°C) for long time (6 hours) (Porter and Merriman, 1983). Finally, even if this technique shows effective results against pathogen only in the superficial layers of the field, these positive effects can last for more than one year. Soil solarization is particularly important where the crop rotation and use of fumigants for the soil sterilization are not do able choices.

Breeding approach – Breeding programs aim to create resistant cultivars so to minimize the negative effects of pathogens on a crop, and consequently reduce the impact chemical products on the environment and human health. Usually, obtaining a new variety is a long and complex process, and for a biennial plant breeding programs require even longer time before release a new variety.

Seed companies around the world are developing several breeding programs in order to release on the market new material with this characteristic. The first step in a breeding program is to obtain powerful screening methods to investigate the physiological behavior of plants when interacting with the pathogen. In the case of onion, different approaches have been applied to evaluate, in greenhouse and open field, the behavior the plant material at all stages (seedling, old plant and bulb). Screening conducted in open field are more reliable, but they required longer times compared to screenings performed in controlled environment like greenhouse or growth chamber.

In fact, Guiterrez and Cramer (2005), spent two years to test and evaluate 20 different local cultivars for resistance to FOC in the field. Conversely, it is possible to halve the time of screening when operating in controlled conditions. In fact, operator has the ability to manage the FOC 115noculums to infest the soil and to control the main environmental parameters to facilitate the infection allowing proceeding faster in the breeding program. Moreover, these screening results correlated well with results from the field trials (Reting et al., 1970). In addition, , it is important to consider that a test carried out in controlled environment allows to obtain data with a small quantity of seed, since the probability that all the plants or seedling are infected by FOC is higher (Fantino and Schiavi, 1987).

After several FOC tests, researchers have formulated several hypothesis regarding how many possible genes are involved in this important trait in onion. Krueger *et al.* (1989) proposed an interaction model between nuclear genes with cytoplasm involved in their regulation. Authors suggested this theory because they observed a difference in the tolerance between the reciprocal hybrids. These results were concordant with the ones obtained by Tsutsui (1991) in subsequent study. On the contrary, Bacher *et al*. (1989) did not register any difference in FOC resistance between reciprocal F1 progeny. Cramer (2000) hypothesized that the resistance to FOC is determined by two partially dominant genes. Finally, Özer *et al*. (2003) observed that during the interaction FOC-onion the pathogen produced pectolytic enzymes while the host produced distinct antifungal compounds, consequently they suggested that the degree of infection was correlated with a different production of compounds by the two organisms.

4.2 Goals

To better study the interaction onion-*Fusarium* and the putative environment influence, a comparison between greenhouse and open field data were analyzed. *In vitro* (greenhouse) experiments showed that there is a correlation between the inoculum concentration of *Fusarium* and symptoms developed on the host. There is not the same evidence in natural systems, most likely because environmental conditions, like temperature and water, influence the pathogen infection. It has been reported how wounded plant or bulb facilitate the entry of the pathogen, consequently during agronomic practices and/or bulb processing in the storage phase should be avoid mechanical damages.

The main goal of this research is to study inheritance of tolerance to *Fusarium oxysporum* f. sp. *cepae* and evaluate its possible parental imprinting in onion crop. The data obtained will be crucial to onion breeders, with the purpose to create new varieties resistant to this pathogenic fungus, through developing and implementing of an efficiency breeding program.

4.3 Material and methods

4.3.1. Plant material

Plant materials used in physiopathological evaluation of onion behavior to *Fusarium oxysporum* f. sp. *cepae* (FOC) have been Double Haploid (DH) lines obtained from Open Pollinated (OP) population belonging to different typologies.

A. FOC test 2012

The material tested in our first test were six different cultivars: a Spanish type (yellow long day onion) with the code 30Y09-2/1 and five DH lines belonging to a Density type (Yellow long day onion) with the code 30Y20-3/14A, 30Y20-3/66, 30Y20-6/11, 30Y20-7/32 and 30Y20-7/49A, The lines listed above were chosen among those with higher availability of seeds. Moreover we used two commercial hybrid declared with a high resistance to *Fusarium*: Vaquero from NUNHEMS and CROCKETT from Bejo. The aim of their use it was to compare physiopatologycal behavior of our DH lines to important commercial hybrids

B. FOC test 2013 (May)

The cultivars used in this second test were thirteen: eleven DH lines (30Y09-2/1, 30Y20-3/43, 30Y20-3/44, 30Y20-3/66, 30Y20-3/69bis, 30Y20-7/28, 30Y20-7/32, 30Y20-7/38, 30Y20-7/43, 30Y20-7/49A and 30Y20-7/65. While controls were always the two commercial hybrids listed before: Vaquero and Crockett.

C. FOC test 2013 (November)

In this second test did in November we analyzed the best tolerant DH line (30Y09-2/1) and the more susceptible DH lines (30Y20-3/14A, 30Y20-3/66) identified in previous tests comparing them to F1/RF1 obtained by crossing them in our field activities:
30Y09-2/1 x 30Y20-3/14A (F1)
30Y20-3/14A x 30Y09-2/1 (RF1)
30Y09-2/1 x 30Y20-3/66 (F1)
30Y20-3/66 x 30Y09-2/1 (RF1)

Moreover, we decided to study also two new genotypes and their combinations with 30Y09-2/1:

30Y20-3/38 (DH)
30Y09-2/1 x 30Y20-3/38A (F1)
30Y20-3/38A x 30Y09-2/1 (RF1)
Fanter (a local diploid OP variety)
30Y09-2/1 x Fanter (F1)
Fanter x 30Y09-2/1 (RF1)
In this test we used jut CROCKETT hybrid as control

4.3.2. Fungal strain(s)

The highly pathogenic isolate of *Fusarium oxysporum* f. sp. *cepae* used to test the susceptible/resistance of all plant material studied in this PhD project was collected by sampling diseased onion in Romagna by a private laboratory during a surveillance activity I 2011. The inoculum code is FOC- 2P/2012. In preliminary experiments, this isolates was able to show his pathogenicity according standard tests on onion.

4.3.3. Growing chamber experiments

Evaluations assays under laboratory conditions were set up in order to analyze the phenotypic responses of plants in particular experimental treatments and evaluate the performance of different genotypes and/or combinations in response to an infection by the pathogenic fungus. All tests were performed in a growing chamber with controlled environment with temperature and day length set in a specific way. Lamps used for illumination of the chamber were neon L58W-77 G13 FLUORA produced by OSRAM and its light spectrum is reported below:

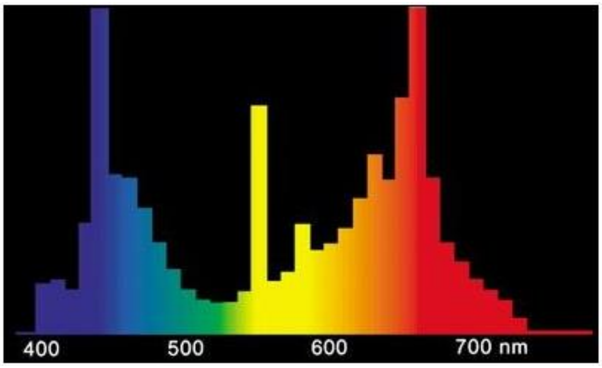
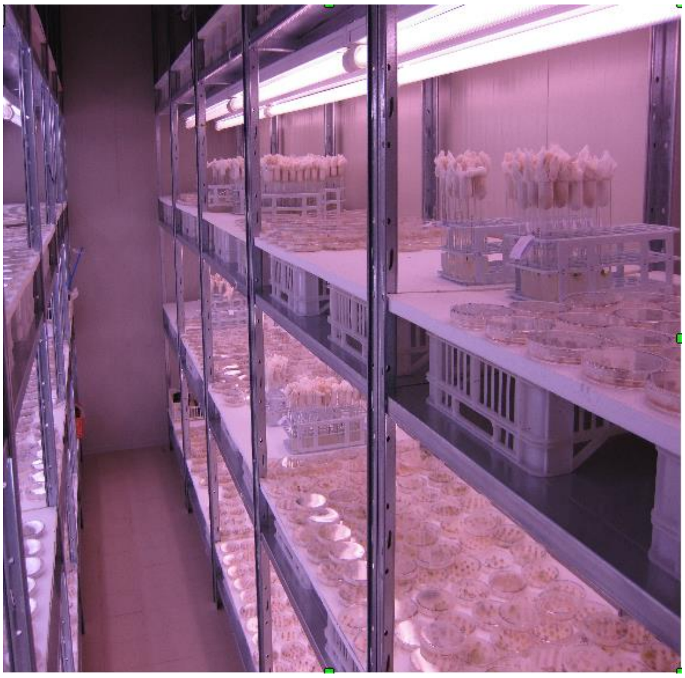


Fig 4.2. Light spectrum of the FLUORA lamp

One of the main peaks was around 680-700 nm, exactly one in each plant absorbs light. In fact photosystem I absorbs at a wavelength of 700 nm, while the photosystem II absorbs around 680 nm.



In each shelf 6 neon lamps were present laid out in 2 rows; seed trays with plants were placed to 30 cm from the lamps. The environment conditions inside the growing chamber during the experiments were a constant temperature of 28°C, with 8 hours of light and 16 hours of night.

Fig 4.1. Growing chamber

4.3.4. Inoculation and disease monitoring

The inoculation procedures followed during experiments are reported below.

A. Inoculum preparation

After receiving the inoculum of FOC by supplier, it was filtered with sterile gauze in order to eliminate most of the mycelium and obtain only the spores of the fungus. The following step was to determine the concentration of the inoculums using the Thoma Cell Counting Chamber. The inoculum was ready for experiments after to have adjusted concentration of the solution to 1 x 10^6 conidia per milliliter of sterile water.

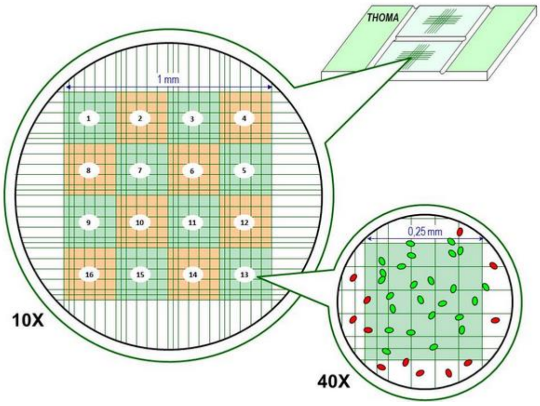


Fig 4.3. Schematic rappresentation of the Thoma cell

B. Plant preparation

After 55-60 days from sowing in a seed tray, plants were removed from tray and their root system was washed in water to remove the peat. Subsequently, with a scissor, a part of roots were cut off to create wounds and facilitate the penetration of the FOC inoculum into the plant, where it could initiate disease development. Needs to notice that one week before the inoculum, part of leaves of all plants were cut in order to reduce the water stress during the treatment and the following transplanting.

C. Plant inoculums and transplanting

After the cutting of roots, plants were immersed in the solution containing the *Fusarium* conidia and, some of them, just in water as control for 5 minutes.

Fig 4.4. Onion plants during pathogen inoculation

Finally, plants inoculated and controls were transplanted in another seed tray with 104 holes using fresh peat.

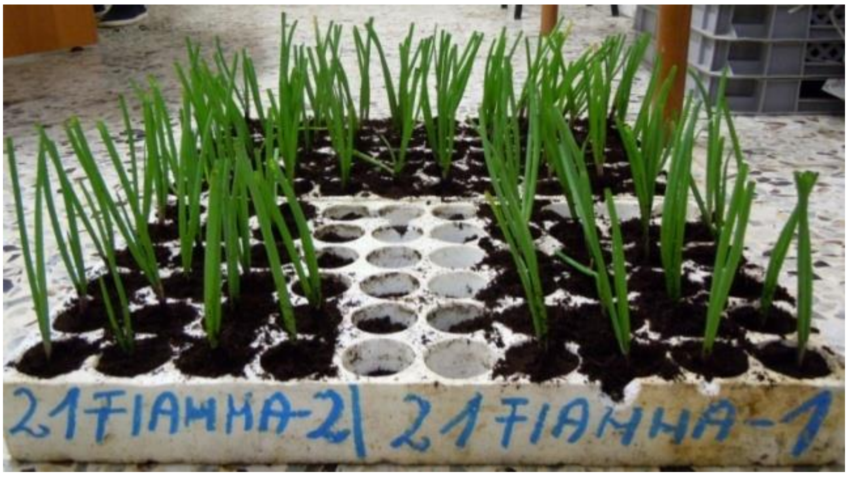


Fig 4.5. Onion plant transplanted after the inoculation step

Monitoring and evaluation of symptom development were done weekly.

For each test phytopathological test, a set of non inoculated plants was used as negative control.

4.3.5. Phytopathometry

In our physiopathological test we used young plant at the 3-4 leaves stage, in order to maximize the number of treated plants in the space available. Seeds availability for several DH lines analyzed was poor some time, forcing us to sow a small number of seeds or postponing their analysis after to have reproduced the seed.

Regarding the agronomical traits considered to evaluate the disease evolution, we made a visual assessment of the following plant parameters:

- Plant development (Plant Growing Rate)
- Leaf chlorosis
- Leaf senescence
- Mortality (incidence)

These specific agronomical traits were chosen because, as mentioned above, the typical symptoms of the disease include stunting, wilting, chlorosis, vascular discoloration, necrosis and death of the plant.

Measurements of the symptoms (disease severity) were made according a disease index scale, with values ranging from 1 (poor) to 5 (optimum). Values took into account following features: plant development, mortality rate, leaf chlorosis and senescence.

Disease index (DI) was calculated as follows:

$$\mathbf{DI} = \frac{[(\sum \text{ \% of death plants}) + (\sum \text{ \% Growing Rate}) + (\sum \text{ \% chlorotic area})]}{(\sum \text{ \% of death plants})}$$

Each assay lasted 3 to 4 week.

4.4 Results

Fungus inocula and methods adopted in this project produced a quantifiable disease incidence and severity in all tests, providing a useful screening system to evaluate the host tolerance to this pest.

For any experiment, first disease symptoms have started to appear after 7-10 days from the date from inoculation. The initial goal to identified tolerance difference among material obtained with gynogenesis it was reached because three DH lines show an intermediate resistance compared to two different commercial hybrids declared tolerant versus *Fusarium oxisporum*.

May 2012

The first test identified one DH line (30Y09-2/1 - Fig 4.6) with an excellent tolerance to the pathogen, especially if compared to commercial hybrids used as controls.

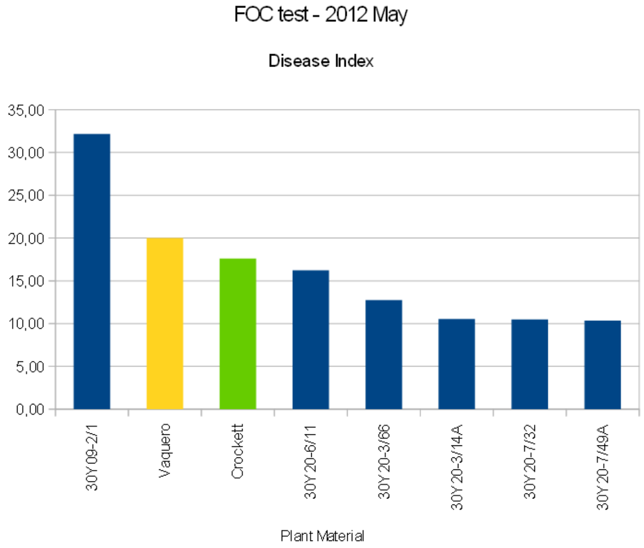


Fig 4.6.

Onion lines ordered according to their value DI obtained during the FOC test 2012 (May)

As it is possible to see in the graphic showing the mortality rate (Fig. 4.7), the tolerance trend of the 30Y09-2/1 was always better than other material during the whole duration of test. In the same studies three DH lines (30Y20-6/11, 30Y20-7/32 and 30Y20-7/49A) were identified as extremely susceptible to fungus useful to our general purpose to create a new population of plant crossing a tolerant with susceptible lines in order to evaluate maternal effect for this specific trait. It should be noted as the plants of the susceptible lines have collapsed quickly after inoculation, while those of the tolerance material show degeneration, from the point of view of the general metabolism, less sudden.

Finally, it was observed an evident correlation among mortality rate, plant growing rate and leaf chlorosis as expected.

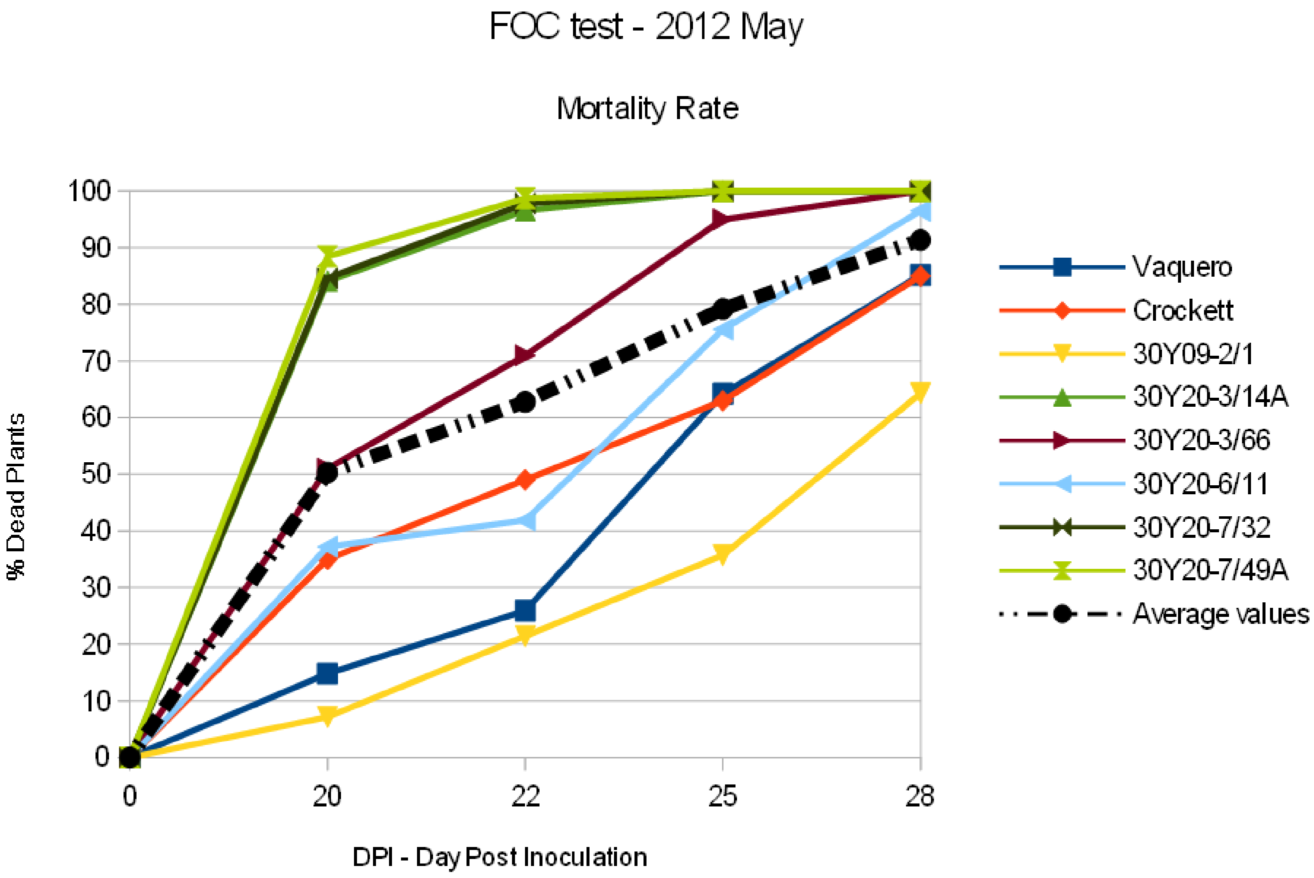


Fig 4.7. Plant Mortality Rate of all lines during the FOC test 2012 (May)

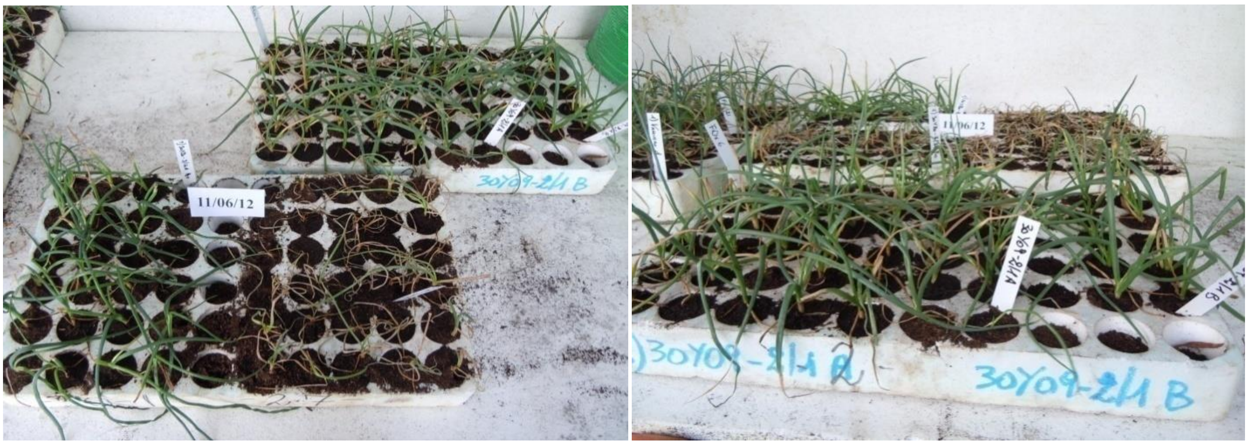


Fig 4.8. Onion plants of some lines after 20 DPI in FOC test 2012 (May)

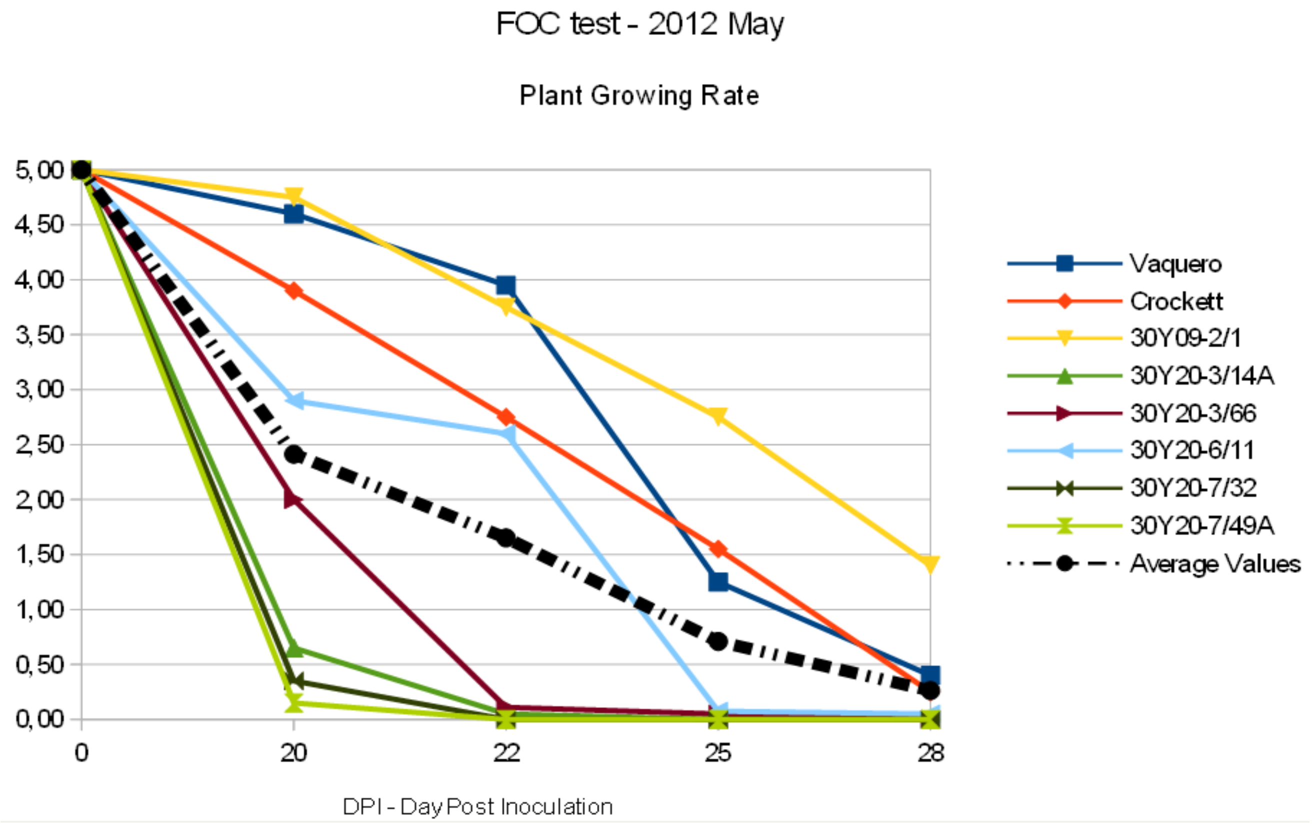


Fig 4.9. Plant Growing Rate of all lines during the FOC test 2012 (May)

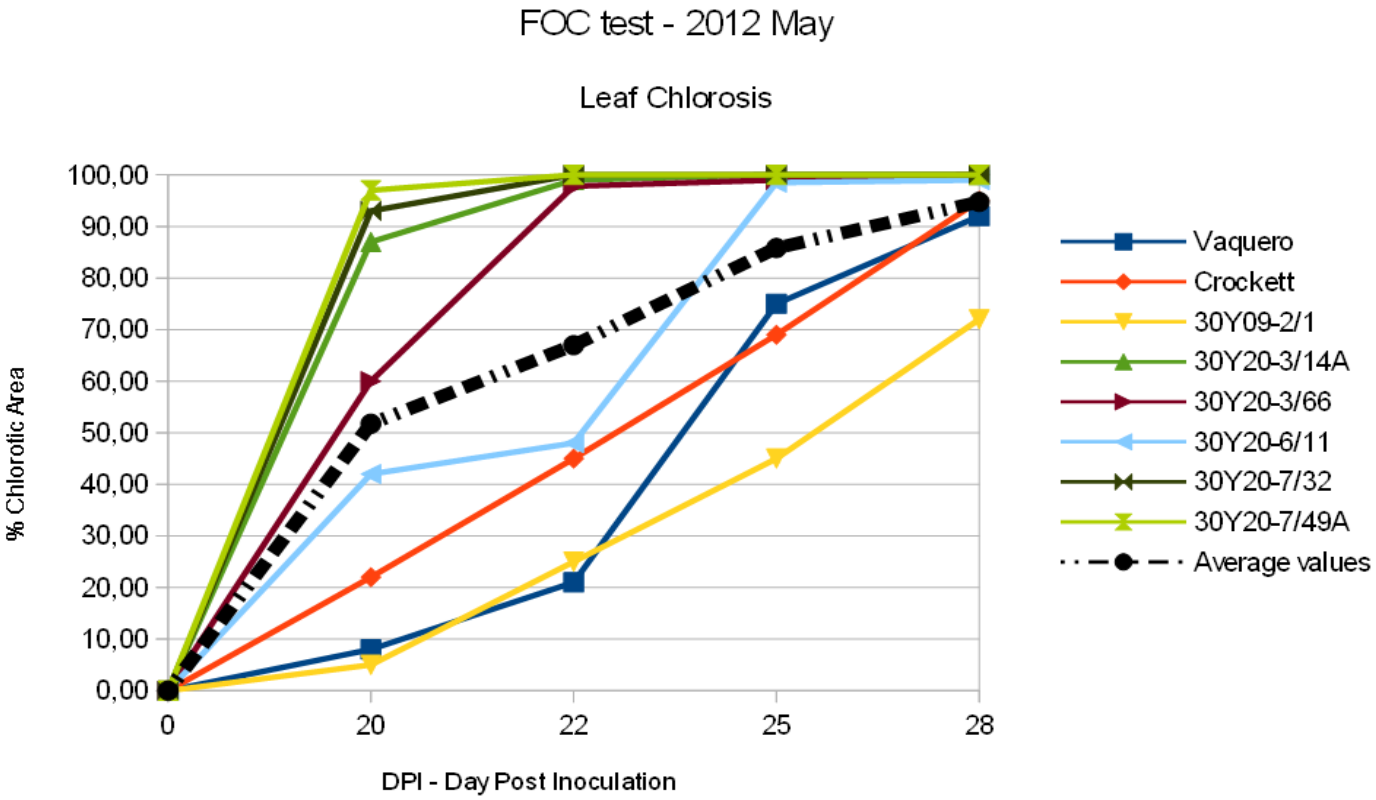


Fig 4.10. Evolution of Leaf Chlorosis in all lines during the FOC test 2012 (May)

After identifying the genotypes useful to the continuation of the project, it was set up some crosses among the best DH line 30Y09-2/1 and 30Y20-3/14A and 30Y20-3/66, using bulbs obtained from plant sowed in the field in the same period to those sowed for the physiopathological test. Moreover, in addition to crosses listed above, 30Y09-2/1 was crossed to a different DH line (30Y20-3/38A) and a diploid Open Pollinated variety to evaluate in a better way all possible genetic and epigenetic interactions.

May 2013

Meanwhile, the following year, a second physiopathological test was performed in order to evaluate again the major part of the same material tested before, besides to new DH lines thanks to the in vitro production of the first year of experiments. The results from the second test confirm what it has been seen in the previous test, namely that the DH line 30Y09-2/1 showed a good reaction to *Fusarium oxysporum* together to commercial hybrids and to two new DH lines. Also in this case there was a correlation among all agronomical traits evaluated, even if the plant behavior of each line showed a slightly difference in the general tolerance to pathogen. In particular, in this test the best genotype was Crockett F1 followed by a new DH line (30Y20-3/43), 30Y09-2/1, a new DH line (30Y20-3/43) and Vaquero F1.

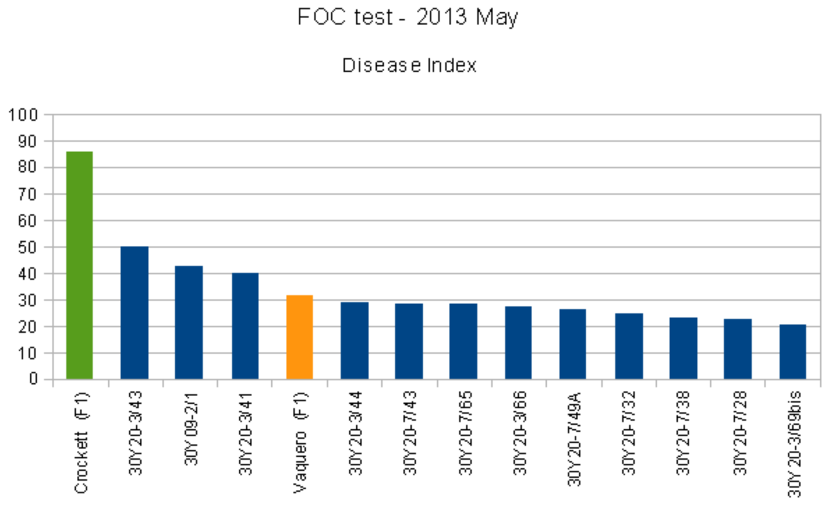


Fig 4.11. Onion lines ordered according to their value DI obtained during the FOC test 2013 (May)

Contrary to what is seen in the previous test, where the best line was 30Y09-2/1 followed by Vaquero and Crockett, now the order of the material and its tolerance to the fungus is changed, even if the inculum of pathogen and environment conditions were the same. The reason of this modification of their behavior is unclear, so it is difficult to make a hypothesis that can explain what it was observed.

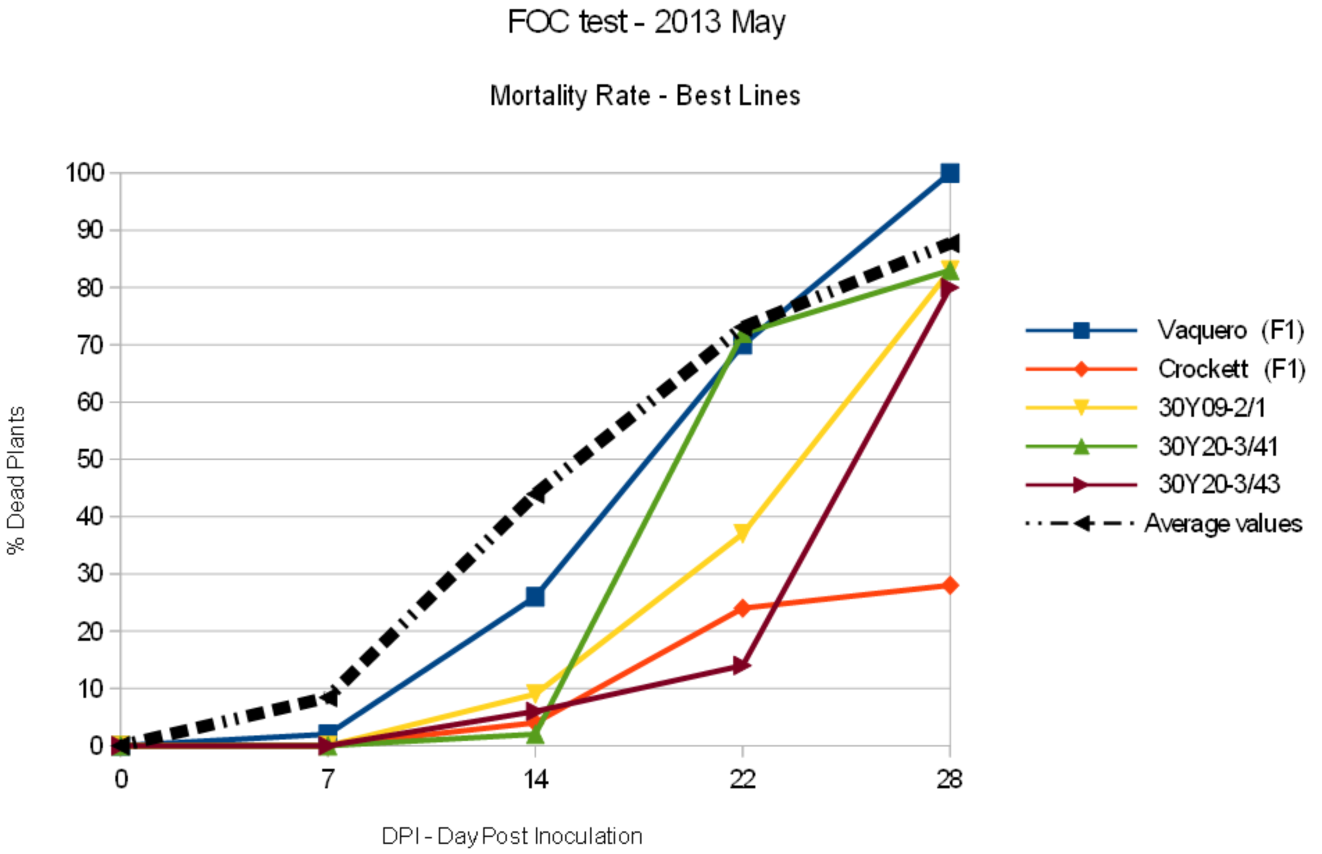


Fig 4.12. Plant Mortality Rate of all best lines during the FOC test 2013 (May)

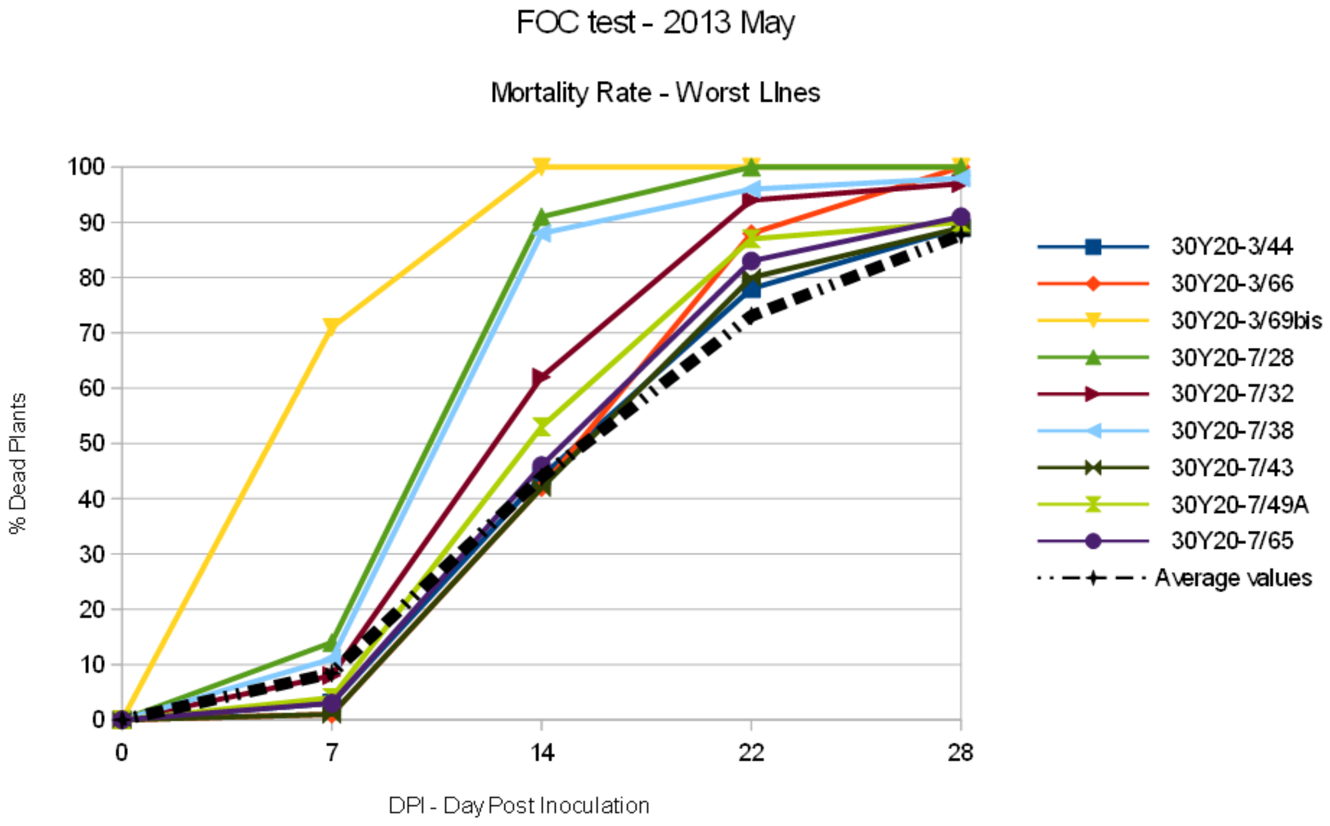


Fig 4.13. Plant Mortality Rate of all worst lines during the FOC test 2013 (May)

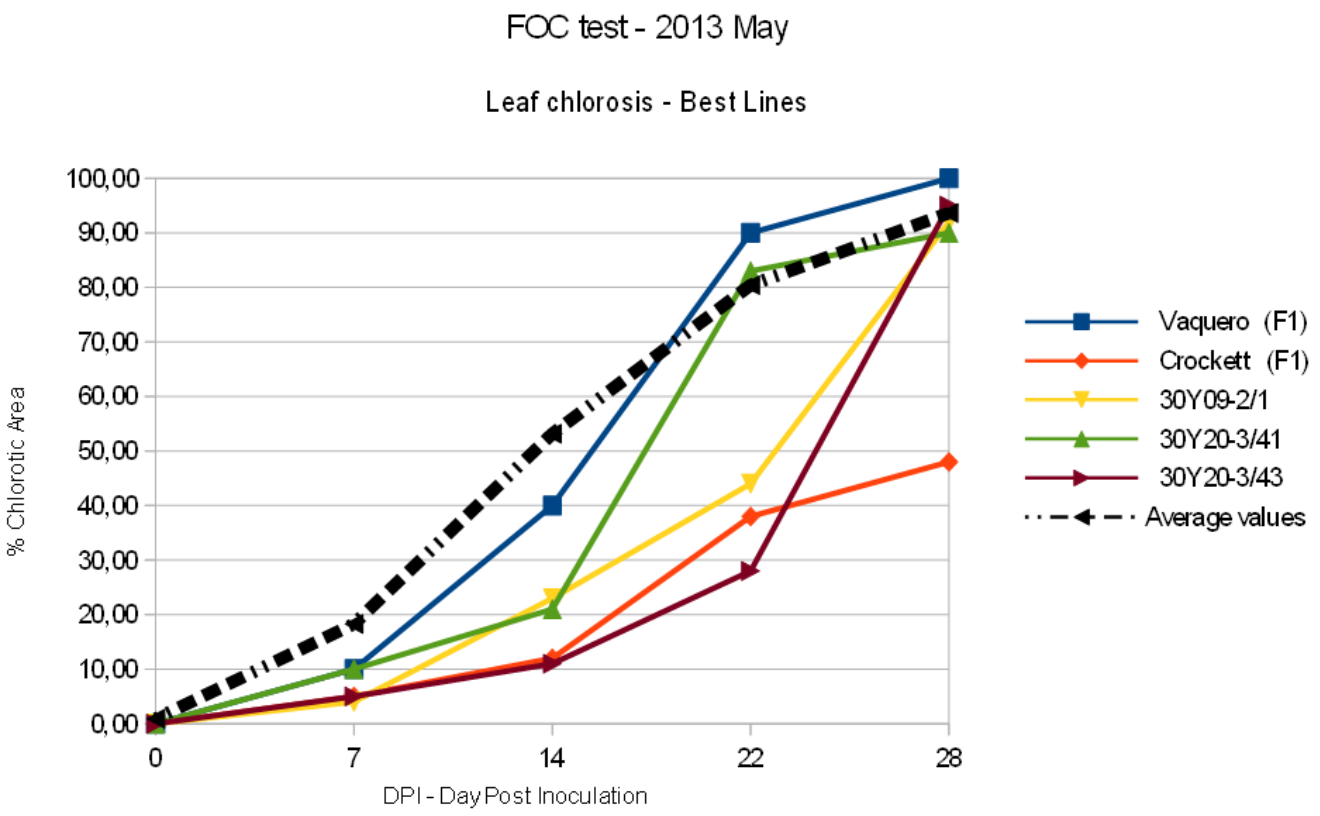


Fig 4.14. Evolution of leaf chlorosis in all best lines during the FOC test 2013 (May)

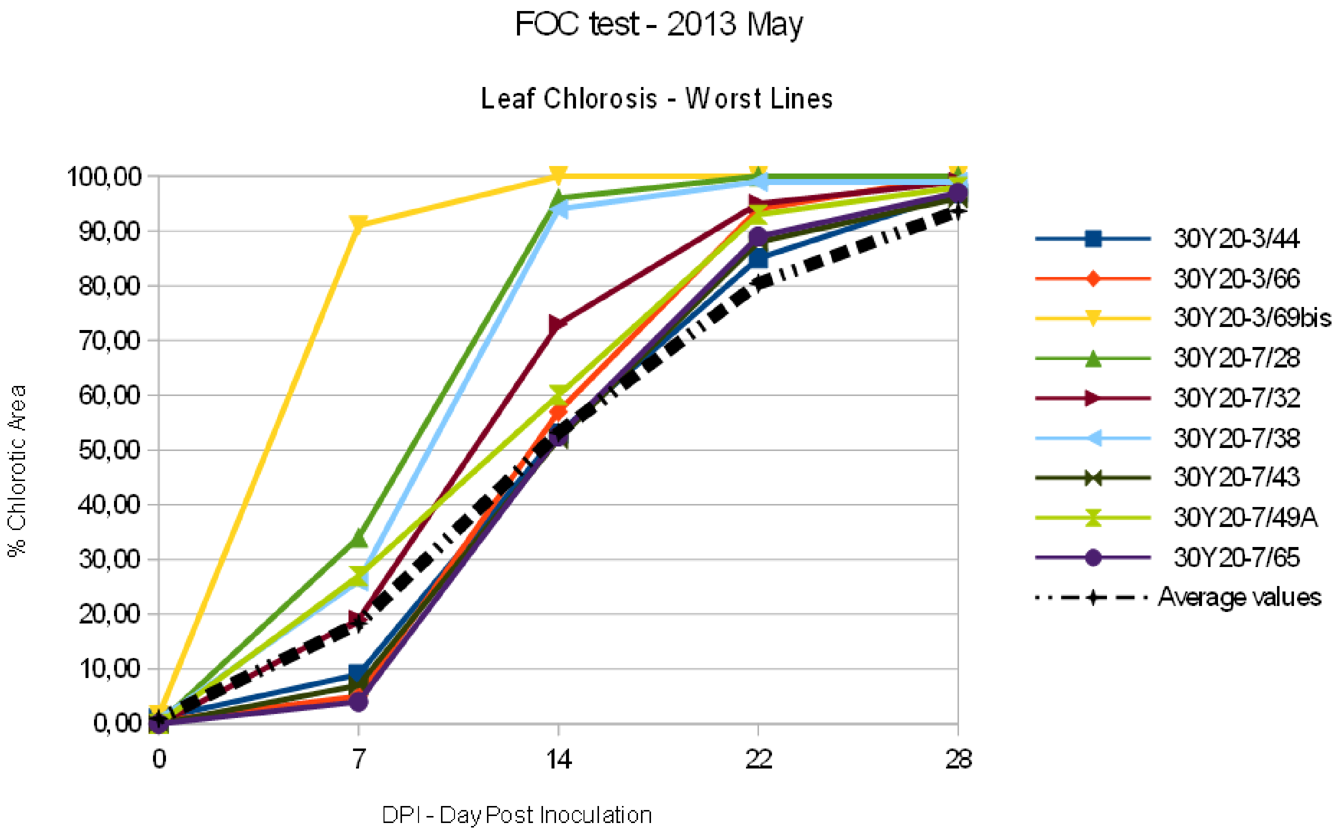


Fig 4.15. Evolution of leaf chlorosis in all worst lines during the FOC test 2013 (May)

Due to high number of DH lines evaluated, the different plant material was plotted for its disease tolerance and evolution in order to highlight the opposite reaction to pathogen. It is evident the difference between the two groups, susceptible and tolerance, in comparison to in comparison to the average values obtained from all the lines analyzed. All parameters showed how the difference between susceptible and tolerant begins to be consistent after just two weeks. In any case, any palnt material tested againt Fusarium was found totally resistant to this pathogen. The same result it was obtained in a similar study in wilt rocket (Gilardi et al., 2007).

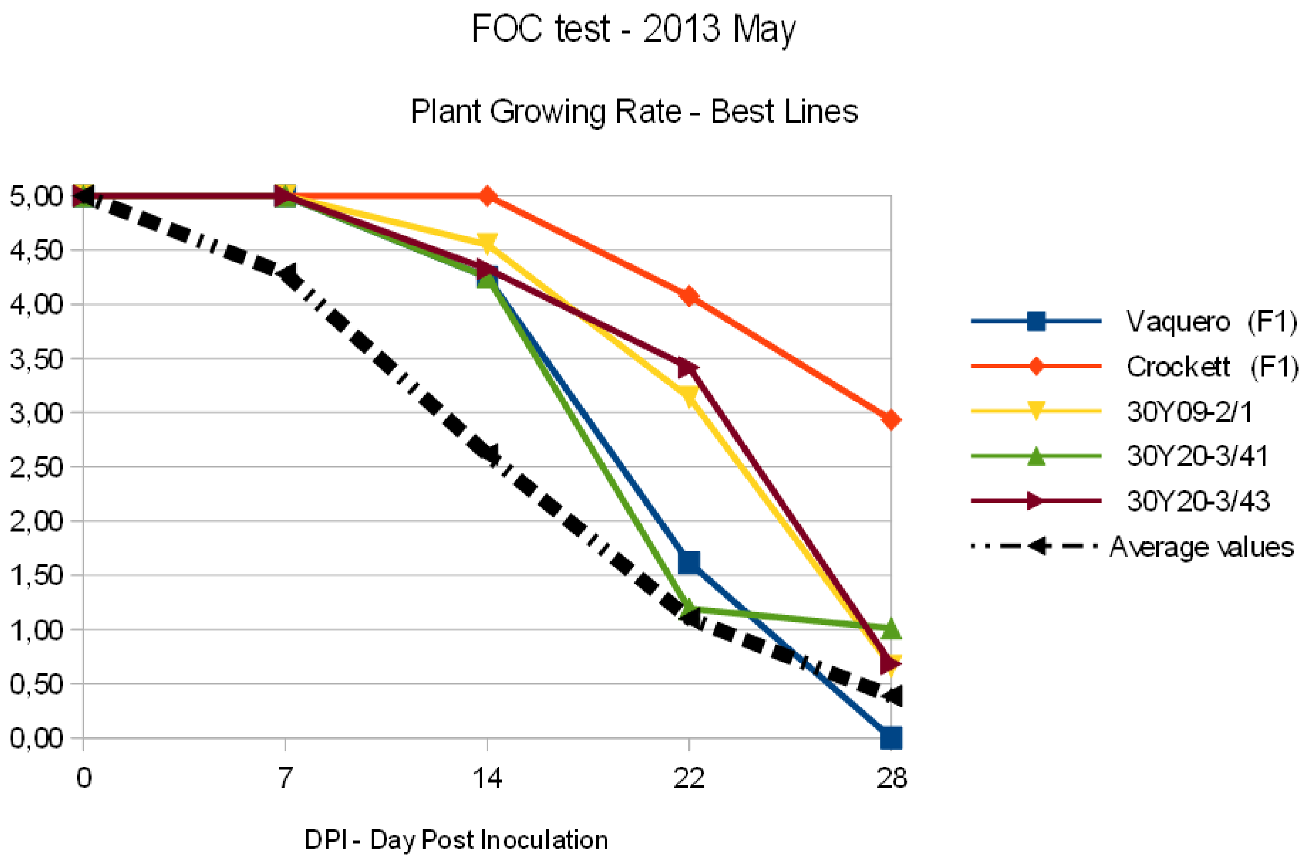


Fig 4.16. Evolution of plant growing rate in all best lines during the FOC test 2013 (May)

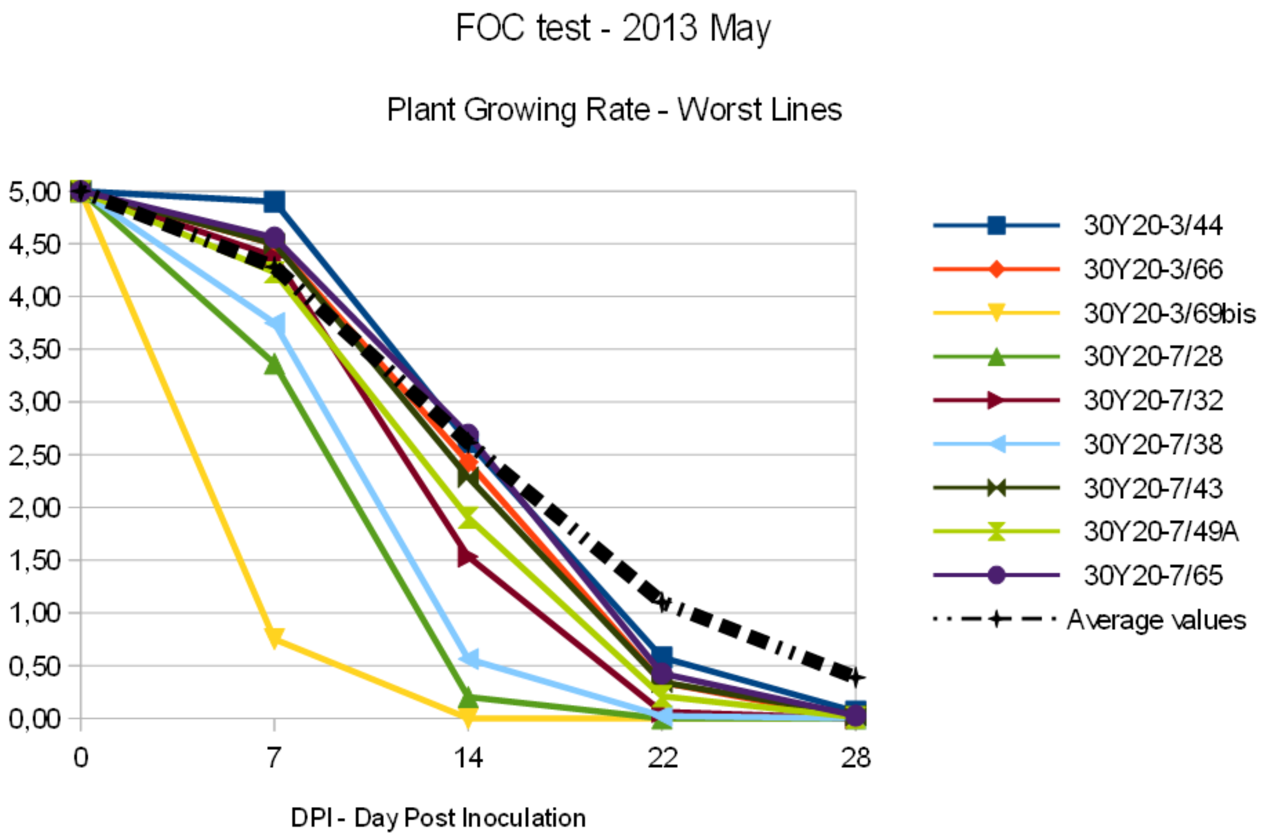
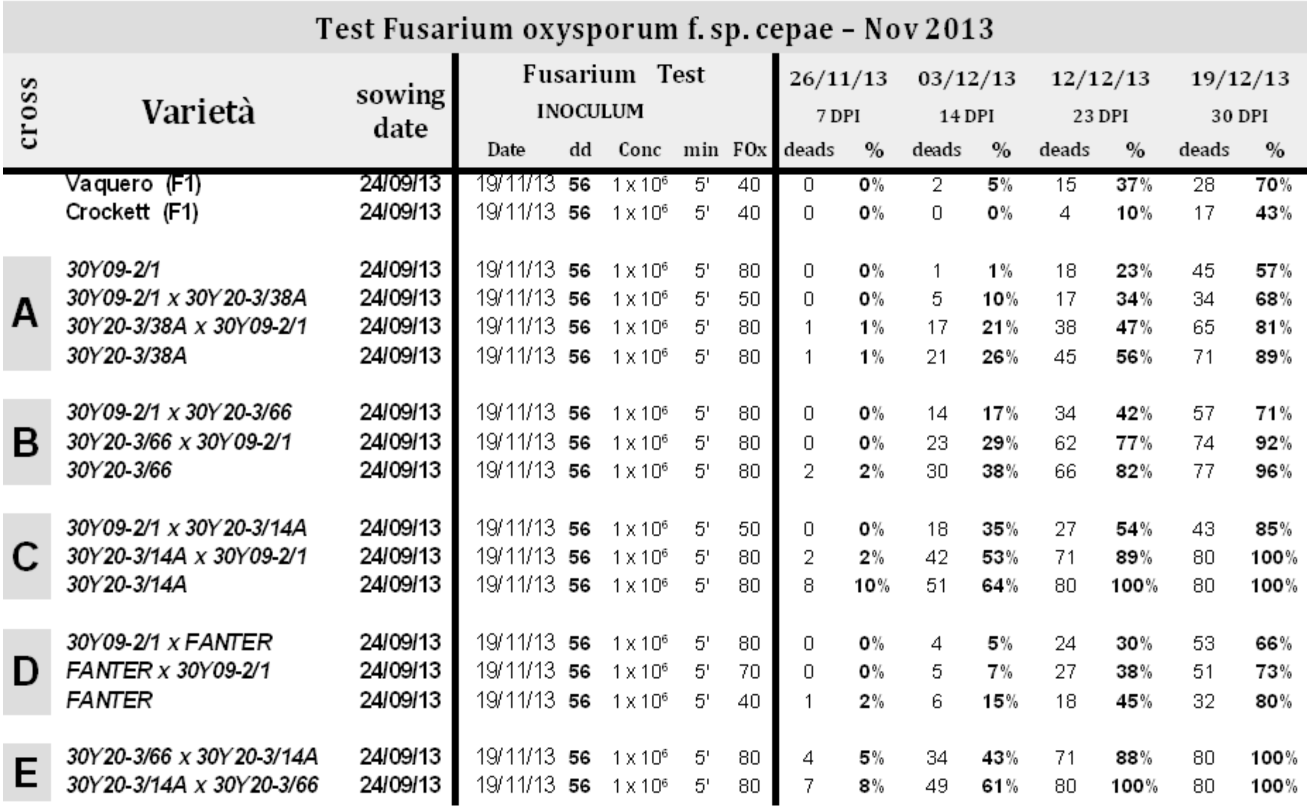


Fig 4.17. Evolution of plant growing rate in all worst lines during the FOC test 2013 (May)

November 2013

In last test, the maternal effect for this specific disease tolerance was evaluated using plant F1 and its reciprocal cross (RF1) arising from crosses between the tolerant line 30Y09-2/1 and other lines, some of which are known as susceptible to *Fusarium oxysporum*.

In the table below some data are reported on the plant material used in this test, in order to check what were the crosses used and their parental lines. Moreover, they are present also some data about inoculum, day post inoculation and mortality rate.



cross	Varietà	sowing date	Fusarium Test INOCULUM					26/11/13 7 DPI		03/12/13 14 DPI		12/12/13 23 DPI		19/12/13 30 DPI	
			Date	dd	Conc	min	FOx	deads	%	deads	%	deads	%	deads	%
	Vaquero (F1)	24/09/13	19/11/13	**56**	1×10^6	5'	40	0	**0%**	2	**5%**	15	**37%**	28	**70%**
	Crockett (F1)	24/09/13	19/11/13	**56**	1×10^6	5'	40	0	**0%**	0	**0%**	4	**10%**	17	**43%**
A	*30Y09-2/1*	24/09/13	19/11/13	**56**	1×10^6	5'	80	0	**0%**	1	**1%**	18	**23%**	45	**57%**
	30Y09-2/1 x 30Y20-3/38A	24/09/13	19/11/13	**56**	1×10^6	5'	50	0	**0%**	5	**10%**	17	**34%**	34	**68%**
	30Y20-3/38A x 30Y09-2/1	24/09/13	19/11/13	**56**	1×10^6	5'	80	1	**1%**	17	**21%**	38	**47%**	65	**81%**
	30Y20-3/38A	24/09/13	19/11/13	**56**	1×10^6	5'	80	1	**1%**	21	**26%**	45	**56%**	71	**89%**
B	*30Y09-2/1 x 30Y20-3/66*	24/09/13	19/11/13	**56**	1×10^6	5'	80	0	**0%**	14	**17%**	34	**42%**	57	**71%**
	30Y20-3/66 x 30Y09-2/1	24/09/13	19/11/13	**56**	1×10^6	5'	80	0	**0%**	23	**29%**	62	**77%**	74	**92%**
	30Y20-3/66	24/09/13	19/11/13	**56**	1×10^6	5'	80	2	**2%**	30	**38%**	66	**82%**	77	**96%**
C	*30Y09-2/1 x 30Y20-3/14A*	24/09/13	19/11/13	**56**	1×10^6	5'	50	0	**0%**	18	**35%**	27	**54%**	43	**85%**
	30Y20-3/14A x 30Y09-2/1	24/09/13	19/11/13	**56**	1×10^6	5'	80	2	**2%**	42	**53%**	71	**89%**	80	**100%**
	30Y20-3/14A	24/09/13	19/11/13	**56**	1×10^6	5'	80	8	**10%**	51	**64%**	80	**100%**	80	**100%**
D	*30Y09-2/1 x FANTER*	24/09/13	19/11/13	**56**	1×10^6	5'	80	0	**0%**	4	**5%**	24	**30%**	53	**66%**
	FANTER x 30Y09-2/1	24/09/13	19/11/13	**56**	1×10^6	5'	70	0	**0%**	5	**7%**	27	**38%**	51	**73%**
	FANTER	24/09/13	19/11/13	**56**	1×10^6	5'	40	1	**2%**	6	**15%**	18	**45%**	32	**80%**
E	*30Y20-3/66 x 30Y20-3/14A*	24/09/13	19/11/13	**56**	1×10^6	5'	80	4	**5%**	34	**43%**	71	**88%**	80	**100%**
	30Y20-3/14A x 30Y20-3/66	24/09/13	19/11/13	**56**	1×10^6	5'	80	7	**8%**	49	**61%**	80	**100%**	80	**100%**

Test Fusarium oxysporum f. sp. cepae – Nov 2013

Fig 4.18. List of plant materials evaluated in FOC test 2013 (November). The 30Y09-2/1 was the maternal line in the following crosses: A, B, C and D. Last combination (E) was obtained between two susceptible lines

Also the results of this test confirmed the good tolerance to *Fusarium oxysporum* of the plant material identified in previously tests. In fact, in the first positions of the list obtained with Disease Index, there are always the commercial hybrids and the DH line 30Y09-2/1 fallowed by all other.

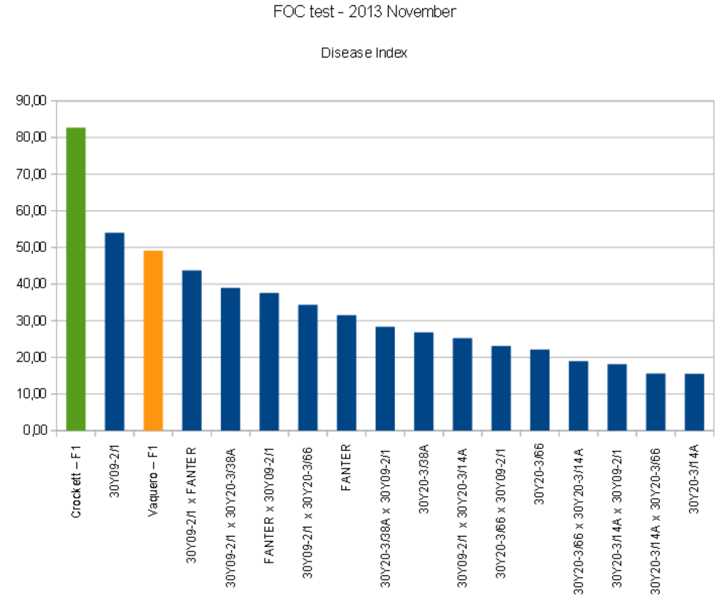


Fig 4.19. Onion lines ordered according to their value DI obtained during the FOC test 2013 (May)

The results obtained in May 2013 were confirmed, where the DH line 30Y09-2/1 showed a tolerance intermediate to fungus if compared to the varieties controls, Crocket and Vaquero. General trend of infection in host plant was the same compared to the other test done previously, as it is possible to see in the graphs reported below.

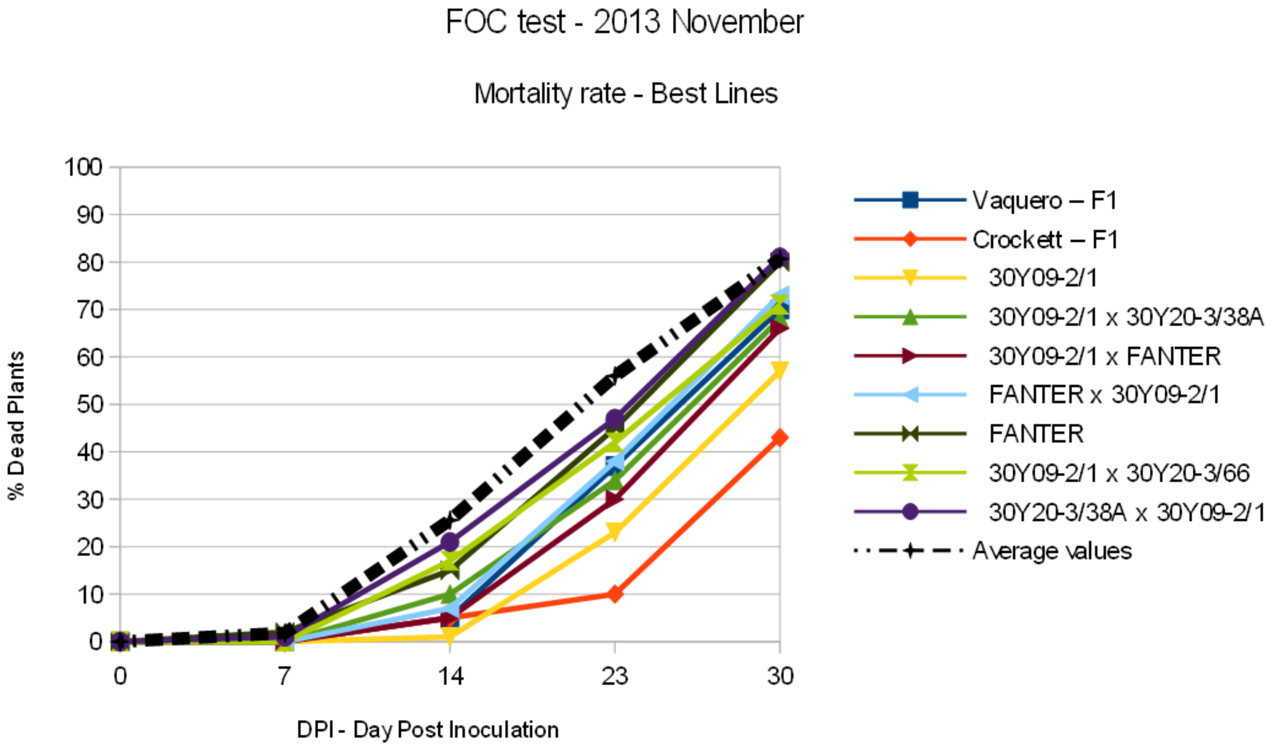


Fig 4.20. Plant Mortality Rate of all best lines during the FOC test 2013 (November)

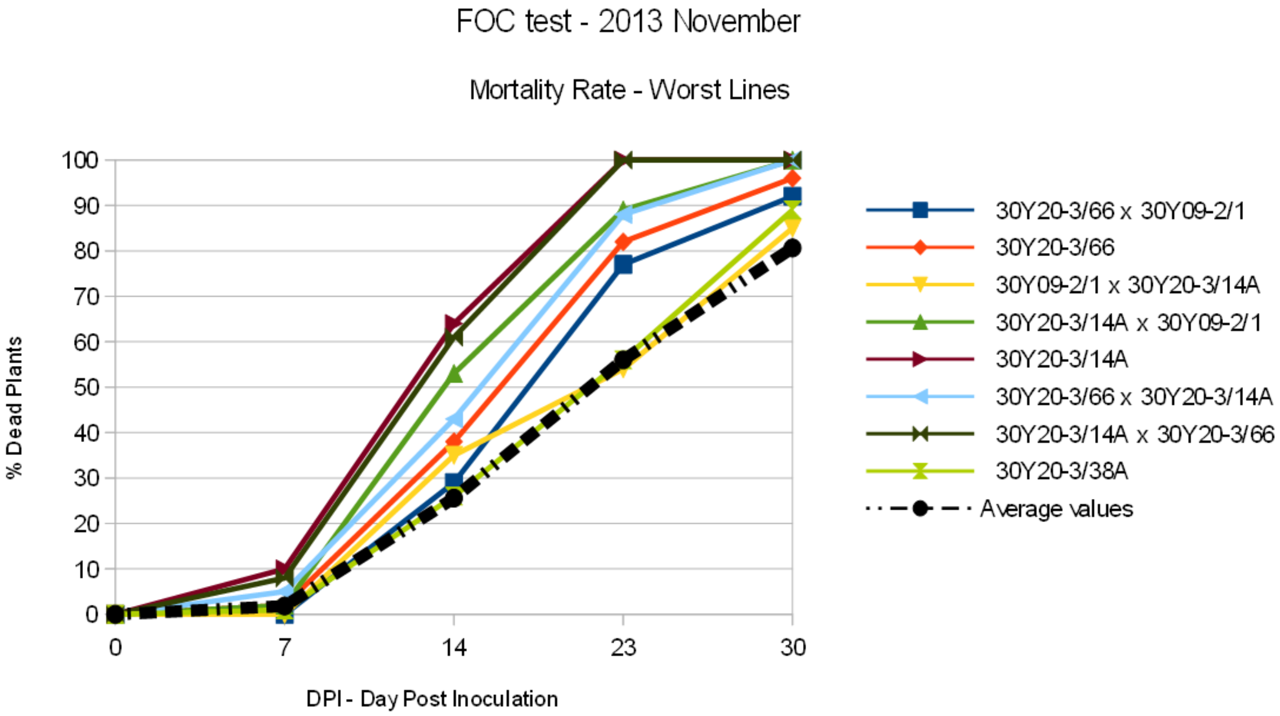


Fig 4.21. Plant Mortality Rate of all worst lines during the FOC test 2013 (November)

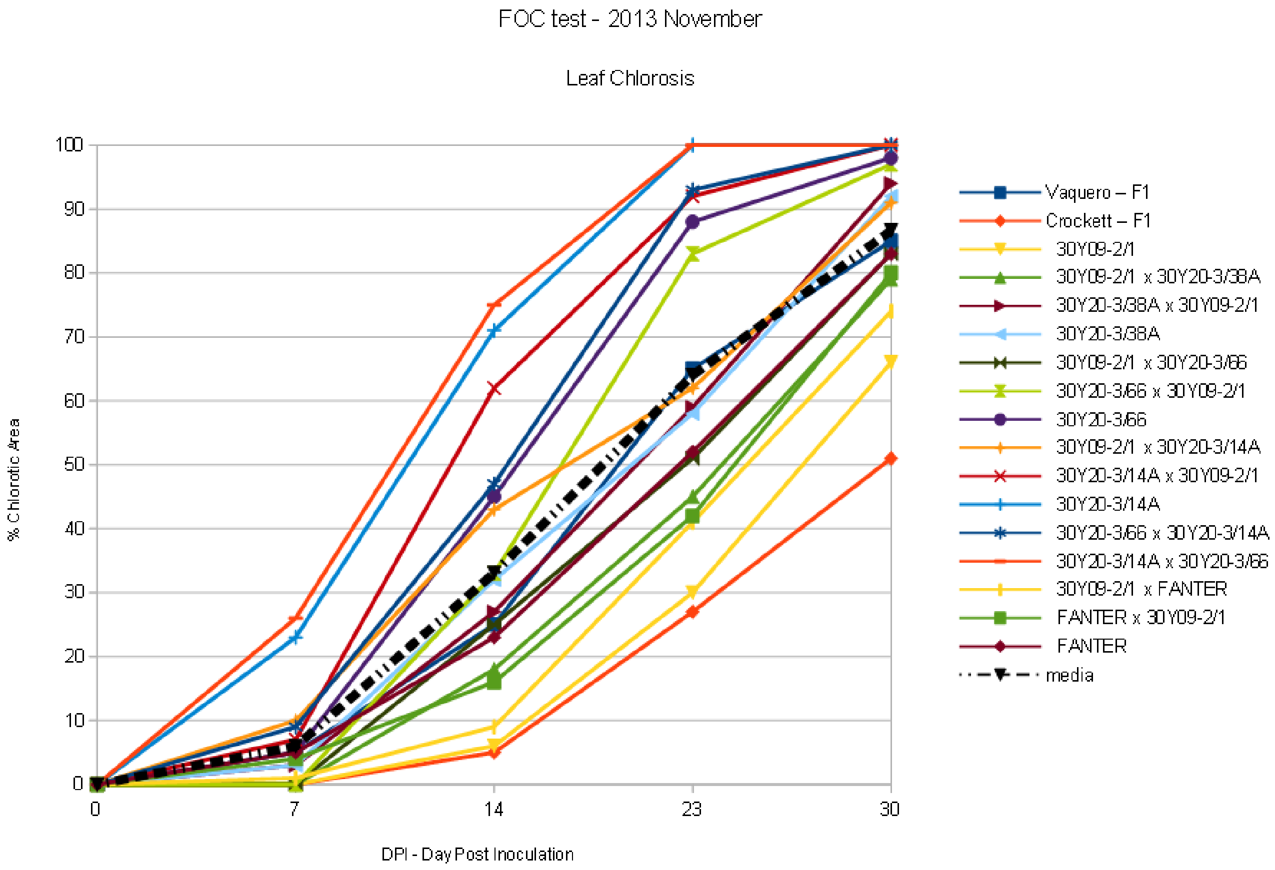


Fig 4.21. Evolution of leaf chlorosis in all lines during the FOC test 2013 (May)

New plant materials created for this in last test, made it possible to do new observations that allowed us to do different assessments. In particular, it evaluated the tolerance to *Fusarium oxysporum* in the reciprocal progenies with an identical genome, but generated from a different maternal line. This has been possible because parental lines were Double Haploids; consequently, there has been no genetic recombination when these have produced gametes. In conclusion, F1 and RF1 obtained in the same cross had the same identical genome.

In the graphs below, it is possible to observe the different behavior of all F1 and RF1 against this fungus pathogen and, in particular, how each progeny is more close to maternal than paternal line.

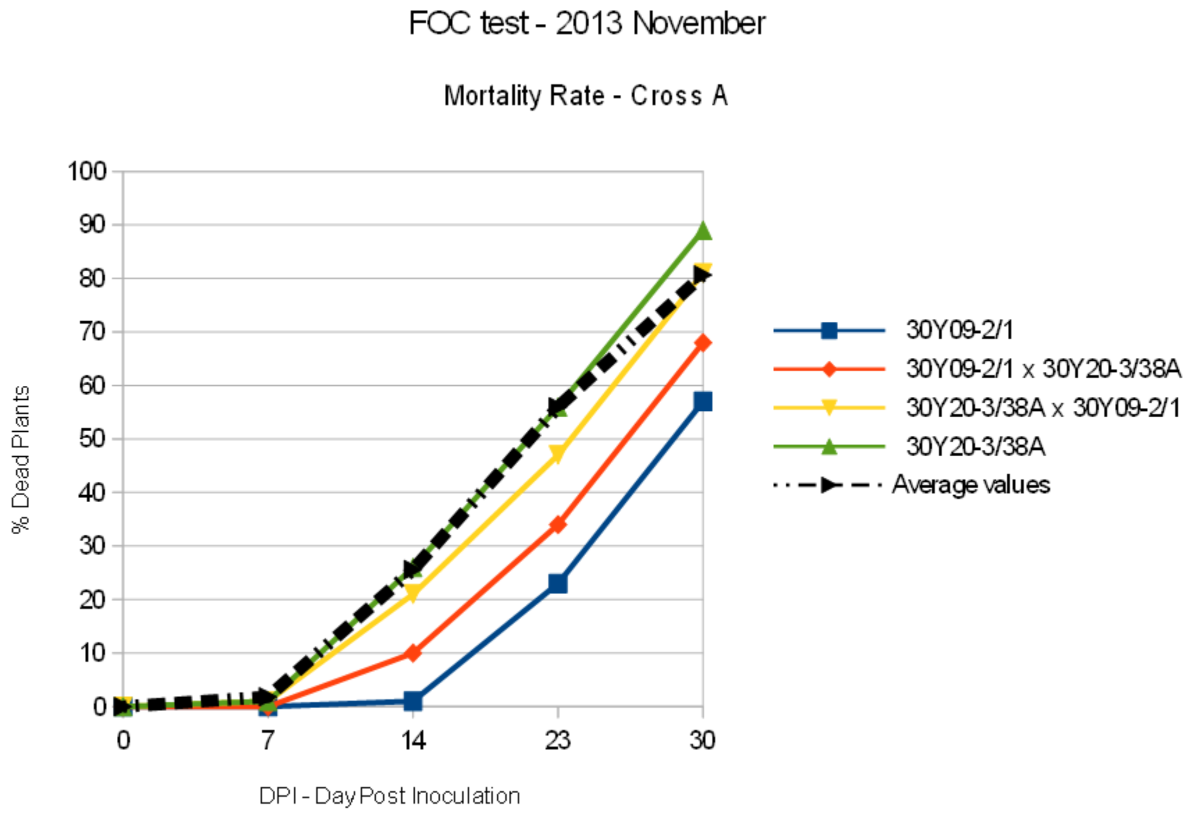


Fig 4.22. Cross A - Plant Mortality Rate of the parental lines and of the progeny (F1/RF1) obtained from their combination during the FOC test 2013 (November)

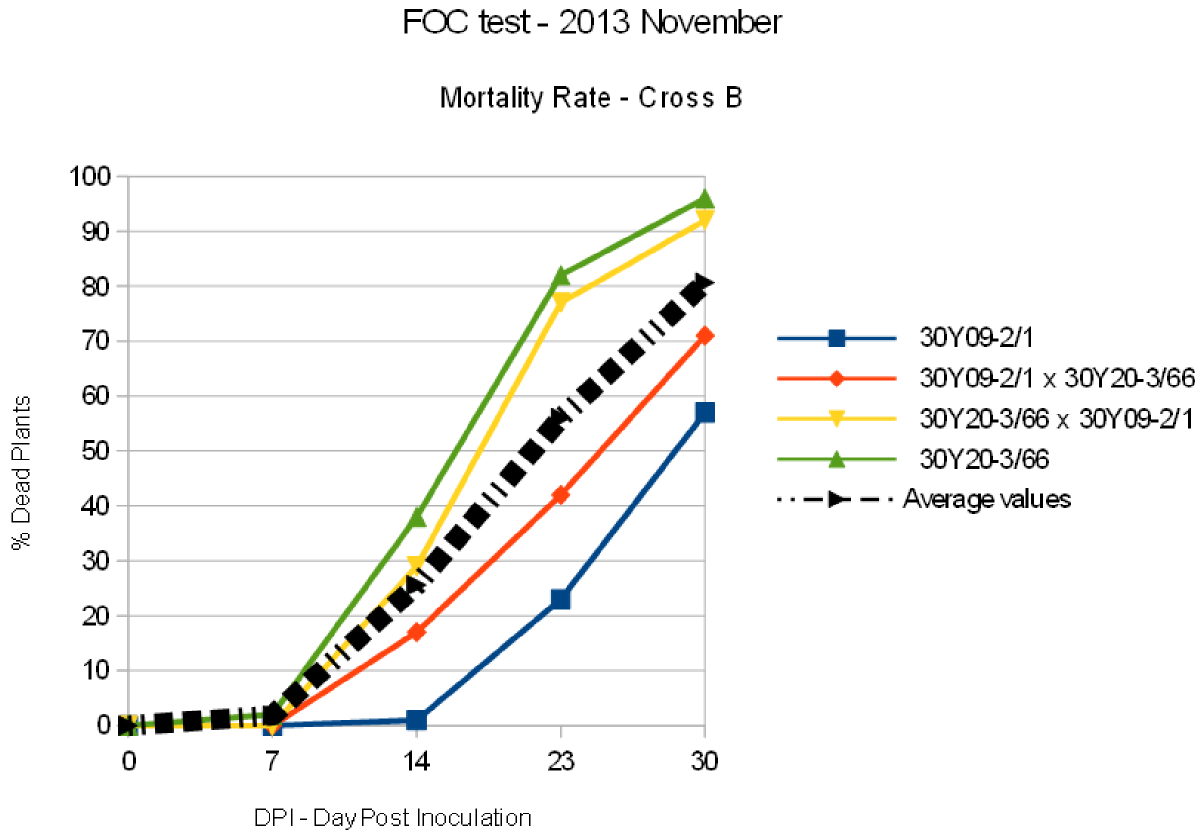


Fig 4.23. Cross B - Plant Mortality Rate of the parental lines and of the progeny (F1/RF1) obtained from their combination during the FOC test 2013 (November)

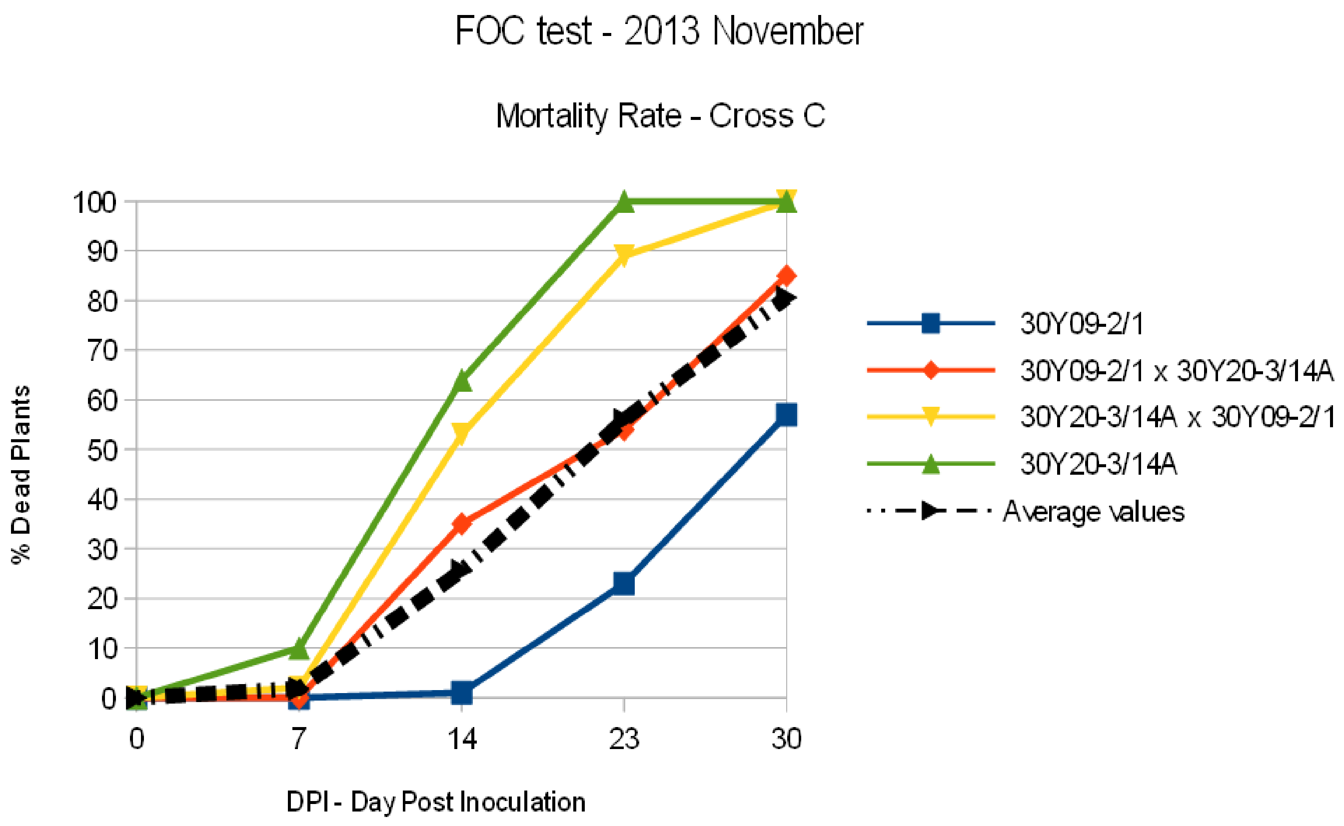


Fig 4.24. Cross C - Plant Mortality Rate of the parental lines and of the progeny (F1/RF1) obtained from their combination during the FOC test 2013 (November)

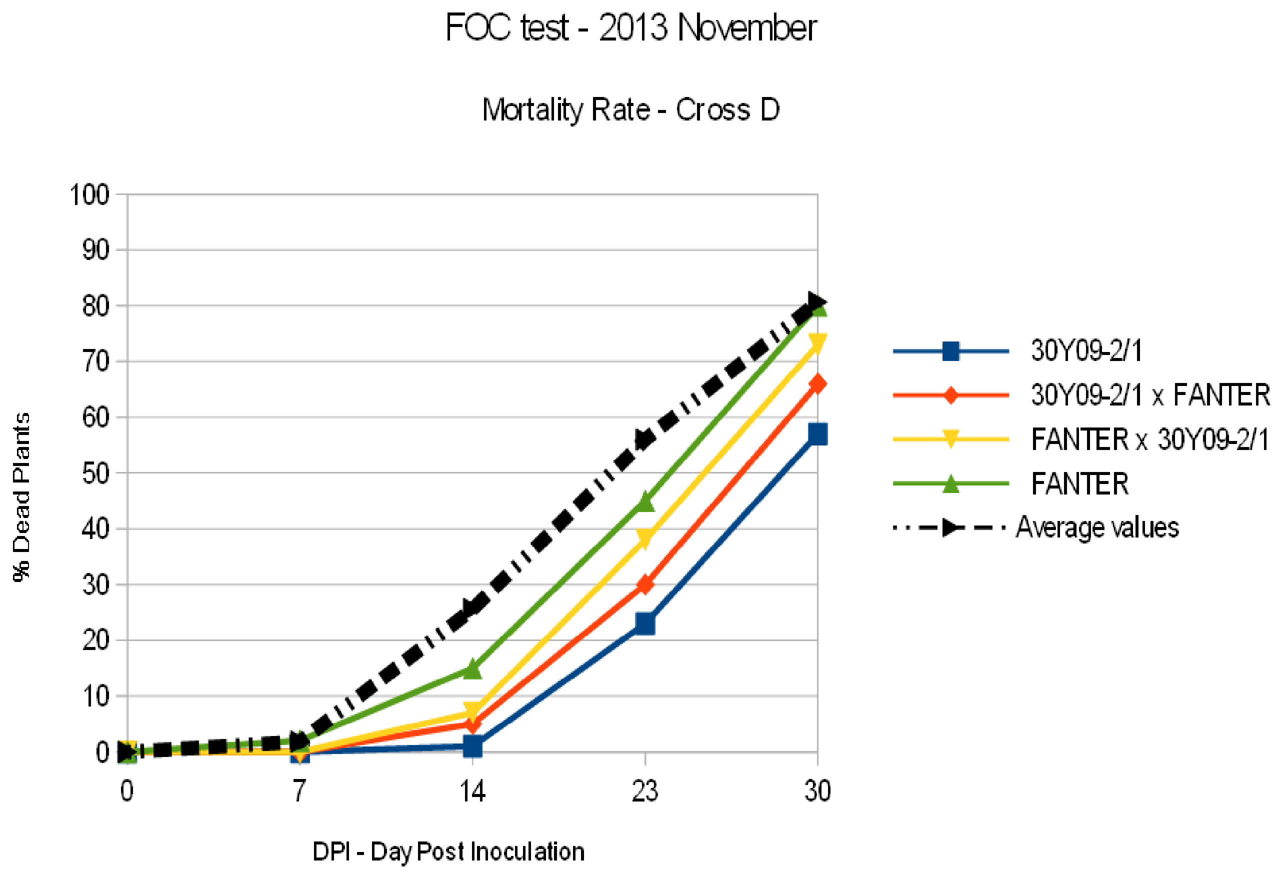


Fig 4.25. Cross D - Plant Mortality Rate of the parental lines and of the progeny (F1/RF1) obtained from their combination during the FOC test 2013 (November)

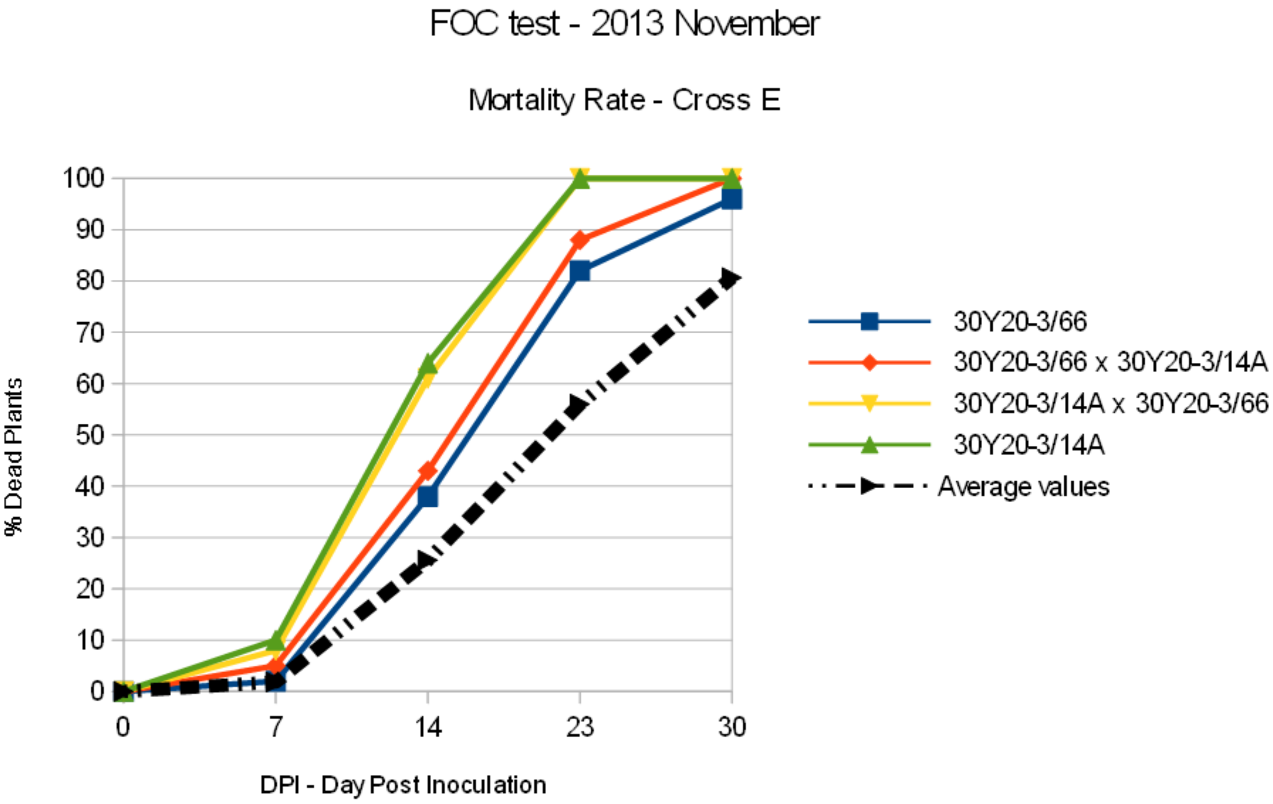


Fig 4.26. Cross E - Plant Mortality Rate of the parental lines and of the progeny (F1/RF1) obtained from their combination during the FOC test 2013 (November)

4.5 Discussion

Little was known about the behavior of the onion population of origin of the DH lines used in this project against *Fusarium oxysporum*, since it had never been characterized. In fact, these cultivars were chosen for *in vitro* culture, because they had other interesting agronomical traits like Dry Skin Color, Dry Skin Retention and Storage Ability.

First goal was to identify DH lines with a different tolerance to *Fusarium oxysporum* in order to use them, in a second step, to produce new different lines to study the maternal influences in the hereditability of this agronomical trait.

We analyzed 5 hybrids (F1) and 5 reciprocal hybrids (RF1) obtained crossing the same parental DH line 30Y09-2/1 to different lines with opposite ability to react to the *Fusarium oxysporum* and different origin. In particular, two different typologies (Density and Spanish) were crossed, besides to use a plant material with different origin (*in vitro* and diploid plant).

In all crosses analyzed, the results of our experiments showed that in all cases progenies have similar tolerances to fusariosis as their maternal lines.

In any case, a maternal effect on inheritance of disease tolerance to *Fusarium oxysporum* has been demonstrated. Finally, it is interesting to note that even if there is a putative strong maternal influence all combinations show a different behavior during interaction host-pathogen.

4.6 References

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4.7 General Discussion

The multidisciplinary approach used in this research project, obtained by the combination of molecular and physiopathological analysis, allowed to study in the overall epigenetic effects on the final phenotype of the plant because the scientific results have confirmed the influence of maternal line on multiple agronomic traits in different environmental conditions.

4.8 Conclusion

The influence of the stockseed (maternal line) on the progeny generated was reflected in many of the molecular analyzes, pathological tests and phenotypic assessments conducted in this PhD project. These results will be of great help to better understand and assume the behavior and performance of the varieties in the field, as well as any deviations from the expected performance. Finally, this scientific approach can be re-used to investigate and understand in depth what happens in onion plants during:

- other types of plant-pathogen interaction
- periods of drought
- lack of nutrients from the soil
- crosses with material of different genetic characteristics