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Characterization of tomato (*Solanum lycopersicum* L.) male sterile mutants putatively affected in class B MADS-box transcription factors

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Chapter 1

General Introduction

1. Hybrid seed production overview

The term "hybrid" can assume different meanings in evolutionary biology; generally it means an organism generated by cross-fertilization between two individuals from different species or individuals that are distinguishable on the basis of several heritable characters (Harrison, 1990). The first description about the hybridization was reported in 1694 by Camerarius, who hypothesized the possibility to fertilize one female plant with pollen from another plant used as male donor (Zirkle, 1935). However, the first artificial hybrid production was ascribed by some authors to the experiments done by Fairchild, who crossed *Dianthus caryophyllus* with *Dianthus barbatus*, while by others to Linnaeus, with experiments on *Tragopogon* in 1759 (Zirkle, 1935). Hybridization experiments done in the past centuries demonstrated that hybrids originated from interspecific cross were characterized in same case by sterility and that they were impossible to observe without the human interference (Roberts, 1929). Normally, hybrid individuals have intermediate characteristics between the two parents used for cross and in the later-generations they tend to return into the parents morphology (Roberts, 1929).

When hybridization occurs, it possibly reveals some mechanisms in reproductive processes that act influencing the viability/fertility of hybrids or viability/fertility in the hybrid later generations. These mechanisms can act at prezygotic or postzygotic level. At prezygotic level it is known that habitat, temporal and ethological barriers, gametic competition and incompatibility influence the viability/fertility of hybrids (Rieseberg and Carney, 1998). The main gametophytic barrier that acts negatively in the evaluation of hybrid fertility is represented by the growth speed of pollen tube (Carney *et al*., 1996; Carney and Arnold, 1997). At postzygotic level, the most common barriers that influence the second and successive generations of hybrids are the weakness, sterility and breakdown that characterize the F_1 and subsequent populations (Rieseberg and Carney, 1998). A different sterility degree is possible to observe in the F_1 generation and this was explained using a standard model proposed by Dobzhansky (1937) and subsequently modified by Wu and Palopoli (1994).

The possibility to observe alterations in viability/fertility is one characteristic typical of hybrid originated by interspecific crosses. Contrarily, hybrids being originated from crosses within closely related species are superior respect to their parents in vegetative vigour and robustness (Grant, 1975). This phenomenon is called heterosis, which is a multigenic complex trait that influences many aspects as rate of vegetative growth, flowering time, yield (in terms of increase in inflorescence size and increase in fruit/seed sets) and resistance to abiotic and biotic stresses (Lippman and Zamir, 2007). The expression of heterosis is mainly due to the dominance and overdominance but also to epistatic interactions and epigenetics factors (Chen, 2010). The dominance model explains the

increase in vigour in the hybrids as the result of accumulation of dominant genes derived from both parents (Davenport, 1908), while the overdominance model suggests that this increase in vegetative growth or in productivity is due to the heterozygous status and it is important for traits controlled by one or few genes (East, 1936). The dominance effect can also be described as the possibility of one parent to have gene copies that are missing in the other parent and, after crossing, the hybrids would have more genes than the parent genotypes (Fu and Dooner, 2002). The successful production of hybrids in maize (1920) and the observation of heterosis was the first step for understanding and use this phenomenon in the breeding of other crop species. After maize, hybrids were produced in eggplant (1924; Nishi, 1967), watermelon (1930), cucumber (1933), radish (1935), tomato (1940) and cabbage (1942) (Liedle and Anderson, 1993). These hybrids were developed using natural crossing, while in 1933 Pearson obtained in cabbage F_1 hybrid generation using self-incompatibility and Jones and Clarke (1943) in onion with the help of cytoplasmic male sterility.

The production of hybrids in terms of procedure is based on three steps: 1) development and identification of suitable parental lines, 2) multiplication of parental lines and 3) crossing between parents and production of hybrid seed. The hybrid seed production requires procedures that render easy and economical to maintain the parental lines and also mechanism necessary to control the pollination phase. Therefore, several procedures to prevent the self-pollination of the female line such as mechanical emasculation, self-incompatibility, gynoecism, auxotrophy, use of chemical hybridizing agents (CHAs) and male sterility were developed (Kumar and Singh, 2004).

Generally, hybrids are produced with emasculation procedures that involve the removal of male organs from the female plants before anthesis. When in the female plants the anther cone is removed, it is possible to proceed with the hand pollination using pollen grains from the line chosen as male parent.

Self-incompatibility (SI) is a mechanism where genic factors inhibit the elongation of self-pollen tubes into the style and consequentially make not possible the fertilization process. The expression of SI is determined by several alleles at the same locus (S alleles): pollen and pistil generate SI when they have the same alleles at the locus S. There are two types of SI, gametophytic (GSI) where the pollen phenotype depends only on the S allele it carriers and sporophytic (SSI) where the pollen phenotype is determined by the genotype of the parent plant on which pollen grains are produced (Kumar and Singh, 2004). The SSI system was observed for the first time in radish (Stout, 1920) and today it is used in *Brassicacae* for hybrid seed production (Pearson, 1983; McCubbin and Dickinson, 1997; Tripathi and Singh 2000; Singh 2000; Singh *et al*. 2001).

The use of gynoecism is a procedure used in cucumber hybrid production and it is coupled with the employment of parthenocarpic genes (Kalloo, 1988) so pollination is not necessary for fruit set. Using chemical reagents, such as silver nitrate or silver thio-sulphate, in gynoeciuos lines several staminate flowers can be induced that are useful to multiply the seeds of these parent plants.

F_1 hybrids in tomato and in barley can be produced using the monogenic-recessive auxotrophic mutants that have a mutation in a gene involved in nutritional requirements (Barabas, 1991). The auxotrophic parental line when is self-pollinated produces a lethal progenies, but the hybrids derived from crosses between the mutant line and a wild type line are characterized by normal phenotype and vegetative growth. In tomato, the utilization of an auxotrophic mutant for thiamine requirement as female line crossed with pollen from a normal genotype produced pure F_1 seed (Barabas, 1991). Unfortunately, today in tomato the research addressed to the use of auxotrophic mutants in hybrid seed production was discontinued, and also for other crop species this procedure remains difficult to use (Barabas, 1991).

Another procedure to control the self-pollination of female plants used in hybrid production is the application of chemical hybridizing agents (CHAs; McRae, 1985), which are able to induce male sterility. The first successful experiment obtained using CHAs, in particular maleic hydrazide, a reagent able to induce male sterility, was done in 1950 in maize plants (Moore, 1950; Naylor, 1950). The CHAs act at different levels and the specific effects that they determine are dependent from time and dosage of treatments. The major effects that they produce involve the arrest of anther development, anthers with normal features producing unviable pollen grains, lack of anther dehiscence and formation of abnormal microspores (McRae, 1985). To use CHAs in hybrid production, they should respond to some requisites: have a wide spectrum of action to induce sterility; determine male sterility without influence in female fertility; not be dangerous for humans and animals and be easy and economically convenient to use (Virmani *et al*., 2003). The use of CHAs also shows several disadvantages such as production of impure seeds if they are not effective when the environmental or growth conditions are not favorable, some chemicals are toxic for human health (as zinc methyl arsenate or sodium methyl arsenate) and some reagents have a high cost (Virmani *et al*., 2003).

The last strategy used to control the pollination phase is the use of male sterility, which is described in detail in the next paragraphs.

1.1. Hybrid seed production in tomato

In tomato, the first hybrid was developed in Bulgaria in 1932 and in USA in the first 1940 by Dr. Shifriss; it was called *Burpee Hybrid* (Daskaloff, 1937). Subsequently, the tomato hybrid production acquired importance and started to grow continuously in the seed market. In the last two decades, the breeding programs and hybrid seed production were addressed to enhance plant tolerance to abiotic or biotic stresses, fruit nutritional value and fruit quality (Atanassova and Georgiev, 2002). Duvick (1997) reported that in the USA the majority of tomato production (100% of fresh market and 80% of processing tomatoes) is obtained with hybrids and also in most of countries such as Europe, Asia and Australia there is the same situation.

Today hybrid tomato varieties are more used then the open pollinated lines; this is due to the many advantages that characterize the hybrids such as high productivity, early maturation, high fruit uniformity, superiority in fruit quality and resistance to different diseases. The tomato hybrid production give plants with desirable and superior characteristics but at the same time the production of hybrid seed is not easy and it requires much labor (Atanassova and Georgiev, 2002).

Tomato is characterized by hermaphrodite flowers that have five or more anthers fused laterally to form a staminal cone that encloses the pistil. The pistil usually is shorter than the anthers and an inserted stigma represents for the flowers a useful characteristic for self-pollination. For hybrid seed production in the line chosen as female parent it is necessary to remove the anther cone through manual emasculation before the anthesis stage; subsequently the stigma is hand pollinated using pollen from the male parent. In order to eliminate these laborious procedures it could be useful to adopt in hybrid production male sterile genotypes.

2. Male sterility

Male sterility is defined as mis-production of functional anthers, pollen or male gametes by the plants, while the female fertility remains unchanged (Kaul, 1988). The phenotypic manifestation of male sterility can involve the complete absence of male organs, abortion of pollen grains in different developmental stages, anther malformations, lack of anther dehiscence and also the inability of mature pollen to germinate on a compatible stigma.

Male sterility is a powerful system in hybrid seed production in monoecious and hermaphrodite crop species because it eliminates the tedious and laborious hand emasculation of the flowers and reduces the cost associated with seed production. This system could be useful particularly in crops such as maize, soybean, pepper and tomato where the emasculation practices determine an increase in the production cost (Sawhney, 1997).

Based on its inheritance, male sterility can be divided into genic male sterility (GMS) and cytoplasmic male sterility (CMS).

2.1 Genic male sterility in hybrid seed production

The nuclear or genetic male sterility (GMS) usually is controlled by one or two genes at the recessive status. GMS was used in hybrid seed production at commercial levels for rice (Singildin, 1979a), sugar-beet (Doney and Theurer, 1978), sorghum (Obilana and Rouby, 1980) and cotton (Weaver, 1979). The main limit associated with the use of GMS in hybrid seed production is the maintenance of the male sterile plants. Generally, the male sterile line is maintained through a backcross with heterozygote plants that originates a 50% fertile and 50% sterile progeny. In the field this creates a problem because it is necessary to select the sterile and remove the fertile plants (Frankel 1973; Frankel and Galun 1977). To bypass this problem, several strategies were proposed for the multiplication of male sterile lines to use as female parent in hybrid seed production (Table 1.1). One of them involves the use of markers that are associated with vegetative characters of plants for

identifying, preferably prior to flowering, the male sterile lines inside a segregating population. The markers linked to male sterile (*ms*) genes provide a useful approach for an early and accurate identification of lines carrying the *Ms* allele and consequently the fertile plants can be removed at the seedling stage and in the field could be grown only the sterile genotypes. Linkage between the *Ms* gene and phenotypic marker can regard different morphological traits and it can become evident in different development stages (seed, seedling and adult plant). In maize, it was proposed the use of one *ms* allele, ms_1, which is associated with a marker for seed characters, particularly with a gene for white endosperm (Galinat, 1975; 1976). In tomato, different *ms* genes were discovered that were linked with markers involved in different phenotypic characters, such as purple pigmentation and potato leaf trait, which allowed an easy identification of sterile plants at the seedlings stage (Philouze, 1973; Woollard and Hernandez, 1979; Durand, 1981). Markers associated with *ms* genes that are visible in adult plants were also described, for example in sunflower, a GMS gene Ms_{10} isolated by Leclercq (1966) was found very close (< 1 cM) to a gene controlling anthocyanin pigmentation (*T*) (Stoenescu and Vranceanu, 1977). This discovery was used in hybrid seed production and has permitted the elimination at early stages of fertile plants trough the observation of red coloration on the hypocotyl.

Table 1.1. List of different strategies used to maintain and multiply male sterile lines (from Perez-Prat and van Lookeren Campagne, 2002; for references within the Table see the original paper).

System	Propagation of female parent line	Advantages	Disadvantages	Refs
Chemical selection of male-sterile plants	Selection by herbicide application	Simple Stable sterility	Need for herbicide treatment in hybrid seed production field Need to eliminate all fertile plants by herbicide spray Requirement for female parent seeds doubles For some crops, asymmetric distribution of the female parent plants in the hybrid seed production field	[25]
Chemical induction of sterility	Self-fertilization	Simple Fertility restorer gene not required[b]	Need for treatment with a chemical in hybrid seed production field Need to achieve 100% male sterility by chemical induction	[4–8]
Chemical induction of fertility	Self-fertilization after treatment with a fertility-restoring chemical	Chemicals applied to a smaller acreage than in the above systems No need to achieve 100% restoration Possibility to multiply recessive male-sterile mutants	Need for treatment with a chemical for multiplication of female parent Chemically inducible promoter required for some systems Fertility restorer gene required[b]	[9–13, 17–19, 21,22]
Environmental induction of fertility	Self-fertilization under permissive environmental conditions	No need for chemical treatment Fertility restorer gene not required[b] Possibility to multiply recessive male sterile mutants	Need for controlled environmental conditions Difficult to find mutants with tight sterility and reliable induction of fertility	[26–30]
Two-component male sterility	Self-fertilization of grand -parent lines crossing to produce male-sterile female parent line	No need for chemical treatment Fertility restorer gene not required[b]	Two lines required to produce the male-sterile female parent Need for a pollination control method to produce the male-sterile female parent	[23,–24]
Maintainer lines for dominant or recessive nuclear male-sterile lines	Crossing with maintainer line	No need for chemical application Possibility to multiply recessive male sterile mutants	A maintainer line required Fertility restorer gene required[b] Seed sorting might be required	[34,35]

[a]The advantages and disadvantages of several strategies for the multiplication of male-sterile lines are listed. The different strategies are described in combination with a pollination control system because the methods to propagate the male-sterile line depend, in most cases, on how male sterility was generated.
[b]The fertility restorer gene mentioned in some systems is needed to propagate the male-sterile line. For clarity, the requirement of a fertility restorer gene to produce fertile F1 hybrids, inherent to many of the described systems, is not included in this review.

Another approach to resolve the maintenance of sterile lines is the use of mutations that are conditionally fertile. In this situation, selfing of male sterile plants is possible to get a progeny that is all male sterile. To achieve the fertility restoration in male sterile lines chemical treatments, metabolite applications and environmental conditions could be used. When male sterility is caused by lack of some metabolites, to restore the fertility it is necessary to supply an exogenous applications of the missing metabolites. In petunia and tobacco male sterile lines characterized by missing production of chalcone synthase, the fertility restoration occurs when the plants are subjected to application in the stigma surface of flavonols (Derksen *et al*., 1999; Taylor and Mo, 1993; Van Tunen *et al*., 1992). Similarly, in *Arabidopsis* the restoration of the fertility in male sterile mutants occurs when jasmonic acid is applied (Browse, 1997; Sanders *et al*., 2000), while in tomato the fertility of some mutants can be restored by gibberellic acid (Sawhney and Greyson, 1973) or silver nitrate (Yardanov, 1983) applications. The fertility could also be restored by changing the environmental factors such as temperature and photoperiod (environment-sensitive genic male sterility, EGMS) (Smith, 1947). When the male sterility is influenced by temperature conditions, it is called temperature-sensitive genic male sterility (TGMS), while when it is influenced by photoperiod it is called photoperiod-sensitive genic male sterility (PGMS) (He *et al*., 1999; Dong *et al*., 2000). Environmental factors that promote sterility could be used for producing F_1 hybrid seeds (Sawhney, 1984), while changing the environmental conditions to favor fertility restoration could be useful when it is necessary to self the seed parent and produce a large amount of male sterile seeds.

2.2 Cytoplasmic and genic-cytoplasmic male sterility

Cytoplasmic male sterility (CMS) is due to mutations that involve the mitochondrial genome and is characterized by maternal inheritance, thus the progeny of a CMS plant will be all sterile. The CMS system can be useful in hybrid seed production (Hanson, 1991) but the sterility of the F_1 seed makes it suitable for crops where the commercial product is a vegetative part of the plant. The CMS sterile line is maintained by crossing with a maintainer, which is genetically isogenic except for the cytoplasm that is fertile. To use CMS in hybrid production of crops where the product is the seed or the fruit it is necessary to have available nuclear fertility restorer (*Rf*) genes (Schnable and Wise, 1998; Kempken and Pring, 1999) which are capable to inhibit the cytoplasmic sterility and restore the fertility in the next generation; this case is referred to as genic-cytoplasmic male sterility (GCMS).

CMS was studied in detail in many plant species such as maize (Levings, 1990), petunia (Bino, 1985) and sorghum (Pring *et al*., 1995; Xu *et al*., 1995a). In maize, in the 1950 it was used for hybrid development the CMS-T (Texas) system that determined an increase in yield and seed productions. After the development of this system in maize, the CMS was applied in other crops such as rice, where the first hybrid seed production was released in 1970 in China and determined an increase of 20% of grain yields.

Generally, the GCMS system makes use of three different breeding lines: the CMS line, the maintainer line and the restorer line (Fig.1.1). The CMS line used has the male sterile cytoplasm,

missing of nuclear fertility restorer genes. The CMS line is used as female parent and it is propagated by crossing with the maintainer line (male parent) that has normal cytoplasm and the same nuclear genome of the female parent. The restorer line functions as pollen parent and is characterized by *Rf* genes, nuclear genes that are capable to restore the fertility. Crossing the CMS line with the restorer line, the result will be a F_1 hybrids progeny that is male fertile and presents a hybrid vigour due to the combination of nuclear genome from both parents (Schnable and Wise, 1998).

Today, the CMS system is used for hybrid seed production in many species such as rice, sorghum, carrot and sunflower, but it is not adopted in all agricultural crops because in many of them *Rf* genes for fertility restoration are not available.

2.3 "Biotechnological" male sterility

The introduction of biotechnology techniques has created a new opportunity to obtain male sterile lines working on the development and metabolism of the tapetum (Mariani *et al*., 1990; Van der Meer *et al*., 1992; Hernould *et al*., 1998) or of the pollen grains (Worrall *et al*., 1992). Transgenic male sterile plants were obtained with the tissue-specific expression of a gene encoding a ribonuclease capable to disrupt the tapetal cells (Mariani *et al*., 1990) or a gene encoding the diphtheria toxin A-chain (Koltunow *et al*., 1990). The system described by Mariani *et al*. (1990) involves the use of a cytotoxic chimeric gene constituted of the sequence encoding a barnase, an extracellular RNase produced by *Bacillus amyloliquefaciens*, under the control of TA29 promoter (*tapetal specific transcriptional activity* gene from tobacco). The expression of this construct causes an ablation of tapetal cells and consequentially renders the plants male sterile. To obtain the F_1 hybrid seed with this system it is necessary cross the male sterile line with a male fertile that is engineered for the expression of the barstar sequence, an intracellular barnase-specific RNase inhibitor produced by *B. amyloliquefaciens*, under the control of TA29 promoter. The progeny produced from this cross will be all fertile. Subsequent researches addressed in the field of engineering male sterility have demonstrated that is possible to use different other strategies to obtain this goal. These different approaches include the use of a chimeric tapetal-specific glucanase gene to determine disruption of microspores (Worral *et al*., 1992), inhibition of flavonoid biosynthesis inside the tapetal cells (Van der Meer *et al*., 1992) and the use of a chimeric *rolB* gene from *Agrobacterium rhizogenes* to increase the auxin activity in anthers (Spena *et al*., 1992).

The genetically engineered system used to produce male sterile plants can be applied a wide range of crops such as oilseed rape, lettuce, tomato and corn. Engineering cytoplasmic male sterility is not used very often and this is due to the difficulty in the transformation process of mitochondria or chloroplasts. Contrarily, the engineering GMS was successfully used to produce engineered male sterile plants in corn and *Brassica napus*.

2.4. Environment-sensitive genic male sterility

Initially, the EGMS lines were not used very often for hybrid seed production because they seemed unstable but in the recent years, they become considered the best instrument for hybrid development. The first EGMS line was discovered in pepper (Martin and Crafword, 1951); successively EGMS was studied in most crop species.

In the *Brassica* genus, particularly in broccoli, it was reported a mutant that is sensitive to low temperature. This mutant, called *ms-6*, after an exposure of 30 days a low temperatures (7-11°C), showed a partial restoration of fertility, while an increase of only 1°C, from 11 to 12°C, determined again sterility conditions (Dickson, 1970). In Brussels sprouts, sterile plants become fertile when they are subject to low temperature (10°C; Nieuwhof, 1968b).

In onion, it was studied a male sterile line that is completely sterile in low temperature condition (14°C) while with intermediate temperature (23°C) the fertility is restored (Van der Meer, 1969).

In rice (*Oryza sativa*), the first PGMS mutant was found in ssp. *japonica*, in a line called Nongken 58S, and it was characterized by male sterility under LD conditions while presented fertility restoration in SD conditions (Shi, 1985). Then, the first TGMS discovered was Annong S-1 in *Oryza sativa* ssp. *indica*, which is completely male sterile under high temperatures while with low temperatures the fertility is restored (Deng *et al*., 1999). Actually, in rice PGMS and TGMS lines are commonly used for hybrid seed production at commercial level with the two-line system (Fig.1.1). Using a two-line system in hybrid seed production there are some advantages such as that the P/TGMS lines can be propagated under specific environmental conditions without the help of maintainer plants and that the negative effects of CMS on important agronomic traits can be eliminated (Virmani *et al*., 2003).

Several conditional male sterile mutants and putative candidate genes were identified in different rice chromosome. For example, for one PGMS mutant it was identified the putative candidate gene *p/tms12-1* that encodes for a precursor of a small RNA, important regulator in pollen development, in which a single nucleotide substitution C/G determines a loss-of-function in this small RNA and subsequently the male sterile phenotype (Zhou *et al*., 2012). Another PGMS in rice, called *carbon starved anther* (*csa*), shows a mutation in a R2R3 MYB transcription factor, which is involved in pollen development. After its characterization, the allele was transferred in *japonica* and *indica* cultivars in order to develop new male sterile lines (Zhang *et al*., 2013).

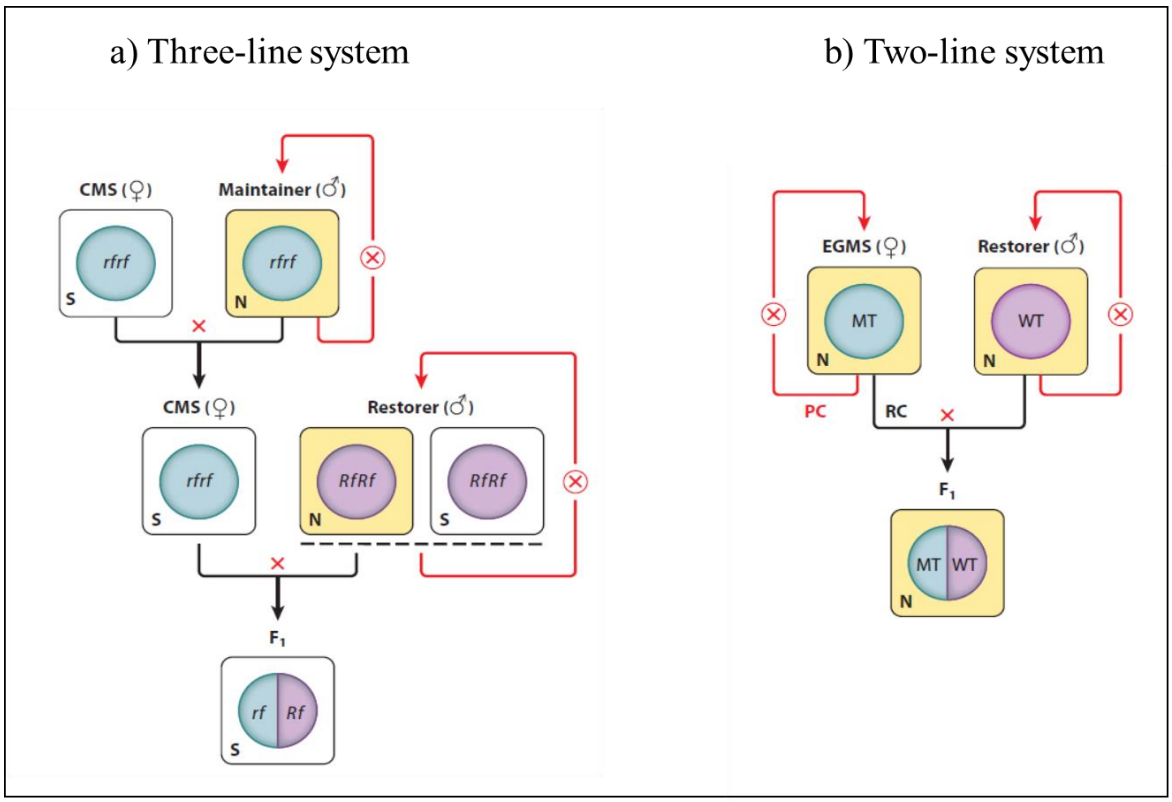

Figure 1.1. Schematic representation of CMS and EGMS in hybrid seed production. a) Application of CMS in hybrid seed production requires three-line system. The CMS line is crossed with the male parent, the progeny obtained will be sterile. The sterile progeny is crossed with the restorer line to produce the fertile hybrid seeds. b) Application of EGMS in hybrid seed production determines a changing from three-line system to two-line system. The EGMS line is characterized by sterility under certain environmental conditions, in which is self-pollinated for propagate itself, while in environmental conditions that favorite the fertility restoration is crossed with the male line for hybrid seed production (Figure from Chen and Liu, 2014).

3. ABC(DE) model of flower development and homeotic MADS-box genes

Flower structure represents a good model to study the association between development, genes and evolution. In some flower model species, such as *Arabidopsis thaliana*, *Antirrhinum majus*, *Petunia hybrida*, *Nicotiana tabacum* and *Oryza sativa*, the flower development was studied in detail to understand how this process functions at genetic level. These studies demonstrated that the inflorescence and flower development are processes that are controlled by a network of regulatory genes, which are organized in a hierarchical fashion (Okada and Shimura, 1994). At the top of this model, there are the *late and early flowering genes* that are influenced by environmental factors and endogenous signals such as day length, light quality, temperature and gibberellins (GA) and that control the transition from vegetative to reproductive status through the activation of the *meristem identity genes*. *Meristem identity genes* control the transition from vegetative to inflorescence (*shoot meristem identity genes*) and from inflorescence to floral status (*floral meristem identity genes*). The main role played by meristem identity genes is to activate the *floral organ identity genes*. *Floral organ identity genes*, called also floral homeotic genes, were identified for the first time in *A. thaliana* and *A. majus* studying mutants where flowers exhibited homeotic organ transformations (Krizek and

Fletcher, 2005; Causier *et al*., 2010; Bowman *et al*., 2012). As in most of Angiosperms, in this two model species flower structure is composed four concentrical whorls of organs, which are in sequence from outermost to innermost sepals, petals, stamens and carpels. Mutants identified in *Arabidopsis* and *Antirrhinum* were grouped in three different classes, called A, B and C. Loss of A function generates a transformation of sepals in the first whorl into carpels and petals in the second whorl into stamens. Class B mutants have petals (second whorl) converted into sepals and stamens (third whorl) into carpels, while a loss in C activity causes transformation of stamens (third whorl) in petals and carpels (fourth whorl) into sepals (Fig. 1.2). Studying mutations in floral structure, where the arrangements of flower organs is changed, the function of the genes necessary for flower organs identity was understood (Haughn and Somerville, 1988; Coen and Meyerowitz, 1991).

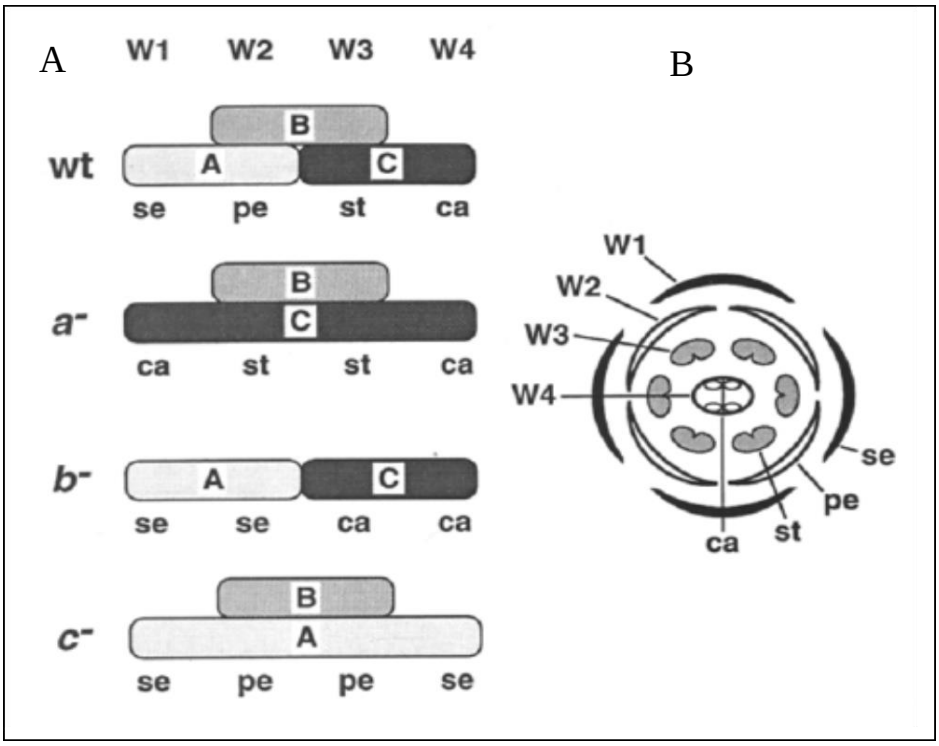

Figure 1.2. Description of floral whorl arrangements in *Arabidopsis thaliana* and the organization of ABC model. A) Action mode of floral organ identity genes according to the ABC model. B) Schematic representation of floral whorl in Arabidopsis, where W1 indicates the first whorl, sepals; W2 the second whorl, petals; W3 third whorl, stamens; W4 fourth whorl, carpels. (Figure from Weigel and Meyerowitz, 1994).

In 1991, a basal model to explain the function and the manner how homeotic genes act to determine floral organ identity was proposed (Coen and Meyerowitz, 1991). This model, called "ABC model" suggests the division of floral homeotic genes in three classes A, B and C that are characterized by different activity. Genes that are member of these three classes specify the identity of different floral whorls. Expression of A function alone determines the sepal formation (first whorl). The combination of A and B functions specifies the petal development (second whorl) while combination of B and C activity the stamens (third whorl). Expression of C genes alone specifies carpels development (fourth whorl). The model also suggests that A and C have an inhibitory activity, they negatively regulate

each other. In fact, when A function is lost in all floral whorls is present the C function, while when C function is missing could be observed a opposite situation (Weigel and Meyerowitz, 1994; Riechmann and Meyerowitz, 1997; Theissen *et al*., 2000).

According to the ABC model, in *Arabidopsis* the A function is controlled by two genes, *APETALA1* (*AP1*) and *APETALA2* (*AP2*); also the B function is regulated by two genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), while C only by the *AGAMOUS* (*AG*) gene. In *Antirrhinum* the class A gene is represented by *SQUAMOSA* (*SQUA*), class B by *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), while C by *PLENA* (*PLE*).

Cloning of these genes revealed that they encode for transcription factors, which are involved in the regulation of other genes that act directly or indirectly in the floral organs formation (Theissen *et al*., 2000; Theissen and Saedler, 1999). In all of these genes, except for *AP2*, it was identified a conserved DNA sequence of 180 bp that corresponds to a conserved region of 60 amino acids, named MADS-box domain which shows a higher sequence similarity between different plant species suggesting that these gene have a common evolutionary ancestor (Theissen *et al*., 1996; Weigel and Meyerowitz, 1994; Schwarz-Sommer *et al*., 1990; Yanofsky *et al*., 1990). Genes characterized by the MADS domain are members of MADS-box family (Ma *et al*., 1991; Davies and Schwarz-Sommer, 1994). The MADS acronym derives from the first four proteins discovered within this group, *MCM1* discovered in yeast, *Saccharomyces cerevisiae*; *AGAMOUS* from *A. thaliana*, *DEFICIENS* from *A. majus* and *Serum Response Factor* (*SRF*), a human protein (Schwarz-Sommer *et al*., 1990). In plants, the MADS-box proteins are characterized by four different domains that are the MADS (M), intervening (I), keratin-like (K) and C-terminal (C) regions (Theissen *et al*., 1996; Purugganan *et al*., 1995; Ma *et al*., 1991) (Fig.1.3). Genes encoding proteins of this type are called MYKC-type MADS-box genes (Münster *et al*., 1997). The MADS domain, located at the N-terminal, is necessary for DNA binding and it works to perform the dimerization and accessory factor-binding functions (McGonigle *et al*., 1996; Riechmann and Meyerowitz, 1997; Immink *et al*., 2002). The I domain is positioned downstream of the MADS and upstream of K regions and it is characterized by a conserved sequence of 30 amino acids (Münster *et al*., 1997; Ma *et al*., 1991). The K domain, presents only in MADS-box proteins in plants, is formed by 70 conserved hydrophobic amino acids necessary to amphipathic helix formation and it is important in protein-protein interaction and dimerization processes (Fan *et al*., 1997; Yang *et al*., 2003). All these domains are conserved among different MADS-box proteins, while the C-terminal region is characterized by high variability in length and sequence (Moon *et al*., 1999; Honma and Goto, 2001). The C-terminal region encodes for a transcriptional activation domain and it was hypothesized as indispensable for DNA binding and protein dimerization (Zachgo *et al*., 1995).

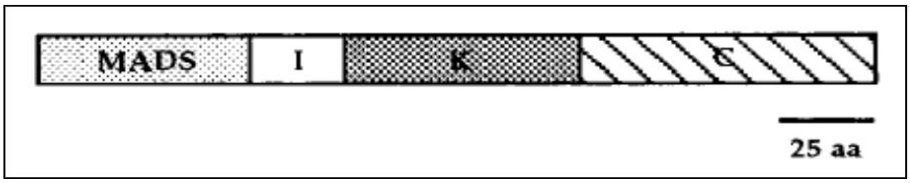

Figure 1.3. Representation of different domains, which characterized the structure of MADS-box genes. Downstream to the N terminal there is the MADS domain that is highly conserved across different eudicots species. The K domain is located after the intervening region (I) and is characterized by 70 amino acids important in protein-protein interaction. The most variable domain is represented by the C-terminal region, which is variable in size and sequence. (Figure from Kramer *et al*., 1998).

More recent studies gave new information about the possibility to extend the classical ABC model (Causier *et al*., 2010; Bowman *et al*., 2012). A main information and revision is represented by the introduction in this basal model of another class of genes that acts in redundant manner and is fundamental for A, B and C function (Pelaz *et al*., 2000; Ditta *et al*., 2004). These genes encode for MADS proteins and for this reason they were incorporated within the ABC model as conferring the E function. The function of these genes was discovered studying the floral morphology of loss of function mutants in petunia and in tomato where the second, third and fourth whorl were transformed into sepals (Angenent *et al*., 1994; Pnueli *et al*., 1994). In *Arabidopsis* class E genes were studied in detail and according to the mutant phenotype they were called *SEPALLATA* (*SEP*) genes. In *Arabidopsis* there are three members of the class E MADS-box genes, *SEP1*, *SEP2* and *SEP3*. In addition, the class D was added to the model, including another set of genes discovered in petunia, *FLORAL BINDING PROTEIN 7* (*FBP7*) and *FLORAL BINDING PROTEIN 11* (*FBP11*) which are involved in the control of ovule development (Colombo *et al*., 1995). In *Arabidopsis*, genes orthologous of *FBP7* and *FBP11* were identified in *AGAMOUS-LIKE 11* (*AGL11*), recently renamed *SEEDSTICK* (*STK*), *SHATTERPROOF 1* (*SHP1*) and *SHP2* (Pinyopich *et al*., 2003; Alvarez and Smyth, 1999). With the introduction of D and E functions the model was extended from ABC to ABC(DE) (Fig. 1.4).

Phylogenetic reconstruction of MADS-box genes show that they cluster into several defined gene clades (Theissen *et al*., 1996). In each clade, are grouped genes which have highly related functions and similar expression patterns. The MADS-box genes that provide the A, B and C function are members of *SQUAMOSA*-like, *DEFICIENS* or *GLOBOSA*-like and *AGAMOUS*-like clade respectively (Theissen *et al*., 1996; Purugganan *et al*., 1995; Doyle, 1994). The genes grouped in D and E class are members of the AGAMOUS-like clade, together with class C genes (Angenent and Colombo, 1996).

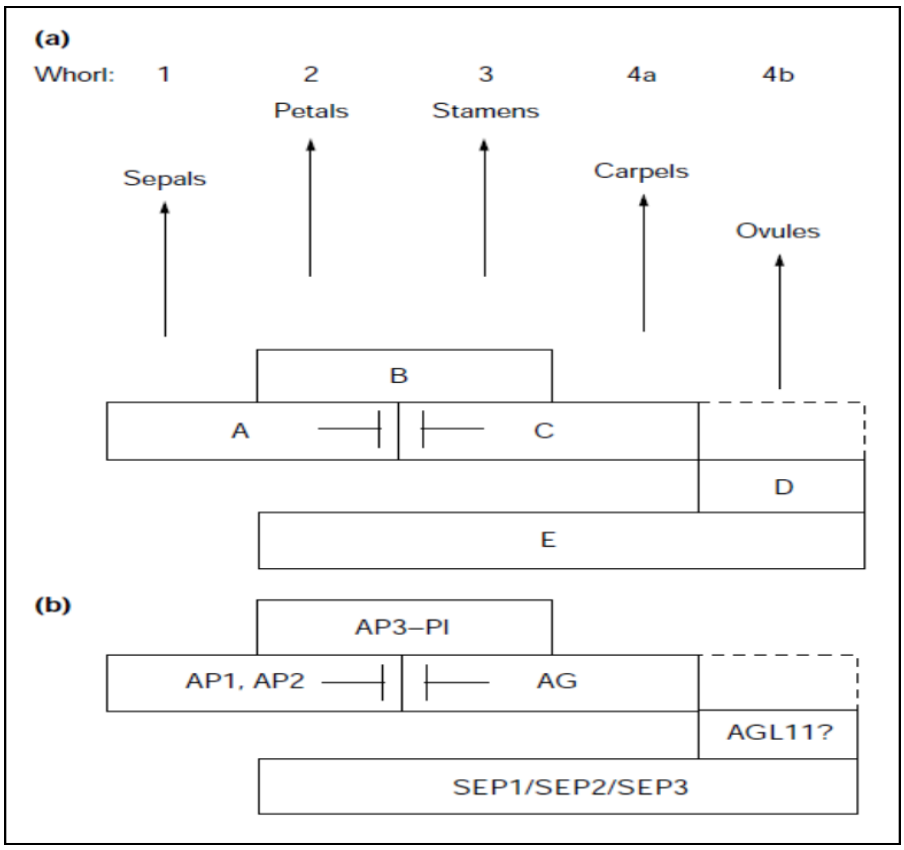

Figure 1.4. ABC(DE) model in *Arabidopsis thaliana*. a) Representation of each class of gene and its function in controlling the identity of floral organs. b) For each class of MADS-box gene were determined in *A. thaliana* the genes that are members of them. (Figure from Theissen, 2001).

All floral identity genes or gene products can interact together for determining the identity of different flower organs. The interaction was studied using the electrophoretic mobility and yeast two-hybrid system in *Arabidopsis* and *Antirrhinum*. These studies revealed that the formation of DNA-binding dimers shows a high degree of partner-specificity (Reichmann *et al*., 1996; Davies *et al*., 1996; Fan *et al*., 1997). In *Arabidopsis* the class B gene *AP3* and *PI* interacts with the class C gene *AG* for specifying stamen identity. The interaction between AP3-PI involved specific amino acids residues that are mapped in the K region such as Glu-97 and Asp-98 for PI and Asp-98 and Arg-102 for AP3 (Yang *et al*., 2003). The class C protein, AG forms DNA-binding dimers with proteins from class E, particularly with AGL2-like (Davies *et al*., 1996; Fan *et al*., 1997). Theissen (2001) has proposed a "quartet model" to explain how MADS-box proteins interact to specify the organ identity. According to the quartet model, the floral organ identity is determined only when the MADS proteins interact developing a tetrameric structure (Fig. 1.5). Each structure is composed of two dimers of MADS proteins, each of which binds to a single DNA binding site. Considering this model, in *Arabidopsis* sepals are specify by interaction AP1-AP1-unknown proteins, petals by AP1-AP3-PI-SEP, stamens by AP3-PI-AG-SEP and carpels by AG-AG-SEP-SEP. Recent studies demonstrated that the AP1 interact with SEP4 protein, which plays an importan role in meristem identity (Ditta *et al*., 2004).

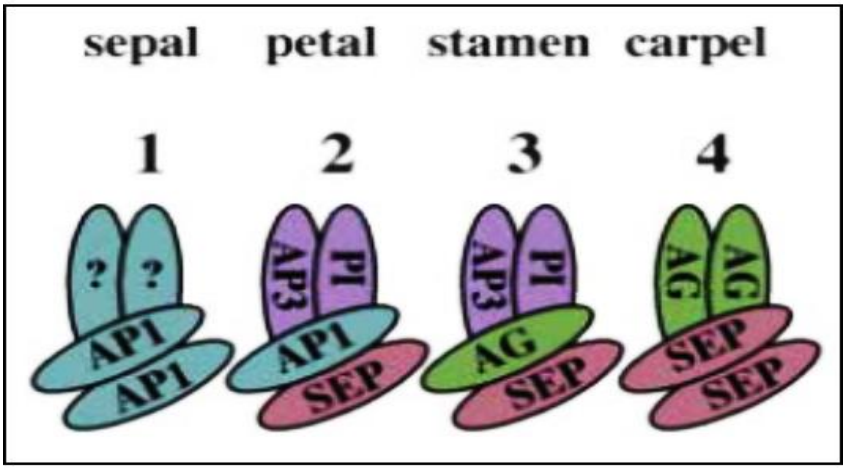

Figure 1.5. Quartet model of MADS-box gene function in *Arabidopsis thaliana*. According to this model the floral identity is determined only when the MADS proteins interact forming a tetrameric structure. (Figure from Jack, 2004).

The class B MADS-box genes are required to specify the identity of second and third whorl and their expression remains active until the development of petals and stamens is complete. The expression of these genes, together with class C genes, is activated by floral meristem identity genes, particularly *LEAFY* (*LFY*), *UNUSUAL FLORAL ORGAN* (*UFO*) and *AP1*. Strong evidence that the expression of class B genes, particularly *AP3*, is activated by *LFY* comes from analysis done in *Arabidopsis* on *lfy* mutant, where the expression of *AP3* was drastically reduced. This experiments given an information about a possible direct regulation of *LFY* on *AP3* (Lamb *et al*., 2002).

3.1 Duplication event in class B MADS-box genes

Studying class B genes in *Arabidopsis* and *Antirrhinum* and according to their high similarity in other plants it was hypothesized that their function and mode of action are conserved in eudicot species. Kramer *et al*., (1998) demonstrated that the expression pattern of class B genes in petals in other eudicot species was different from that detected in *Arabidopsis* and *Antirrhinum*, while in stamens it was similar. The authors also reported a possible duplication event that occurred in the *AP3*/*DEF* clade at the origin of higher eudicots radiation. These studies have demonstrates that for the *AP3*/*DEF* lineage in some higher eudicots exist two paralogues, one corresponds to the eu*AP3* and the second one is represented by tomato *AP3* paralog *TM6* (*TOMATO MADS BOX GENE 6*; the first gene characterized in tomato for this lineage; Pnueli *et al*., 1991). eu*AP3* and *TM6* lineages are distinguishable on the basis of structure of C-terminal domain. The C-terminal region of *TM6* is very similar to the paleo*AP3* motif, which is characteristic of AP3/DEF proteins from lower eudicots, magnolid dicots, monocots and basal angiosperms, while the motif of eu*AP3* seems to be specific of higher eudicots and maybe it originated from a frameshift mutation (Kramer and Irish, 1999; Vandenbussche *et al*., 2003). Lamb and Irish (2003) reported that eu*AP3* in higher eudicots has probably developed a novel function in floral morphogenesis with respect to the paleo*AP3* motif. Similarly, two paralogues were also discovered in the *PI* lineage that shows a higher conservation in sequence than the members of the *AP3* lineage (Kramer *et al*., 1998).

Successive studies revealed in several eudicots species the presence of more than one gene for *AP3*/*DEF* and for *PI*/*GLO* suggesting a possible subsequent subfunctionalization of class B function (Kramer *et al*., 1998). A duplication event when occurs can results into different consequences as silencing of one gene copy (nonfunctionalization), acquisition of novel function by one gene copy (neofunctionalization) or partitioning of original function between the two gene copies (subfunctionalization) (Lynch and Conery, 2000). Subfunctionalization as consequence of a duplication is typical for genes that are characterized by multiple regulatory regions, because a partitioning of function in two genes derived from a loss of function mutations is more frequent than a gain of function mutations that generate a neofunctionalization (Lynch and Force, 2000).

The *Solanacee*, such as petunia, tobacco and tomato have both duplications, in the *AP3* and *PI* lineages and represent a useful instrument for studying the duplication events and the possible diversification in function inside these two different clades.

In *Petunia hybrida* studies conducted by van der Krol *et al*., (1993) demonstrated that the class B MADS-box genes have a different organization respect to that described in *Arabidopsis* and *Antirrhinum*. The *DEF* paralog, called also *Green Petals* (*GP*), plays a different function. A mutation in the *PhDEF* gene causes a complete conversion of petals into sepals while the stamens remain with normal morphology. Successive analyses conducted on different petunia mutants revealed that the *TM6* gene is involved in the determination of stamen identity acting in redundant manner with *DEF* (Rijpkema *et al*., 2006). In this work the authors selected a *tm6 def* double mutant that showed a complete conversion of petals into sepals and stamens into carpels like the phenotype obtained in *def* and *ap3* mutants in *Antirrhinum* and *Arabidopsis*. In *Petunia*, like in tomato, were also discovered two paralogues for the *GLO*/*PI* lineage, called *PhGLO1* and *PhGLO2* and in this conditions the interaction between class B MADS-box genes became more complex. The petal development is controlled by heterodimers formation between *DEF*/*GLO1* and *DEF*/*GLO2* that are necessary also for the stamens together with the complex between *TM6*/*GLO2* (Rijpkema *et al*., 2006). The analysis of *PhDEF* and *PhTM6* together with *SlDEF* and *TM6* promoter region sequences revealed the presence of highly conserved domains inside the eu*AP3* lineage, which are missing in paleo*AP3* (*TM6*) clade (Rijpkema *et al*., 2006).

In tomato as in petunia there are four members for the class B MADS-box genes: *Tomato MADS box gene 6* (*TM6*) (syn. *TDR6*; Busi *et al*., 2003; Pnueli *et al*., 1991) and *Tomato APETALA 3* (*TAP3*) (syn. *SlDEF*, *LeAP3*; Kramer *et al*., 1998; de Martino *et al*., 2006) for the AP3 clade and *Tomato PISTILLATA* (*TPI*) (de Martino *et al*., 2006; syn. *SlGLO2*; Mazzucato *et al*., 2008) and *Solanum lycopersicum GLOBOSA* (*SlGLO*) (Mazzucato *et al*., 2008; syn. *SlGLO1*, *LePI*, *TPIB*; Leseberg *et al*., 2008; Geuten and Irish, 2010) for the PI clade. Studies conducted on loss-of-function mutants for the *DEF* function have showed that *SlDEF* gene is necessary to specify the identity of petals and stamens. Contrarily to *def* mutant in *Petunia*, *tap3* mutants are characterized by complete transformation on petals into sepals and stamens into carpels, while RNA interference mutants for

TM6 gene show a phenotype where only stamen morphology is defective (de Martino *et al*., 2006). Stamens of *TM6* mutants are characterized by conversion into carpelloid organs with external ovules in the surface. The mutant phenotypes observed in tomato and the expression of these two genes in WT plants revealed that both, *SlDEF* and *TM6* have developed different functions. *SlDEF* expression is detected in petals and stamens while that of *TM6* in stamens and carpels (de Martino *et al*., 2006). Overexpression of *TM6* in *tap3* mutant shows a flower phenotype with a partial restoration of petal morphology. Thus indicating that, when the *DEF* function is missing, *TM6* can substituted it but not in equal manner (de Martino *et al*., 2006).

To understand the role played by the two paralogues of the *PI* clade, loss-of-function tomato mutants for *SlGLO1* and *SlGLO2* were obtained (Geuten and Irish, 2010). In *Nicotiana benthamiana* and *P. hybrida* mutants for both *GLO* genes show phenotypic alteration in petals and stamens (Vandenbussche *et al*., 2004), while knock-out mutants for *SlGLO1* or *SlGLO2* in tomato show only stamen alterations, thus indicating no involvement of them in petal formation and also that they have a similar expression pattern (Geuten and Irish, 2010). When the function of both orthologues is completely missing, the flower acquires a typical phenotype, where the petals are converted into sepals and stamens into carpels, phenocopying the previously described *tap3* mutant (de Martino *et al*., 2006). This alteration was also observed in *N. benthamiana* when the function of both genes is lost.

To summarize, the experiment conducted by de Martino *et al*., (2006) and Geuten and Irish (2010) in tomato, reported that a phenotype typical of class B mutants is the results of missing expression of *SlDEF*. In addition, these works also suggest the possible interaction that occurs between proteins encoded by members of class B MADS-box genes. In tomato, contrarily to petunia and tobacco, the DEF protein forms heterodimers with GLO1, while TM6 interacts only with GLO2 (Leseberg *et al*., 2008) suggesting that the protein interaction process itself differs between species (Fig.1.6).

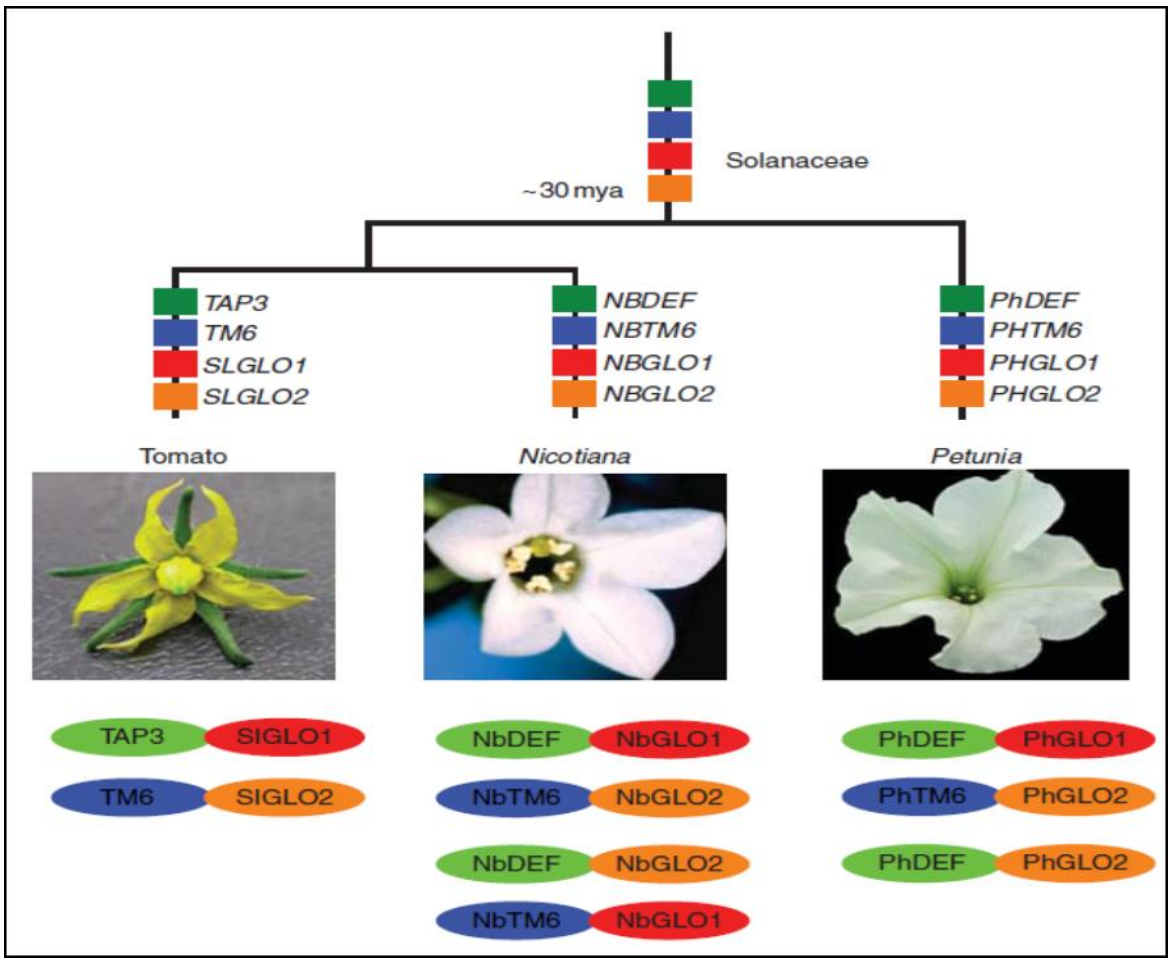

Figure 1.6. Origin and evolution of class B MADS-box genes in *Solanacee* family. In some species of the *Solanacee* family, the duplication event that occurs in class B MADS-box genes originated a subfunctionalization, which causes a partition of the ancestral function in both paralogues of the *AP3* and *PI* lineages. The heterodimers formation in these species is different, indicating a different protein-protein interaction between species. (Figure from Geuten *et al*., 2011).

4. Male sterility in tomato

The use of male sterile lines of tomato to facilitate and decrease the cost of hybrid seed production was frequently suggested in the past (Barrons, 1943; Currence, 1944; Rick, 1945). The male sterile phenotype mainly affects the male organs, which appear reduced in size and sometimes modified in color intensity and pollen grains are absent or no viable. The remaining part of the plant, except for the flower size that is usually smaller than the wild type, has normal features (Rick, 1950). In addition, good male sterile tomato lines should have a style sufficiently protruding beyond the anthers (exerted) to facilitate the pollination process and the female fertility should not be affected. When viable pollen from other genotypes is applied on the stigma surface of the sterile plants, it is possible have fruit and seed sets in a normal fashion (Rick, 1950).

Genic male sterility occurs naturally in tomato contrarily to the male sterility controlled by cytoplasmic factors. More than 55 male sterile (*ms*) genes/alleles were studied and identified and they were grouped mainly in two classes according to the alteration formed in the staminal cone (Kaul, 1988). In the first class, are grouped all mutants that present structural defects in the anther

cone, such as all *stamenless* genotypes, while in the second one there are all other mutant genotypes, such as *positional sterile* (*ps*), *positional sterile-2* (*ps-2*) and *exerted stigma* (*ex*), that show functional sterility, so they have a normal anther cone with lack of dehiscence of pollen grains. Using structural sterility in tomato hybrid production there are some advantages associated with the elimination of hand emasculation, because the stigma is accessible for pollination without removal of the anther cone, due to the malformation in the morphology of male organs and stable sterility expression. The main disadvantage is due to the procedure for the maintenance of the sterile line, which could be resolved using marker associated with the male sterile gene (Philouze, 1974; Durand, 1981; Tanksley and Zamir, 1988) or using environmental factors (Sawhney, 1997; Masuda *et al*., 2000) or chemical reagents to restore the fertility (Von Schmidt and Schmidt, 1981). This limit is the same that could be observed using genotypes with functional sterility, where there could also be the disadvantage associated with the possibility that in some mutants like *ps* and *ps-2* is necessary operate with hand emasculation. The male sterile mutants like some members of the *stamenless* series and others, such as *male sterile-10* (*ms-10*), *ms-15* and *ms-32*, which are all characterized by defects in anther morphology and exerted stigma seem to be more applicable in breeding programs and in hybrid seed production.

An easier and more rapid anther emasculation is not the only aspect that is needed in order to have an efficient system for hybrid seed production. Hybrid yield production is another aspect that plays an important role. Is necessary to use a male sterile system in tomato seed production that joins an easy emasculation with a higher production, characteristics that are expressed by environmental-sensitive genic male sterile lines.

In tomato mutants that are sensitive to temperature conditions were described. Mutations such as *stamenless* (*sl*) and *stamenless-2* (*sl-2*), both members of the *stamenless* series, are sterile in high temperature conditions, while low temperatures determine a fertility restoration, similarly to *ms-15* and *ms-33* (Gomez *et al*., 1999; Sawhney and Greyson, 1973; Sawhney, 1997). Another TGMS mutant is *variable male sterile* (*vms*), which is characterized by sterility in high temperature conditions, particularly with temperature of 30°C or above (Rick and Boynton, 1967). Recently, a photoperiod-sensitive male sterile mutant was discovered by Prof. V.K. Sawhney (2004), called *7B-1*, which show complete sterility in long day (LD) conditions, while in short day (SD) conditions the sterility is restored and the mutant is able to produce fruits and seeds.

The male sterile phenotype characteristic of these mutants, particularly *sl*, *sl-2*, *pistillata-2* (*pi-2*), *vms* and *7B-1* and their advantages and disadvantages in hybrid seed production are described below.

4.1. Conditional male sterile mutants in tomato

The *stamenless* and *TAP3* mutants

The tomato monogenic male sterile mutant *stamenless* (*sl*) was described for the first time by Bishop in 1954. The phenotype of this mutant is characterized by complete transformation of stamens into carpels and partial transformation of petals into sepals. The transformed stamens are fused with the

proper carpels to form a unique gynoecium. Initially, this mutant was described as a recessive mutation (Bishop, 1954), while successive studies showed that *sl* is semidominant (Gomez *et al*., 1999). Gomez *et al*. (1999) found three plant mutant phenotypes, wild type (WT), homozygous and heterozygous for the *sl* allele. The homozygous *sl* plants produce the typical phenotype, while the heterozygotes have an intermediate phenotype, with WT petals, short and distorted stamens and external ovules on the basis of the adaxial surface (Fig. 1.7 A, B and C). The *sl* phenotype is very similar to those shown by the homeotic mutants *pistillata* (*pi*) or *apetala3* (*ap-3*) in *Arabidopsis thaliana* and *deficiens* (*def*) or *globosa* (*glo*) in *Anthirrinum majus*, all belonging to B-class MADS box genes. In tomato, the B-class MADS box genes are represented by four members, *TM6* and *SlDEF* (located respectively on chr. 2 and 4) orthologous of *AP3*, and *SlGLO1* and *SlGLO2* (located respectively on chr. 8 and 6) orthologous of *PI*. The *Sl* locus was located on chromosome 4 (Khush, 1965); Gomez *et al*. (1999) found that the *sl* mutation affects the *SlDEF* gene, using a *St-deficiens* probe from *Solanum tuberosum*.

The male sterile mutation *sl* has a phenotype very useful for the tomato hybrid seed production. A similar *sl* mutation was obteined in Primabel cultivar (*sl-Pr*) after irradiation (Philouze, 1991) and it showed a phenotype with stamens completely absent and fruits with modified form. The *sl-Pr* phenotype was characterized for the first time by Quinet *et al*. (2014). The mutant, named also *TAP3* for the similarity with the knock out mutant for the *TAP3* gene (de Martino *et al*., 2006), shows a strong phenotype, the petals are completely transformed into sepals and the stamens are absent and transformed into carpels (Fig.1.7 D and E). The homozygous plants showed a phenotype stronger than that described by Gomez (1999), while both heterozygous plants were, more or less similar to the WT, depending on the growth conditions. The results of the characterization of the *sl-Pr* mutant confirmed, through the allelism test and sequencing that *sl* and *TAP3* are allelic and involved the same gene, *SlDEF*, located on chr. 4.

The *stamenless-2* mutant

The tomato male sterile mutant *stamenless-2* (*sl-2*) was first described by Hafen and Stevenson in 1958 as an allelic mutant of *sl*, and as member of an allelic series with different degree of homeotic transformation in the second and third floral whorl. These studies indicated that the *sl-2* mutation was due a single recessive gene. The phenotype of the *sl-2* mutant showed alteration only in the structure of the anther cone, in agreement with the first description made by Hafen and Stevenson (1955). They described the mutant flowers having twisted, distorted and laterally free anthers and with the stigma protruding beyond them. Later, Sawhney and Greyson (1969) found also the presence of external ovules in the adaxial surface of the stamens. The anther modification is expressed only in homozygous plants, with a high variability in alteration degree. Heterozygotes have the same anther morphology of the WT plants, this to confirm that the mutation is due to a single recessive gene (Sawhney and Greyson, 1973). The *sl-2* plants showed a different degree in the alteration of anther structure and in the number of external ovules, when they were grown in different conditions. The

mutants showed a higher number of external ovules and a higher degree of anther alterations when they were grown in summer field conditions, while these traits were reduced in winter greenhouse conditions (Sawhney and Greyson, 1973). This indicated that the mutation was influenced by environmental factors. Successive studies confirmed the first observation about the environmental factors influence on the expression of *sl-2*. In detail, it was reported that this mutation was temperature sensitive, so with an intermediate temperature (23/18°C) the plants showed male sterile flowers with no viable pollen, while in low temperature conditions (18/15°C) the plants produced several male fertile flowers with viable pollen (Sawhney, 1983). These results were observed also after treatments with exogenous hormones such as gibberellins and auxins. However, applications of gibberellic acid (GA_3) to *sl-2* plants induced the conversion into WT flowers as occurred in low temperatures. In contrast, applications of indole-3-acetic acid (IAA) produced completely male sterile flowers in agreement with the phenotype in high temperature conditions (Sawhney and Greyson, 1973). These observations demonstrated that the *sl-2* expression can be modified by different temperature conditions and hormones applications.

The *7B-1* mutant

The tomato male sterile *7B-1* mutant was described for the first time by Sawhney (1997) as a homozygous recessive photoperiod-sensitive phenotype. The *7B-1* phenotype is very similar to that of *sl-2* mutants but its expression is affected by two different environmental factors, in one case by the photoperiod and in the second one by the temperature. The *7B-1* mutant shows male sterility, with a strong phenotype, in long days (LD), while in short days (SD) it produces several male fertile flowers and the phenotype is less strong. The phenotype of *7B-1* plants grown in LD conditions (16 hours light/ 8 hours dark at 23/18°C) includes flowers with short, retracted and distorted anthers, the style and stigma elongated and no fruit set (in some cases, production of small parthenocarpic fruits was reported; Sawhney, 2004). In contrast, in SD conditions (8 hours light/ 16 hours dark at 23/18°C) the mutant produces flowers with normal phenotype and viable pollen, so it is possible to observe the production of several fruits with many seeds. (Sawhney, 1997; 2004; Fig.1.7 F and G). This spontaneous mutant represents an important system for tomato hybrid seed production, because its particular male sterile phenotype makes possible the pollination without the manual emasculation (Sawhney, 2004) and at the same time, the elimination of this operation determines a strong decrease of production costs.

Successive studies showed that the *7B-1* seed germination presents a high resistance to abiotic stresses, such as osmotic, salt and low temperature stress. The capacity of *7B-1* seeds to grown with various stresses and in the presence of abscisic acid (ABA) was due, probably, to the high level of endogenous ABA accumulated in the seeds and it was reported that this competence matches with a lower sensitivity to the light, particularly to the blue light (Fellner and Sawhney, 2001; 2002). In addition, *7B-1* seedlings showed under blue light conditions lower sensitivity to coronatine, a toxin produce by *Pseudomonas syringae* strains (Bergougnoux *et al*., 2009). In summary, the particular

male sterile phenotype, together with the improved resistance to biotic and abiotic stress of *7B-1* makes this genotype a very interesting system for hybrid seed production in tomato.

The *pistillate-2* (*pi-2*) mutant

The *pistillate-2* (*pi-2*) male sterile mutant, also called *green pistillate* (*gp*) (Rasmussen and Green, 1993), was originated from treatment with ethyl methanesulfonate (EMS) of the tomato cultivar Castelmart (Rick, 1993). The *pi-2* mutant shows a strong sterile phenotype, petals are completely converted into sepals and stamens into carpels. This unique phenotype makes it easy to recognize the homozygous recessive genotype. Flowers of these mutants present a protruding stigma, which allows a manual pollination with pollen from other genotypes. This operation produces irregular fruits resulting in abnormal seed production and for this reason *pi-2* is not very useful in tomato hybrid production (Fig. 1.7 H, I and J).

The phenotype of *pi-2* is similar to that of another male sterile mutant, called *pistillate* (*pi*) (Rick and Robinson, 1951), which shows a weaker modification of the corolla structure, in comparison to *pi-2* (Fig. 1.7 K). Both mutants, *pi* and *pi-2* recall the phenotype of MADS-box homeotic mutants. Data from allelism tests demonstrate that these mutations are not allelic and the *pi* mutation maps in the long arm of chromosome 3, position not compatible with anyone of the class B and E MADS box transcription factors (Olimpieri and Mazzucato, 2008). Recent studies confirmed the early hypothesis that *pi* represents a weak allele of *Falsiflora* (*FA*), the orthologous of *LEAFY* in tomato (Ruiu and Mazzucato, unpublished). These results indicate also that the phenotype of *pi-2* is more comparable with the phenotype showed by mutants of the *sl* series.

The *variable male sterile* (*vms*) mutant

variable male sterile (*vms*) is a spontaneous mutant discovered in *S. lycopersicum* cultivar San Marzano by Rick and Boynton (1967). They described it as a male sterile mutant, which showed sterility in summer field conditions with temperatures of 30°C, while in greenhouse temperatures up to 32°C are required. Abnormalities were found in the structure of the anther cone and some alterations affected also the corolla. Stamens presented distorted shape, separation from each other, discoloration and high variability in their structure (Rick and Boynton, 1967). Stainable pollen was not produced at anthesis and this reflected in plants completely unfruitful during all the summer season. The structure of the pistil appears normal; pollination with pollen from other genotypes determines production of normal fruits and seeds. The male sterile phenotype was observed only in summer period, from June to October in field conditions in the interior valley of California, while in the other months, in normal greenhouse growth conditions (experimental station of University of California, Davis, USA), the sterile phenotype reverted in fertile flowers and some viable pollen grains were produced. In the mentioned study (Rick and Boynton, 1967), it was also reported that the *vms* mutation is likely due to one single recessive gene, which is strongly linked with the *bushy* (*bu*) and *dialytic* (*dl*) markers. Previous studies (Rick and Khush, 1966) demonstrated that *bu* and *dl* are localized in the long arm of chr. 8, so it is derived that *vms* is localized also in the same

chromosome, probably in the region between these two markers. The *vms* mutant is characterized by a normal development of the tapetum cell and pollen until the microspore stage, when the major number of microspores aborts (Rick and Boynton, 1967). For this reason, *vms* is classified as a sporigenous (or "pollinic") sterile mutant and it is grouped in the class where the abort involved the microspore (see Table 3.1, Rick and Butler, 1956).

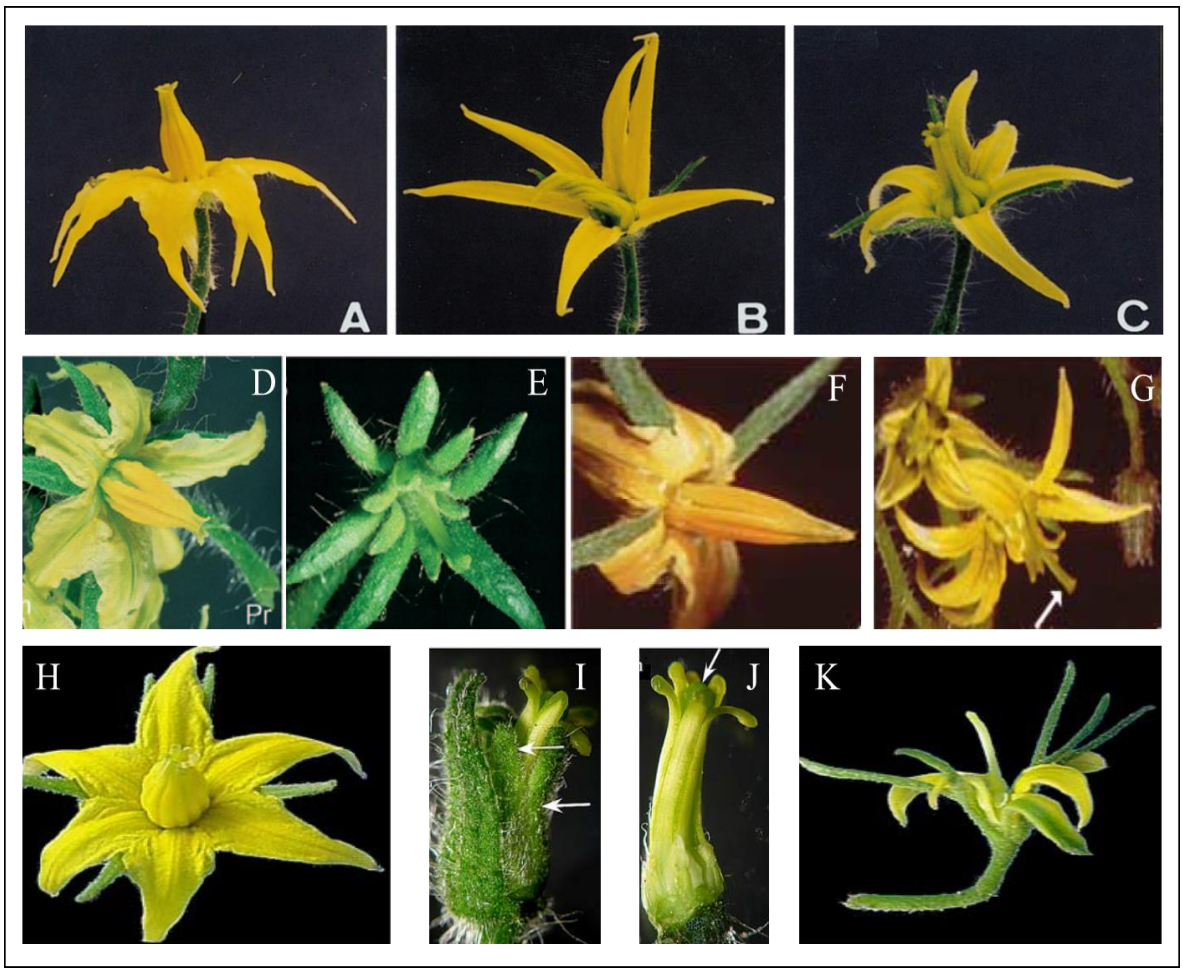

Figure 1.8. Flower phenotype of several conditional male sterile mutants discovered in tomato. A and B-C, respectively WT and *sl* flowers described by Gomez *et al*., (1999). The flower of *sl* plants grow under high temperature showed anthers converted into carpelloid organs. D and E, WT and *TAP3* mutant obtained in cultivar Primabel (Quinet *et al*., 2014). The *TAP3* mutant is characterized by complete transformation of petals into sepals and stamens into carpels. F and G, WT and *7B-1* mutant described by Sawhney (2004). The mutant flower is characterized by retracted and distorted anthers and the stigma is exerted above them. H and I-J, WT and *pi-2* flowers phenotype (Olimpieri and Mazzucato, 2008). In *pi-2*, like in *TAP3*, there is the complete conversion of second and third whorl in first and fourth whorl. K, flower of *pi* that shows petals with some green spots (Olimpieri and Mazzucato, 2008).

5. Similarity between class B homeotic mutants and conditional male sterile mutants in tomato

In *Arabidopsis* the homeotic mutants for all classes of genes that are involved in floral organ identity were studied and described in details (Bowman *et al*., 1989). Particularly, analysis of the flower

morphology of *Arabidopsis* mutants for *AP3* reported by Bowman *et al*., (1989) shows a strong similarity between these mutant phenotypes and the conditional male sterile mutants described in different crop species. The phenotype of *AP3* mutant in *Arabidopsis* is characterized from conversion of petals into sepals, which are not distinguishable from the true sepals in the first whorl except for the size that is small in mutant plants. The stamens in the third whorl show a different degree of carpelloid transformation, which becomes stronger when there is an increase in temperature (25/29°C). A decrease in temperature (e.g. to 16°C) determines the formation of organs in the second whorl, which have features similar to the wild type flowers, but cannot be considered as true petals while in the third whorl stamens return normal. This mutation resembles phenotypes showed by male sterile mutants where the sterility is influenced by environmental factors such as temperature and photoperiod. When the male sterile phenotype is due to specific environmental factors, changing them is possible have a partial or complete restoration of the phenotype which becomes likely normal. In tomato, were characterized different conditional male sterile mutants that had different degree of homeotic transformation of stamens and petals and it was hypothesized that they were members of an allelic series. Initially, this allelic series, called *stamenless* series, was characterized by five alleles that had different alterations in anther structure and also partial or complete transformation of petals into sepaloid organs (Hafen and Stevensons, 1958). The first mutant of the *stamenless* series analyzed was *stamenless (sl*) (Bishop, 1954) and successively *sl-2* which is characterized, contrarily to *sl* by petals with normal morphology. The *sl-2* anther cone phenotype depends from the temperature conditions showing different alterations such as formation of carpelloid structures or external ovules in the adaxial surface (Sawhney and Greyson, 1973a, 1973b; Sawhney, 1983). The *sl* phenotype observed initially by Bishop (1954) and successively by Gomez *et a*l., (1999) was very similar to those reported by de Martino *et al*., (2006) in loss-of-function mutants for *SlDEF* gene. The first molecular analysis for identifing the underlying gene, demonstrated that *sl* was due to a mutation in *SlDEF*, a class B MADS-box gene located on chromosome 4 (Gomez *et al*., 1999). These results were confirmed during the phenotypic and molecular study of the *TAP3* mutant, another conditional male sterile tomato mutant sensitive to the temperature (Quinet *et al*., 2014). The authors demonstrated that *TAP3* is allelic to *sl*, and that it is due to two point mutations in the coding region of *SlDEF*. The mutations are not located into the MADS domain but in K and C region. Differently, for *sl* the mutation was localized in the promoter region of *SlDEF*.

In tomato, conditional male sterile mutants where the phenotype and fertility restoration is dependent on a change in temperature and/or photoperiod conditions, are also influenced by treatments with plant growth regulators. In *Arabidopsis* it was observed that for the development of each floral organ hormones play an important role. For example stamen development reliant on almost all hormones, while in petal formation act gibberellins (GAs), auxin and jasmonic acid (JA). Differently, gynoecium development is influenced mainly by auxin action (Chandler, 2011). In male sterile tomato mutants, particularly in *sl-2* is known that the fertility restoration occurs when plants grown under low temperature but also when they are subjected a GA_3 treatments. Contrarily, high

temperature conditions and treatments with indole acetic acid (IAA) induced sterility (Sawhney, 1983, 1997). Successive studies demonstrated that in stamens of *sl-2* plants the amount of abscisic acid (ABA) is more abundant than in the other vegetative parts and with respect to the wild type genotype. The increase of ABA in stamens corresponds to alteration in anther structure and consequentially to the sterility condition; in contrast when the sterility is restored the amount of ABA inside the stamens decreases. These results suggested that the sterility condition in *sl-2* genotype is due to an imbalance of ABA level that is returned towards normality when the plants grow under low temperature (Singh and Sawhney, 1998). These data indicate that *sl-2* is associated with high levels of IAA and ABA and lower levels of GA_3, and probably it involves also changes in the biosynthesis and catabolism of these hormones, which have a common signal transduction pathway that affects pollen and stamen development (Singh and Sawhney, 1998).

6. Objectives and outline of the thesis

In addition to *sl* and *sl-2*, in tomato other male sterile mutants sensitive to the temperature and/or photoperiod, such as *pistillate-2* (*pi-2*), *variable male sterile* (*vms*) and *7B-1*, were studied and characterized at phenotypic level. The genes underlying them have not yet been identified.

This thesis aimed to the morphological and molecular characterization of environmental-sensitive genic male sterile genotypes in tomato. The main objective of the research was the phenotypic characterization of the *7B-1* mutant and the identification of the putative gene underlying the mutation. The other goals of this work were represented by studies conducted in order to characterize other tomato male sterility mutations, such as *sl-2*, *pi-2*, *TAP3* and *vms* in environmental conditions that favor or not the expression of the sterile phenotype.

In **Chapter 2,** it was described the morphological characterization of the *7B-1* and *sl-2* mutants, under environmental conditions that favor the sterility expression (long day and high temperature) and environmental conditions that determine a partial or complete fertility restoration (short day and low temperature). The phenotypic analysis was aimed to verify if the mechanism that controls the sterility/fertility restoration remains unaltered changing the geographic area.

In **Chapter 3,** it was described the phenotypic analysis of *sl*, *sl-2*, *TAP3*, *7B-1*, *pi-2* and *vms* and the allelism test done between all these mutant genotypes to gain more information about the relationship and the genetic nature of these mutations.

In **Chapter 4,** the results about the molecular analysis and the putative gene candidate of *7B-1* mutation and for its allele *sl-2* were reported. Mapping populations for the two mutations were developed and analyzed at molecular level using markers for all four members of class B MADS-box genes in order to identify a possible candidate gene. The putative gene involved in the *7B-1* mutation, *SlGLO2* located on chr. 6, was sequenced in WT and mutant samples, analyzing the whole genomic sequence including part of the promoter region. *7B-1* and WT flower buds and anthers

collected at different development stages were used to analyze the expression of the candidate gene and of the other three members of the class B MADS-box family by semi quantitative RT-PCR. The same plant samples were used in qPCR experiments to validate the expression of some genes found differentially expressed in WT and *7B-1* stamens after RNA-seq analysis. Results reported in this chapter supported the idea that the candidate gene for *7B-1* mutant is a member of class B MADS-box. The complementation experiment using the WT allele of *SlGLO2* gene to transform *7B-1* plants will is on going and will possibly confirm that.

In **Chapter 5,** two other male sterile mutations sensitive to the temperature, *pi-2* and *vms* were analyzed at morphological and molecular levels. For the *pi-2* mutation, it was performed an allelism test with the *TAP3* mutants described by Quinet *et al*., (2014) which demonstrated that the two mutations are allelic. Because the *TAP3* mutation is due to two point mutations found in the coding region of *SlDEF*, the *SlDEF* genomic sequence of WT and *pi-2* mutants was sequenced. The sequence analysis revealed that a single nucleotide substitution in the splicing site could determine the male sterile phenotype. For the second mutation, *vms*, it was screened and classified a mapping population classified according to the phenotype in WT and mutant individuals. The *vms* phenotype, which is sterile under summer season conditions with high temperature, was observed at different time intervals because it is influenced by subtle temperature variations. After the molecular screening, it was demonstrated that the candidate gene for this mutation is possibly another class B MADS-box gene, *SlGLO1*, located on chr.8. The WT and *vms* samples were sequenced and the analysis showed a nucleotidic substitution in the *SlGLO1* coding region, which causes changing in amino acid sequence that was predicted not tolerable.

Chapter 2

Morphological characterization of *7B-1* and *sl-2* tomato male sterile mutants

Introduction

Male sterility is defined as the inability of plants to produce or release functional pollen grains (Kaul, 1988). This inability is the result of missing production of functional anthers, microspores or male gametes (Kaul, 1988). Male sterility can result in mutations that involve nuclear or cytoplasmic genes and it can be classified into nuclear or genic, cytoplasmic and genetic-cytoplasmic male sterility (Kaul, 1988; see details in Chapter 1). The nuclear or genic male sterility (GMS) is controlled by nuclear genes (Sawhney, 1997) and it is characterized by Mendelian inheritance (Kaul, 1988). This kind of male sterility is known in a large number of monocotyledon and dicotyledon plant species. Normally, it is controlled by recessive genes (*ms*), but it can also be regulated, in few cases, by genes at the dominant status (*Ms*) (Kaul, 1988). Usually GMS is originated through spontaneous mutations, but it can also be obtained using chemicals mutagens (Fujimaki *et al*., 1977; Cross and Ladyman, 1991), radiations (Driscoll and Barlow 1976), genetic engineering (Mariani *et al*., 1990; Worrall *et al*., 1992; Goldberg *et al*., 1993), protoplast fusion (Kofer *et al*., 1990; Gourret *et al*., 1992), t-DNA transposon tagging (Aarts *et al*., 1993) and modifications in flavonoid pathway (Van Tunen *et al*., 1994). GMS is mainly due to recessive genes (*ms*), which were identified in a high number of plant species, for example in corn (Coe *et al*., 1987; Palmer *et al*., 1992), soybean (Palmer *et al*., 1992) and tomato (Stevens and Rick, 1986). Most *ms* genes act in the early phases of pollen development, at level of microsporogenesis or microgametogenesis. At the phenotypic level, the genic male sterile mutants are characterized by normal morphology of the whole plant with the exception of the flower structure, which shows different degree of stamen aberrations (Kaul, 1988). For example, in tomato male sterile mutants the flower and anther morphology results in a difference in size that is smaller in ms than in the WT plants. Anthers can show modifications that involve their transformation into carpelloid-like structures as in the *sl*, *sl-2, 7B-1* or *vms* mutants (Sawhney, 1994).

The expression of the GMS can be influenced by the action of environmental factors and in this case it is called environment-sensitive genic male sterility (EGMS, Smith, 1947). The main environmental factors that can influence GMS are temperature and photoperiod (He *et al*., 1999; Wu *et al*., 2003). The EGMS when occurs in a specific range of temperature is called temperature sensitive genic male sterility (TGMS), when is due to the action of photoperiod is called photoperiod-sensitive genic male sterility (PGMS) and when is due to both factors, we have the photo-thermo-sensitive genic male sterility (TPGMS). The EGMS can be seen as desirable strategy to overcome some limits that are associated with the utilization of the GMS in hybrid seed production (see Chapter 1, 2.4), because it determines sterility in certain conditions while in other the fertility is restored (Smith, 1947). The major problem linked with GMS for hybrid seed production is the maintenance of the male sterile

line (Perez-Prat and van Lookeren Campagne, 2002). The sterile line is maintained by backcrossing with heterozygous plants (maintainer) originating a progeny that is 50% fertile and 50% sterile (Frankel, 1973; Frankel and Galun 1977). Using the EGMS there is no need to have restorer gene in the male parents in two-line hybrids and this makes the seed production very simpler and more cost effective (Virmani *et al*., 2003). In addition, the EGMS have two limits, one is that the sterility is conditioned by the influence of environmental factors and the second one is that the multiplication of these lines is restricted by season and space (Virmani *et al*., 2003). The ideal EMGS line to use in hybrid seed production must respond to some important points: sterile plants should have complete sterility, the sterile-time should be at least 30 days a year, the mechanism of fertility restoration should be known very well and the seed set of fertile plant should be more than 30% (Siddiq and Ali, 1999). The environment male sterility can be also influenced by a decrease in nutritive elements. A complete sterility can be observed in wheat as consequence of copper (Agarwala *et al*., 1980) and boron deficiency (Rerkasem and Jamjod, 1997). The sensitive genotypes in conditions of microelements deficiency are used as female parents, while the tolerant as male parents for hybrid seed production (Virmani and Ilyas-Ahmed, 2001). Copper deficiency has effect in the first phase of flower bud development in *Chrysanthemum* (Graves and Sutcliffe, 1974), while in wheat it determines a reduction in yield production (Graham, 1976). In conditions where the nutritive reduction is very severe the flowers are characterized by no pollen production or no anther dehiscence (Agarwala *et al*., 1980).

For the male sterility influenced by environmental factors, the first evidence that temperature influenced the fertility was described in pepper (Martin and Crawford, 1951) and successively the EGMS was object of many works in a large number of plant species (Table 2.1).

Table 2.1. List of different environment-sensitive genic male sterile sources found in different plant species (from Virmani *et al*., 2003; for references within the Table see the original paper).

Crop	Environmental factor	Reference
Pepper	Temperature	Martin and Crawford (1951)
	Temperature	Peterson (1958)
	Temperature	Daskaloff (1972)
Cabbage	Temperature	Rundfeldt (1960)
Maize	Temperature	Duvick (1966)
	Temperature	He et al (1992, c.f. Yuan 1997)
Tomato	Temperature	Rick and Boynton (1967)
	Temperature	Abdallah and Verkerk (1968)
	Temperature	Steven and Rudich (1978)
	Temperature	Sawhney (1983)
Wheat	Daylength	Fisher (1972)
	Temperature	Jan (1974)
	Daylength and temperature	He et al (1992, c.f. Yuan 1997)
	Daylength and temperature	Tan et al (1992, c.f. Yuan 1997)
Barley	Daylength	Batch and Morgan (1974)
	Temperature	Sharma and Reinbergs (1976)
	Daylength	Ahokas and Hocket (1977)
Vicia faba	Temperature	Berthelem and Le Guen (1975)
	Light intensity	Duc (1980)
Cucurbits	Temperature	Rudich and Peles (1976)
Rice	Daylength and temperature	Shi (1981, 1985)
	Temperature	Zhou et al (1988), Sun et al (1989)
	Temperature	Maruyama et al (1991)
	Temperature	Virmani and Voc (1991)
Sesame	Temperature	Brar (1982)
Sorghum	Daylength and temperature	Murty (1995)
Soybean	Daylength	Wei et al (1994)
Brassica napus	Temperature	Xi et al (1997)
Wheat	Copper, boron, and molybdenum deficiency in soil	Agarwala et al (1979, 1980)
	Boron deficiency in soil	Rerkasem and Jamjod (1997)
Maize, barley, oats, and sunflower	Copper deficiency in soil	Dell (1981)

In rice, this system is very well studied and very used for hybrid seed production (Virmani *et al*., 2003). The first mutant isolated by Shi and Deng (1986) in the *Japonica* variety Nongken 58 was a PGMS mutant which is characterized in long day (LD, > 14 hours) by complete sterility that is restored when the line is grown under short day conditions (SD, < 13 hours). Similarly, in another rice mutant the fertility is increased when the plants are transferred from field conditions into growth chambers under a photoperiod of 12 hours of light (Oard *et al*., 1991). Another rice mutant had complete male sterility in LD conditions while the restoration of the fertility happened in SD (Dong *et al*., 1993). This latter mutant differed from the first Nongken 58, because the fertility was maintained in low temperature conditions (Dong *et al*., 1993). Although the first TGMS source found in rice was the *S-1* spontaneous mutant isolated in China that had male sterility with temperatures comprised between 28°C and 33°C while it showed fertility with a temperature range of 27-22°C (Yang and Wang, 1990; Sun *et al*., 1989). In rice was also studied the biochemical mechanisms that control the EGMS and they are more or less the same that influenced the GMS. In rice it was found that the sterility in LD may be due to reduction in content of proline in anthers (Chen and Xiao, 1987) and in peroxidase and esterase activity during microspores formation (Qian and Zhu, 1987). The transition of sterility into fertility, although, seems due to changes in carbohydrate level (Wang *et al*., 1990), in minerals up-take (Yang and Zhu, 1987) and in hormone balance (Xu et al., 1990; Yang *et al*., 1990; Luo *et al*., 1993).

In wheat, the first PGMS identified was a mutant that showed transformed anthers with presence of external ovules when grown in a 10 h of photoperiod, while with 16 h of light the morphology returns normal (Fisher, 1972). Other wheat male sterile mutants were identified, but several lines are difficult to use because they need an extreme range of temperatures or photoperiods (Yang and He, 1997; Luo *et al*., 1998; Ma and Shi, 2002). Recently in wheat a line, called 337S, was found that has sterility in SD and in low temperature conditions (Guo *et al*., 2006).

The first PGMS source observed in barley was a mutant where the fertility was drastically reduced when it was exposed at 10 hours of photoperiod (Batch and Morgan, 1974). Successive studies conducted on the *ms9* mutant demonstrated that it was completely sterile when grown in Finland while it showed partially restoration of fertility when it grown in Bozeman (Montana, USA) (Ahokas and Hockett, 1977), suggesting the influence of photoperiod in the restoration of fertility.

In maize, a particular male sterile mutant was discovered, that showed sterility in autumn and winter season, while it returned fertile in summer; however the expression was not due to the photoperiod action (He *et al*., 1997). In *Brassica oleracea*, a TGMS mutant showed conversion from sterility into fertility with temperatures of 10/11°C in 30 days, while with slightly different temperatures (12°C) the conversion into partial fertility took over two months (Dickson, 1970). In watermelon, it is known that the sex expression is influenced by temperature and photoperiod (Rudich and Peles, 1976), which, also play an important role, together with other environmental factors, in the ratio of female/male flowers in melon. Usually, low temperatures, high concentrations of microelements in the soil and SD conditions favor the formation of female flowers, while opposite conditions stimulate the production of male flowers (Kaul, 1988).

In tomato, the first observation about the influence of temperature in the expression of male sterility was done on the *sl* mutants, which showed complete absence of stamens when the plants were grown in summer field conditions (Bishop 1954). This mutant grown in winter greenhouse conditions showed always alterations in anthers structure, but to a lesser extent and with some production of viable pollen (Bishop, 1954). Gomez *et al*., (1999) have reported that *sl* shows more than 15% of flowers with restoration of fertility when the mutant genotype is grown in low temperature conditions. The same consideration was done for plants of *male sterile-15* (*ms-15*) and *male sterile-33* (*ms-33*) mutants grown with low temperatures. In this case, these environmental conditions determined a complete restoration of fertility (Schmidt and Schmidt, 1981). Starting from these first observations, other tomato male sterile mutants were found, where the sterility was controlled by temperature, such as *vms* (Rick and Boynton, 1967) (see Chapter 5) and *sl-2* (Sawhney and Greyson, 1973; Sawhney, 1983). Sawhney (1983) found that in a temperature-controlled experiment *sl-2* plants grown with temperatures between 28 and 23°C showed stamens with carpelloid features, while with a range of temperatures comprised between 18 and 15°C they had a normal anther morphology. The same plants grown in intermediate temperatures (between 23°C and 18°C) produced flowers with half-normal anthers and half anthers with carpelloid features (Sawhney, 1983). These data

suggested that a difference of 5°C in temperature were sufficient to determine a change in the fertility of *sl-2* plants. Together with morphological defects that occur in anthers, the *sl-2* mutant is also characterized by abnormalities in pollen development, which involve the tapetum degeneration and consequentially the formation of microspores (Sawhney and Bhadula, 1988). These data were supported from the evidence that in *sl-2* the activity and number of esterase enzyme, which play a role in hydrolysis of sporopollenin (Ahokas, 1976), are reduced like in the GMS and cytoplasmic male sterility (CMS) systems (van Marrewijk *et al*., 1986; Bhadula and Sawhney, 1987). In contrast, the *T-4* male sterile mutant obtained with irradiation of the cultivar First, a Japanese commercial tomato variety (Masuda *et al*., 1999) shows a complete fertility restoration and good seeds set in SD and a partial restoration and a low seed set in LD under low night temperature (12-18°C) (Masuda *et al*., 2007). These data demonstrate that this mutant is not a PGMS as it was initially hypothesized (Masuda *et al*., 2000) but is sensitive to changes in temperature conditions.

Recently, in tomato a new recessive male sterile mutant sensitive to photoperiod, called *7B-1*, was discovered (Sawhney, 2004). *7B-1* mutant is completely male sterile in LD when it is grown in summer field conditions. The flowers showed some production of viable pollen when the plants are grown under SD conditions with 8-10 hours of light. In sterility conditions, the *7B-1* flowers show anthers retracted, with few pollen grains. The protruding stigma makes easy the operations of pollination using pollen from others genotypes. Female fertility is not affected. Therefore, this capacity renders *7B-1* plants suitable as female parent for tomato hybrid seed production. In SD conditions, the fertility is restored and consequentially flowers are able to produce fruits and induce the seeds set. This PGMS mutant is able to maintain the capacity to show sterility in LD and to restore fertility in SD in different geographic areas, such as California and Chile (Sawhney, 1997).

Starting from these considerations about the possibility in tomato to restore fertility by the action of environmental factors, this Chapter aims to focalize the attention on this recently described mutant where the fertility can be restored thought the action of the photoperiod. *7B-1* has a particular phenotype that makes it an interesting instrument to use in tomato hybrid seed production (Sawhney, 2004). Starting from this observation about *7B-1*, the growth experiments described in this Chapter are focalized to understand the expression level of the sterility in Italy growth conditions. To obtain this information growth experiments were set up in different seasons in order to gain more information about the sterility/fertility restoration.

Together with the phenotypic characterization of *7B-1*, it was also described the phenotyping of another tomato male sterile mutant, *sl-2*, which shows a phenotype very similar to *7B-1*, but in contrast it is sensitive to the effect of temperatures (Sawhney and Greyson, 1973; Sawhney, 1983). The morphological characterization of both mutants was the first step, which is followed by allelism tests (Chapter 3) and a molecular analyses (Chapter 4) in order to understand if *7B-1* and *sl-2* involved a mutation at the same locus.

Materials and Methods

Plant material and morphological characterization of the 7B-1 *mutant*

A fixed line carrying the *7B-1* mutation and its isogenic wild-type (WT) in the background of cultivar Rutgers was kindly provided by Prof. V.K. Sawhney, (University of Saskatoon, Canada). *7B-1* is a photoperiod-sensitive male sterile mutant and it shows male sterility in LD conditions while it returns male fertile in SD conditions. In order to characterize this mutant in both conditions, it was grown in different seasons together with its near-isogenic WT. Twenty plants for *7B-1* and its WT counterpart were grown in summer field and in autumn greenhouse conditions in the experimental farm of Tuscia University (Viterbo, Italy) to observe the flower morphology.The flower morphology was observed at the anthesis stage (Stage 4; flowering staging according to Mazzucato *et al*., 1998), sampling two flowers per plant using the stereomicroscope. For each flower, the number of sepals and petals was counted and on one representative sepal and petal was used to measure the length and width. The anther, the ovary and the pistil length and width were also measured. Anthers were counted and classified according to their structure as normal, carpelloid, carpelloid with external ovules or styliform and adnate to the pistil. The fruit set in the first four inflorescence (derived by open pollination, OP) were counted and weighted in 12 WT and *7B-1* plants grown in summer and autumn conditions.The same fertility evaluation was done in fruits from four WT and mutant plants derived by hand pollination (HP) and shaking of the inflorescence. For HP, flowers were emasculated before anthesis and pollinated with fertile pollen grains of WT parents. From the ripe fruits collected, seed was extracted and counted. All the recorded data about the fruit and seed set were analyzed through descriptive statistics and presented as mean ± standard error of the mean (SEM).

Microscopy

To determine the *7B-1* pollen phenotype, two flowers at Stage 4 per plant were sampled for the evaluation of viability and capacity of germination. Each parameter evaluated in the mutant was compared with data from the isogenic WT line. Pollen viability was evaluated by light microscopy after staining the pollen with two drops of 1% acetic orcein solution. Pollen grains were classified as viable (V) or not viable (NV) based on their staining capability and morphology. The ratio of germination was determined after incubating mature fresh pollen in 200 µL of germination medium [sucrose 10% (w/v), boric acid 100 mg/L, calcium nitrate 300 mg/L, magnesium sulfate 200 mg/L and potassium nitrate 100 mg/L] at 37°C for 2 h. After the incubation in germination medium the pollen grains were stained with few drops of 0.005% aniline blue and observed using the UV light microscope. Pollen grains were classified as germinated or not germinated according to the possibility to observe the growth of pollen tube. All the recorded data about the pollen viability and germination were analyzed through descriptive statistics and presented as mean ± standard error of the mean (SEM).

Scanning electron microscopy (SEM) was used to better characterize the floral phenotype of the *7B-1* mutant in short and long day conditions. Anthers of WT and *7B-1* plants were sampled at Stage 3 and Stage 4 (according to Mazzucato *et al*., 1998). The explants were prepared by removing sepals and petals, individual anthers were separated from the androecium and then were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 h, rinsed overnight in cacodylate buffer, post-fixed in 1.0% osmium tetroxide for 10 h, rinsed again and dehydrated in a graded ethanol series. The plant material from WT and mutant flowers was observed by a 5200 JEOL JSMSEM (JEOL Ltd, Tokyo, Japan) as reported earlier (Mazzucato *et al.*, 1998).

WT and *7B-1* flower buds sampled at the Stage 0 (3-4 mm long flower bud) were used to analyze the meiotic process. In order to observe the meiosis in anthers and ovules each floral organ was prepared removing sepals and petals, individual anthers were separated from the androecium and for the ovules was removed part of the pericarp. Specimens were stained with one or two drops of 0.005% aniline blue and then the meiosis process was observed with UV fluorescence using a photomicroscope equipped with an epifluorescence illuminator (DM-455 dichroic mirror, EX-435/10 excitation filter, BA-460 barrier filter).

Plant material and morphological characterization of the sl-2 *mutant*

An F_2 population segregating the mutation *sl-2* (background not specified) was obtained from the C.M. Rick Tomato Genetics Resource Center (TGRC; University of California, Davis, USA) with accession number LA2-137. One line fixed for the WT (*Sl-2*) and one fixed for the mutant allele (*sl-2*) were selected from the original seed stock and used thereafter to study the mutant phenotype. Twenty plants for the WT and the *sl-2* mutat line were grown in field conditions during summer and in greenhouse conditions during autumn season to analyze the floral morphology and to obtain seeds. The floral phenotype, in both conditions, was observed by taking two flowers per plant at the flowering time (Stage 4) using the stereomicroscope. The flower morphology and anther structure was observed in details, as described before for the *7B-1* mutant. Anthers were classified according to their structure in normal, carpelloid and carpelloid with external ovules or carpelloid styliform adnate to the pistil.

Results and Discussion

Morphological characterization of 7B-1 *in LD and SD conditions*

The *7B-1* mutant is characterized by complete male sterility in LD when it grows in summer field conditions, while the fertility restoration can be observed when the plants grown under SD conditions with 8-10 hours of light (Sawhney, 2004). In sterility conditions, the *7B-1* flowers show anthers retracted, with few pollen grains and the stigma is protruding, while in SD the flowers return normal and some viable pollen was produced. To confirm this floral morphology in our growth conditions, 20 plants of WT and mutant genotype were grown in summer field and in autumn greenhouse conditions. All mutant plants grown under 15 h of photoperiod (LD, summer field conditions)

showed complete male sterility while under 8-10 h (SD, autumn greenhouse conditions) of light partially fertility restoration was observed. To confirm that in more detail, flowers were analyzed using the stereomicroscope. Flowers from the WT counterpart, observed in LD and SD conditions, has a typical morphology with green sepals, yellow petals, and yellow anthers laterally fused to form a staminal cone that encloses the gynoecium. The stigma is not protruding in this genetic background (inserted stigma) and for the manual pollination these flowers need to be emasculated (Fig. 2.1 A and D). The *7B-1* flowers observed in LD conditions showed a particular morphology, sepals and petals remained normal while anthers have heavy alterations. The anthers have a typical yellow color but they are short, retracted, laterally free and the stigma is exposed (exerted stigma, Fig. 2.1 B and E). Mutant plants grown in SD conditions show flowers with the anther alteration weaker than in the LD conditions (Fig. 2.1 C and F).Together at the alterations found in the anther structure was also observed a difference in pistil length that is due at interaction between genotype and environmental conditions. In LD conditions, *7B-1* flowers have a pistil more length than the WT while in SD the situation is inverted. The pistil length in *7B-1* is shorter than the WT. In both case, the pistil length is a consequence of a style elongation. Except alteration in the anthers morphology and pistil length no other difference between WT and *7B-1* in the floral or vegetative characters were found.

Each floral organ was measured, revealing differences in sepal dimensions in different environments and in petal dimensions between WT and *7B-1* (Table 2.2). Both genotypes showed longer and wider sepals in LD than in SD. For petal length and width, the difference is between genotypes, and it remains the same in both growth conditions. The dimensions are higher in WT than in mutated flowers (Table 2.2). For the anthers, the mutant showed a higher number, whereas the WT had longer anthers in both growth conditions. Strong differences between the two genotypes were evident in anther structure; the WT's anther showed a normal feature, structured with two lobes each containing two pollen sacs with forming pollen grains inside (Fig. 2.1 G). All mutated flowers observed showed anthers with carpelloid structures that are also present in the explants from SD but with lower frequency together with external ovules in the adaxial surface (Fig. 2.1 H and I; Table 2.2). Traits related to the female part of the flower showed significant GxE interaction. Pistil length was higher in the mutant in LD and in the WT in SD. This was essentially due to style elongation, as ovary length followed an opposite trend and was significant only in SD (Table 2.2).

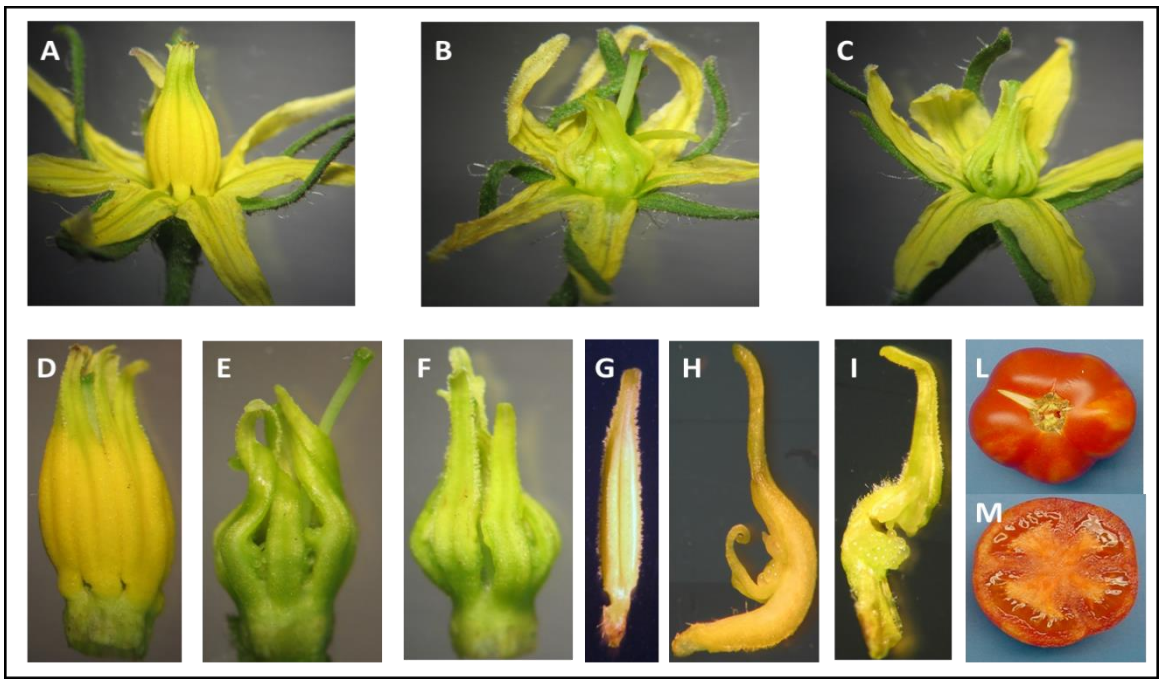

Figure 2.1. Flower phenotype of *7B-1* mutant and its WT counterpart. A, D and G, classical flower phenotype of WT (A), with anthers laterally fused to form the staminal cone (D) which appeared with normal morphology (G). B and E, *7B-1* flower and anther cone in LD conditions with anthers short, retracted and laterally free and the stigma protruding between them. C and F, *7B-1* flower in SD conditions with staminal cone alteration less strong than in LD. H and I, particular of *7B-1* anther morphology, presence of a carpelloid-like structure (H) or external ovules (I) in adaxial surface. L and M, fruits derived by open pollination of *7B-1* plants grown in LD with normal shape and parthenocarpic features.

These results are in agreement with the morphological data reported by Sawhney (2004) and with the observation that the *7B-1* mutation involved only a change in the structure of the anthers while the whole vegetative and floral structures remained unchanged. The modified anther conformation observed is the same that is reported in literature data and also the elongation of the stigma between the stamens which is only visible when the plants grow in summer season under LD. The protruding stigma outside the anthers is very interesting and useful characteristic because it makes easy the pollination techniques, bypassing the emasculation phase, which is very laborious and expensive for tomato hybrid seed production.

Table 2.2. Morphometric data of flowers from the tomato *7B-1* mutant and its near isogenic WT in the original seed stocks.

Floral organ	**Trait**	**WT LD**	**WT SD**	***7B-1 LD***	***7B-1 SD***	**P[1]**		
						G	E	GxE
Sepals	No.	6.0	6.3	6.8	6.3	ns	Ns	Ns
	Length	18.3	9.8	16.3	11.0	ns	***	Ns
	Width	2.1	1.6	1.9	1.4	ns	**	Ns
Petals	No.	6.9	6.1	6.0	6.4	ns	ns	Ns
	Length	20.8	16.7	16.0	15.4	*	ns	Ns
	Width	5.2	5.7	4.3	4.0	**	ns	Ns
Anthers	No.	6.2	6.1	6.6	6.4	*	ns	Ns
	Length	11.7	9.4	9.5	8.4	**	**	Ns
	Normal ant. (%)	96.8	100	0	0			
	Carpelloidy (%)	0	0	100	64.4			
	Carpelloid with EO (%)	3.2	0	0	35.6			
Pistil	Length	8.3	7.5	9.7	6.3	**, **		***[2]
Ovary	Length	2.4	2.0	2.1	2.4	ns, *		*[2]
	Width	3.1	1.9	2.6	2.2	ns, *		*[2]

[1] Two-ways ANOVA statistics for differences between Genotypes (G), Environments (E) and their interaction (GxE): ns, not significant; *, ** and ***, significant for P≤0.05, 0.01 and 0.001 respectively.

[2] For traits with significant interaction, differences between Genotypes within Environment (LD and SD) are reported as estimated by Student's t test.

Twelve WT and *7B-1* plants, in LD and SD, were grown until the maturation phase to observe the fruit and seed sets. The WT plants showed a good percentage of fruit set (~ 60% in LD and ~ 50% in SD) and seeds set (Fig.2.2). The only source of variation that showed significant differences in fruit set was "Genotype" (P≤0.001, not shown). For the other traits, all the interactions involving "pollination type" were significant thus the analysis of variance was carried out separately for each type of pollination. Under open pollination, WT fruits were significantly bigger and richer of seeds compared with the mutant. Fruits were also bigger in LD than in SD (Table 2.3). Under cross pollination, no difference was found in seed weight, whereas the mutant still produced less seed than the WT. Finally, shaking treatment produced the highest number of seeds in the WT (~140) (Fig.2.2 D and H) and was characterized by significant GxE interaction. The *7B-1* genotype, as expected, in LD produced no fruit set, except for a few parthenocarpic (seedless) fruits (Fig. 2.1 L and M; Fig. 2.2 D and H). Differently, when the *7B-1* flowers are hand-pollinated with pollen from themself or from the WT genotype they produce fruits with a weight near to those of WT and with an average of 40 seeds per fruit (Fig. 2.2 D and H). In SD, when the plants are subjected to OP the *7B-1* mutant produced a few parthenocarpic fruits like in the LD conditions. In contrast, when HP is made the situation changes, flowers produce fruits with a good weight and a good seed set, with an average of 60 seeds per fruit (Fig. 2.2 D and H). In SD with inflorescence shaking, the mutant flowers induced a seed set of about 6 seeds per fruit.

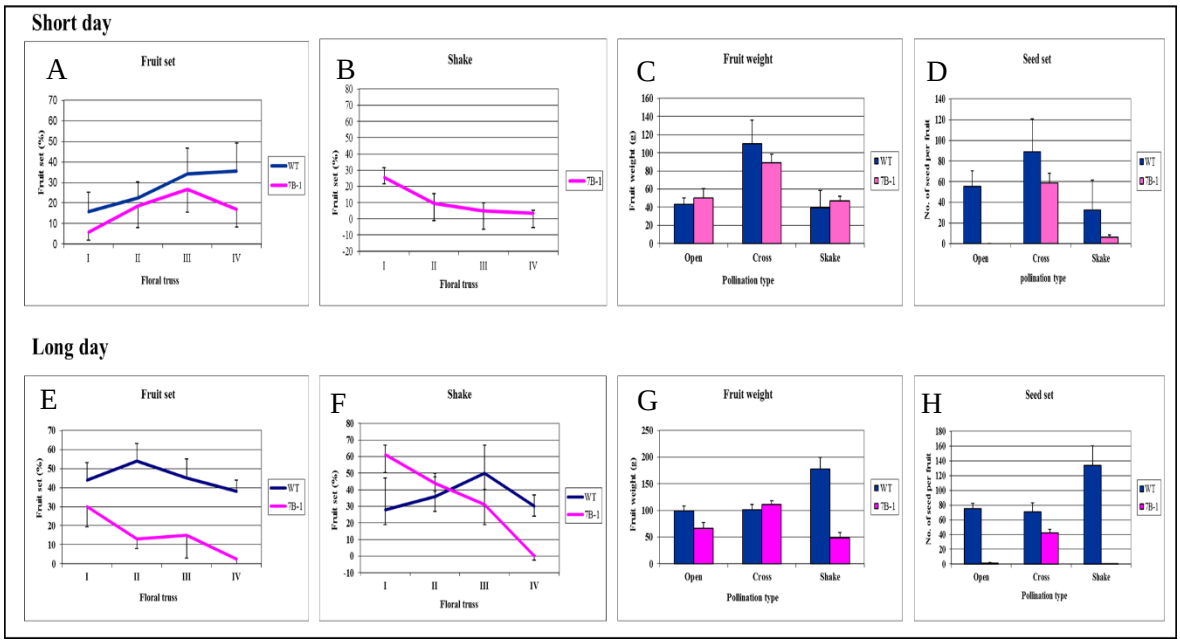

Figure 2.2. Fruit set and shake at the first four floral trusses in the *7B- 1*mutant (pink line) and in its near-isogenic WT line (blue line) in SD (A and B) and LD conditions (E and F). Fruit weight (C and G) and number of seeds per fruit (D and H) in the two genotypes in SD and LD conditions, considering three different type of pollination (open, cross and shake)

Table 2.3. Statistical analysis of fruit weight and number of seeds per fruit in the WT and the *7B-1* mutant under different pollination regimes.

Trait	**Type of pollination**	**Source of variation[1]**		
		G	E	G*E
Fruit weight	Open	**	**	ns
	Cross	ns	ns	ns
	Shaken	-	-	**
No. of seeds per fruit	Open	***	ns	ns
	Cross	**	ns	ns
	Shaken	-	-	*

[1]Two-ways ANOVA statistics for differences between Genotypes (G), Environments (E) and their interaction (GxE): ns, not significant; *, ** and ***, significant for P≤0.05, 0.01 and 0.001 respectively.

The observation of missing fruit set in *7B-1* plants under Italian LD growth conditions and a restored production in SD confirm that the mechanism which control sterility/fertility restoration of this mutant remaining unchanged and stable when the geographic area is different (Sawhney, 1997). The data obtained analyzing the fruit and seed set in *7B-1* plants grown in SD is in agreement with the data reported by Sawhney (2004). The author observed a production of several fruits with have an average of 75 seed per fruit. In both cases, the average of number of seed per fruit is less respect to the WT counterpart, but higher than in those observed in LD and this is due to lower pollen quantity produced by mutated anthers, which is enough in SD for fruit and seed set (Sawhney, 2004). However, the fruit set reported in *7B-1* in SD conditions under shaking in our conditions resulted much lower than that reported in Canada. The difference was that those authors used obscuration to

impose SD whereas our treatment was based on natural photoperiod conditions. SD conditions in Italy correspond also with low light intensity and lower temperatures that may hamper pollen production in the mutant. Thus the use of *7B-1* seed parents in hybrid seed production in Italy would imply the maintenance of the seed parent through hand self-pollination or the use of growth conditions where SD are artificially obtained through obscuration. In LD conditions, fruits from *7B-1* plants grown in field, which were hand self-pollinated demonstrate to have a good fruit and seed set. The percentage of seed per fruit (44 ±8.71) obtained, suggesting that the utilization of this system in the maintenance of *7B-1* genotype during summer season is an ideal approach.

Morphological characterization of the sl-2 *mutant*

The male sterile *sl-2* tomato mutant is characterized by the formation of aberrant anthers when it is grown under high temperatures (28°C day/23°C night), while a partial WT phenotype can be restored when it is grown under low temperatures (18°C day/15°C night) (Sawhney, 1983). The anther malformations involve the possibility to observe carpelloid structures and external ovules in the adaxial anther surface (Sawhney, 1983). This phenotype is very similar to that observed in the *7B-1* tomato mutant. In order to characterize this mutation in parallel with *7B-1*, 20 WT and *sl-2* plants were grown in summer field conditions and in autumn greenhouse conditions to characterize the flower phenotype in environments where it is possible to observe the maximum and minimum expression level of the mutation. Flowers from WT and *sl-2* plants were characterized using the stereomicroscope. The flower conformation remains unchanged in WT and *sl-2* genotypes, except for the anthers cone structure that shows alterations in *sl-2* mutant flowers. (Table 2.4). The morphology of *sl-2* staminal cone is characterized by twisted, distorted and laterally free anthers with the stigma protruding beyond them (Fig. 2.3 A).

The morphometric analysis was done only in flowers taken from *sl-2* and WT plants grown under summer field conditions, while the anther morphology was observed in detail also in plants grown in low temperature conditions. In both cases WT anthers had a completely normal morphology. In the mutant in high temperature conditions all the anthers showed some malformation: about 85% of the anthers presented carpelloid changes and in the remaining 15% they assumed a styliform structure that remained free or was adnate to the pistil (Table 2.4). In autumn, all *sl-2* anthers remained aberrant, and the main aberrations present were the presence of carpelloid structures (57.5 %) and external ovules in the adaxial surface (42.5 %) (Fig. 2.3 B, C and D).

Table 2.4. Morphometric analysis of the *sl-2* mutant flowers compared with those of the near-isogenic wild-type (WT) line

Floral organ	**Trait**	**WT**	***sl-2***	**P[1]**
Sepals	No.	6.1	6.0	ns
	Length	11.3	13.4	ns
	Width	1.3	1.3	ns
Petals	No.	6.1	6.1	ns
	Length	9.1	10.5	ns
	Width	2.2	2.6	ns
Anthers morphology (%)	No.	6.2	6.10	ns
	Normal	100	0	
	Carpelloid	0	46.4	
	Carpelloid with external ovules	0	39.1	
	Carpelloid styliform	0	4.3	
	Carpelloid styliform adnate to the pistil	0	10.1	
Ovary	Length	1.44	1.42	ns
	Width	1.20	1.38	ns

[1] Statistical analysis after Student's *t* test; ns, not significant.

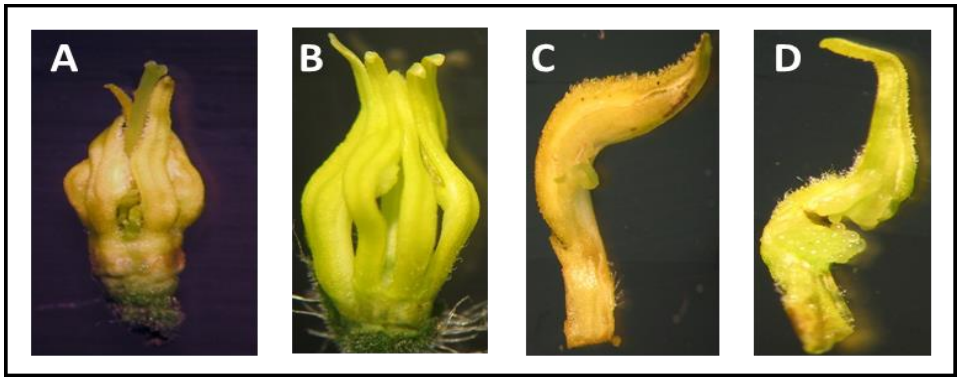

Figure 2.3. Staminal cone and anther phenotype of *sl-2* flowers. A, staminal cone with stigma exerted outside the anthers in flowers from plants grown in high temperature conditions. B, staminal cone with anthers laterally free from flowers grown in low temperatures. C and D, anther features, carpelloid structure and carpelloid structure with the external ovules.

Differences in fruit set were reported between the *sl-2* mutant and its near-isogenic WT. The mutant setting was sharply decreasing with the floral truss (Fig. 2.4 A). A significant GxE interaction was found in the analysis of fruit weight and seed number; the fruits set in summer were smaller in the mutant whereas in autumn they were similar to the WT. In both seasons, *sl-2* fruits were often seedless or had a low number of seeds (Fig. 2.4 B, C). Although the number of seeds per fruit was less than one half in the WT in autumn compared with the spring conditions, the mutant set about 8 seeds per fruit after open pollination independently of growth conditions.

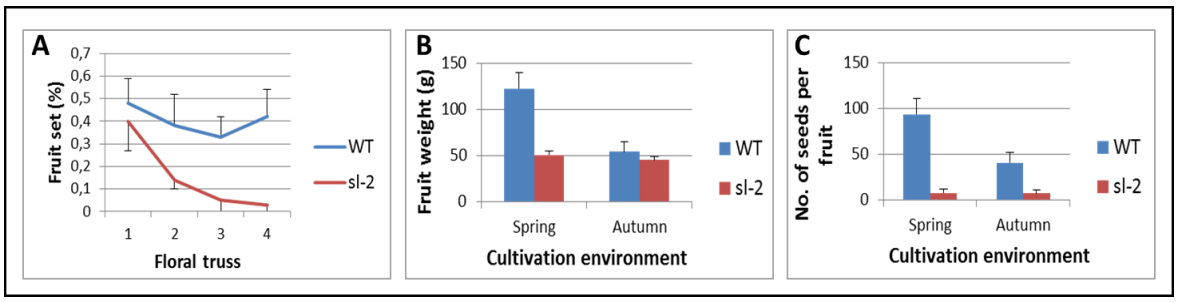

Figure 2.4. Fruit set at the first four floral trusses in the *sl-2* mutant and in its near-isogenic WT line in summer conditions (A). Fruit weight (B) and number of seeds per fruit (C) in the two genotypes in spring and autumn conditions.

The phenotypic characterization of *sl-2* confirmed the previous observations about the similarity with the tomato photoperiod-sensitive male sterile *7B-1* mutant. Both mutants have a particular morphology of the staminal cone where the anthers are retracted and the stigma is exposed allowing the hand pollination without emasculation. This conformation is useful for hybrid seed production, but *sl-2* results more difficult to use because this mutation is sensitive to high temperatures (Sawhney and Greyson, 1973). The high temperatures determined the typical *sl-2* sterile phenotype but only a change of 5°C in temperature determines the development of a weakly fertile phenotype (Sawhney, 1983) which is not characterized by protruding stigma, and this makes not possible the hand pollination without emasculation. The presence of external ovules in the adaxial surface observed in our *sl-2* plants is in agreement with the data reported from Sawhney and Greyson (1973) who reported the presence of these structures that were not found in the first study conducted by Hafen and Stevenson (1955). It is know that the sex expression and development of external ovules is influenced by change in environmental factors and growth regulators (Heslop-Harrison, 1957; 1959); the reason that explains the capacity from Sawhney and Greyson to observe external ovules in *sl-2* may reside in their experimental conditions that included different growth temperature.

In our conditions, the *sl-2* anther phenotype was not much different in the two different adopted growing seasons indicating that the low temperatures experienced in autumn were not enough permissive to induce a substantial recovering of fertility. However, *sl-2* represents a system less convenient than *7B-1* for hybrid seed production because the mutant is able of some seed set under open pollination also in the conditions that should maximize the sterile phenotype.

Microscopic analysis of WT and 7B-1 *flower phenotype*

The fruit set data in *7B-1* plants in LD and SD indicated that the low percentage of fruits production is due to the small quantity of viable pollen inside the anther locules. In order to confirm this, the pollen grains from *7B-1* flowers grown under different conditions were sampled to determine the viability and germination capacity. The viability observation highlighted that *7B-1* anthers in SD have pollen grains of normal size and shape, viable with a percentage less than the WT but higher than the mutant in LD conditions (Fig. 2.5 D). In LD, *7B-1* showed pollen grains wrinkled and aborted with a very low percentage of viable pollen (Fig. 2.5 B). These data were supported also

from the germination test that reported a low number of germinated pollen grains in *7B-1* in both conditions, SD and LD. Compared with the WT in SD the difference of in germinated pollen grains is not very big, possibly due to the low temperatures in autumn/winter season that influenced negatively the germination process. In contrast, in LD conditions in *7B-1* pollen the growth of pollen tube is very low respect to WT. To check the presence of aberrations in the meiotic process in anthers and also in ovules from WT and *7B-1*, male and female sporogenesis was observed under light-microscopy. The analysis show not big difference in each stage of meiosis process between WT and *7B-1* organs, indicating that the meiosis occurs normally.

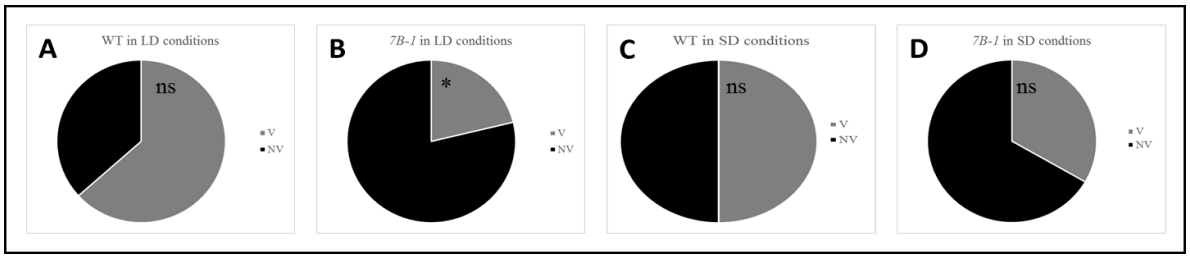

Figure 2.5. Pollen viability in WT and *7B-1* plants grown under LD (A, B) and SD (C and D) conditions. V (green) and NV (black) stand for viable and not-viable pollen, respectively. Pies show mean percentages (n = 30). * means significant different with P≤0.005.

In summary, all these observation about the behavior of the pollen in *7B-1* support the hypothesis that the absence of fruit set is due to the missing production or germination of pollen grains and may be it is linked to a decrease in production of some molecules that are essential for pollen development such as enzymes, lipids, starch and pollen wall materials (Goldberg *et a*l., 1993; Zhang *et al*., 2006). However, the absence of apparent aberrations in meiosis process, in particular in microspore mother cells, is in contrast with the observation reported in literature. Sawhney (2004) reported in his histological studies done on *7B-1* developing anthers the presence of callose around the microspore mother cells and no meiotic process and no microspores formation were observed. The data reported here can be explained through the hypothesis that the microscopic analysis was done only in organs from SD conditions and in different growth stage. The meiotic process together with the tapetum development is important in pollen formation. In *A. thaliana* and rice the anther development was well studied and many genes involved in this process were identified. Mutations in genes involved in the meiotic process determine the production of defective meiocytes with consequentially male sterility conditions.

Results about the lack of differences in meiosis in ovules, supported by the nearly normal number of seeds produced by the mutant under HP, indicated that the female fertility in this mutant is not affected, as reported early (Sawhney, 2004).

The *7B-1* phenotype was studied in detail at morphological level using ultrastructural analysis. The SEM analysis involved the study of the anthers morphology in samples taken at Stage 3 and 4 (Mazzucato *et al*., 1998), stages where the anther is near to or in anthesis. This kind of study was

conducted to investigate the possible differences in the surface features of anthers from WT and *7B-1* in conditions where the mutant expressed sterility and where it is restored.

The WT anthers in LD conditions are characterized by production of rows of hairs on lateral and adaxial surface (Fig. 2.6 C) that are also present in the explants from SD (Fig.2.6 A). In mutant anthers the hairs are completely absent in both surfaces when plants are grown in LD (Fig. 2.6 D) while in SD they are present but their production is lower than in the WT grown in the same conditions (Fig. 2.6 C). Mutant samples produce carpel-like structures visible in the adaxial surface in SD and in LD (Fig. 2.6 C and D). In LD conditions, anthers of *7B-1* flowers produce together with the carpelloid structures also external ovules at the base of them (Fig. 2.6 D). WT anther structures are also characterized by the presence of two techae that are completely absent in *7B-1* in LD, while in SD mutant anthers have only partially developed staminal techae (Fig. 2.6 C).

The ultrastructural study demonstrated that there are significant difference in the morphology that involved the growth of hairs and production of particular structures in the adaxial surface between normal and *7B-1* anthers in LD and SD. In addition, the structure of *7B-1* anthers observed with SEM in LD conditions can be compared with the SEM phenotype of *sl-2* anthers, confirming the similitude between these two male sterile mutants. *sl-2* anthers were well studied with the SEM (Sawhney and Polowick, 1985; Mazzucato *et al*., 2008) and these data reported observation of similar alterations in the surface features of this mutant. The major alteration observed involve the hairs formation and the carpel-like structures. For the hairs production, *sl-2* grown with high temperatures appears without them as *7B-1* in LD, while in intermediate conditions the production is lower in agreement with those found in *7B-1* in SD. Carpelloid structures associated with external ovules formation, the same defect observed in *7B-1* in LD, was found as the main alteration in anthers of *sl-2* mutants in high temperature conditions.

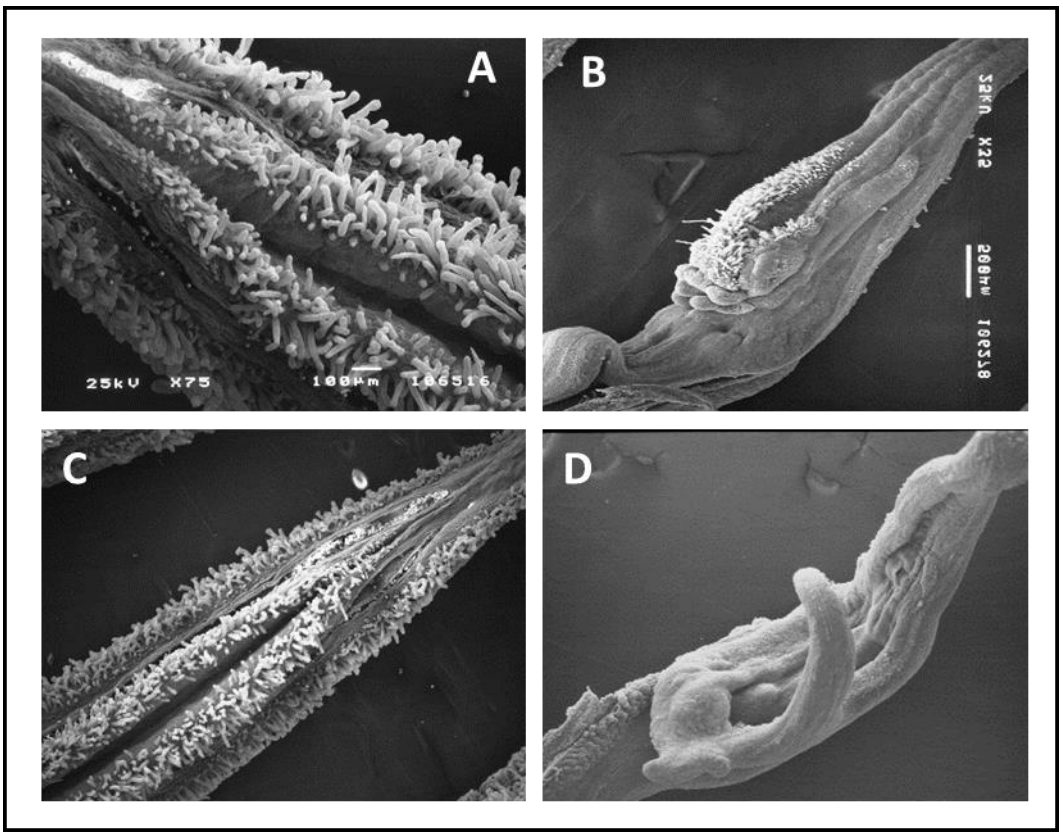

Figure 2.6. Ultrastructural characterization of anthers in WT and *7B-1* flowers grown in SD and LD conditions. WT anther features in SD (A) and LD (C). B, *7B-1* anthers in SD with carpelloid structures grown on the adaxial surface, they showed a lower presence of hairs in the lateral and adaxial surface. D, *7B-1* anthers in LD with carpelloid structures and external ovules at the base of the adaxial surface.

Together these results suggest a very high similarity between *7B-1* and *sl-2* tomato male sterile mutants and the possibility that the mutations are allelic and involve an alteration at the same locus. Further analyses were set up to investigate the association between them and are described in the next chapters (Chapter 3 and Chapter 4).

Chapter 3

Characterization of tomato male sterile mutants and allelism tests

Introduction

The male sterility in tomato was reported for the first time in 1915 by Crane and these spontaneous or induced mutants were grouped in three classes by Kaul (1988): structural, sporogenous and functional. The sporogenous or pollen sterility mutants are characterized by production of WT flowers with abnormal pollen grains. The mutants present in this class are classified into different groups according to the pollen development aborting stage (see Table 3.1; Rick and Butler, 1956). In the group where pollen abortion occurs after microspores formation there is the *vms* mutant, which is described in details in the next chapter (see Chapter 5). The functional sterile mutants were described as plants with flowers able to produce viable pollen but the anthers do not have the capacity to release it. The first functional mutants described were the *positional sterile* (*ps*) (Larson and Paur, 1948) and *positional sterile-2* (*ps-2*) (Tronickova, 1962). Both mutants, *ps* and *ps-2*, were characterized by production of viable pollen but it was trapped inside the anthers locule. *ps-2* male sterile phenotype is due to a single nucleotide mutation in a *polygalacturonase* (*PG*) gene that determines an alternative splicing, therefore the mRNA is aberrant, lacking of one exons (Gorguet *et al*., 2006; 2008).

Table 3.1. Classification of sporogenous mutants into five different groups according to the different pollen development aborting stage (from Rick and Butler, 1956).

Premeiotic	Meiotic	Tetrad	Microspore	Not determined
ms-3	*ms-1*	*ms-2*	*ms-9*	*ms19*
ms-15	*ms-5*	*ms-4*	*ms-13*	*ms20*
ms-29	*ms-7*	*ms-6*	*ms-14*	*ms21*
ms-32	*ms-8*	*ms-11*	*ms-24*	*ms22*
	ms-10	*ms-17*	*ms-27*	*ms25*
	ms-12	*ms-23*	*ms-28*	*ms38*
	ms-16	*ms-34*	*ms-31*	*ms39*
	ms-18	*ms-45*	*ms-37*	*ms44*
	ms-30	*ms-46*	*ms-41*	*ms48*
	ms-33		*ms-43*	
			Digenic mutant	
			vms	

Structural or staminal mutants shown abnormal anther cone and no pollen or very little is produced. The abnormalities, sometimes, can involve also sepal, petal and carpel formation, suggesting a strong similarity with the homeotic mutants for MADS-box transcription factors. Bowman *et al*. (1989) described a recessive temperature sensitive mutant in *A. thaliana* for the *ap3* gene that showed a complete conversion of sepals and petals into stamens and carpels. Similar phenotypes were found

in tomato mutants, *pi* (Rick and Robinson, 1951) and *pi-2* (Rick, 1993). Recently, literature data and examination of the phenotype indicated members of the class B MADS-box transcription factors family as candidates for the tomato structural male sterile mutants. These transcription factors specify the identity of petals and stamens. The *sl* mutant (Bishop, 1954) presents a severe phenotype where the stamens are completely absent. Heterozygous plants for this mutation showed anthers with defects in the structure and sometimes they produced some viable pollen grains (Gomez *et al*., 1999). Recently studies were reported that the *sl* and one of its alleles, *TAP3* were defective in the sequence of the class B MADS-box gene, *Sldef* located on chromosome 4 (Gomez *et al*., 1999; Quinet *et al*., 2014). In the structural mutant group, the most studied mutant is *sl-2* (Hafen and Stevenson, 1958), which has a phenotype similar to that of *sl* mutant but it is less severe. The phenotype can be reverted into normal phenotype growing *sl-2* plants in low temperature conditions or with treatment with gibberellic acid (Sawhney and Greyson, 1973b; Sawhney, 1983). For *sl-2* and *sl*, together with other male sterile mutants, it was hypothesized that they are members of an allelic series (Hafen and Stevenson, 1958).

Studied conducted by Hafen and Stevenson (1955; 1958) described five *sl* mutants, which are members of an allelic series. These mutants, called *sl, sl-2, sl-3, sl-4* and *sl-5*, were characterized by a different degree of abnormalities in anther structure. All mutants present petals with typical yellow color, except *sl-5*, which has a sepaloid petal structure; *sl-2*, *sl-3* and *sl-4* have vestigial development of the anthers and the stigma is exposed, while *sl* and *sl-5* have sterile anthers and no pollen grains is produced. The production of viable pollen is observed at high levels in *sl-3* and *sl-4* while is less in *sl-2*. Hafen and Stevenson described also, the allelism test done between all five mutants and the results hypothesize that *sl* and *sl-5* are allelic for one factor that acts in petal and anther development and that *sl-2* may be due to the same factor. Successive allelism studies (Nash *et al*., 1985) observed eight staminal mutants, *sl, sl-2, sl-5, corollaless* (*cs*), *floradel* (*fl*), *blunt* (*bn*), *stamenless?* (*sl?*) and *pelican male sterile* (*pms*), which are grouped into two different allelic series. In the first series, they included inside *sl, sl-2, sl-5, cs* and *fl*, while in the second they included *bn, sl?* and *pms*. The data confirmed the previous results reported by Hafen and Stevenson (1955; 1958) about the strong allelism between *sl* and *sl-5*, the different segregation ratio of *sl-2* plants during screening of allelism populations, and also that the all mutations were due to a single recessive gene. New observations were done about the fruit set of the *sl* series. None of all *sl* mutants is sufficiently fertile for the self-maintenance but all, except *cs* are very easy to maintain in heterozygous form. None of all mutants from *sl* series is very useful for tomato seed production, except *sl-2*, due, specifically, at the stamens structure, which are not adnate to the pistil.

Together, these results suggest that the mutations were due to different loci, maybe a different class B MADS-box transcription factor that acts in the control of petal and stamen development (Gomez *et al*., 1999).

Starting from considerations about the possible allelism between different male sterile mutants and the possible implication of class B MADS-box transcription factors, the experiments described in

this Chapter were performed to gain more information about the relationship and the genetic nature of these mutations. The tomato mutants that we have studied in details are *sl*, *sl-2*, *TAP3*, *7B-1*, *pi-2* and *vms*; in particular in this Chapter it is described the morphological characterization and the allelism test done between all these mutant genotypes.

Materials and Methods

Plant material used for allelism test

For each mutation studied appropriate vegetable material stock obtained from different donors was used.

For *7B-1* mutation was used a fixed line and its near-isogenic WT in the background of cultivar Rutgers, which was obtained from Prof. V.K. Sawhney (University of Saaskatoon, Canada). For *sl-2* mutant, was used a fixed line in a background not specified, which was obtained from the TGRC with accession number LA2-137 and an appropriate WT (Mazzucato *et al*., 2008). An F_2 sample segregating the *sl* mutation in background of cultivar Ailsa Craig was obtained from TGRC, with accession number LA3816. A fixed line was used for *vms* in background of cultivar San Marzano, which was obtained from TGRC with accession number LA2-219. For *pi-2* mutation was used a segregating F_2 sample, which was obtained from TGRC, with accession number LA3-802. Relative cultivars were used as WT for each mutation.

Allelism test between 7B-1 *and* sl-2

Starting from the consideration that *7B-1* and *sl-2* mutants present a similar phenotype was done an allelism test between them. Cross between *7B-1* and *sl-2* resulted in a F_1 population, where 26 plants were grown in summer field conditions in the experimental farm of the Tuscia University (Viterbo, Italy) to analyze the flower morphology and to obtained F_2 seeds. The phenotype observation was done at flowering time and all plants were classified according to flower morphology in WTs or mutants. Twenty F_2 plants derivate from F_1 seed stock were grown in the same conditions for screening again the phenotype.

Allelism test between 7B-1 *and* sl

To explore the possible allelism between *7B-1* and *sl*, the seed stock segregating *sl* was grown and plants screened for the mutant phenotype. Three F_1 populations were obtained after crossing three WT plants of this population (seed parents) and *7B-1* mutant plants (pollen donors) were already available for the phenotyping. WT seed parents were also selfed in order to detect their genotype (SlSl or Slsl) at the *Sl* locus. Twelve seeds from the three F_1 and the three S_1 populations were grown in summer field conditions to examine the phenotype and to obtain F_2 seeds. The morphological analysis was done at flowering time, taking two flowers per plant at the anthesis (stage 4; flowering staging according to Mazzucato *et al*., 1998). Using a stereomicroscope in each flower was observed the corolla morphology, its color and the structure of anther cone. Plants were classified according to the phenotype. The F_2 seed was harvested from four plants showing a mutant phenotype and from

one WT plant. F_2 populations were grown in summer field conditions for screening again the phenotype as detailed before.

Allelism test between sl *and* sl-2

The same experiment was done for confirm the data reported in the past about the allelism between *sl* and *sl-2* (Hafen and Stevenson, 1955; 1958; Nash *et al*., 1985). The population used for the allelism test was previously obtained crossing mutant plant for *sl* with WT plant of *sl-2* mutant. Four seeds were obtained and three plants were grown to screen the phenotype. The morphological screening was done with the same method described above, analyzing the anther structure and the corolla morphology of all plants observed.

Plant material and morphological characterization of the TAP3 *mutant*

An F_2 seed sample segregating the *TAP3* mutation in the background of cultivar Primabel was kindly provided by Dr. M. Quinet (Louvain Univeristy, Belgium). Nineteen plants were grown in summer field conditions to examine the phenotype. The *TAP3* mutant shows a particular phenotype, petals are converted to sepals and anthers into carpels and this makes it easy to discriminate the recessive homozygous mutant from the WT or heterozygous plants. The morphological characterization was done at flowering time, taking one flower per plant at stage 4 using the stereomicroscope. For each flower was counted the number of sepals and petals, was measured the length and width of them and for petals was visually screened the color intensity. The anther and the ovary length and width were measured. Anthers were counted and classified according to their structure in normal, carpelloid and carpelloid with external ovules. This observation was done only for the WT and heterozygous flowers, not for the recessive mutants because they show anthers transformed into carpelloid-styliform structures completely adnate to the pistil. Seed was recovered from five WT and eight heterozygous plants to obtain the F_3 generation for progeny test. Twelve F_3 plants for each progeny were grown in summer season in field or in greenhouse and then classified according to flower morphology into WT, heterozygous and mutants for confirm the results obtained with the F_2 progeny. Several F_3 plants were grown until the time to harvest seeds for obtaining the F_4 stock. For each plant, fruits were counted, weighted and the number of seed was recorded.

Allelism test between 7B-1 *and* TAP3 *and* sl-2 *and* TAP3

One heterozygous plant for the *TAP3* mutation was crossed with *7B-1* or *sl-2* pollen to originate two different segregating populations. For the first population, obtained crossing *TAP3* heterozygous plant with *7B-1* pollen, eight plants were screened for the phenotype; successively in summer field conditions other 20 plants were observed. For the second one, were growing eight plants and successively other 14 were analyzed to discriminate *sl-2*, *TAP3*, WT, heterozygous or double mutant phenotype.

Genetic relationship between 7B-1, sl-2 *and* sl *assed by mapping*

Allelism test showed that the mutation *sl-2* and *7B-1* are probably allelic. The *sl* mutation, involved in the locus *SlDEF* on the long arm of chromosome 4 (Gomez *et al*., 1999), was reported as allelic to *sl-2* (Hafen and Stevenson, 1958; Nash *et al*., 1985). For assessing this hypothesis were screened two F_2 mapping populations, which were obtained in previous years after crossing the mutants *sl-2* and *7B-1* with the introgression line 4-4 (IL 4-4), carrying a segment from *S. pennellii* (Eshed and Zamir, 1995) in the target region of chromosome 4. F_2 populations were grown in greenhouse during the spring and in the field conditions in summer (experimental station of Tuscia University, Viterbo). In total 200 plants were scored for the phenotype and for the marker T0360 (Fulton *et al*., 2002a) which is located near (10 cM) to *SlDEF*. Total DNA was extracted from each plant according to the Doyle & Doyle protocol (1990), amplified by PCR reaction using the following primers: T0360 forward (5'-AAA CTC TCC GAG CTA GTG CGG-3') and T0360 reverse (5'-ACC CAA CAC CAA ATT GTC CAA-3'). PCR amplification was performed in a 20 µl of total volume, containing 75 ng of genomic DNA template, 10 µM for each primer, 200 µM of dNTPs, 1X DreamTaq Buffer and 1 U of DreamTaq polymerase (Fermentas). The PCR reaction was conducted with an initial denaturation of 4 min at 94°C that was followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, extention for 2 min at 72°C, plus 7 min of final extention at 72°C. Difference on size of the amplicons (for T0360 marker the amplicons size are 600bp for *7B-1*/*sl-2* parents and 650 bp for the IL4-4) were visualized by 1.5% agarose gel and stained with ethidium bromide. In addition, a smaller number of plants were screened with marker *C2_At4g33350*, positioned opposite to T0360 respect the *SlDEF* locus, and with a marker (LeAP3; Mazzucato et al., 2008) located in the *SlDEF* gene itself. PCR amplification was performed using primers specific for marker C2_At4g33350 (forward: 5'- TAT CAT CTA TCT CTT CCG TTC GCT TC-3' and revers: 5'- ATG CGA TTC CTT CAA CCT TCA AC-3') and for *LeAP3* (forward: 5'- ATG GCT CGT GGT AAG ACT CAG-3' and reverse: 5'-AGG CTT TCT CCC ATC CTC TGT-3'). The PCR experiment was conducted using the same volume and same reagents described above, but using a different amplification protocol. The PCR conditions for C2_At4g33350 were 35 cycles of 1 min at 94°C, 1 min at 57°C and 2 min of 72°C, while for *LeAP3* were 30 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. To observe a difference on size in each amplicons was used for *LeAP3* 1.5% agarose gel (amplicons size are 1050 bp for *7B-1*/*sl-2* parents and 1000 bp for IL 4-4), while for C2_At4g33350, which is a cleaved amplified polymorphic sequence (CAPS) marker, it was necessary to cut each product using *HinfI* as restriction enzyme. Restriction experiments were performed by digesting 10 µl of PCR product with 1U of *HinfI* (New England Biolabs) and resolving the restriction fragments on 2% agarose gel (restriction fragments size are 1000bp + 500 bp for *7B-1*/*sl-2* parents and 800+500+300 for IL 4-4) . Association analysis between *7B-1* or *sl-2*, T0360 and *SlDEF*, was done using a Joinmap 3.0 software (Ooijen and Voorrips, 2001). Some F_2 plants have been subjected a progeny test to check the phenotype classification and to confirm the independence between the mutations *sl-2*/*7B-1* and the *sl*/*SlDEF* locus.

Allelism test between 7B-1 *and* vms

Allelism test between *7B-1* and *vms* was done by crossing a *7B-1* plant with pollen from *vms* mutant. Fourteen F_1 plants were grown and screened for the flower morphology and classified according to the phenotype in WT, heterozygous or mutants.

Results and Discussion

Allelic interaction between sl, sl-2 *and* 7B-1

sl mutation is a member of an allelic series, where is grouped also the *sl-2* mutant. Results from different allelism test between them indicated that *sl* and *sl-2* are strongly linked, so suggest that they are allelic (Hafen and Stevenson, 1955; 1958; Nash *et al*., 1985). *7B-1* mutant is characterized from anther structure modification, it is different from *sl* phenotype plants which showed greenish petals and green anther completely adnate to the pistil but it is very similar to the male sterile *sl-2*. Literature data mentioned above reported that *sl* and *sl-2* are allelic, then considering the phenotype similitude between *7B-1* and *sl-2* maybe it is also an allele of *sl* series and it is grouped into structural sterile mutants class, where both mutations are inside.

To genetically characterize the *7B-1* mutant it was developed an allelism test between this mutation and *sl*. Progeny tests on the seed parents used showed that two of them were heterozygous for the *sl* mutation. The *sl* mutation segregated in a Mendelian fashion in the selfed progenies that showed to derive from heterozygous parents (8 WTs vs 4 *sl* mutants). All the anther cone elements in mutant plants were completely converted into carpels and tightly fused to the pistil (Fig. 3.1 A). In all cases, petals showed greenish portion thus confirming a class B phenotype. The ovary in mutant plants had wider equatorial diameter due to the contribution of illegitime anthers.

Twelve seeds from the F_1 populations derived from these seed parents grown in summer field conditions segregated five plants showing a mutant phenotype and seven WTs. Comparing morphometric data of these segregants with those from the *sl* mutant and its near isogenic WT (Table 3.2), it was clear that no major difference was present except for the anther cone. Whereas the anther cone of *sl* mutants is composed of 100% anthers carpelloid and fused to the pistil, anther cones of flowers showing a "mutant" phenotype in F_1s showed carpelloid anthers of various features but never fused to the pistil (Table 3.3).

These morphometric data suggest that *sl* mutant segregated in a Mendelian way and it was completely recessive because WT plants were all normal.

Table 3.2. Morphometric data of sepals, petals, anthers and ovary of tomato *sl* mutant flowers and near isogenic WT in the original seed stock. Each value represents the average ± SE of 12 flowers per phenotype.

Floral organ	**Trait**	**WT**	***sl***	**P[1]**
Sepals	No.	6.2±0.1	6	ns
	Length	11.8±0.5	12.2±0.6	ns
	Width	1.2±0.1	1.1±0.1	ns
Petals	No.	6.4±0.14	6.3±0.15	ns
	Length	13.3±0.7	12.4±0.7	ns
	Width	2,6±0.2	1.8±0,19	**
Anthers morphology (%)	No.	6.2±0.1	6.2±0.13	ns
	Normal	91.9		
	Carpelloid	6.8		
	Carpelloid with external ovules			
	Carpelloid styliform	1.4		
	Carpelloid styliform adnate to the pistil		100	
Ovary	Lenght	1.5±0.06	1.8±0.14	*
	Width	1.4±0.05	2.6±0.12	***

[1] Student's t test statistics: ns, not significant; *, ** and ***, significant for P≤0.05, 0.01 and 0.001 respectively

Table 3.3. Morphometric data of sepals, petals, anthers and ovary of tomato flowers in WT and "mutant" plants derived after cross between *sl* heterozygotes and *7B-1* mutants (F_1). Each value represents the average ± SE of 12 flowers per phenotype.

Floral organ	**Trait**	**WT**	**"mutant"**	**P[1]**
Sepals	No.	6.1±0.1	6.6±0.14	*
	Length	11.5±0.4	12.5±0.7	ns
	Width	1.5±0.1	1.1±0.04	***
Petals	No.	6.2±0.12	6.5±0.15	ns
	Length	12.6±0.6	11.5±0.4	ns
	Width	2.8±0.17	2.5±0.2	ns
Anthers morphology (%)	No.	6.3±0.14	6.8±0.17	*
	Normal	100	14.6	
	Carpelloid		29.3	
	Carpelloid with external ovules		24.4	
	Carpelloid styliform		31.7	
	Carpelloid styliform adnate to the pistil			
Ovary	Length	1.6±0.05	1.5±0.07	ns
	Width	1.6±0.1	1.5±0.08	ns

[1] Student's t test statistics: ns, not significant; * and ***, significant for P≤0.05 and 0.001 respectively

Allelism test between sl *and* sl-2

To test the allelism between *sl* and *sl-2*, we succeeded in pollinating a plant homozygous for *sl* with pollen from an *Sl-2sl-2* heterozygote. Similarly to the results described above, the F_1 progeny never showed the strong *sl* phenotype, but instead anther cones composed of normal (17.5%), carpelloid (30.2%), carpelloid with external ovules (23.8%) and carpelloid styliform (28.6%) stamens. All plants showed this phenotype being, in the hypothesis of no allelism, all di-hybrids.

Segregation data suggest no allelism between *7B-1* and *sl*, because is not possible to observe presence of half population (50%) with strong mutant phenotype. The expression of mutant phenotype was possible to observe only in (double) heterozygous plants, which showed a phenotype very different to those of *sl* mutants. The flower morphology of heterozygous plants is characterized by absence of greenish petals, typical of *sl*, and anthers have a yellow color and a structure similar to those of WT, with some alterations (Fig. 3.1 D and E).

Plants from four F_2 seed stock, growing always in summer field conditions, showed together the WT and WT/2 also *7B-1* and some flowers with *sl* phenotype. These data indicated that in F_2 is possible observe the segregation of both phenotype at the homozygous level which not possible in F_1 status, because progeny used was from double heterozygous mutants.

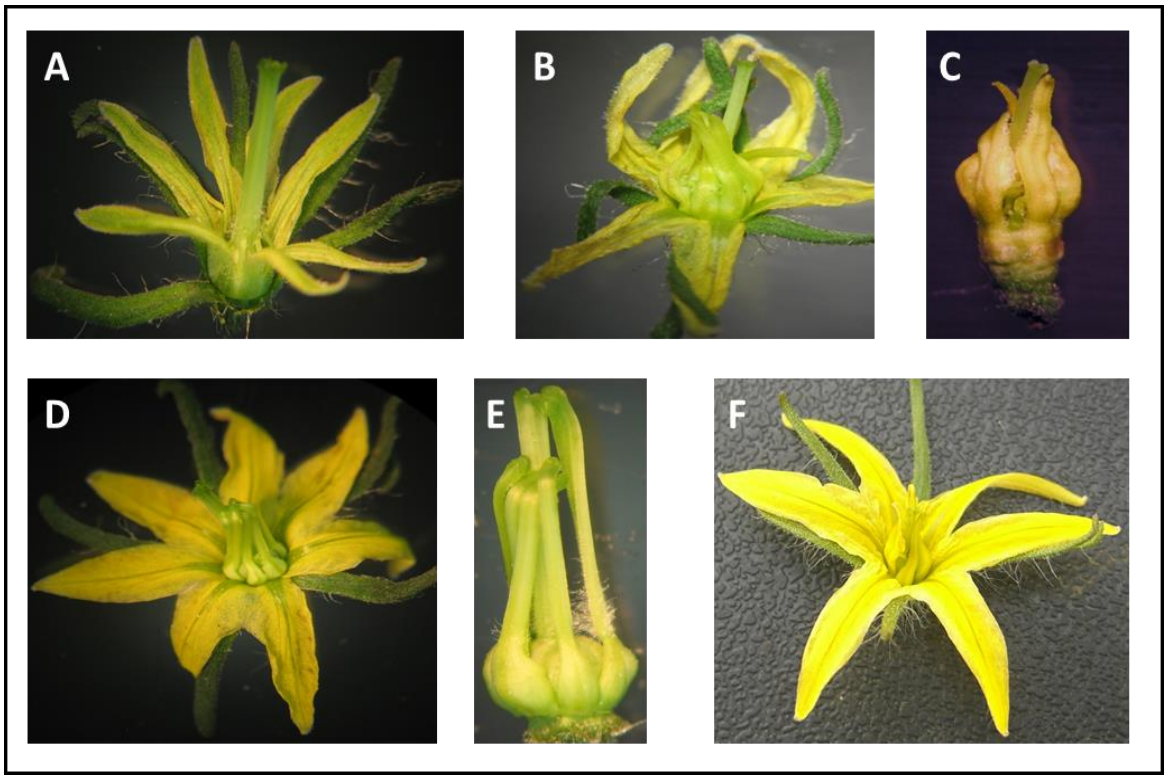

Figure 3.1. Flower phenotype of parent plants and the progeny used for allelism test between *sl*, *7B-1* and *sl-2*. A, typical *sl* phenotype with greenish petals and anthers completely transformed in carpels and adnate to the pistil. B and C, *7B-1* and *sl-2* mutants respectively, which have a similar phenotype with short, retracted and distorted anthers with the stigma protruding between them. D and E, flowers of heterozygous plants derived from F_2 obtained after cross between *sl* and *7B-1*; flower phenotype is different from the parents, showing a WT typical corolla with anthers filiform, which are different from the carpelloid stamens typical of *sl* mutants. F, flower from a plant derived from allelism between *7B-1* and *sl-2*, the morphology is very similar a both parents.

In order to explain the data reported here and confirm that *7B-1* and *sl* are not allelic we can consider the Mendel segregation for the F_2 progenies (Table 3.4). All F_2 progenies segregated WT plants and mutants having the *sl* or *7B-1* phenotype showing that the parent plants were di-hybrids. Thus F_2 progeny plants from different parents were pooled to form a population of 64 individuals. Hypothetically, Mendelian segregation for a population with 64 plants would originate 36 WTs, 12 mutants for *7B-1*, 12 for *sl* and four double mutants. As we expect that the double mutant phenotype will be similar to *sl*, then *sl*-like plants are expected to be 16. Globally, our four F_2 populations segregated 32 WTs, seven plants having the *7B-1* phenotype and 25 plants having the *sl* phenotype (Table 3.4) These results suggest that the two loci are independent, giving support to a status of double heterozygosity more than heteroallelism in the parent plants. However, segregation ratios are not completely in line with the Mendelian segregation, because an excess of plants with *sl* phenotype was observed. These plants derived probably from the fact that double heterozygous plants showed a *sl*-like phenotype. This hypothesis would explain that in allelism tests done in the past (Hafen and Stevenson, 1955; 1958; Nash *et al*., 1985) a large number of mutants were erroneously considered allelic and members of the same series.

Table 3.4. Genotypic and phenotypic segregation data according to the Mendelian segregation of two loci in a population composed of 64 plants. Segregations data derived from phenotype screening of F_2 populations derived from cross between *sl* with *7B-1*/*sl-2*. WT= WT homozygous, H=heterozygous and M= mutant homozygous.

Genotype at the *7B-1* locus	**Genotype at the *Sl* locus**	**No. of plants expected for a Mendelian segregation (N=64)**	**Phenotype**	**No. of plants expected for a Mendelian segregation (N=64)**	**No. of plants observed (N=64)**
WT	WT	4	WT	36	32
WT	H	8			
H	WT	8			
H	H	16			
M	H	8	*7B-1*	12	7
M	WT	4			
H	M	8	*sl*	12	25
WT	M	4			
M	M	4	*7B-1 sl*	4	0

These data rule out the results obtained in the past about the possible allelism between *sl* and *sl-2*. The absence of strong mutants in the F_1 populations indicated that both mutations have an independent segregation. In summary, *sl* and *sl-2*, considering our results, are not allelic, as *sl* and *7B-1*.

It remains to investigate the relationship between *sl-2* and *7B-1* by developing another allelism test. F_1 plants obtained from the cross between *7B-1* and *sl-2* mutants were grown until the flowering time to evaluate the flower morphology. All flowers from 12 plants of the F_1 populations showed short, retracted and distorted anthers, the typical morphology reported in both mutations, indicating allelism

between them. Analysis of flower morphology of 20 F_2 plants from various F_1s showed the same phenotype, confirming the hypothesis of allelism between *7B-1* and *sl-2* (Fig. 3.1 F).

Genetic relationships between sl, sl-2 *and* 7B-1 *assessed by mapping*

Allelism test showed that *7B-1* is allelic to *sl-2* and both are not allelic to *sl*. To confirm these results and finally exclude *SlDEF* gene as candidate for these male sterile mutants, we used two mapping populations (already available) developed after cross between the *7B-1* and *sl-2* mutations with a suitable introgression line (IL4-4; Eshed and Zamir 1995), where an introgression from *S. pennellii* spanned the target region where *SlDEF* is located. One hundred-thirteen plants for the population segregating *7B-1* and 78 for that segregating *sl-2* were screened at flowering time in order to classify each individual according to the flower morphology. For the first population, we scored 86 WTs and 23 *7B-1* plants while for the second one, 61 WTs and 17 *sl-2* plants were scored. The classification of the phenotype for both F_2 populations is concordant to the Mendelian segregation. In 70 plants per population we screened the T0360 marker, localized on chromosome 4, very close to the region where is mapped the *SlDEF* gene, candidate for the *sl* mutation (Gomez *et al*., 1999). The analysis indicated that the mutations are not linked to the *SlDEF* region (Table 3.5), because the marker does not segregates with the phenotype. To confirm this, a smaller number of plants was screened with other molecular markers, C2_At4g33350, positioned opposite to T0360, and a polymorphism located in the *SlDEF* gene itself. The molecular screening showed no co-segregation between markers and mutant phenotype indicating the independence between *sl-2*/*7B-1* and the *sl*/*SlDEF* locus (data not shown). These data confirmed that the *SlDEF* gene is not the candidate for both mutations ruling definitely out an allelism between *sl-2* and *sl*. More information and insights about the molecular characterization and the study of the candidate gene for *7B-1/sl-2* mutants are reported in Chapter 4.

Table 3.5. Morphological and molecular classification of individuals from two mapping populations segregating respectively the *sl-2* and *7B-1* mutation and molecular markers located on the long arm of chromosome 4.

Mutation involved	**Phenotype**	**Marker T0360[1]**	**No. of F_2 plants**	**P value[2]**
sl-2	WT	A	18	0.25-0.50
		H	26	
		B	12	
	sl-2	A	3	
		H	9	
		B	1	
7B-1	WT	A	12	0.75-0.90
		H	31	
		B	12	
	7B-1	A	5	
		H	7	
		B	3	

[1] A, marker phenotype of the mutant parent; B, marker phenotype of the IL4-4 parent; H, heterozygous marker phenotype.
[2] Significance of χ^2 test for independent segregation (3:6:3:1:2:1).

Morphological characterization of TAP3 *mutant*

Nineteen plants from a F_2 seed sample segregating the *TAP3* mutation were grown in the field in summer conditions until to the flowering time, when the plants were screened for the phenotype. Five plants were classified as WT (completely normal), 12 in putative heterozygous (weak phenotype) and two in homozygous mutants (strong phenotype). WT plants showed a typical flower morphology with green sepals, yellow petals and anthers cone with yellow stamens (Fig.3.2 A and D). Homozygous recessive mutants showed the first whorl with sepals, the second and third whorl appeared transformed, petals were converted into six green short sepals and anthers into green carpels completely adnate to the pistil (Fig. 3.2 C and F). Heterozygous plants had a phenotype similar to the WT, with normal structure of the corolla, but petals showed lower color intensity, and yellow anthers, which sometimes showed abnormalities, such as carpelloid-like structures or presence of external ovules (Fig. 3.2 B and E).

This putatively heterozygous phenotype observed in our growth conditions is compatible with recently published data about the *sl* mutant and its allele *TAP3* (Quinet *et al*., 2014). They have analyzed both mutants in two different growth conditions, in Belgium and Spain, and they found difference in expression of heterozygous phenotype. In Belgium, *TAP3* and *sl* showed an heterozygous phenotype more similar to WT plants, while in Spain, heterozygotes showed a phenotype with some defects in anthers morphology, mainly for *sl* mutant. These observations are concordant with the first observation about the *sl* phenotype done by Gomez *et al*., (1999). *sl* recessive mutant is characterized by conversion of stamens into carpels, like *TAP3*, but petals are not converted into sepals, they showed yellow color with only a few green spots (Bishop, 1954; Gomez *et al*., 1999). The heterozygous *sl* and *TAP3* plants, growing in Spain, showed a phenotype similar to our heterozygous *TAP3*, growing in Italy and this can be explained with the similar environmental conditions. It is know that environmental factors, like temperature, can affect the expression of the mutant and heterozygous phenotype (Gomez *et al*., 1999). Maybe light intensity can also affect the phenotype expression and our observations are different from the mutant phenotype observed in Belgium because in Italy the light intensity is higher than in Belgium, but it is similar to Spain. The similitude in light intensity, between Spain and Italy, can be explain our capacity to observe an intermediate phenotype between the homozygous dominant and the homozygous recessive mutants.

A morphometric analysis was done in each plant classified (data not shown). Sepal length results higher in mutants than in WT and heterozygous plants, while petals from heterozygotes are shorter than the WTs. Sepaolid petals from the mutant are shorter than the sepal in the first whorl, in agreement with the observation done by Quinet *et al*., 2014. Regarding the anther structure, the WT showed normal anthers, mutants had stamens completely converted into carpels and heterozygotes had some anthers with normal features and others with some abnormalities, particularly stamens with external ovules. Ovary length and width were lower in heterozygotes respect with to the WT flowers. Progeny derived from 13 F_2 plants confirmed the results obtained with the first phenotype

classification. Each F_2 plants classified as WT gave a fixed WT progeny, while the F_2 heterozygous showed F_3 progeny segregating WT, heterozygous and mutant plants. Flowers from heterozygous plants were observed at the stereomicroscope to analyze the anthers morphology for confirming the previous results. The anther morphology of heterozygous samples showed more or less the same phenotype results, the majority of anthers having carpelloid features with presence of external ovules. One F_3 mutant plant developed some partenocarpic fruits with abnormal shape being originated from carpels of third and fourth whorl (Fig. 3.2 G and H).

The development of a few parthenocarpic fruits from *TAP3* mutant plants in summer field conditions was in agreement with different literature data (Quinet *et al*., 2014; de Martino *et al*., 2006; Gomez *et al*., 1999). Gomez *et al*., (1999) observed a rare production of parthenocarpic fruits from *sl* mutants, that are allelic to *TAP3*. The same observation was reported in two *TAP3* antisense lines, where the fruits were characterized by attachment of transformed sepals from second whorl and carpels of third whorl (Quinet *et al*., 2014; de Martino *et al*., 2006). The fruits from transgenic plants, where the expression of *SlDEF* is silenced, showed an aberrant conformation with abnormal locule size and no seed production (de Martino *et al*., 2006). These data gave information that the production of parthenocarpic fruits with particular conformation is depending from the growth conditions and it is controlled by the expression of *SlDEF* (Quinet *et al*., 2014).

Fig. 3.2. Floral morphology and details of anthers cone of WT (A and D), heterozygous (B and E) and homozygous recessive mutant (C and F). WT flowers showed a typical structure of the corolla with green sepals, yellow petals and anthers cone with yellow stamens and normal features (A and D). Heterozygous flowers showed normal corolla but some alteration in structure of the anthers, which are light yellow, shorter, distorted and the stigma is protruding (B and E). *TAP3* mutant has second whorl converted in sepals and third whorl in carpels, which are filiform and completely adnate to the pistil (C and F). G and H, unripe fruits of a mutant plant where is possible to see sepals of first and second whorl and fused carpels remain attached to the partenocarpic fruits.

Allelism test between 7B-1 *and* TAP3 *and* sl-2 *and* TAP3

Allelism test described below indicated that *7B-1* and *sl-2* are allelic and in the same time demonstrated that they are not allelic to *sl* mutation, which is allelic to *TAP3*. In order to confirming that *7B-1* and *sl-2* are not allelic to *sl* series, an allelism test was performed using two segregating populations, obtained by crossing an heterozygous plant for *TAP3* with pollen from *7B-1* or *sl-2*. The first population obtained crossing a *TAP3* heterozygous plant as female parent with *7B-1* as male parent, segregated with a ratio that indicated no allelism between them. From 28 plants, were obtained 15 plants WT, 10 similar to *7B-1* and three WT with few anther alterations. The segregation ratio and the missing observation of *TAP3* phenotype suggested that the mutations are not allelic. The same results were obtained crossing *TAP3* heterozygous plants with *sl-2* as male parent. Twenty-two plants segregated with a ratio of 15 WTs, four similar to *sl-2* and three classified as near-WT, indicated that there is not allelism. In both segregating populations it was not possible to see the mutant with strong (*TAP3*) phenotype, but 30 to 50 % of the progeny showed an intermediate phenotype. These individuals were likely the double heterozygotes that like the other allelism test described below indicated that all two phenotype are expressed. The double heterozygotes showed anthers with some defects, in the first cross mainly carpelloid structures with external ovules, while in the second one also carpelloid filiform stamen was observed (Fig. 3.3). These results confirm the hypothesis that *7B-1* and *sl-2* are not allelic to the *TAP3* mutant and with the *sl* series and excluded the involvement of *SlDEF* gene. However, the phenotype shown by double heterozygotes indicates that the two loci may encode functional partners and therefore indicates as candidate for these mutations other class B MADS-box transcription factors, like *TM6* (chr.2), *SlGLO1* (chr.8) and *SlGLO2* (chr.6).

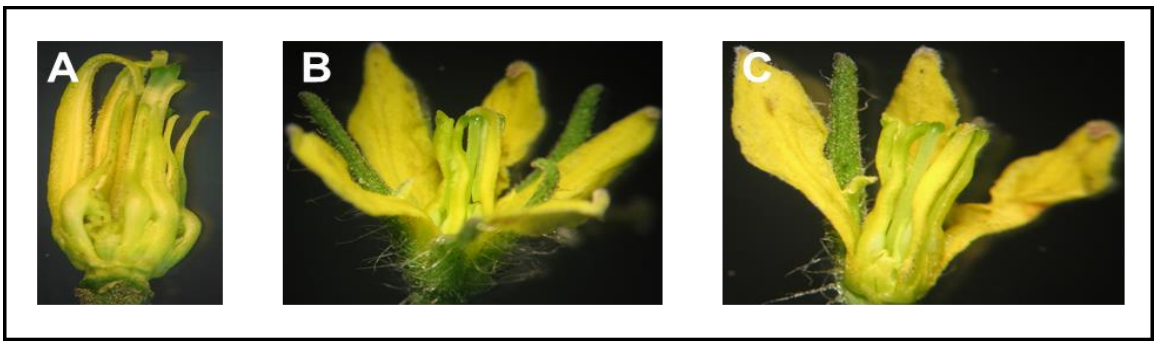

Fig. 3.3. Floral phenotype of double heterozygotes from cross between *7B-1* and *TAP3* and *sl-2* and *TAP3*. A, anthers cone of flower from cross between *7B-1* and *TAP3* showing some alteration, parts are WT and the other part are carpelloid like *7B-1* mutant. B and C, floral phenotype of plants originated crossing *sl-2* with *TAP3*. Heterozygous flowers are characterized from carpelloid filiform stamens.

Allelism test between 7B-1 *and* vms

Data obtained from several allelism tests demonstrated that *7B-1*, main object of this work, is not comparable with *sl* series and consequentially it is not due to mutation in *SlDEF* sequence (more details are explained in Chapter 4). The tomato male sterile phenotype *vms* probably is due a mutation in one gene positioned near two molecular marker, *bushy* (*bu*) and *dialytic* (*dl*), localized on chromosome 8. In this region is localized one class B MADS box gene, *SlGLO1* which is the best candidate for this mutation (more details about the candidate gene in Chapter 5). *vms* showed alteration of anther structure, a phenotype similar to *7B-1* mutant, and a floral morphology similar to those observed in mutants obtained through silencing of *SlGLO1* and *SlGLO2* (Geuten and Irish, 2010). To understand if *7B-1* and *vms* mutations are allelic, was performed an allelism test using a F_1 crossing population, which was obtained previously and was grown in two different season and years. In the first year, eight plants were grown in autumn in greenhouse conditions, segregating five WT and three mutant plants with weakly expression of the phenotype. Mutant plants showed mainly anthers with WT features alternating some carpelloid anthers with external ovules. The presence of WTs and mutants with only weaky phenotype indicates no allelism between *vms* and *7B-1*. It is know that *vms* showed a strongly expression of the phenotype under summer season, particularly with a temperature range between 28°C and 32°C (Rick and Boynton, 1967). This may indicate that autumn and the greenhouse conditions are not ideal to see a possible mutant expression. To confirm the data obtained in autumn, six F_1 plants were grown in summer field condition and they segregated five WTs and only one plant, with weak phenotype. Phenotype observation confirms that *vms* and *7B-1* are not allelic excluding at the same time a mistake in classification of flowers because in summer season there is a good expression of the *vms* mutation. Therefore, on the contrary if *vms* and *7B-1* were allelic, in summer field conditions, with temperature of 28°C-30°C (maximum expression of the mutation) the mutant phenotype would be easily identified.

Our results indicated no allelism between them and excluded the involvement of *SlGLO1* gene as candidate for *7B-1* mutant. The weak phenotype seen in few individuals of this cross may be explained with incomplete recessivity of one of the two mutations or, alternatively, assuming that the two mutations are involve functional partners and express some mutant phenotype in double heterozygotes.

Chapter 4

Molecular characterization of *7B-1* and *sl-2* tomato male sterile mutants

Introduction

Flower formation is the basis of plant reproduction and was carefully studied in model species such as *A. thaliana* and *A. majus*. The identity of different floral organs is specified by *floral organ identity genes*, which were identified studying mutants that showed alteration in flower development and type, number and position of floral organs (Bowman *et al*., 2012). The activity performed by proteins encoded by these genes is explained by the combinatorial "ABC model" according to which each class of gene specifies for the identity of a specific floral organ (Coen and Meyerowitz, 1991). The class A proteins specify for sepal formation, the class B together with A for petals, class B and C for stamens and class C alone for carpels. In addition, the class A and C have an antagonistic activity (Coen and Meyerowitz, 1991). Recently the ABC model was integrated with two other classes of genes, class D that specifies for ovules identity (Colombo *et al*., 1995) and class E that concurs in the development of all floral organs (Pelaz *et al*., 2001). In *Arabidopsis*, these genes were identified as *AP1* and *AP2* for class A, *AP3* and *PI* for class B, *AG* for C class, *STK*, *SHP1* and *SHP2* for class D and *SEP1*, *SEP2* and *SEP3* for class E. In snapdragon (*A. majus*), the orthologous genes are *SQUA* for class A, *DEF* and *GLO* for class B and *PLE* for class C. It is known that proteins encoded by these genes for accomplish their function must form obligatorily a heterodimeric structure with interacts with other proteins of this type in order to determine floral organ formation (Honma and Goto, 2001; Pelaz *et al*., 2001). All of these genes, except *AP2*, encode for transcription factors that are members of the MADS-box family (Theissen *et al*., 2000), which is characterized by presence of a DNA-binding domain (MADS domain) that is highly conserved among flowering plants. In some higher eudicots it was reported for class B MADS-box genes a duplication event for *AP3* and *PI* lineages that determined the formation of two paralogues for each clade that are characterized by a partition of the ancestral function (subfunctionalization; Geuten *et al*., 2011). Particularly, this process was studied in details in *Solanacee* family, paying attention to three species, tomato, petunia (*Petunia hybrida*) and tobacco (*Nicotiana benthamiana*) (Geuten *et al*., 2011).

In tomato, MADS-box genes belonging at all classes of these families were identified; *MACROCALYX* (*MC*) is a member of class A and it is important for sepal formation and inflorescence determinacy (Vrebalov *et al*., 2002). For class B, as mentioned earlier, there are four members, *SlDEF* and *TM6* for the *AP3/DEF* lineage and *SlGLO1* and *SlGLO2* for *the PI/GLO* clade (de Martino et al., 2006; Geuten and Irish, 2010). The C function in tomato is performed by *TOMATO AGAMOUS 1* (*TAG1*) (Pnueli *et al*., 1994a); in the D class there are two genes, *TAGL1* or *ARLEQUIN* (*ALQ*) and *TAGL11* (Busi *et al*., 2003; Vrebalov *et al*., 2009; Giménez *et al*., 2010), while the E action is played by *TOMATO MADS-BOX 5* (*TM5*) and *TOMATO AGAMOUS-LIKE 2* (*TAGL2*; synonymous *TM29*) (Pnueli *et al*., 1994b; Ampomah-Dwamena *et al*., 2002; Busi *et al*.,

2003). Another MADS-box gene was characterized, *TM4* or *TDR4*, which is homologous of *FRUITFULL* (*FUL*) (Pnueli *et al*., 1991; Busi *et al*., 2003; Lozano *et al*., 2009) and *TM8*, which is important for anthers, ovary and fruit development (Pnueli *et al*., 1991; Daminato *et al*., 2014).

The floral organ identity genes, particularly members of class B and C are activated by floral meristem identity genes (Lohmann and Weigel, 2002). In *Arabidopsis* it was observed that *AP3* and *PI* are activated through the action of these genes in the meristems precursor of petals and stamens, while *AG* in those precursor of stamens and carpels (Lohmann and Weigel, 2002). *LFY* promotes directly the expression of floral organ identity genes suppressing the expression of negative regulators for MADS-box genes, such as EMBRYONIC FLOWER 1 (EMF1) protein (Winter *et al*., 2011). It is known that, for the activation of class B MADS-box genes, *LFY* requires other factors that act in the same pathway (Jack, 2004). The class B genes are also activated by the UNUSUAL FLORAL ORGAN (UFO) protein (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995) and also by the action of *AP1* (Weigel and Meyerowitz, 1993). Recently, through the use of new technologies such as microarray analysis and ChIP-Seq, it was understood that the MADS-box factors influenced directly or indirectly the expression of an enormous number of downstream genes (Ito *et al*., 2004; Wellmer *et al*., 2004, 2006; Gomez-Mena *et al*., 2005; Mara and Irish, 2008; Kaufmann *et al*., 2009, 2010a; Jiao and Meyerowitz, 2010; Wuest *et al*., 2012). An example of genes that are directly activated by *AP3*/*PI* and also *AG* expression is represented by *NOZZLE*/*SPOROCYTELESS* (*NZZ*/*SPL*), a transcription factor that is involved in the regulation of sporogenesis (Yang *et al*., 1999). *AP3* and *PI* suppress the expression of genes that are necessary in carpel and ovule development (Wuest *et al*., 2012), such as *CRABS CLAW* (*CRC*). *CRC* is involved in carpel development and is expressed early in the third whorl of knock-out mutants for class B MADS-box genes (Bowman and Smyth, 1999). The multitude of downstream genes regulated by class B MADS-box factors encode for proteins involved in cell wall formation (polygalacturonases, pectate lyases and cell wall structural proteins), in stresses response, proteins similar to receptor-like kinases and phosphatases (Zik and Irish, 2003). These genes controlled downstream by MADS-boxes are necessary for different processes and indicate that in floral organ specification are involved a multitude of development pathways (Gomez-Mena *et al*., 2005; Mara and Irish, 2008; Kaufmann *et al*., 2010; Wuest *et al*., 2012).

Studying the tomato knock-out mutants for class B MADS-box genes it was possible to understand how these genes interact and function (de Martino *et al*., 2006; Geuten and Irish, 2010). The *SlDEF* gene is involved in petal and stamen development while *TM6*, *SlGLO1* and *SlGLO2* are required for stamen development (Geuten *et al*., 2011). Knock-out mutants for each class B MADS-box gene showed alteration only in one floral organ, particularly in the stamen morphology that presents carpelloid structures, except *SlDEF* which is characterized by a more severe phenotype with complete homeotic transformation of petals into sepals and stamens into carpels (de Martino *et al*., 2006). This phenotype resembled that of the tomato male sterile mutant *sl* and its allele *TAP3* (Bishop, 1954; Gomez *et al*., 1999; Quinet *et al*., 2014). The *sl* phenotype is influenced by

temperature conditions, high temperatures determine male sterility. Under high temperature conditions Gomez *et al*., (1999) observed three type of segregants, the WT plants with normal flower morphology, heterozygotes with normal petals and some alteration in the anther cone and homozygous for *sl* with carpelloid structures on stamens. The mutation was also characterized at molecular level, indicating that the putative gene was a class B MADS-box gene, localized on chromosome 4, *SlDEF* (Gomez *et al*., 1999). Successive studies conducted on *sl* and *TAP3* confirmed the previous data and gave new information about these mutations (Quinet *et al*., 2014). *TAP3* presents a male sterile phenotype influenced like *sl* by temperature conditions; under high temperatures it presents a flower morphology very similar to that of *sl*, except for the petals that are completely converted into sepals. The phenotypic analysis, carried out in two different geographic areas, Belgium and Spain, under high temperature conditions, revealed that in Belgium for both mutations it was not observed heterozygous phenotype, while in Spain the heterozygotes were evident with petals with normal features and anthers with some alterations. The phenotypic similarity suggested the possibility that *TAP3* was an allele of *sl*, and this was confirmed by allelism test (Quinet *et al*., 2014). Analyzing the sequence of *SlDEF* in both genotypes, it resulted that this gene is involved in both mutations. *TAP3* phenotype is due to two point mutations in the coding region, one causing an amino acid substitution and the other producing a stop codon with consequential production of a truncated protein. Differently, in *sl* genotype it was found an insertion of three small sequences in the promoter region that determines a rearrangement which is probably the cause of this mutation (Quinet *et al*., 2014).

In tomato, there are other male sterility mutations that have a phenotype which presents malformation in anthers structure, like *sl-2* and *7B-1* and it is of interest for both to identify the candidate gene. Data reported in literature suggest that *sl-2*, a mutant sensitive to the temperature, is an allele of *sl*, but no definitive evidence has been provided so far. Interestingly, *sl-2* is phenotypically similar to *7B-1*, a recently described photoperiod sensitive male sterile mutant. *sl-2* and *7B-1* are characterized by alterations in anther structure resulting in anthers that are short, retracted, distorted, laterally free and the stigma is protruding beyond them, while the remaining part of the flower is likely normal (Sawhney, 1983; Sawhney, 2004). This phenotype is very similar to those reported by de Martino *et al*., (2006) and Geuten and Irish (2010) for the knock-out mutants for class B MADS-box genes, particularly for *TM6*, *SlGLO1* and *SlGLO2*, which are involved mainly in stamen development.

This Chapter provides a the molecular characterization of *7B-1* (and consequentially of *sl-2*), in order to understand the genetic nature of the mutations and identify the putative candidate gene(s).

Materials and Methods

Morphological and molecular screening of the 7B-1 *mapping population*

A back-cross (BC_1F_1) mapping population was developed in the previous years after crossing the *7B-1* male sterile mutant with pollen of *Solanum pennellii* from TGRC (accession number LA0716). Ninety-nine plants from the BC_1F_1 population were grown in summer field conditions (experimental station of Tuscia University, Viterbo), the season when it is maximum the expression of the *7B-1* male sterile phenotype, in order to classify them according to the anther cone morphology in wild-types (WTs) and mutants. For each plant, two flowers were taken at anthesis (Stage 4) and the anther morphology was observed using the stereomicroscope. The structure of the anthers for WT and *7B-1* flowers was classified in normal, carpelloid and carpelloid with external ovules.

The same BC_1F_1 population was also characterized at molecular level using markers associated to the four class B MADS-box genes. For each target gene molecular markers located into the gene itself or associated to it were used (all markers used are summarized in Table 4.1). Initially, 43 WT and *7B-1* BC_1 plants were scored with all the selected markers. Total DNA was extracted from each plant according to the Doyle and Doyle protocol (1990) and amplified by PCR using primers and amplification conditions listed in Table 4.2. PCR amplification was performed in a 20 µL of total volume, containing 50 ng of genomic DNA template, 10 µM for each primer, 200 µM of dNTPs, 1 X Dream *Taq* Buffer and 1 U of Dream *Taq* polymerase (Fermentas). The PCR reaction was conducted with an initial denaturation of 4 min at 94°C that was followed by 30/35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at the temperature reported in Table 4.2 (Ta), extension for time as reported in Table 4.2 at 72°C, plus 7 min of final extension. Differences in size of the amplicons were visualized by 1.5% (w/v) agarose gels stained with ethidium bromide. *TM6* and C2At_4g33350 were used as Cleaved Amplified Polymorphic Sequence (CAPS) markers after cutting the amplicons with *Ase*I (New England Biolabs, Beverly, MA, USA) for the first marker and *Hinf*I (Invitrogen, Carslbad, CA, USA) for the second one. The CAPS polymorphism was obtained after digesting 10 µL of amplification product for both markers with 1 U of the appropriate enzyme in a final volume of 20 µL. The restriction fragments were visualized in a 1.5% (w/v) agarose gel.

Table 4.1. List of molecular makers for each class B MADS-box gene used in screening of the BC_1F_1 mapping population.

Class B MADS-box gene	Marker name	SGN or Solyc ID	Marker type
TM6	SSR356	SGN-M1029	In/del
	TM6	Solyc02g084630.2.1	CAPS (*Ase*I)
SlDEF	T0360	Solyc04g081570.2.1	In/del
	C2At_4g33350	Solyc04g079000.2.1	CAPS (*Hinf*I)
SlGLO2	C2At_1g52200	SGN-U221468	In/del
SlGLO1	SSR38	Solyc08g077480.2.1	In/del
	LePI	Solyc08g067230.2.1	In/del

Table 4.2. List of primers and amplification conditions used in PCR experiments.

Marker name	Primer sequence (5'-3')	Ta (°C)	Extension time (min)	No. of cycles	PCR product length (bp)[a]
SSR356	F: ACCATCGAGGCTGCATAAAG R: AACCATCCACTGCCTCAATC	57	1	32	259_E 200_P
TM6	F: TCGACAAACAGGCAGGTCAC R: CGTTCACGAATCTCAGCAAC	60	1	35	$630+200_E$ $400+200_P$
T0360	F: AAACTCTCCGAGCTAGTGCGG R: ACCCAACACCAAATTGTCCAA	56	1	30	600_E 650_P
C2At_4g33350	F: TATCATCTATCTCTTCCGTTCGCTTC R: ATGCGATTCCTTCAACCTTCAAC	57	2	35	$1000+500_E$ $800+500+300_P$
C2At_1g52200	F: ACATTTGGACAAATAGCAGAAGTC R: TGAGAGCAGACAGCAGGCATCATC	55	1	35	250_E 200_P
SSR38	F: GTTTCTATAGCTGAAACTCAACCTG R: GGGTTCATCAAATCTACCATCA	57	1	32	750_E 700_P
LePI	F: GGATTGTAAACAGAACTTGGAC R: GTTGTCTCTGTCATTAATTTGCCC	57	2	35	2100_E 1950_P

[a] Values are PCR product lengths in base pairs (bp) approximated by comparison with molecular weight ladders. Pedices indicate product length in *Solanum lycopersicum* (E) or *S. pennellii* (P) for in/del markers or the banding pattern for CAPS markers.

For confirming the results observed with the first molecular analysis (n=43), it was screened the whole segregant population (n=99) with markers flanking the class B MADS- box gene *SlGLO2* located on chr. 6. In this analysis, other four molecular markers were used, two associated to the *SlGLO2* gene and two located in two different introns of the gene itself (Table 4.3). The PCR reaction was performed as described before using primers and PCR conditions described in Table 4.4. For T0834, a touch-down cycle was adopted in order to avoid aspecific amplification products, while the *LeGLO2* marker was used as CAPS using the *Taq*αI (Invitrogen, Carslbad, CA, USA) restriction enzyme. The last marker, located into the gene itself, named *LeGlO2 6* was obtained from a primer combination designed to amplify the last intron, the last exons and part of the 3' UTR in the WT and *7B-1* DNA in order to obtain the genomic sequence of *SlGLO2* gene. In *7B-1* samples, these specific primers do not produce any amplification product so they were used as molecular marker for screening the whole BC_1F_1 population.

Table 4.3. List of molecular markers used to screen the BC_1F_1 population for the genomic region harboring the *SlGLO2* gene.

Marker name	Marker type	Map position[a]	Physical position (bp)	Solyc or marker code
T0834	In/del	32,00	33353218.. 33354040	Solyc06g053980
LeGLO2	CAPS (*Taq*αI)	38,00	34289235.. 34286205	M13/PI
LeGLO2 6	Pres/Abs	38,00	34289235.. 34286205	M13/PI
C2At_1g77470	In/del	39,10	34501206.. 34504819	Solyc06g060150

[a] Marker positions on chr. 6 according to the Tomato-EXPEN 2000 map, (http://www.sgn.cornell.edu)

Table 4.4. List of primers and amplification conditions used for PCR experiments.

Marker name	Primer sequence (5'-3')	Ta (°C)	No. of cycles	PCR product length (bp)[a]
T0834	F: TAATTGGGACCCCATCAGAA R: CCCTTTAGGAGCACAAGCAG	60-50	15-25	800_E 900_P
LeGLO2	F:GTTTTCTTCGAGTGATAGTGACAAA R: ACCATGGGAAGAGCCCATA	60	36	$240+100_E$ $250+90_P$
LeGLO2 6	F: GGTTTTCTCGGGGATTAGTATG R: GTTTTCAGAATCCATCTAGACC	60	36	600_P
C2At_1g77470	F: TGCCCTACAATCACGATGTACACG R: AAACCACCCTCAGGGACATCAAG	60	40	2500_E 2000_P

[a] Values are PCR product lengths in base pairs (bp) approximated by comparison with molecular weight ladders. Pedices indicate product length in *Solanum lycopersicum* (E) or *S. pennellii* (P) for in/del markers or the banding pattern for CAPS markers

The BC_1F_1 population was grown until fruit maturation in order to harvest seed from all WT plants and from some *7B-1* plants, which were capable to produce fruits. The seed was used to set up a progeny test for seven *7B-1* plants to confirm the phenotypic classification. The progeny test was carried out growing 12 plants for accession in summer field conditions in order to score them for the phenotype and for the molecular markers. The phenotypic screening was conducted through the classification of the plants in WT or mutant according to the anther morphology. The molecular analysis was conducted using the markers for the class B MADS-box gene *SlGLO2* (Table 4.3) with the PCR conditions described above (Table 4.4).

Molecular identification of 7B-1 *mutation*

The entire genomic sequence of the *SlGLO2* gene (3041 bp) in the WT and *7B-1* line was amplified using five different primer combinations (Table 4.5). The primers were manually designed to cover the whole sequence and controlled with the IDT DNA Tecnology online software to check for the presence of harpins and the possibility that they formed heterodimers or homodimers. Four different

primer combinations were used to amplify the first part of genomic sequence of the *SlGLO2* gene (Table 4.5), containing 418 bp of promoter region and the first four exons and introns and the sequence was obtained from the PCR product. The second part of the gene (last three exons and introns plus 365 bp of the 3' UTR) was obtained using one primer combination (Table 4.5) and the amplicon was cloned into pGEM T-easy vector. The fragment was sequenced using the universal primers for the pGEM vector T7 as forward and SP6 as reverse.

At the same time, we sequenced the entire coding region (645 bp) of the *SlGLO2* gene in cDNA samples (the procedure for the cDNA synthesis is described in the next paragraph) of WT and mutant plants using two specific primer combinations (Table 4.5).

To complete the sequence of the *SlGLO2* gene in the WT and *7B-1* mutant, it was also obtained the sequence of the promoter region (2079 bp) using three different primer combinations (Table 4.5).

Table 4.5. List of the primer combinations used to amplify the genomic sequence, the cDNA and the promoter region of the *SlGLO2* gene in WT and *7B-1* genotypes.

Marker name	**Description**	**Primer sequence (5'-3')**
LeGLO2 1	418 bp promoter region-5' UTR-first exon	F: TCACCACAGCCCAATACTCA R: CTTGCCTGTTGTTTGTGTTTTC
LeGLO2 2	First and second exons-first and second intron	F: GTGATCATCATGGGGAGAGG R: TTCTGCTCCTTAAATAAGCATTC
LeGLO2 3	Second intron	F: GGGAGGAGACTATGGGATG R: CGAGATCGAGTGATAATTTTCG
LeGLO2 4	Last part of the second intron-third exon and intron-fourth exon	F: TTCGAAAATTATCACTCGATCTC R: GTCCATTTTGTAAGGCTTC
LeGLO2 5	4^{th} to 7^{th} exon- 4^{th} to 6^{th} intron-3'UTR- 365 bp downstream	F: TGTATGTGTACAGGCACCTCAA R: TTTTGTGTCCATTTTCTTTGC
LeGLO2 cDNA 1	5' portion of the cDNA	F: GTGATCATCATGGGGAGAGG R: TCCTCCTCCAGAATTTGATCC
LeGLO2 cDNA 2	3' portion of the cDNA	F: TTCTAGTATCAGTGCCAAGCAGG R: ACCATGGGAAGAGCCCATA
LeGLO2 p1	Promoter region	F: GAATAGTCCAAAACGCCCCT R: GTCTTGTCTAGGTGCCTTGA
LeGLO2 p2	Promoter region	F: GTCCACAAGGCAAATGATTGAG R: GCTTACCAAGGGGTTGAATGAC
LeGLO2 p3	Promoter region	F: TGAAACGGAGGAAGTACTCGT R: TGTGAGTATTGGGCTGTGGT

Total RNA isolation and cDNA synthesis

In spring conditions, 15 plants of the WT and of the fixed *7B-1* mutant line were grown with standard horticultural practices in an unheated tunnel under environmental light conditions at the experimental station of the Tuscia University (Viterbo, Italy). During flowering, young flower buds, anthers and stems of different plants from both genotypes were sampled. Flower buds were sampled at two

different stages, Stage -1 (length of flower bud 1-2 mm) and Stage 0 (length of flower bud 3-4 mm), while dissected anthers were sampled at Stage 1 (anther length 5-6 mm; flower stadiation according to Mazzucato *et al*., 1998). For the stem, about 3 cm in length of tissue were sampled from a young lateral branch of three-months-old plants. Stem tissue was cut in small pieces. The tissue samples were collected and immediately frozen in liquid nitrogen. Total RNA was extracted from 100 mg of plant material from each stage using TRIzol reagent (Invitrogen, Carslbad, CA, USA) according to the manufacturer's instructions. RNA integrity was checked by 1% (w/v) agarose gel, while the concentration and purity of each sample was controlled measuring the absorbance at 260 nm and 280 nm using the Agilent RNA 6000 Nano Kit with an Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA was used only if the ratio between absorbance at 260 nm and 280 nm was higher than 1.8. cDNA was synthesized from 2 µg of total RNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. For the analysis, three biological replicates for each genotype and stage were used.

RNA transcriptome analysis by RNA-seq

Sample preparation for transcriptomic analysis and the RNA-seq procedure was performed at Palacky University (Olomouc, Czech Republic) by Dr. V. Omidvar.

The *7B-1* mutant and WT seedlings were grown in growth chamber under controlled temperature and photoperiod (16 h light/ 8 h dark) conditions. Flower buds of different sizes smaller, equal and bigger than 4-5 mm (hereafter referred to as stages 1, 2, and 3) were collected and stamens were dissected under a microscope. Stages of flower buds were based on those described by Sheoran *et al*., (2009); flower buds at stage 1 represents pre-meiotic anthers, stage 2 is where tetrads are formed in WT anthers (meiotic anthers), but meiosis breaks down in MMCs in *7B-1* and stage 3 represents post-meiotic anthers (Sheoran *et al*., 2009). Stems from three-month old seedlings were used for qPCR. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) from *7B-1* and WT anthers of different stages and pooled separately in equimolar ratio. Two sRNA libraries were constructed using the TruSeq Small RNA Sample Preparation Kit (Illumina). The final PCR products were purified from the gel and sequenced using Illumina Hiseq2000 platform (Illumina). Adaptor sequences were trimmed and reads mapped (no mismatch allowed) to the tomato genome ITAG 2.4 Release using PatMaN (Prufer *et al*., 2008) and a customized Perl script.

Real-time PCR and RNA-seq validation

To analyze the expression of all four members of class B MADS-box genes a semi-quantitative RT-PCR was performed using four-fold dilutions of the first-strand cDNA. Two biological replicates from WT and *7B-1* and stage -1, 0 and 1 were used in this experiment with specific primers for these transcription factors (Table 4.6). Amplification reactions were performed in a total volume of 20 µl using the same reagents and concentration used for the PCR experiments described above. The RT-

PCR was conducted using an initial denaturation of 4 min at 94°C followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 s and extension at 72°C for 30 sec. The *α-Tubulin* gene was used as HK gene (Solyc04g077020.2 as housekeeping (HK) gene; Coker and Davies, 2003).The number of cycles used in the RT-PCR experiments was the same for all class B MADS-box genes, except for the HK gene where 20 cycles were adopted. Amplified products were visualized on a 1% (w/v) agarose gel stained with ethidium bromide.

Real time PCR (qRT-PCR) analysis was applied in order to validate selected genes which were found differentially expressed with RNA-seq analysis in WT and *7B-1* and to investigate other six genes, the other three members of class B MADS-box transcription factors, plus other three involved in the regulation network downstream to class B genes (ò'Maoiléidigh *et al*., 2013; Wellmer *et al*., 2014; Mara *et al*., 2010).

The same cDNA used for semi-quantitative RT-PCR was also used for qRT-PCR. Two biological replicates from each genotype, WT and *7B-1*, and each stage, -1, 0, 1 and stem were used with the SSO ADV UNIVERSAL SYBR GREEN Master Mix (Bio-Rad, USA) according to the manufacturer's instruction in the Bio-Rad CFX96 Manager system (Bio-Rad, USA). The primers used for validating the expression of selected genes are listed in Table 4.6. The amplification experiments were performed in a total volume of 15 µL containing 1.75 µL of four-fold diluted cDNA, 1 X SSO ADV UNIVERSAL SYBR GREEN mix and 300 nM of each primer. The experiment was conducted using an amplification program that consisted in one step of denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 58°C for 30 s. Each amplification event was conducted in three technical replicates. To evaluate the gene expression level, results were normalized using *α-Tubulin* as HK gene. Relative expression was calculated as log2Ratio of the fold change (FC) calculated with the ΔΔCt method as follows: $2^{[(\Delta Ct \text{ value of target gene in the mutant})-(\Delta Ct \text{ value of the target gene in the WT})]}$.

Table 4.6. Gene description and primer sets used for qRT-PCR to validate the expression pattern of selected genes.

SGN solyc ID or LeGI v. 11 code	Gene name	Gene function description	Primer sequences (5'-3')
Solyc02g062870.2	*Polygalacturonase-5*	cell wall modifying enzyme	F: ATGGAACATCCGCTACAGAAG R: GAGTTCTTTGAAACCAGAAGC
Solyc01g079200.2	*Gibberellin 2-oxidase*	anther development	F: GGCTCCCTTTGGTTATGGTAG R: GTCACCGAGCTGAAAGTAGAG
Solyc06g059820.1	*F-box*	tapetum degeneration	F: GGTGGAACCCTGCTACTAAAG R: CATCATACCCTACCGCTGTTAC
Solyc01g005310.2	*Dynamin like protein*	meiosis regulator	F: AACAATGATGCTGGTGAAGG R: GCAGTGTAGATGGTGTTCCTG
Solyc07g053460.2	*Cysteine proteinase*	cell wall modifying enzyme	F: GGTGTCGATTGGAGGAAGGA R: TGATGCCCTCTATTGCTGCT
Solyc07g044870.2	*Polygalacturonase-*like	cell wall modifying enzyme	F: CAGAATCCACCTGTCTGTGCTG R: CTTGGGAGCTAGGTCGTATCTGG
Solyc05g051250.2	*Glutamine synthetase*	male sterility gene	F: TTGGTCCTGCTGTTGGTATC R: CCAATCACCAGGGATAGGTTT
Solyc10g086460.1	*Actin*	meiosis regulator	F: CACTTCCTCACGCTATCCTTC R: TTCCTTCTCAGCACTGGTTG
Solyc06g059970.2	Class B MADS-box transcription factor *SlGLO2*	petal and anther development	F: TTCTAGTATCAGTGCCAAGCAG R:CCTCTCATATTTCCACTTCCACCA
Solyc02g062870.2	*Solute carrier family 2*	pollen development	F: ACACCAAATTCTTGCCACATG R: TCCCAAGTCCAGTTAATGCC
Solyc08g067230.2	Class B MADS-box transcription factor *SlGLO1*	petal and anther development	F: GGAGCAGGAGCAAGATCAAC R:GTTGGACTCTGAAAGCAAAAGGC
Solyc02g084630.2	Class B MADS-box transcription factor *TM6*	petal and anther development	F: CAAGCCCAAACACTACGACA R: CTTCCCCTGTTCTCTGCCTT
XM_004233310	Transcription factor *bHLH135*	involved in light signalling	F: TGTCTGGGAGAAGGTCAAGG R: CTTGTTGGAGCGACGATTACG
Solyc04g081000.2	Class B MADS-box transcription factor *SlDEF*	petal and anther development	F:TAAGTCCCTCTATCACGACCAAC R:TCCTATTCACATCCTTTAGCTTCC
Solyc05g012150.2	*Crabs Claw*	Carpel development	F:GCACCTTTTGTTGTAAAACCTCCT R:AGCTTCTCTATGTGGTATCTCTGG
ABX82930	*transcription factor Style 2*	involving in light signaling	F: CCTTACCACAACTTCCTCTCCT R: CGACCTTCTGCTCGACATCC
Solyc04g077020.2	*α-Tubulin*	housekeeping	F:TGAGGTCTTCTCACGCATTGACCA R: AATCCTTCTCGAGGGCAGCAAGAT

The "Solyc" codes underlined identified genes selected from the literature.

Preparation of the construct for complementation experiments

For attempting complementation experiments of the *7B-1* mutant, the full length cDNA of the *SlGLO2* gene (645 bp) was obtained as described before (paragraph *Total RNA isolation and cDNA synthesis*) with amplification from anthers (Stage 1) of WT flowers. At the extremity of the complete CDS sequence were added by PCR reaction the recognition sites of two restriction enzymes, *Bam*HI and *Sac*I using the following primers: forward (5'- GGATCCATGGGGAGAGGTAAAATAGAG-3'; *Bam*HI restriction site is underlined) and reverse (5'-GAGCTCTTACATTCTTTCATGTAGATTTGGCTGC-3'; *Sac*I restriction site is underlined). The PCR reaction was performed in a total volume of 50 µL containing 5 µL of ten-fold diluted cDNA, 200 µM of dNTPs, 0.5 µM of each primer, 1 X of Q5 Reaction Buffer and 1 U of Q5 High-Fidelity DNA Polymerase (New England Biolabs, Beverly, MA, USA). The PCR experiments were conducted using an initial denaturation of 30 s at 98°C, followed by 35 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 60°C and extension for 30 s at 72°C, plus a final extension of 2 min at 72°C. The amplified product was purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and ligated into pBI121 vector under the control of the *Cauliflower Mosaic Virus* (*CaMV*) *35S* promoter and the *nopaline synthase* (*NOS*) terminator (Chen *et al*., 2003), using T4 DNA ligase (Fermentas) following the manufacturer's protocol. The pBI121 vector was linearized with the restriction enzymes *Bam*HI and *Sac*I to remove the *β-glucuronidase* (*GUS*) gene and the complete CDS of *SlGLO2* gene was ligated into the plasmid to generate the complementation construct. Ten µL of ligase reaction was used to transform 100 µL of *Escherichia coli* DH5α competent cells. The positive colonies were validated by PCR using specific primers for the sequence of the CDS (the same primers used for the first amplification), primers for the *CaMV 35S* promoter (forward: 5'- CCCACAGATGGTTAGAGAGGC -3') and *NOS* terminator (5'- AAGACCGGCAACAGGATTC-3') and some restriction reactions with different enzyme combination for confirm the gene insertion. Plasmid minipreparations of colonies resulted positive for PCR and restriction reactions were sequenced (Macrogen Europe, Amsterdam, The Netherlands). One of the sequenced colonies was used for transformation of *Agrobacterium tumefaciens* competent cells strain GV3101. Together with the construct preparation for the complementation experiment, the plasmid pBI121 was also used to transform *Agrobacterium* to use as negative control. Positive colonies for both constructs were checked by PCR and using restriction enzymes and then used for plant transformation.

Plant transformation procedure

The plant material used for the transformation was cotyledons from WT and *7B-1* seedlings. *7B-1* seeds used were obtained from hand-pollination of 21 mutant flowers with self pollen, while for WT was grown seed derived from open-pollination. To check the mutant condition of seed harvested on *7B-1* plants, a few seedlings from each hand pollination event were screened by PCR using specific

primers for *SlGLO2* gene that discriminate the mutant genotype as they produce amplification only in the WT genotype (see Table 4.3 and Table 4.4).

The plant transformation procedure was performed at the Plant Lab, Scuola Superiore Sant'Anna (Pisa, Italy) using the protocol described by Zuluaga *et al*., (2008). One hundred and fifty-five seeds of WT and 125 of *7B-1* were surface sterilized with 25 mL of 4% (v/v) solution of sodium hypochlorite and then grown in germination medium (25 g/L of MS powder, 0.5 mL/L MS vitamin solution, 15 g/L sucrose and 7 g/L agar, pH 5.7) for 15 d at 24°C in light conditions. When the seedlings reached an optimum size, cotyledons were cut and placed in the co-culture medium (4.3 g/L MS powder, 1 mL/L MS vitamin solution, 20 g/L glucose, 1 mM MES and 7 g/L agar, pH 5.7; after autoclaving 0.75 mg/L trans-zeatin, 1 mg/L indole-acetic acid (IAA) and 200 µM acetosyringone solution) for 2 d in dark conditions at 24°C. The cotyledons of both, WT and *7B-1*, were co-cultivated with *Agrobacterium* transformed with *pBI121::SlGLO2* and *pBI121::GUS*. After 2 d, the explants were transferred in the induction medium (4.3 g/L MS powder, 1 mL/L MS vitamin solution, 20 g/L glucose, 1 mM MES and 7 g/L agar, pH 5.7; after autoclaving 0.75 mg/L trans-zeatin, 1 mg/L IAA, 300 mg/L cefotaxime and 100 mg/L kanamycin) until the shoots begin to form (sub-cultured into fresh medium every 15 d) at 24°C under 16 h of light with a light intensity of 80 µmol $m^{-2}s^{-1}$.

As the transformation experiments were not successful in giving engineered plants, the following steps of the protocol were only carried out in preliminary experiments on WT and *7B-1* untransformed regenerants.

When the shoots reached the length of 1-2 cm they were transferred in the elongation medium (4.3 g/L MS powder, 1 mL/L MS vitamin solution, 20 g/L glucose, 1 mM MES and 7 g/L agar, pH 5.7; after autoclaving 0.1 mg/L trans-zeatin, 0.05 mg/L IAA, 300 mg/L cefotaxime and 100 mg/L kanamycin) until the explants reached about length of 3-4 cm. After this phase the shoots were placed in rooting medium (4.3 g/L MS powder, 1 mL/L MS vitamin solution, 20 g/L glucose, 1 mM MES and 7 g/L agar, pH 5.7; after autoclaving 0.2 mg/L indole butyric acid (IBA), 400 mg/L carbenicilline, and 100 mg/L kanamycin) where they grow until the root development. Then, the small plants obtained were transferred in soil and covered with a plastic cup for 8 days under growth chamber conditions. Acclimatized T_1 plants were transplanted in pots and grown in confined environment until the flowering time to observe the reversion of *7B-1* phenotype into WT morphology.

In silico analysis of the promoter region of class B MADS-box genes

Approximately 2000 bp of sequence corresponding to the promoter region of *SlDEF*, *TM6*, *SlGLO1* and *SlGLO2* were retrieved from Solanum Genomic Network database (http://www.sgn.cornell.edu) and analyzed in order to find *cis*-acting regulatory elements that could be linked with the environmental perceptions. All promoter sequences were analyzed using the SOFTBERRY

(linux1.softberry.com) an online software for prediction of regulatory elements inside the promoter region and the findings were submitted in RARGE database (http://rarge.psc.riken.jp/) (Sakurai *et al*., 2005) in order to find some information about the signaling pathway where the regulatory elements are involved.

Results and discussion

Morphological and molecular screening of the 7B-1 *mapping population*

The *7B-1* tomato male sterile mutant shows sterility in summer season under LD conditions, while it returns fertile in autumn under SD conditions (Sawhney 1997; 2004). As described in Chapter 2, in this mutation the mechanisms that control sterility and fertility restoration remains unchanged also in Italian growth conditions. In order to characterize the mutation at molecular level and identify a candidate gene, a BC_1F_1 mapping population was constituted. The whole population (n=99) was characterized at phenotypic level according to anthers morphology and classified into WT and *7B-1* plants. The phenotypic screening and classification gave 62 WT and 37 *7B-1* plants with an apparent defect in the frequency of mutant plants (χ^2=6.31, P<0.05). All WT plants showed a classical flower morphology and all anthers had normal features, while *7B-1* flowers were characterized by typical morphology of this mutation, anthers were retracted, distorted, separated from each other and the stigma was exerted. The anthers analyzed using the stereomicroscope showed different types of abnormalities; anthers WT-like (2.9%), with carpelloid structures (34.3%), with carpelloid structures plus external ovules on the adaxial surface (57.4%) and carpelloid filiform adnate to the pistil (5.4%). For the molecular analysis, initially 43 plants were analyzed using molecular markers associated with class B MADS-box genes or located into the gene itself). This molecular analysis showed that all markers used segregated independently from the mutation (P >0.05), except C2At_1g52200, that appeared associated with the *SlGLO2* gene (P<0.01, data not shown). This marker, located at 6 cM from *SlGLO2* gene, suggested the hypothesis that the candidate gene was *SlGLO2*. To confirm this, the entire mapping population (n=99) was screened using C2At_1g52200 molecular marker with the result of 20% recombination between the marker and the *7B-1* locus.

To better understand if the presence of these recombinants was due to the distance from the gene, which is positioned at 38 cM, other two markers were chosen, one T0834, located upstream to *SlGLO2* (pos.32 cM) and the second one C2At_1g77470, located downstream to the gene (pos.39.10 cM). Together with these two markers associated to the gene, other two polymorphisms located into the gene itself were used, *LeGLO2* and *LeGLO2 6* (Table 4.3 and Table 4.4). The analyses showed that there is a co-segregation of the molecular phenotype with the mutation, but also in this case were found recombinant individuals (Table 4. 7). Seventy-six percent of the BC_1 plants showed the parental haplotype in the target region. Out of the seven plants showing recombination among markers, none was a *7B-1*/*GLO2* recombinant reinforcing the idea that *7B-1* represents a *GLO2* mutation (Table 4.7). The only recombination beween the *7B-1* phenotype and polymorphisms at the *SlGLO2* gene were found in 16 individuals that had the "A" haplotype at the molecular analysis, but

were classified as WT according to the flower phenotype (Table 4.7), suggesting initially a mistake in the classification.

Table 4.7. Classification of BC_1F_1 mapping population (n=99) according to the phenotype in WT and *7B-1* plants and results of molecular screening using markers for the genomic region of the *SlGLO2* class B MADS-box gene (chr.6). The molecular markers are sorted from upstream to downstream respect to *SlGLO2*. A and H indicate the different marker haplotype: A corresponds to the polymorphism found in *Solanum lycopersicum* and consequentially to the *7B-1* parent, while H corresponds to polymorphism of *S. pennellii* and to the WT parent.

Phenotype at *7B-1* locus	**Phenotype at molecular markers**					**No° of plants**
	T0834	**LeGlo2**	**LeGLO2 6**	**C2_At1g77470**	**C2At_1g52200**	
7B-1 (A)	A	A	A	A	A	**35**
WT (H)	H	H	H	H	H	**41**
WT (H)	A	H	H	H	H	**3**
WT (H)	H	H	H	H	A	**1**
WT (H)	A	H	H	A	A	**1**
7B-1 (A)	A	A	A	H	H	**1**
7B-1 (A)	A	A	A	A	H	**1**
WT (H)	A	A	A	A	A	**16**

Flowers from WT plants that showed a *7B-1* haplotype at molecular level were observed again at the stereomicroscope, revealing a correct phenotyping and at the same time suggesting the possibility in a mistake in molecular analysis. In addition it was set up a progeny test using seed recovered from seven of the 16 plants. For each progeny were observed 12 plants and classified according to the phenotype in WT and *7B-1* mutants and also they were analyzed at molecular level using only the two molecular markers located into the *SlGLO2* gene itself. The progeny test for the phenotype classification revealed that all progenies, except one, segregated the mutant phenotype; most of WT individuals showed anther structure with normal features but some anthers possess small defects. The molecular analysis conducted on progeny plants and repeated on respective samples from the BC_1F_1 mapping population confirmed the previous results that these individuals at molecular level behave as mutants (data not show).

The data obtained with the screening on the mapping population and those obtained with the progeny test suggest that these 16 plants are anomalous "recombinant". They are anomalous because it was observed only one type of recombination; all individuals are WT at phenotypic level, but "mutants" at molecular level. This could be explained with the presence of some modifiers genes that may act at transcriptional or post-transcriptional level, which hamper the expression of the *7B-1* phenotype. Taken together, the data show that the two loci, *7B-1* and *SlGLO2*, co-segregated supporting the hypothesis that the candidate gene for this male sterile mutation is *SlGLO2*.

These suppositions are also supported by the fact that the fruit set was not recovered for all 16 "false recombinant" individuals, but only for seven plants while if they were really WT genotype, a good fruit and seed set had to be observed in all plants.

In the molecular analysis we have started using markers linked to each class B MADS-box gene because literature data about the characterization of several knock-out mutants for these genes (de Martino *et al*., 2006; Geuten and Irish, 2010) and also the identification of *SlDEF* as causative gene of *sl* and *TAP3* male sterile mutations (Gomez *et al*., 1999; Quinet *et al*., 2014) suggest that the putative candidate for *7B-1* was a class B member.

The analysis showed that the putative gene involved in this mutation is *SlGLO2* gene and this is corroborated also by the phenotype of knock-out lines for this gene, which are characterized by alteration only in third whorl with anthers laterally free and with carpelloid structure (Geuten and Irish, 2010). Mapping data presented here exclude an involvement of the other members of the B class in the *7B-1* mutation. The exclusion of *SlDEF* as candidate gene for *7B-1* validates also the results obtained from allelism tests, described in Chapter 3, which demonstrated that *7B-1* and its allele *sl-2* are not allelic to *sl*, where the gene involved was actually identified in *SlDEF* (Gomez *et al*., 1999; Quinet *et al*., 2014).

Molecular analysis of SlGLO2*, the candidate gene for the* 7B-1 *mutation*

To demonstrate that the candidate gene for *7B-1* mutation is *SlGLO2*, the entire genomic sequence was sequenced in WT and mutant samples. The whole sequence (3041 bp), constituted from seven exons and six introns, was obtained for WT and *7B-1* genotypes, except the second intron region (Supplementari Figure S1 reported in the Appendix) where different primer combinations and amplification strategies were unsuccessful due to the amplification of aspecific PCR products; consequentially this part of the gene is missing in both genotypes. Similar problems were found during the amplification of the last exon and part of 3' UTR region, about 610 bp; particularly with a pairs of primers, called *SlGLO2 6*, amplification was not obtained in the *7B-1* but only in the WT genotype. To verify if the lack of amplification in was really a prerogative of the mutant genotype, some individuals of the BC_1F_1 mapping population were screened using this primer combination and this resulted in an amplification product of the correct size (610 bp) in WT samples, while no amplicon was detected in *7B-1* plants (Fig.4.2). Starting from the missing amplification on *7B-1* genotype, the whole mapping population was analyzed using *LeGLO2 6* as molecular marker as described above.

In Chapter 3, it was described an allelism test between *7B-1* and *sl-2* mutations that demonstrated that they are allelic. Therefore, the amplification experiment using *LeGLO2 6* primers was conducted also in the *sl-2* mutant and its respective WT. The result shows that also *sl-2* lack the amplification product and this indicates that non only the mutation is due to the same gene, but also that the genetic lesions have similar consequences (Fig. 4.1).

In order to understand if in this region there was a deletion other different primer combinations were designed to try to amplify it. An amplification product was obtained for the mutant samples using primers designed to amplify a portion of the gene of about 1800 bp (from 4^{th} exon until 365 bp of the

3’ UTR), then cloned into pGEM T-easy vector and sequenced using T7 and SP6 primers. The sequence did not show differences between WT and mutant samples (Fig. S1).

The results obtained from the *SlGLO2* sequence indicate that there are not important differences in the coding region between the two genotypes, except for four nucleotidc substitutions found in intronic regions and probably these data did not support the initial hypothesis about *SlGLO2* as candidate gene for *7B-1* mutation. The SNPs found in different introns (Fig. S1) are localized far from the splicing sites and to confirm if they are a true mutations or are due to some problems associated with the reading of the sequence is necessary repeat the sequencing of them.

To validate our assumption that *SlGLO2* still remains the putative gene involved in this mutation we tried to amplify and sequence the cDNA of WT and mutant samples. cDNA of WT and *7B-1* samples from three different stages of flower development, was amplified using two primers combinations. The cDNA and amplification products were obtained only in the WT line while in *7B-1*, changing also primers and amplification method, it was not detected any amplification product and obviously it was not possible to make a comparison between the two genotypes.

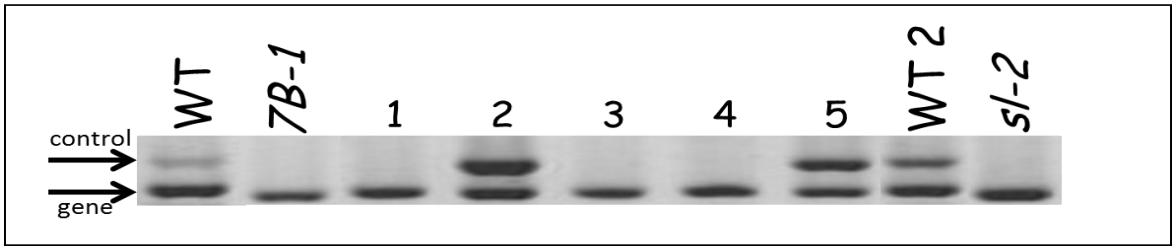

Figure 4.1. Amplification results with *LeGLO2 6* primer combination. WT is the respective normal genotype for *7B-1* mutation, while WT2 is the respective wild-type for *sl-2* mutation. The numbers from 1 to 5 represent some samples from the BC_1F_1 mapping population analyzed with these primers. The WT parents and the WT samples show the expected amplification product (610 bp, second black arrow), while the mutant genotypes and segregants do not have the product. The first black arrow indicates the amplification product relative to another primer combination used as control to verify that the PCR experiment occurs correctly.

The missing detection of *SlGLO2* amplification product from cDNA of *7B-1* plants at different stages of flower development indicates a possible lack of expression of this gene.

The lack of expression of the gene could be a consequence of mutations, insertion or deletion that can reside in the genomic sequence (exonic or intronic region), in promoter or in the downstream region of the gene. Literature data conducted on different species belonging to the *Ranunculaceae* family, particularly in apetalous species, demonstrated that the loss of petals is associated with a decrease or elimination of expression of the *AP3-3* gene. Particularly, it was observed that in *Nigella* an apetalous mutant do not show expression of *AP3-3* due to an insertion of a transposable element in the second intron that determines a silencing of the gene with consequential transformation of petals into sepals (Zhang *et al*., 2013). In naturally apetalous mutants that occur in *Beesia* and *Enemion* genera, a defect in exon-intron structure or a deletion in the regulation region near the CArG box motif, typical of the MADS-box transcription factors, causes a down regulation of *AP3-3* gene (Zhang *et al*., 2013).

In our case, the failure to obtain the cDNA sequence of *SlGLO2* gene is not probably due to a mutation in the genomic sequence, because it was obtained a complete identical sequence for WT and *7B-1* sample, except for the second intron that was not amplified in both genotypes and for the four SNPs found in the introns. Maybe the mutation can reside inside the second intron, but the difficulty in obtaining the sequence in WT and *7B-1* individuals could indicate that the missing of amplification product is due in this case to technical problems.

Alternatively, the mutation could involve the promoter region and for this reason 2000 bp of promoter from WT and *7B-1* genotypes were amplified and sequenced. No differences were found between the two sequences in the sequenced region (Fig. S2). Attempts to sequence a wide region upstream were not successful in both genotypes due to technical problems during amplification procedures.

In order to better understand if there is some difference in the promoter region, it will be necessary to complete the sequencing to cover at least 3000 bp upstream to the starting codon. Sequencing reported in literature about the obtainment of *SlDEF* promoter region (2.3 kb), which was pursued using an anchor-PCR approach (Schupp *et al*., 1999) due to difficulties through the normal PCR experiment (Quinet *et al*., 2014), suggests the hypothesis to try this experiment adopting this new method. The difficulty to have the entire sequence of the *SlGLO2* promoter may be associated with the observation that in the original sequence, which was used to design primers, there were approximately 700 bp not identified (designed as N) positioned upstream to the first part of the promoter that we have tried to amplify without success. In this contest, an anchor-PCR approach can be useful to explain the problems that occurred during the various experiments and to understand if there are insertions in the sequence that cause a genomic rearrangement of the region such as it was described for the *sl* mutation (Quinet *et al*., 2014). In *sl*, it was demonstrated recently that the mutation is due to insertions of three small regions in different parts of the *SlDEF* promoter. These insertions are homologues to the sequences located in different position of chr. 3 and this rearrangements maybe is the cause of *sl* phenotype (Gomez *et al*., 1999; Quinet *et al*., 2014).

Summarizing the results relative to the sequencing of *SlGLO2*, the genomic sequence did not show differences between WT and *7B-1* genotype, except for the second intron that was not amplified in both and consequentially it cannot be excluded the hypothesis that the lack of expression observed in the mutant is associated to one lesion in the genomic sequence. At the same time, the existence of genomic sequence of *SlGLO2* indicates that the lack of amplification product on cDNA in *7B-1* samples is not due to the lack of the entire gene as described for *Thalictrum* (Zhang *et al*., 2013). Then, the main hypothesis that remains valid is a rearrangement in the promoter region that maybe determines a down-regulation of the gene or a change also in the region downstream at the 3' UTR.

The lack of expression of this class B MADS-box gene in *7B-1* plants was also demonstrated, as described in the next paragraph, with qRT-PCR experiments, supporting the idea that the main candidate for this male sterile mutation remains *SlGLO2*.

Comparison of global gene expression between WT and 7B-1 *mutant*

To investigate and identify genes that are differentially expressed in tomato male sterile mutant *7B-1* grown under LD conditions, comparative transcriptomic analysis between the mutant and its corresponding WT was performed by RNA-seq. The RNA-seq experiment was performed at Palacky University (Olomouc, Czech Republic) by Dr. V. Omidvar and within this collaboration our work included the validation through qRT-PCR of the expression of 10 genes that were differentially expressed in *7B-1* with respect to the WT.

The transcriptomic analysis was done using pooled samples, which consisted of a bulk of anthers at three different flower development stages plus stem (see Materials and methods) and differential expression value of these 10 genes was expressed as $\log_2$ fold change (Table 4. 9). Out of these 10 genes, two are up-regulated while the other eight are down-regulated in the mutant with respect to the WT sample. To validate the RNA-seq analysis results, the expression pattern of these genes, was tested using qRT-PCR procedure.

Table 4.9. RNA-seq data relative to 10 genes objective of the qRT-PCR validation. In the last columns is reported the normalized expression relative to the WT and *7B-1* and the differential expression value calculated as Log2 fold change [DE(log2)] respectively for each gene.

Gene code	**Gene annotation**	**Normalized expression**		
		WT	***7B-1***	**DE(log2)**
Solyc02g062870.2	*Polygalacturonase-5*	0.82	51.47	-1.78
Solyc01g079200.2	*Gibberellin 2-oxidase*	32.79	152.49	-1.71
Solyc06g059820.1	*F-box*	165.79	3.00	3.01
Solyc01g005310.2	*Dynamin like protein*	655.50	12.29	4.39
Solyc07g053460.2	*Cysteine proteinase*	733.66	11.33	4.59
Solyc07g044870.2	*Polygalacturonase*-like	43724	161.10	4.60
Solyc05g051250.2	*Glutamine synthetase*	3229.85	107.16	4.68
Solyc10g086460.1	*Actin*	2580.13	80.82	4.69
Solyc06g059970.2	Class B MADS-box transcription factor *SlGLO2*	1677.09	18.02	5.48
Solyc02g062870.2	*Solute carrier family 2*	5473.77	24.16	6.96

Differently from the RNA-seq analysis, the qRT-PCR validation was performed analyzing the expression patterns not in pooled samples but in each different flower developmental stage and in the stem separately (see Materials and methods) in order to understand how the gene expression changes during flower development.

The 10 genes were all validated with qRT-PCR experiments, two were upregulated in *7B-1* sample in the RNA-seq analysis, *polygalacturonase-5* (*TAPG-5*) and *gibberellin 2-oxidase* (*GA2-ox*) and they only partially showed similar expression pattern by qPCR. The *TAPG-5* gene did not confirm the previous results, it did not show significant differences with the WT sample in the single stages observed, while *GA2-ox* resulted upregulated in *7B-1* in stage -1, 1 and stem, while in stage 0 was downregulated but not significantly (Fig. 4.4 A and B).

For the other eight genes that were downregulated according to RNA-seq, the most significant differences inside different flower developmental stages were observed for *glutamine synthetase, actin* and *SlGLO2*. *Glutamine synthetase* is significantly downregulated in anthers and stem, while in two flower bud stages was not different (Fig. 4.4 C), while *actin* is characterized by expression pattern completely opposite to those of glutamine synthetase, the two flower bud being strongly downregulated, while anthers and stem are not different (Fig. 4.4 D). *SlGLO2* shows a strong downregulation in the mutant in all three stages that represent the floral organ while in stem there is no difference (Fig. 4.4 E).

The other genes show differences between *7B-1* and WT (Fig. 4.4 F, G and H), except the *cysteine proteinase* (Fig. 4.4 I and J) that is similar in WT and mutant genotypes and consequentially it did not confirme the downregulation described in RNA-seq data. The remaining four genes all showed significant down-regulation in the mutant only in flower buds at Stage 0.

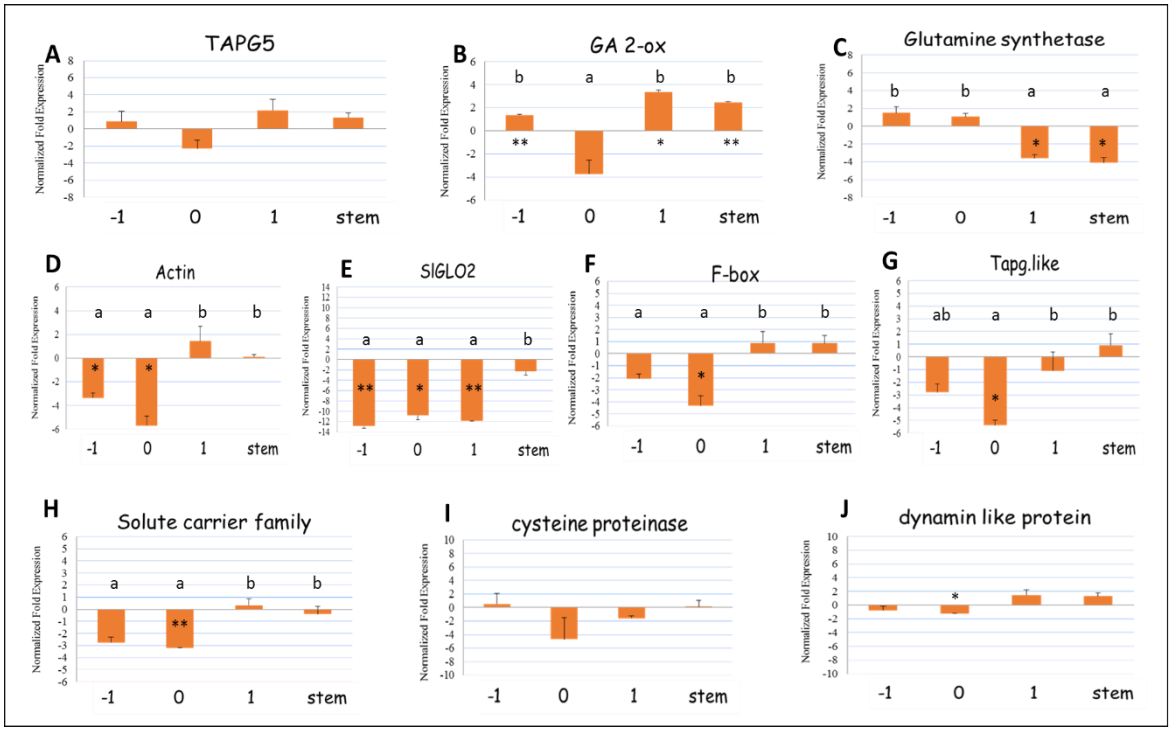

Figure 4.2. qRT-PCR validation of 10 genes resulting differential expressed in the *7B-1* mutant from RNA-seq data. The y-axis indicates the expression calculated as log2 fold change. The x-axis indicates the different stages used for validation, two flower buds (-1 and 0), dissected anthers (1) and stem. The results are averages of two independent biological experiments with three technical replicates. Error bars show the standard error value of two biological replicates. Significant differences between stages were calculated according to ANOVA and Duncan test; those within stage according to Student's *t* test

The *GA2-ox* gene analyzed in this experiment is a member of a genic family that regulates the plant growth by inactivating endogenous bioactive gibberellins (GAs). GAs are important for different processes, particularly are involved in stamen development, including filament elongation and anther dehiscence (Hu *et al*., 2008; Rieu *et al*., 2008). In tomato, were found five members of the *GA2-ox* family, which are expressed in different manner in different parts of the plant (Serrani *et al*., 2007). The gene found upregulated in *7B-1* mutant plants corresponds to *GA2ox-3*, which is expressed in internodes (young and adult), in petals and stamens at anthesis and in root (Serrani *et al*., 2007). The up-regulation of this gene in this mutant is an interesting result because studies conducted on *7B-1* seedlings showed that they are characterized by a decrease in level of endogenous GAs (Fellner and Sawhney, 2001). *7B-1* is a photoperiod-sensitive male sterile mutant and it was hypothesized that it is characterized by a defect in light perception and signaling, which determines an increase in endogenous ABA level and consequentially a decrease in ethylene and GA levels (Fellner *et al*., 2005).

Other interesting result is represented by the discovery that the gene with higher down-regulation in the mutant is *SlGLO2*, the best candidate for the *7B-1* mutation. The validation with qRT-PCR procedures confirmed the results obtained with RNA-seq, in each different flower developmental stages analyzed, the gene being expressed at very low levels. The lack of expression of *SlGLO2* in different phase of flower development is in agreement also with the previous data regarding the missing amplification product on cDNA of *7B-1* samples. These data altogether support further the hypothesis that *SlGLO2*, a class B MADS-box gene mainly involved in stamen development (Geuten and Irish 2010) is the candidate for *7B-1* mutation. This mutant is characterized by alteration in anther structure that resembles the phenotype of knock-out mutants for *SlGLO2*, where the expression is completely abolished, indicating that the lack of expression detected in early phases of flower development (stage -1 and 0) and in anthers is the main cause of the phenotype that characterizes the *7B-1* genotype grown under LD conditions.

The validation of RNA-seq revealed the downregulation of *SlGLO2*, suggesting a lack of expression of this gene in the *7B-1* mutation, which was demonstrated with experiment of semi-quantitative RT-PCR. The semi-quantitative RT-PCR was carried out for all class B MADS-box genes in order to understand the effective lack of expression of *SlGLO2* and how change the expression of the other three genes (Fig. 4.3). The experiment shows that for *SlGLO2* no transcript was detected in all three development stages analyzed (-1, 0 and 1), while for the other class B MADS-box genes, *SlDEF*, *SlGLO1* and *TM6* good levels of expression were observed, demonstrated that *7B-1* mutation is due to a missing expression of this class B MADS-box gene.

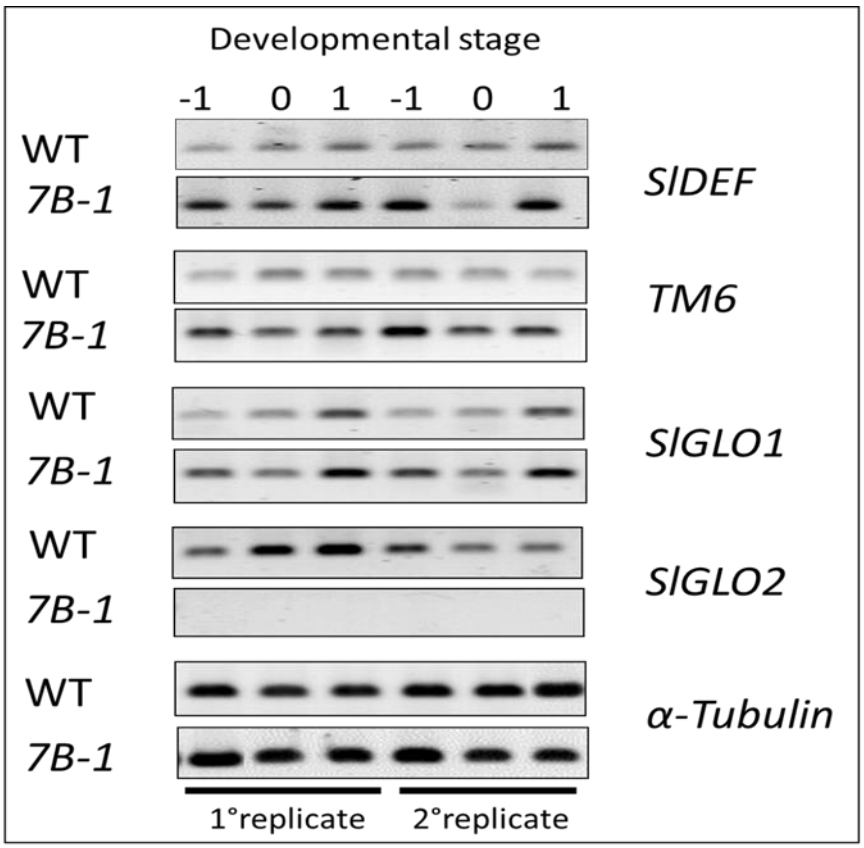

Figure 4.3. Expression patterns of class B MADS-box genes in WT and *7B-1* genotype. Semi-quantitative RT-PCR results are representative of two biological replicates experiments. *α-Tubulin* was used as a housekeeping gene. -1, 0 and 1 stages were used (-1 and 0 two flower bud and 1 dissected anthers). Transcript was detected for *SlDEF*, *TM6* and *SlGLO1* for both genotype and all stages, while for *SlGLO2* only in WT genotype the expression was observed

To confirm the results obtained with semi-quantitative RT-PCR, the expression of other class B MADS-box genes and some genes involved in the regulation network of them was investigated using qRT-PCR methodology in WT and mutant plants grown under LD conditions in the same flower developmental stages used for validation of RNA-seq data. The genes analyzed are listed in Table 4.6. For the class B MADS-box genes, it was found that the *SlGLO1* gene is down-regulated in *7B-1* at Stage 0 whereas in the stem it was up-regulated (Fig. 4.4 A). *TM6* shows differences in expression in different developmental phases analyzed on *7B-1* genotype, it is down-regulated in first flower bud stage (-1), up regulated in anthers, while in the second flower bud stage and stem is similar to the WT (Fig. 4.4 B). For *SlDEF* there are significant differences in expression in floral bud stages (Fig. 4.4 C). Two genes involved downstream to class B MADS-box genes were chosen and both showed significant difference in the *7B-1* genotype respect to the WT counterpart in most developmental stage. *Crabs Claw* (*CRC*) show a high up-regulation in the stage 1, while the transcription factor *bHLH135* was down-regulated in all flower bud stages while it was similar to the WT in anthers and stem (Fig. 4.4 D and E).

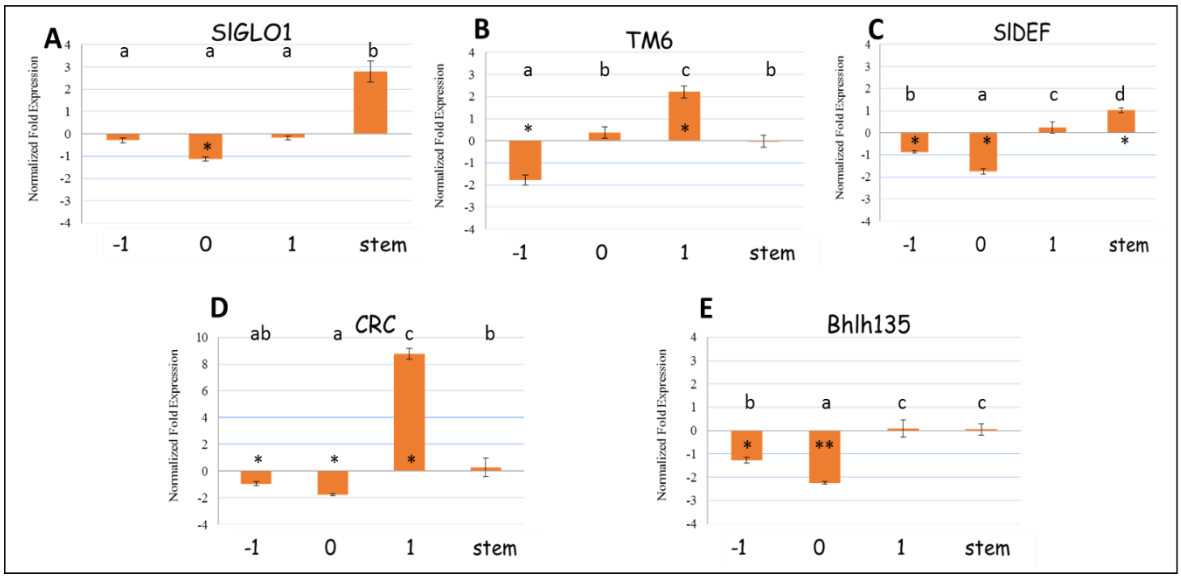

Figure 4.4. Expression pattern of genes member of class B MADS-box family and involved in their regulation network. The y-axis indicates the expression calculated as log2 fold change. The x-axis indicates the different stages used, two flower buds (-1 and 0), dissected anthers (1) and stem. The results are averages of two independent biological experiments and three technical replicates. Error bars show the standard error value of two replicates. Significant differences between stages was calculated according to ANOVA and Duncan test; those within stage according to Student's t test.

The expression pattern observed for *SlGLO1* is in agreement with the results reported by Geuten and Irish (2010) about the expression of class B MADS-box genes in knock out mutants for *SlGLO2* gene. The authors observed that *SlGLO1* expression is reduced in the second floral whorl (petals), similar to those observed in our experiments but in this case, the major down-regulation was in the stage -1 and 0, entire flower buds that included sepals and petals. In anthers, the expression visualized in *7B-1* mutant is different from that reported in knock-out mutants. In *7B-1* anthers, there are not significant differences with the respective WT, while in *SlGLO2* knock-out plants *SlGLO1* was more expressed than in the WT line (Geuten and Irish, 2010). This difference in expression could be due to the complete lack of expression of *SlGLO2* in the lines used by Geuten and Irish (2010), while the tomato male sterile mutant object of this work is defective in the expression of this gene but its transcription is not completely lost. In *SlGLO1* knock-out mutants it was described that *SlGLO2* expression decreases in the second whorl as it happens in knock-out plants for *SlGLO2*, while in the third whorl the expression is similar to the WT, suggesting cross-activation of *SlGLO1* and *SlGLO2* in petals (Geuten and Irish, 2010).

SlDEF gene in *7B-1* is down-regulated in stage -1 and 0, particularly in stage 0, while in the anthers is similar to the WT. Similarly, in knock-out line of *SlGLO2*, *SlDEF* expression decreases in petals, while in anthers is up-regulated respect to the WT line (Geuten and Irsih, 2010). In plants where *SlDEF* expression is completely lost, the *SlGLO2* gene, together with *SlGLO1*, is not expressed in petals and anthers and this suggests that *SlDEF* plays a main role in establish floral organ identity, particularly for petals and stamens (Geuten and Irsih, 2010) and this could explain the decrease of this gene in the *7B-1* mutation.

Interesting is the expression pattern observed for *CRC*. *CRC* is a transcription factor involved in the control of carpel development (Bowman and Smyth, 1999). In *A. thaliana*, ChIP-Seq and transcriptomic analysis showed that *CRC* is activated by *AG* and directly suppressed by *AP3* and *PI* (Wuest *et al*., 2012; O'Maoileidigh *et al*., 2013b). These data are supported by the findings that in B functions mutants, this gene is precociously expressed in anthers (Bowman and Smyth, 1999). In *ap3* and *pi Arabidopsis* mutants where stamens are converted into carpels, the ectopic expression of *CRC* is early evident in the modified third whorl, whereas in WT individuals it is repressed in this whorl by these two genes (Bowman and Smith, 1999). These results are in agreement with those observed in *7B-1*, where the mutant show in anthers an high up-regolation of *CRC*. How observed in *A. thaliana*, an high expression of *CRC* in anthers is accomplished with lack of expression of one of two class B MADS-box genes and here we described a similar expression pattern observed in a tomato male sterile mutant, which is defective in anther structure and putatively in B function. This finding supported, together with the data relative to the downregulation of *SlGLO2* and the trend of the other B MADS-box genes, that *7B-1* mutation could be actually due to a lack of expression of *SlGLO2* gene.

The other gene found significant different is the transcription factor bHLH135 that is homologue to the *BANQUO3 (BNQ3*) gene of *A. thaliana*. *BNQ3* together with *BNQ1* and *BNQ2* encodes for proteins that are members of the basic helix-loop-helix (bHLH) family of transcription regulators (Mara *et al*., 2010) and that are negatively regulated by *AP3* and *PI*. These genes are involved in light signaling and in developmental transition. In situ hybridization studies showed that *BNQ3* is expressed in early developmental stages in floral organ primordia, particularly in sepals, but in late stages its expression was detected in anthers and carpels (Mara *et al*., 2010). In *ap3* and *pi* mutants, *BNQ3* is expressed until stage 12 of flower development in *Arabidopsis* in sepals, after this stage it is also expressed in petals that are converted into sepals, suggesting that AP3 and PI repressed its expression in the second whorl (Mara *et al*., 2010). The qRT-PCR results of *bHLH135* demonstrated that this gene in *7B-1* is downregulated in stages -1 and 0, so in entire flower bud and it is similar with those observed in *Arabidopsis*. *7B-1* mutation affected only the anthers morphology, not petals and also the putative candidate gene, *SlGLO2* was observed to be involved mainly in the stamen identity and consequentially this is not congruent with the data reported in literature but it could be explained with the possibility that *bHLH135* in tomato could be expressed in different floral whorl respect to *A. thaliana*.

Complementation of the 7B-1 *mutant with the* SlGLO2 *WT allele*

In order to confirm that the class B MADS-box gene *SlGLO2* is responsible for *7B-1* tomato male sterile mutation it was attempted a complementation experiment using the WT allele of this gene to transform the mutant genotype. The *pBI121-SlGLO2* construct obtained for complementation approaches is presented in Fig. 4.5. So far, it was possible to carry out only one preliminary

transformation experiment and this was due to delays associated to the obtainment of seed from the *7B-1* genotype.

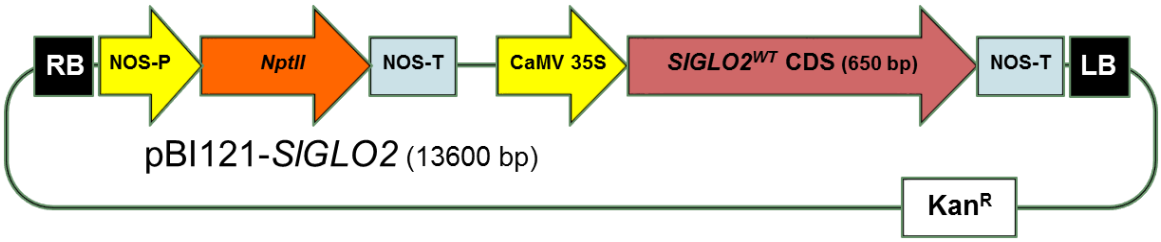

Figure 4.5. Schematic representation of the T-DNA region of the complementation construct *pBI121-SlGLO2* (13600 bp). RB and LB represent right and left T-DNA borders, respectively; CaMV 35S and NOS-T, represent respectively promoter and terminator driving the *SlGLO2* WT CDS (650 bp); NOS-P and NOS-T, represent respectively the promoter and terminator driving the nopaline phosphotrasferase II (nptII) gene conferring resistance to Kanamycin.

How explained previously, *7B-1* is a genotype that in LD is completely male sterile and to obtain seed is necessary to proceed in SD and to self it with hand pollination. The complementation experiment requires a consistent amount of seed, so was necessary to hand pollinate in different times and in different years in such a way to produce the good quantity of *7B-1* seed. Then, it was necessary to proceed with a trial to understand how the *7B-1* genotype and the respective WT reacted to in vitro culture, because these are not standard genotypes used in tomato transformation. The trial was carried out for WT and the mutant genotype using the same protocol required for the transformation without antibodies, but only with hormones. Several small plants were obtained for WT and *7B-1* genotype by regenerating from cotyledon explants in two months (Fig. 4.6). This trial gave positive information about the regeneration capacity of these two genotypes.

Overall, we tried to genetically transform about 150 cotyledons from WT seedlings and 250 from *7B-1* with the pBI121-*SlGLO2* construct and 100 for WT and 150 for *7B-1* using pBI121 with GUS gene as control. This experiment failed to give any putative transformant. After two months, explants from positive control plates formed shoots, contrarily to cotyledons from negative controls and co-cultivated plates. The cotyledons from WT and *7B-1* co-cultivated with *Agrobacterium* containing both constructs after two months lost the green color, the consistence due to a vitrification process. On the one hand, this result suggested that there could be some technical problems related to the transformation phase, but on the other hand, it indicated that the employed transformation medium had the right composition, because explants from positive control were able to develop shoots and successively roots.

In conclusions, to resolve these technical problems, other experiments using pBI121-*SlGLO2* construct have to be perform. The complementation of the mutant, consequentially the possibility to observe a restoration of the WT phenotype in *7B-1* genotype could provide the definite proof that *SlGLO2* is the gene responsible for this male sterility mutation.

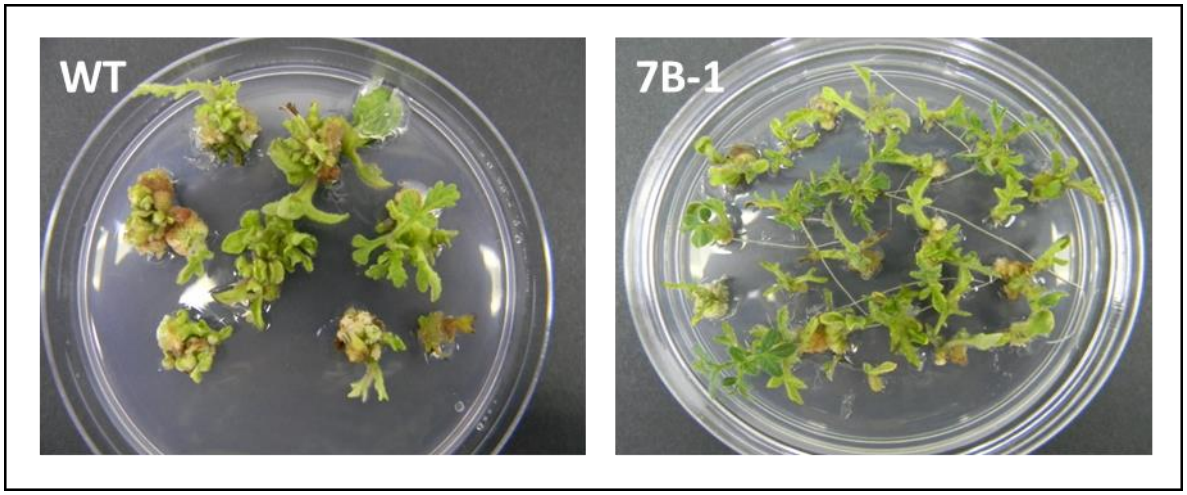

Figure 4.6. Plant regeneration experiment of WT and *7B-1* genotype. Explants of both genotypes were growth in medium culture to asses the regeneration capacity. The regenerated plants were obtained after two months of growth in different culture media. The culture media used for this trial have the same composition of those used for transformation procedures without antibiotics.

In silico promoter analysis

To find putative cis-acting regulatory elements that are connected with the environmental perception and explain how class B MADS-box genes are candidate for environment-sensitive male sterility mutations, we searched the promoter region of these genes using SOFTBERRY. In all promoter regions of the four class B MADS-box genes were analyzed 2000 bp upstream to the ATG.

For *SlGLO1*, *SlDEF* and *TM6* were found more putative regulatory elements than in the promoter region of *SlGLO2.* In *SlDEF* among all regulatory elements observed, noticeable is the presence of an AT-box motif (Terzaghi and Cashmore, 1995), which is a light-responsive element (LREs) and an ASF-1 binding site that can ligate two motifs, one found in many promoter of genes that are involved in auxin and salicylic acid pathways and the second one found in the promoter region of genes involved in abiotic and biotic stress resistance (Benfey and Chua, 1990). In *SlGLO1*, were observed motifs associated with ABA response and G-box LREs elements, while for *TM6* were found elements like the 3AF1 binding site, which is found in several promoters associated with light-response. In *SlGLO2* it was observed an ABA responsive element (ABRE), which was conserved in ABA-responsive (Guiltinan *et al*., 1990). Multiples ABRE elements were found in the 5' flanking region of the Em gene in wheat (Marcotte *et al*., 1989). ABRE is a major *cis*-acting regulatory element that plays an important role in adapting vegetative tissue to abiotic stress such as drought and high salinity, as well as in seed maturation and dormancy (Shinozaki *et al*., 2003). In *SlGLO2* promoter motifs associated with light perception were not discovered but it is known that *7B-1* is characterized by resistance to abiotic stresses due to an accumulation of endogenous ABA, which is more evident under light. Fellner *et al*. (2005) proposed a model to explain the effects of the *7B-1* mutation on light and hormone signaling during hypocotyl and shoot growth (Fig. 4.7). This model hypothesized that the mutant has a defect in light perception and signaling that determines an increase

in endogenous level of ABA and consequentially a reduction in ethylene and endogenous GAs. The reduction of ethylene is a direct consequence of accumulation of ABA and also plays an important role in the light signaling. *7B-1* was described as a photomorphogenic mutant that is characterized by reduction in light perception, particularly under blue light, which leads effects in hypocotyl growth and seed germination (Fellner and Sawhney, 2001; 2002). The light influenced the conversion of 1-amminocyclopropane-1- carboxylic acid (ACC) to ethylene (Weckx and van Pouke, 1989) and in tomato is known that a photoperiod typical of the summer season causes an increase in ethylene production (Jensen and Vaierskov, 1998); thus presumably a problem in light signaling and perception could be responsible of a decrease in ethylene production (Fellner *et al*., 2005).

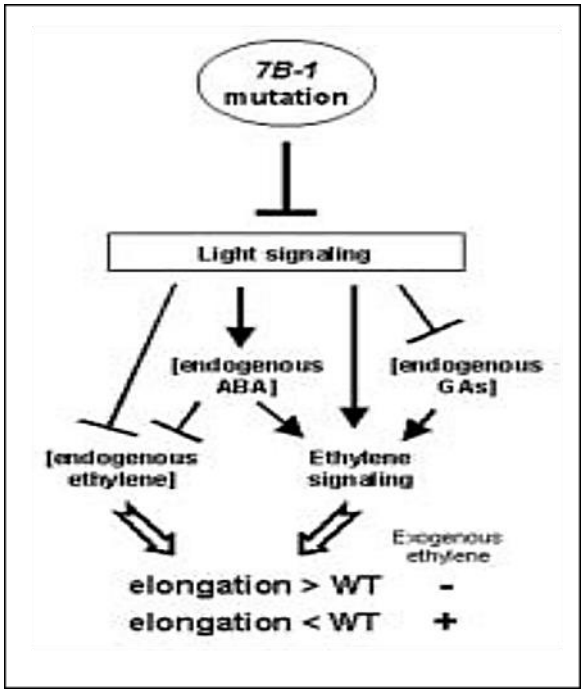

Figure 4.7. A working model suggesting to explain how the defects in light signaling and perception that characterized *7B-1* mutation could leads an increase in endogenous level of ABA and consequentially a decrease in ethylene and GA levels (figure from Fellner *et al*., 2005).

The presence of regulatory elements in the promoter region of class B MADS-box genes that are connected with environmental perception is not a new information because in literature are present data that suggest the hypothesis that the expression of floral organ identity genes is influenced by environmental factors. Recently, it was described that the transcriptional activity of MADS-box genes is controlled by temperature in many plants and this synchronizes flowering with changing seasons (Hemming and Trevaskis, 2010). In tomato, it is known that flower and fruit development is negatively influenced when the plants are grown under low temperature conditions and the main defects that they show are homeotic transformation of one organ into another (Lozano *et al*., 1998). Under low temperatures, it was observed that the homeotic transformations are more frequently produced in the reproductive whorls, particularly are produced petaloid and carpelloid stamens, staminoid carpels and petals transformed into stamens (Lozano *et al*., 1998). These information

indicated that MADS-box genes, particularly those involved in B function, are candidate for conditional male sterile mutations, where the sterility is influenced by environmental factors. Recently, this hypothesis was confirmed from the discovery that the *sl* and *TAP3* mutations, which are characterized by transformation of petals into sepals and stamens into carpels, are due to a mutation in the promoter and coding region of *SlDEF* genes (Gomez *et al*., 1999; Quinet *et al*., 2014).

In conclusion, the data reported in this Chapter about the morphological and molecular characterization of the conditional male sterile tomato*7B-1* mutant indicated how the candidate gene responsible for this mutation is another class B MADS-box, *SlGLO2*, and further supported the influence that temperature and photoperiod have on the expression of this transcription factor family.

Chapter 5

Genetic characterization of *pi-2* and *vms* tomato male sterile mutants

Introduction

Tomato male sterile mutants, particularly those influenced by environmental factors, such as temperature and photoperiod, are characterized by various degrees of petal conversion into sepals and stamens into carpels (Hafen and Stevenson, 1958; Nash *et al*., 1985; Philouze, 1991; Rasmussen and Green, 1993; Gómez *et al*., 1999; Quinet *et al*., 2014). Most of the genes involved in the male sterility mutations have not yet been characterized and identified. The phenotype of these mutants resembles those of homeotic mutants for class B MADS-box genes. In tomato, four class B MADS-box genes are identified, *SlDEF* and *TM6* that are homologues of *AP3* and *SlGLO1* and *SlGLO2* homologous of *PI* (Pnueli *et al*., 1991; Busi *et al*., 2003; de Martino *et al*., 2006; Mazzucato *et al*., 2008; Geuten and Irish, 2010). Recent data reported the characterization and identification of the gene responsible for the *sl* and *TAP3* mutations, which is *SlDEF* (Gomez *et al*., 1999; Quinet *et al*., 2014). The *sl* mutation was studied in detail in the past years and it is characterized by various degrees of alterations in petals morphology and complete transformation of stamens into carpels, while its allele *TAP3* shows the same alterations except for the petals, that are completely converted into sepals (Bishop, 1954; Gomez *et al*., 1999; Quinet *et al*., 2014). The phenotype of *TAP3* is very similar to that of another tomato male sterile mutant, *pi-2*, described by Rick (1993) as a recessive mutation originated in cultivar Castelmart after treatment with EMS. This mutant, that was initially called *green pistillate,* has petals converted into sepals and stamens into carpels. When it is pollinated with pollen from other genotypes, it produces irregular fruits and subnormal seed production. These characteristics are not ideal to use this male sterility system in tomato hybrid seed production (Rick, 1993). The *pi-2* phenotype was successively studied in details using SEM and this ultrastructural analysis showed that the petals are completely absent in the mutant but are replaced by green pistillate petals that have the same density and shape of true sepals, remain green for all development process and attached to carpels when the fruits are produced (Rasmussen and Green, 1993). The SEM analysis revealed that the number, form, placement and timing of early primordia observed in *pi-2* do not differ from those in WT flowers. These observations suggested that the processes involved in determination of organ identity are separated from those involved in organ initiation mechanisms (Rasmussen and Green, 1993). Contrarily, in homeotic mutants of *A. majus* and *A. thaliana* it was observed a difference with the respective WT in patterning and timing of organ initiation (Hill and Lord, 1989; Bowman *et al*., 1991; Coen, 1991).

The phenotypic similarity between *TAP3* and *pi-2* suggested the hypothesis that *SlDEF* could also be involved in the *pi-2* mutation.

Another environment-sensitive tomato male sterile mutant, *vms*, sensitive to high temperature was described in the past, as a spontaneous mutation found in cultivar San Marzano. *vms* is due to a single

recessive allele, which was mapped in the long arm of chr. 8 between the *bu* and *dl* markers (Rick and Boynton, 1967; Tanksley *et al*., 1992). This mutant was characterized at morphological level under low and high temperature conditions in field and in greenhouse in California. In the period from June to October under field conditions the male sterile phenotype was visible, particularly the reduction and malformation in anther structure and a defective corolla were observed. This strong expressivity of the phenotype is not observed in early summer season and when *vms* plants are grown in the winter period in greenhouse conditions, where the only change in the flower structure is represented by a reduction in width of sepals and petals. Therefore, to observe the male sterility it is required a minimal temperature of 30°C in field and 32°C in greenhouse (Rick and Boynton, 1967). The same authors hypothesized that the reaction of *vms* high temperatures is associated with the heat-lability of an altered enzyme encoded by the mutant, while in the WT genotype the enzyme involved remains stable at the same environmental conditions.

The phenotype of *vms*, contrarily to *pi-2*, affects mainly the stamen morphology and resembles the phenotypes of knock-out mutants for class B MADS-box genes, *SlGLO1* and *SlGLO2* (Geuten and Irish, 2010). *vms*, like *7B-1* presents defects in the anther morphology, but an allelism test demonstated that both mutations are not allelic, excluding the involvement of *SlGLO2* gene. To identify the gene underlying the *vms* mutation, starting from the position of *vms* locus on chr. 8, it was hypothesize that the best candidate for it was *SlGLO1*.

In this Chapter, are described our achievements in the morphological and molecular characterization of *pi-2* and *vms* in order to define the genes underlying these mutations.

Materials and methods

Plant material and morphological characterization of the pi-2 *mutant*

For the *pi-2* mutation, it was used a segregating population obtained from the TGRC, with accession number LA3-802. A number of WT plants from the original stock were crossed in the previous years with pollen from plants of a male fertile line (Olimpieri and Mazzucato, 2008). F_1 hybrids were all normal confirming the recessivity of the *pi-2* phenotype. F_2 seed was obtained and one segregating progeny was used in order to morphologically characterize the phenotype of this male sterility mutation. Sixteen plants were grown in greenhouse during the spring-summer season in the experimental station of Tuscia University (Viterbo, Italy). The *pi-2* mutant showed as expected petals converted into sepals and stamens into carpels and this made it easy to discriminate the recessive homozygous mutant from the WT or heterozygous plants. The morphological characterization was done at flowering time, sampling two flowers per plant at anthesis (Stage 4; flowering staging according to Mazzucato *et al*., 1998). For each flower, it was counted the number and measured the length and width of sepals and petals. The anther length and width were also measured. Anthers were counted and classified according to their structure into normal, carpelloid and carpelloid with external ovules. This observation was done only for the WT and heterozygous flowers, not for the

recessive mutants because they showed all anthers transformed into carpelloid-styliform structures completely adnate to the pistil. Seed was recovered from four WTs, two heterozygotes and one mutant homozygous mutant plants to obtain the F_3 generation for progeny test. Fruits were weighted and the number of seed was recorded. Twelve F_3 plants for each progeny were grown in summer season in field or in greenhouse and classified according to flower morphology to confirm the classification made in F_2.

Allelism test between TAP3 *and* pi-2

To investigate if the *TAP3* and *pi-2*mutants are allelic, it was set up a genetic test. Five plants heterozygous for the *TAP3* mutation were crossed in various combinations with plants heterozygous for *pi-2*. The heterozygous status of all parent plants was checked a posteriori by progeny test. Fruits obtained from these crosses were weighted and number of seed was recorded. In winter season, in greenhouse conditions, 12 plants for each cross were grown until the flowering time and the phenotype was observed.

Molecular characterization of the pi-2 *mutation*

Starting from the results obtained with the allelism test, it was decided to sequence the entire genomic sequence of *SlDEF* gene in the *pi-2* genotype in order to confirm the hypothesis that this mutation is due to the *SlDEF*. One WT (homozygous) and one *pi-2* individuals were sampled from the F_2 segregating population to extract total DNA according to the Doyle and Doyle protocol (1990). *SlDEF* was amplified by PCR using primers and amplification conditions listed in Table 5.1. To obtain the entire sequence of the gene (about 3200 bp) four primer combinations were designed according to the genic sequence available on the website Solanum Genomic Network (http://www.sgn.cornell.edu). The primers were manually designed to cover the whole sequence and controlled with the IDT DNA Technology online software to check for the presence of harpins and the possibility that they formed heterodimers or homodimers. PCR amplification was performed in 20 µL of total volume, containing 50 ng of genomic DNA template, 10 µM for each primer, 200 µM of dNTPs, 1 X Dream *Taq* Buffer and 1 U of Dream *Taq* polymerase (Fermentas). The PCR reaction was conducted with an initial denaturation of 4 min at 94°C that was followed by 36 cycles of denaturation for 1 min at 94°C, annealing for 1 min at the temperature reported in Table 5.1 (Ta), extension for 1 min at 72°C, plus 7 min of final extension. The amplification product was visualized by 1.5% (w/v) agarose gels stained with ethidium bromide and successively prepared and purified for sequencing.

Table 5.1. List of the primer combinations and PCR conditions used to amplify the genomic sequence of the *SlDEF* gene in WT and *pi-2* genotypes.

Primers combinations	**Primer sequence (5'-3')**	**Ta (°C)**
SlDEF 3+6	F: ATGGCTCGTGGTAAGATCCAGA R: CTAACCGTAAAGGGCATGTTG	57
SlDEF 7+8	F: CACGTTGAATTGTATGTGCGTCTC R: GTGAGCTTGTTCACATCATCATTCTC	60
SlDEF 9+10	F: GCCTTCCATAAAGTTGGTCA R: GCATTTTGAATAGTCCGAGCA	54
SlDEF 11+12	F: GTTTCCTTCCCTCTTTCTCTG R: CTAGTGAACAAACATTGCATGTG	56

The *SlDEF* genomic sequence in the *pi-2* mutation resulted in the generation of a cleaved amplified polymorphic sequence (CAPS) marker that was developed using the PCR conditions and primers *SlDEF 3+6* as described in Table 5.1. The CAPS polymorphism was obtained after digesting 10 µL of amplification product with 2 U of *NlaIV* (New England Biolabs Beverly, MA, USA) in a final volume of 20 µL. The restriction fragments were visualized in a 1.5% (w/v) agarose gel. The size of fragments expected was 520 bp for WT (no cutting), 280 + 240 bp for *pi-2* and 520 + 280 + 240 bp for heterozygous genotypes.

Plant material and morphological characterization of the vms *mutant*

A line homozygous for the *vms* mutation in the background of cultivar San Marzano, which was obtained from TGRC (accession number LA2-219) and an appropriate WT were used. In order to characterize the mutant in high and normal temperature conditions, *vms* was grown in different seasons together with its near-isogenic WT. Twelve plants for *vms* and its WT counterpart were grown in summer field and in autumn greenhouse conditions in the experimental farm of Tuscia University (Viterbo, Italy). The flower morphology was observed at the Stage 4, sampling two flowers per plant and using the stereomicroscope. For each flower it was counted the number of sepals and petals, and was measured their length and width. The ovary and the pistil length and width were also measured. The anthers were counted and classified according to their structure into normal, likely-normal, carpelloid, carpelloid with external ovules and carpelloid filiform adnate to the pistil. Seed was recovered from fruits of four *vms* plants, which were harvested after open pollination; fruits were weighted and the number of seed was recorded.

Morphological and molecular screening of vms *mapping population*

Starting from literature data that indicated that *vms* maps on long arm of chr. 8, between the markers *bu* and *dl* (Rick and Boynton, 1967), a mapping population was developed in the previous years after crossing the *vms* male sterile mutant with pollen of the introgression line IL8-2. Fifty-four F_2 plants were grown in summer field conditions (experimental station of Tuscia University, Viterbo), the season when the mutation has high expressivity, in order to classify them according to the anther

cone morphology. For each plant, two flowers were sampled at the anthesis (Stage 4) and the anther morphology was observed using the stereomicroscope. The observation of the phenotype was performed in different time intervals in order to better classify the phenotype. For each individual observation, the anthers of WT and *vms* flowers were classified in normal, carpelloid, carpelloid with external ovules and carpelloid filiform adnate to the pistil.

The mapping population was also characterized at molecular level using a marker located into the class B MADS-box gene *SlGLO1*. Total DNA was extracted from each plant according to the Doyle and Doyle protocol (1990), amplified by PCR reaction using the primer combination *SlGLO1 1* as forward (5' AGA ACT CAG GCA CCT AAA GGG T 3') and *SlGLO1 2* as reverse (5' GTT GTC TCT GTC ATT AAT TTG CCC 3'). PCR amplification was performed in 20 µL of total volume, containing 50 ng of genomic DNA template, 10 µM for each primer, 200 µM of dNTPs, 1 X Dream Taq Buffer and 1 U of Dream Taq polymerase (Fermentas). The PCR reaction was conducted with an initial denaturation of 4 min at 94°C that was followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 57°C and extension for 2 min at 72°C plus 7 min of final extension. Differences on size of the amplicons were visualized by 1.5% (w/v) agarose gels stained with ethidium bromide. The size of PCR products expected were 1400 bp as in *S. lycopersicum* for the mutant individuals, 1330 bp as in *S. pennellii* for the homozygous WT and both amplicons for the heterozygotes.

The mapping population was grown until fruit maturation in order to harvest seed from all plants able to produce fruits. The seed was used to set up a progeny test for 11 plants that produced doubtful results to confirm the phenotypic classification. The progeny test was carried out growing 12 plants for accession in summer field conditions in order to score them for the phenotype and for the molecular markers. The phenotypic screening was conducted through the classification of the plants according to the anther morphology, always sampling the flowers at different times. The molecular analysis was conducted using the same marker and same PCR conditions described above.

The coding sequence of the *SlGLO1* gene (4618 bp) of WT and *vms* was amplified using four different primer combinations (Table 5.2). The primers were manually designed to cover the whole sequence and controlled as described above. The primers were designed on the genomic sequence at the end of intronic regions in order to amplify whole exons.

Table 5.2. List of the primer combinations used to amplify the genomic sequence of the *SlGLO1* gene in WT and *vms* genotypes.

Marker name	**Primer sequence (5'-3')**	
SlGLO1 7+8	F: GAAAATGGGAAGAGGAAAGATAG	R: TACCTCATGCTTAGCATCCC
SlGLO1 9+10	F: GGATTGTAAACAGAACTTGGAC	R:CTGTTTTTCACGGATACCAGTGAGTCC
SlGLO1 11+12	F: TTTGACCTACTGTGGTGTTTGG	R: TCTAACGACGTGCCTATGGA
SlGLO1 13+14	F: GACAGAGACAACTAGAGATAGC	R: CGAGGCCATTATCCACCG

Results and discussion

Morphological characterization of the pi-2 *mutation*

The *pi-2* male sterile mutant is characterized by complete conversion of petals into sepals and stamens into carpels, a phenotype that allows an easy identification of homozygous recessive mutants in segregating populations. The molecular characterization was conducted on a F_2 progeny derived from crossing *pi-2* with the IL 3-5 line that was performed in the past in order to understand if this mutation was allelic to *pi* mutant (Olimpieri and Mazzucato, 2008). The allelism test and mapping data demonstrated that the two mutations were not allelic (Olimpieri and Mazzucato, 2008). A progeny from a heterozygous plant was used to characterize at morphological level the *pi-2* mutation and also for successive molecular studies.

Sixteen plants grown in summer season were classified as eight WT, three heterozygotes and five homozygous recessive mutants. Flowers from the WT have a normal morphology with green sepals, yellow petals, and yellow anthers laterally fused to form a staminal cone that encloses the gynoecium. The homozygous mutant flower is characterized by a particular phenotype, the first and second whorls are sepals while the third and fourth are carpels, that resemble the phenotype of homeotic mutants for class B MADS-box genes, particularly those showed from *TAP3* mutant described in the Chapter 3 (Fig. 5.1 A and B). Flowers of heterozygous plants seldom have the floral morphology likely the WT, except that for the anthers, which show some alteration in morphology or production of ectopic organs like carpels or external ovules (Table 5.3).

Table 5.3. Morphometric data of sepals, petals and anthers of tomato flowers in WT, heterozygotes and mutant plants from the F_2 population segregating the *pi-2* mutation. Each value represents the average ± SE of 12 flowers per genotype.

Floral organ	**Trait**	**WT**	**heterozygotes**	***pi-2***
Sepals	No.	6.2±0.3	6.3±0.2	6.0±0.0
	Length	8.2±0.7	8.3±1.0	7.5±0.5
	Width	1.0±0.0	1.0±0.0	1.0±0.0
Petals	No.	6.2±0.3	5.8±0.3	5.0±0.0
	Length	10.4±0.5	10.7±0.4	3.5±0.5
	Width	3.2±0.4	2.9±0.1	1.0±0.0
Anther morphology (%)	No.	6.1±0.28	6.0±0.44	*
	Normal	100.0	83.3	
	Likely-normal		4.8	
	Carpelloid			
	Carpelloid with external ovules		11.9	
	Carpelloid styliform			
	Carpelloid styliform adnate to the pistil			100.0

*The total number of anthers for the *pi-2* flowers is not detectable; all anthers are fused to the pistil

The morphometric data reported in Table 5.3 show that there are not important differences between flowers from the different individuals, except for the length and width of petals in the *pi-2* genotype. The size of petals in WT and heterozygotes individuals is similar, while in homozygous mutants petals are converted completely in sepals and they are characterized by a reduction in size. This reduction is evident when comparing the length and width of the sepaloid petals in the second whorl between those of WT, heterozygous and mutants but it also observed comparing the measurement data reported for transformed petals with the true sepals in the first whorl of the *pi-2* flowers (Fig. 5.1 B).

The *pi-2* phenotype, as reported early, is similar to that of another male sterile mutant, *TAP3*, which was described recently in detail by Quinet *et al*., (2014) and also characterized in this work in Italian growth conditions. *pi-2* shows complete conversion of petals into sepals and stamens into carpels, but flowers are smaller than in the *TAP3* recessive mutant and sepals have a dark green color (Fig. 5.1 A and B). Both mutant phenotypes are characterized by reduction in size of sepaloid petals that are smaller than the respective true sepals in the first floral whorl. This different organ elongation was observed and described in the past by studying the floral organ architecture in *pi-2* (Rasmussen and Green, 1993). The sepaloid petals and carpelloid stamens in the mutant flowers of *pi-2* were measured and the data obtained demonstrate that their elongation occurred with the same rates of the corresponding organs in the WT genotype, suggesting that some aspects of growth are independent from the mechanism that governs floral organ identity (Rasmussen and Green, 1993).

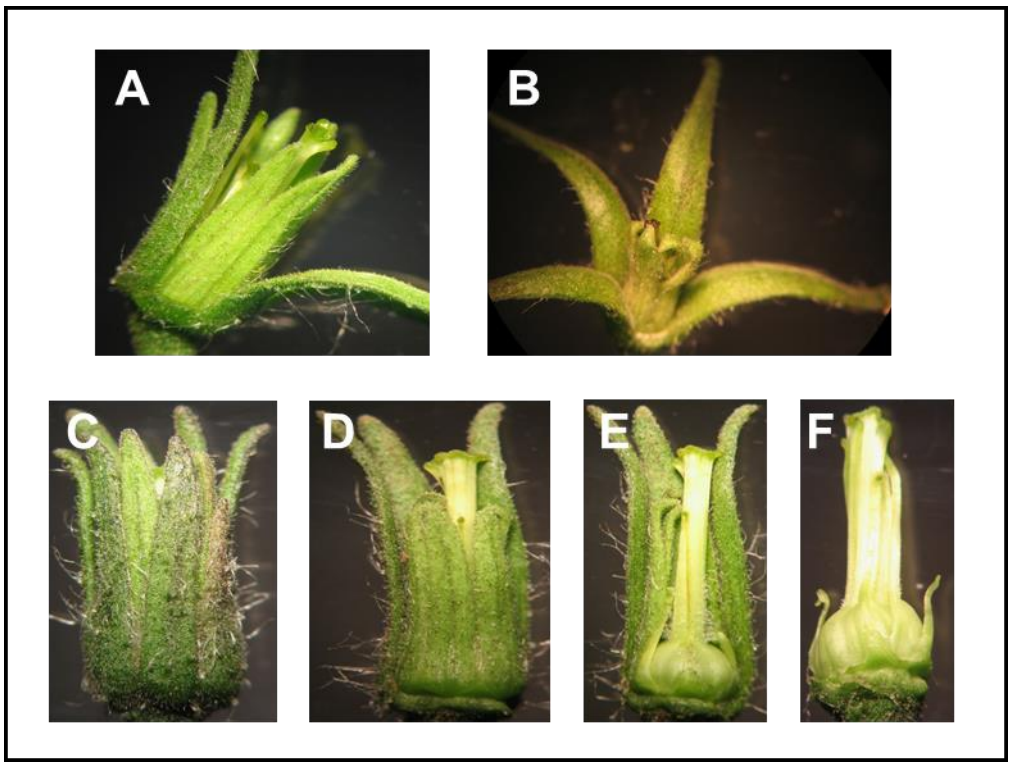

Fig. 5.1. Floral phenotype of *TAP3* (A) and *pi-2* (B) recessive homozygous mutant. Both mutants show a similar phenotype with conversion of petal into sepals and stamens into carpels and difference in color intensity. C, D, E and F represent the heteroallelic mutants phenotype originated from crossing heterozygous *TAP3* with pollen from heterozygous *pi-2* plants that indicated allelism between them. C and D show sepals in the first and second whorl, while in E and F there are details of anthers converted into carpels completely adnate to the pistil.

Allelism test between TAP3 *and* pi-2

Given the phenotypic similarity between *pi-2* and *TAP3* mutants, an allelism test was performed by crossing heterozygous *TAP3* and *pi-2* plants. From these crossed were originated five populations that were phenotypically screened; lack of segregation of the mutant phenotype would have indicated independence, whether segregation of the mutant phenotype would indicate allelism. In all the progenies derived from two heterozygous parents it was seen the mutant phenotype, thus indicating that *pi-2* is allelic to the *TAP3* mutation. From one heteroallelic mutant plant, it was obtained a single parthenocarpic fruit with small size and irregular shape. The phenotype of heteroallelic mutants showed little differences with the parents (Fig. 5.1 from C to F). Sepals in the first whorl have a length intermediate between *TAP3* and *pi-2*, petals converted into sepals are shorter than in *TAP3* but longer than in *pi-2*, both have a green color more intense than the parents (data not shown).

Recently, allelism test and molecular analysis demonstrated that *TAP3* is an allele of *sl* and both have a lesion in the *SlDEF* gene (Quinet *et al.*, 2014). Results reported here, demonstated allelism between *TAP3* and *pi-2* and indicate that *SlDEF* is involved also in this mutation.

Molecular identification of the pi-2 *mutation*

The genomic sequence of *SlDEF* gene (3200 bp) is constituted of seven exons and six introns. The primer combinations used were designed to amplify the sequence of all exons and compare it in WT and homozygous mutant individuals. The entire coding sequence, from the start to the stop codon, was obtained, except for the third and fourth exons that were not sequenced in both genotypes due to problems occur during PCR amplification procedures. In the genomic sequence of the *pi-2* sample was found one point mutation, a nucleotidic substitution (A to G) inside the splicing site at 3' of the first intron (Fig. S3). For confirming the co-segregation of the *pi-2* phenotype and this mutation, a CAPS marker based on *NlaIV* restriction was used. The restriction experiment confirmed at molecular level the phenotypic classification carried out on segregating population and also heterozygous individuals were discriminated (Fig. 5.2). The CAPS marker confirmed a complete co-segregation with the *pi-2* phenotype and plant classified as heterozygotes by the CAPS were validated by progeny test.

The determination of a mutation in *SlDEF* sequence in *pi-2* genotype confirms the hypothesis that this class B MADS-box gene is involved in this mutation. The nucleotidic substitution found at 3' of the first intron located into the splicing site was validated using a restriction enzyme but would be useful to demonstrate its effects through the sequencing of cDNA of WT and mutant genotypes. The mutation could likely cause lack of removal of the first intron and its inclusion in the coding sequence. Literature data reported that in Purple Flowering Stalk (*Brassica campestris* L. ssp. *chinensis* L. var. *purpurea* Bailey) a mutation in the splicing site of a MADS box genes responsible for the variation in flowering time. This nucleotidic mutation is located on the 5' extremity of the sixth intron of the *BrpFLC1* gene, which is homologous to the MADS-box *FLOWERING LOCUS C* (*FLC*) gene from *Arabidopsis* (Hu *et al*., 2010).

These results gave new information about the morphological and molecular characterization of tomato male sterile mutation, *pi-2*. The morphological characterization, together the allelism test confirmed that *TAP3* and *pi-2* are allelic, as was hypothesized initially with only the observation of the mutant phenotype that characterized both male sterility mutations. On the other hand, the first molecular analysis gave a new result, demonstrating that the gene involved in *pi-2* mutation is *SlDEF*. Successive molecular experiments will be necessary to confirm the involvement of this class B MADS-box gene in *pi-2* mutation and the actual causative genetic lesion.

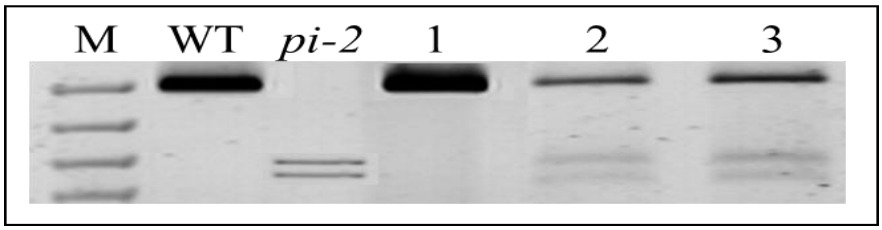

Figure 5.2. Restriction results with *NlaIV* enzyme on amplification products obtained with *SlDEF 3+6* primer combination. WT is the respective normal genotype for *pi-2* mutation. The numbers from 1 to 3 represent some samples from the F_2 segregating population. The WT and the sample number 1 present the restriction product not digested, while *pi-2* show digesting product. Sample 2 and 3 are heterozygous samples and show the codominant pattern.

Morphological characterization of the vms *mutation*

The *vms* mutant is sensitive to high temperature and under these conditions are observed defects in flower morphology, particularly alterations in the staminal cone. Small anthers, with distorted shape, separate each other, laterally free and with the formation of ectopic organs sometimes was reported (Fig. 5.3 A and B; Rick and Boynton, 1967). These malformations are stronger when the mutant is grown in field conditions with temperatures of 30°C or higher and they are accomplished with no production of pollen grains (Rick and Boynton, 1967). To observe if the sterile phenotype of this mutation is stable under different temperature conditions and different geographic areas it was performed a phenotypic screening on *vms* genotype and the respective WT counterpart.

Figure 5.3. Flower phenotype of *vms* plants grown in open field in high temperature conditions. A, *vms* flower with weak manifestation of the male sterile phenotype. B, typical *vms* phenotype with discolored, retracted and distorted anthers.

The morphometric data relative to size of each floral organs were taken for both genotypes in 12 plants grown under high temperature conditions. The WT flowers showed the typical flower morphology of tomato at anthesis, while those of *vms* presented the typical sterile phenotype (Fig. 5.3 A and B). The difference observed in flower morphology between the two genotype involved only the anther cone, the length and width of petals and sepals being similar (data not shown). Anthers of the mutant showed different kinds of alterations, being normal (63%), likely-normal (6.3%), carpelloid (6.3%), carpelloid with external ovules on the adaxial surface (20.8%). In low

temperature conditions the mutated flowers, as expected, resembled the WT structure, so all anthers observed were characterized by normal features. Fruits from open pollination of four *vms* plants grown under high temperature conditions were harvested and seed set was recorded. The production observed was on average of five fruits per plants with a mean production of four seeds per fruit.

The morphometric data about the phenotypic classification of the mutant are in agreement with the first morphological characterization reported in literature carried out in the interior valley of California in summer in field and in winter in greenhouse. The phenotypic observation demonstrated that in both conditions the mutant deviates only in the structure of the flower, with alterations of the corolla and the anther cone. The corolla of flowers from plants grown in field and in greenhouse is reduced to a ring of slender organs with a coloration less intense than the WT, while stamens are strongly malformed, small, distorted, separated from each other, and discolored (Rick and Boynton, 1967). These characteristics are more accentuated under high temperature.

The fruit production in *vms* genotype observed in our experiment was also described by Rick and Boynton (1967), where it was reported only for the oldest inflorescences, those that had flowered in early summer (May and June), while was described a good fruit and seed set when *vms* stigmas are pollinated with pollen from other fertile genotypes, demonstrating that the female fertility is not influenced.

Molecular studies on the vms *mutation*

The *vms* locus was previously mapped on chr. 8 between two molecular markers, *bu* and *dl*, which are located on the long arm (Fig. 5.4; Rick and Khush, 1966; Rick and Boynton, 1967). This region corresponds to the position occupied by the class B MADS-box gene *SlGLO1*. Considering that, a mapping population was obtained by crossing a mutant plant with pollen from the IL 8-2 line in order to confirm the possible involvement of *SlGLO1* in the *vms* mutation.

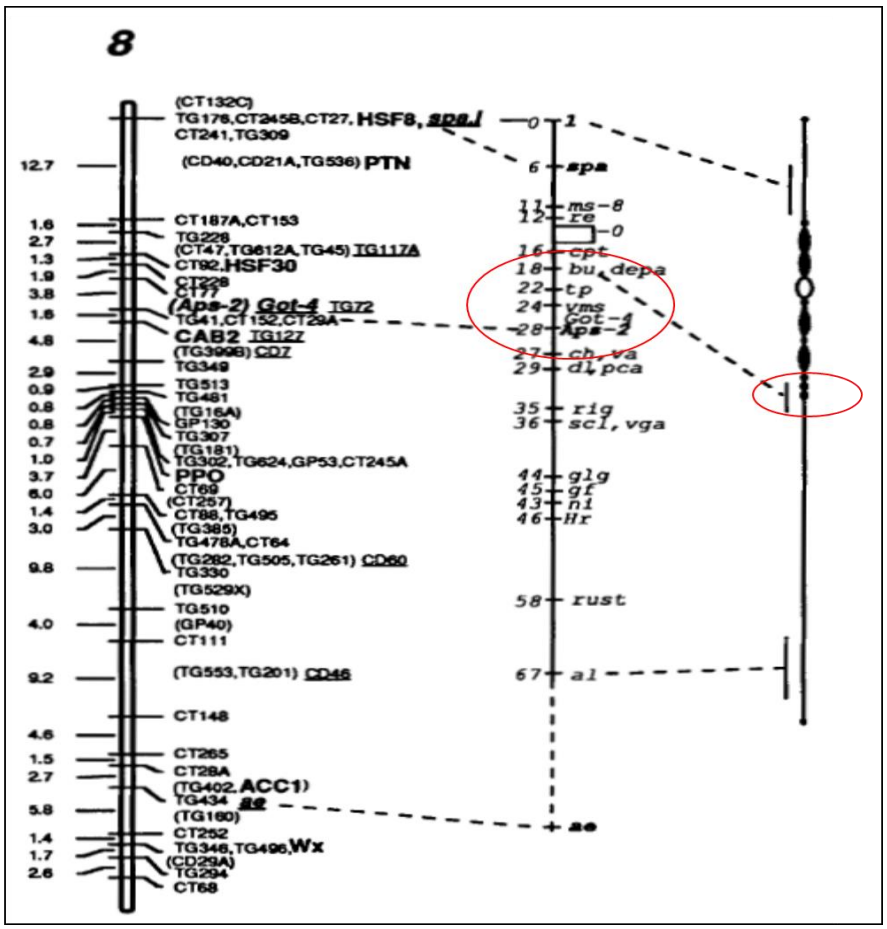

Figure 5.4. Representation of the molecular linkage map of tomato (left), the classical map (center) and the cytological map (right) of chr. 8. The red circle indicates the position of the *Vms* locus on the classical map (Figure from Tanksley *et al.*, 1992).

Fifty-four plants from the mapping population were classified according to the phenotype in mutants and WT at different time intervals in such a way to better distinguish the individuals, since that the mutant phenotype changes easily with the fluctuation in temperature values, particularly during summer season. After five different phenotypic screenings, were identified 37 WT and 17 *vms* plants. The anther morphology of the plants classified as *vms* showed abnormalities as carpelloid structures and external ovules, the same alterations found in the parental genotype. For the WT flowers, most of the anthers have normal features; in a few segregants, flowers showed sometimes alterations similar to the mutants; in order to understand if these individuals were true WT or heterozygotes, a progeny test with their seed was carried out.

The difficult identification of the *vms* phenotype was reported also in the original population described by Rick and Boynton (1967). The authors associated this ambiguous classification of the phenotype in field during summer season with the fluctuations of temperature that are recorded during this period.

The phenotypic similarity with the knock-out mutants for *SlGLO1* and the information about the position of *Vms* locus on chr. 8 addressed our research to hypothesize that the best candidate for this mutation was such gene. Genomic sequence of WT and mutant genotypes was obtained and a single

nucleotidic substitution was found in the *vms* sample. The mutation was a G to A substitution in position 367 of coding sequence resulting in a lysine (K) instead of glutamic acid (E) (Fig. S4). The E to K change in position 123 of the amino acid sequence of SlGLO1 protein was predicted to affect the K box domain and to be not tolerated, supporting the hypothesis that the substitution would affect the functionality of the protein itself.

The sequencing result showed that *SlGLO1* could be involved in the *vms* mutation; to confirm this, all plants from the mapping population were analyzed using a molecular marker located into the gene itself that shows polymorphism between San Marzano and *S. pennellii*. Out of 54 plants analyzed, 45 showed a parental combination, while nine were recombinants between *SlGLO1* and *Vms*. Progeny tests to resolve doubtful cases and to assess heterozygosity at the *Vms* locus showed that at least eight recombinants occurred.

To summarize, the data from the sequencing of *SlGLO1* gene on the *vms* genotype are interesting, because they revealed the presence of a mutation in the coding region, which implies the production of a protein that is predicted to be not tolerated. The E to K substitution involves the K box domain. To understand and explain the effect that this mutation determines in the expression pattern and in protein function it could be useful to proceed with successive expression analyses. Particularly, was observed that the changed amino acid is a conserved residue and probably this influenced the structure of the protein encoded by *SlGLO1* gene. A mutation in the K box domain was found also for the *SlDEF* gene in the *TAP3* genotype, in this case a deletion of the last part of the K box was discovered and it was suggested that it could influence the capacity of the protein to form multimeric MADS complexes (Quinet *et al*., 2014). It is known that the K region is important and is involved in protein-protein interaction (Leseberg *et al*., 2008) and it is likely that changing one conserved amino acid could affect this interaction.

On the other hand, the results obtained from the molecular screening show an independent segregation between the phenotype and *SlGLO1* marker, suggesting that the *SlGLO1* gene may be not the candidate for representing the *vms* mutation. At the same time, these results do not completely invalidate the involvement of *SlGLO1* gene in the mutation, because an independent segregation between phenotype and marker may be apparent due to the incorrect classification of the phenotype. As explained above, the *vms* phenotype is influenced by fluctuation in temperature values, mainly when the plants are grown in field in summer period and this may occasionally render unreliable the phenotyping (Rick and Boynton, 1967).

These variations observed in the phenotype intensity makes difficult to use this system in hybrid seed production. The male sterility is stable in the environmental conditions where the mutation was discovered, but in our growth it does not seems a powerful and useful system to use in tomato hybrid production.

Further experiments are necessary in order to confirm if *GLO1* remains a candidate for *Vms* or it has to be definitely excluded.

Chapter 6

General Discussion

Male sterility is a powerful system in hybrid seed production and it could be useful particularly in crops such as maize, soybean, pepper and tomato, where it eliminates the tedious and laborious hand emasculation of the flowers and reduces the cost associated with seed production (Sawhney, 1997). The expression of male sterility can be influenced by the action of environmental factors such as temperature and photoperiod and it is called environment-sensitive genic male sterility (EGMS). In EGMS, specific ranges of temperature or photoperiod favor the sterility, while other determine the fertility restoration (Smith, 1947). Male sterile mutants present at phenotypic level abnormalities that involve only the flower structure, particularly the anthers morphology but sometimes also defects in the corolla were observed. These phenotypes resemble those of homeotic mutants for the expression of floral organ identity genes, particularly those involved in the identity of petals and stamens, which are members of class B MADS-box transcription factors family (Gomez *et al*., 1999; de Martino *et al*., 2006; Geuten and Irish 2010; Quinet *et al*., 2014). In tomato, recent literature data reported for two male sterility mutations sensitive to temperature, *sl* and *TAP3*, which are allelic, the involvement of the *SlDEF* gene that control the identity of petals and stamens.

The data reported in this thesis described the achievements about the characterization at morphological and molecular level of several tomato male sterile mutants sensitive to temperature and photoperiod. The mutants analyzed are *sl-2*, *TAP3*, *pi-2* and *vms*, where the sterility is influenced by temperature conditions and *7B-1*, where the sterility is expressed under LD conditions. The morphological characterization of these mutants in environmental conditions that favor the sterility expression and in those where the fertility is restored allowed to observe if the stability and the mechanisms that controlled the male sterile phenotype in Italian growth conditions are different from those described in geographic areas where the original mutations were discovered. Particularly, for *7B-1* this characterization gave support about the stability of the mechanisms that regulated the fertility/sterility conditions, confirming the possibility to use this male sterility system in tomato hybrid seed production in Italy.

On the other hand, the molecular characterization permitted to identify for all mutations object of this work the best candidate gene, within the family of MADS-box genes, particularly into the class B clade. The class B MADS-box genes represent the best candidate for this typology of mutations, for the phenotypic similary with plants silenced for this transcription factors and for the influence that temperature have on their expressivity. The identification of the genes underlying the male sterility mutations would be very important in order to pursue marker-assisted selection strategies and the discovery of new alleles by screening of large mutagenized populations.

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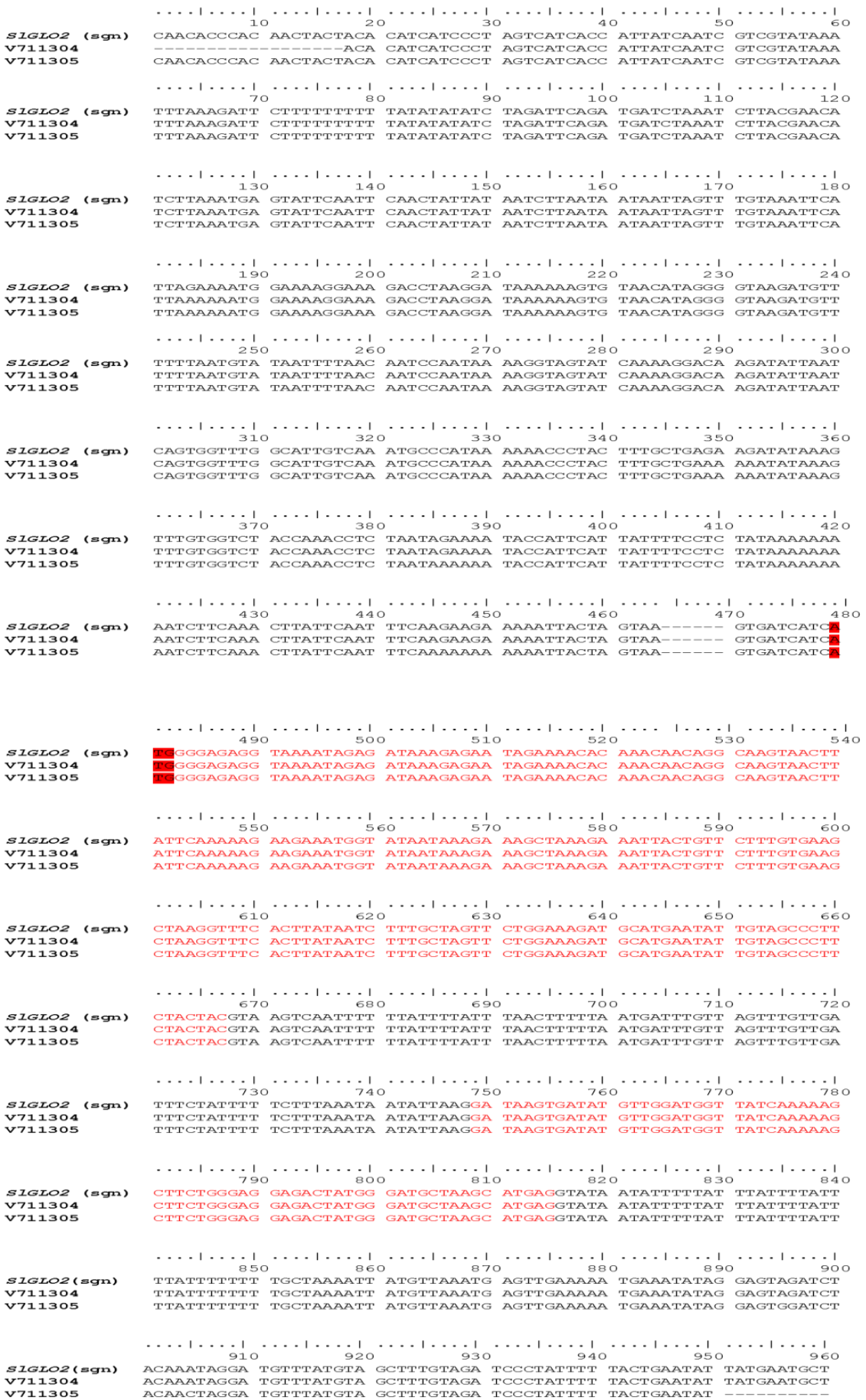
Appendix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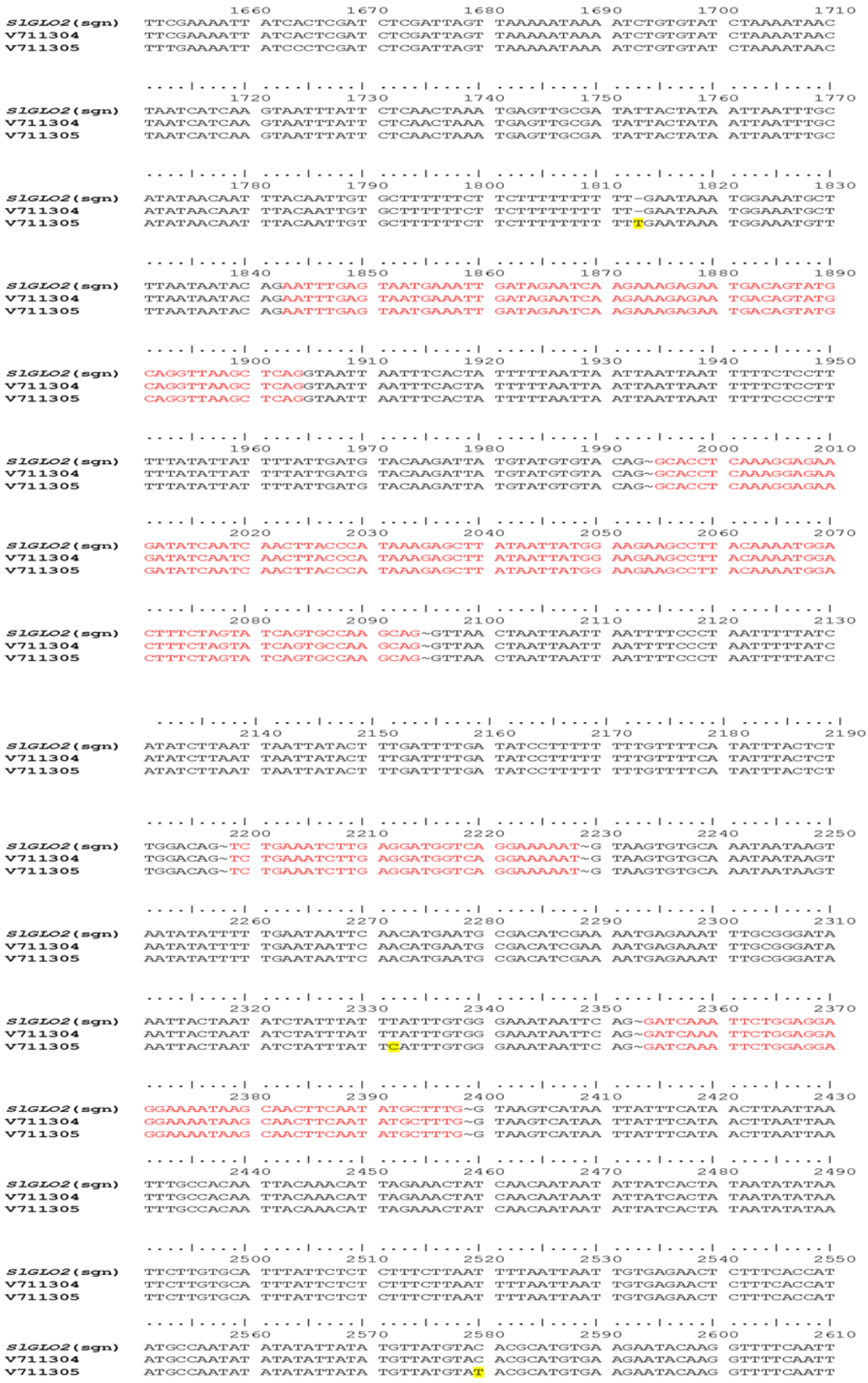
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V711304 GGAAAATAAG CAACTTCAAT ATGCTTTG~G TAAGTCATAA TTATTTCATA ACTTAATTAA
V711305 GGAAAATAAG CAACTTCAAT ATGCTTTG~G TAAGTCATAA TTATTTCATA ACTTAATTAA
2440 2450 2460 2470 2480 2490
S1GLO2(sgn) TTTGCCACAA TTACAAACAT TAGAAACTAT CAACAATAAT ATTATCACTA TAATATATAA
V711304 TTTGCCACAA TTACAAACAT TAGAAACTAT CAACAATAAT ATTATCACTA TAATATATAA
V711305 TTTGCCACAA TTACAAACAT TAGAAACTAT CAACAATAAT ATTATCACTA TAATATATAA
2500 2510 2520 2530 2540 2550
S1GLO2(sgn) TTCTTGTGCA TTTATTCTCT CTTTCTTAAT TTTAATTAAT TGTGAGAACT CTTTCACCAT
V711304 TTCTTGTGCA TTTATTCTCT CTTTCTTAAT TTTAATTAAT TGTGAGAACT CTTTCACCAT
V711305 TTCTTGTGCA TTTATTCTCT CTTTCTTAAT TTTAATTAAT TGTGAGAACT CTTTCACCAT
2560 2570 2580 2590 2600 2610
S1GLO2(sgn) ATGCCAATAT ATATATTATA TGTTATGTAC ACGCATGTGA AGAATACAAG GTTTTCAATT
V711304 ATGCCAATAT ATATATTATA TGTTATGTAC ACGCATGTGA AGAATACAAG GTTTTCAATT
V711305 ATGCCAATAT ATATATTATA TGTTATGTAT ACGCATGTGA AGAATACAAG GTTTTCAATT


2620 2630 2640 2650 2660 2670
S1GLO2(sgn) TTCACACTGC TTATATAGTA AAAACAATTA TACAATGTTT TTATATGTTA CTCGAGAAAA
V711304 TTCACACTGC TTATATAGTA AAAACAATTA TACAATGTTT TTATATGTTA CTCGAGAAAA
V711305 TTCACACTGC TTATATAGTA AAAACAATTA TACAATGTTT TTATATGTTA CTCGAGAAAA
2680 2690 2700 2710 2720 2730
S1GLO2(sgn) AAAAACTAAA TCACCAAACA CCAGTCTATT ATCGAATTTC TAACTACACA CAAATACTTT
V711304 AAAAACTAAA TCACCAAACA CCAGTCTATT ATCGAATTTC TAACTACACA CAAATACTTT
V711305 AAAAACTAAA TCACCAAACA CCAGTCTATT ATCGAATTTC TAACTACACA CAAATACTTT
2740 2750 2760 2770 2780 2790
S1GLO2(sgn) ACGGAAGTTC TATTACCCCT AAACCTTTAA AAGTGGACTA TATATAAACC CCTCAAGTAC
V711304 ACGGAAGTTC TATTACCCCT AAACCTTTAA AAGTGGACTA TATATAAACC CCTCAAGTAC
V711305 ACGGAAGTTC TATTACCCCT AAACCTTTAA AAGTGGACTA TATATAAACC CCTCAAGTAC
2800 2810 2820 2830 2840 2850
S1GLO2(sgn) TAAGTTGTGT GTAGTTAAAA ATTCGAATAT TTTATGTATG TTACTCCCTT TGTCTCATAT
V711304 TAAGTTGTGT GTAGTTAAAA ATTCGAATAT TTTATGTATG TTACTCCCTT TGTCTCATAC
V711305 TAAGTTGTGT GTAGTTAAAA ATTCGAATAT TTTATGTATG TTACTCCCTT TGTCTCATAT
2860 2870 2880 2890 2900 2910
S1GLO2(sgn) TAGTTGTCAA GTCTATTAAA ACTCTAAAAG TACTTGCTCC AGTTTACTTG TTATCTGAAT
V711304 TAGTTGTCAA GTCTATTAAA ACTCTAAAAG TACTTGCTCC AGTTTACTTG TTATCTGAAT
V711305 TAGTTGTCAA GTCTATTAAA ACTCTAAAAG TACTTGCTCC AGTTTACTTG TTATCTGAAT
2920 2930 2940 2950 2960 2970
S1GLO2(sgn) GTACATACAC TATCAGAAAA GTGTGAATTG CTTGGAGGTT TTCTCGGGGA TTAGTATGAA
V711304 GTACATACAC TATCAGAAAA GTGTGAATTG CTTGGAGGTT TTCTCGGGGA TTAGTATGAA
V711305 GTACATACAC TATCAGAAAA GTGTGAATTG CTTGGAGGTT TTCTCGGGGA TTAGTATGAA
2980 2990 3000 3010 3020 3030
S1GLO2(sgn) AATTTACAGG AAAATAAGTT TCATGCGAAT TTTCATACTA ATTTCTACGA AAATCTCC~A
V711304 AATTTACAGG AAAATAAGTT TCATGCGAAT TTTCATACTA ATTTCTACGA AAATCTCC~A
V711305 AATTTACAGG AAAATAAGTT TCATGCGAAT TTTCATACTA ATTTCTACGA AAATCTCCCA
3040 3050 3060 3070 3080 3090
S1GLO2(sgn) TATTATTCAT AGTTTTCTTC GAGTGATAGT GACAAATTAA AAATAGATGT TTGGTTAACT
V711304 TATTATTCAT AGTTTTCTTC GAGTGATAGT GACAAATTAA AAATAGATGT TTGGTTAACT
V711305 TATTATTTAT AGTTTTCTTC GAGTGATAGT GACAAATTAA AAATAGATGT TTGGTTAACT
3100 3110 3120 3130 3140 3150
S1GLO2(sgn) ATTTTGTCTC TTCAAATTGT ATGTTTTTGG ATTCCTAAAG CACCAAAAGG AGATGGGAGC
V711304 ATTTTGTCTC TTCAAATTGT ATGTTTTTGG ATTCCTAAAG CACCAAAAGG AGATGGGAGC
V711305 ATTTTGTCTC TTCAAATTGT ATGTTTTTGG ATTCCTAAAG CACCAAAAGG AGATGGGAGC
3160 3170 3180 3190 3200 3210
S1GLO2(sgn) CATTGGTGGA AGTGGAAATA TGAGAGGAAT TCATGAAGAA GTGTATCATC AAAGAGAAAG
V711304 CATTGGTGGA AGTGGAAATA TGAGAGGAAT TCATGAAGAA GTGTATCATC AAAGAGAAAG
V711305 CATTGGTGGA AGTGGAAATA TGAGAGGAAT TCATGAAGAA GTGTATCATC AAAGAGAAAG
3220 3230 3240 3250 3260 3270
S1GLO2(sgn) GGATTATGAG TACCAAATGC CATTTGGCCT ACGAGTTCAG CCAATGCAGC CAAATCTACA
V711304 GGATTATGAG TACCAAATGC CATTTGGCCT ACGAGTTCAG CCAATGCAGC CAAATCTACA
V711305 GGATTATGAG TACCAAATGC CATTTGGCCT ACGAGTTCAG CCAATGCAGC CAAATCTACA
3280 3290 3300 3310 3320 3330
S1GLO2(sgn) TGAAAGAATG TAAAATTATA TTAATTCCCA CTTTAAATTA AGAACTCTAA TAATTTTAAT
V711304 TGAAAGAATG TAAAATTATA TTAATTCCCA CTTTAAATTA AGAACTCTAA TAATTTTAAT
V711305 TGAAAGAATG TAAAATTATA TTAATTCCCA CTTTAAATTA AGAACTCTAA TAATTTTAAT
3340 3350 3360 3370 3380 3390
S1GLO2(sgn) TTGGGTTTCT ACTATAGCTA GAAACACTAA TTAATCAATT CTAGTTATAT GTAATGAGTA
V711304 TTGGGTTTCT ACTATAGCTA GAAACACTAA TTAATCAATT CTAGTTATAT GTAATGAGTA
V711305 TTGGGTTTCT ACTATAGCTA GAAACACTAA TTAATCAATT CTAGTTATAT GTAATGAGTA
3400 3410 3420 3430 3440 3450
S1GLO2(sgn) TTAGCTAAGA CTTTGTTTCA TGTTGGAATT ATTACCTAAT TTTATGGGCT CTTCCCATGG
V711304 TTAGCTAAGA CTTTGTTTCA TGTTGGAATT ATTACCTAAT TTTATGGGCT CTTCCCATGG
V711305 TTAGCTAAGA CTTTGTTTCA TGTTGGAATT ATTACCTAAT TTTATGGGCT CTTCCCATGG
3460 3470 3480 3490 3500 3510
S1GLO2(sgn) TTTTAATTAA GTACTGAAAT GTAGTGACAT ATTTATATGA ATTGTTCTAC TTACCTAGCT
V711304 TTTTAATTAA GTACTGAAAT GTAGTGACAT ATTTATATGA ATTGTTCTAC TTACCTAGCT
V711305 TTTTAATTAA GTACTGAAAT GTAGTGACAT ATTTATATGA ATTGTTCTAC TTACCTAGCT
3520 3530 3540 3550 3560 3570
S1GLO2(sgn) ACTACATTTT TTTCAATTTG ATTTTGGTCT AGATGGATTC TGAAAACTAG TAGTGAATCT
V711304 ACTACATTTT TTTCAATTTG ATTTTGGTCT AGATGGATTC TGAAAACTAG TAGTGAATCT
V711305 ACTACATTTT TTTCAATTTG ATTTTGGTCT AGATGGATTC TGAAAACTAG TAGTGAATCT
3580 3590 3600 3610 3620 3630
S1GLO2(sgn) ACTTGTTTAT ATATCTAGGG TTTGTACTAC AATATTGAAC TATTTATTCT AGCGTCATGA
V711304 ACTTGTTTAT ATATCTAGGG TTTGTACTAC AATATTGAAC TATTTATTCT AGCGTCATGA
V711305 ACTTGTTTAT ATATCTAGGG TTTGTACTAC AATATTGAAC TATTTATTCT AGCGTCATGA

Supplementari Figure S1. Sequence of entire genomic sequence of the *SlGLO2* gene, including 460 bp of the promoter region and 360 bp of 3' UTR. The first line represents the sequence from Solanum Genomic Network website; V711304 and V711305 are respectively the WT and *7B-1* sequence. The letters highlighted with red color represent the start and stop codon; those highlighted in yellow represent SNP between sequences. The regions of the alignment that are written in red represent the exons while those written in black the introns.

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            670        680        690        700        710        720
Promoter(sgn)       GATTGAGTTT TTGCCTAAAA CCCAATGCAA TTAAACATTA GAACGTCAAT CAAAACCTAA
V711304             ---------- --------------------------CATTA GAACGTCAAT CAAAACCTAA
V711305             ---------- --------------------------CATTA CAACGTCCAT CAAAACCTAA


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            730        740        750        760        770        780
Promoter(sgn)       ACCTA-GGTC TACTACTTAT ATTCTTGGTC TTTTGTTTGA TTTTTTATCT CAGAATTTTC
V711304             ACCTA-GGTC TACTACTTAT ATTCTTGGTC TTTTGTTTGA TTTTTTATCT CAGAATTTTC
V711305             ACCTAAGGTC TACTACTTAT ATTCTTGGTC TTTTGTTTGA TTTTTTATCT CAGAATTTTC


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            790        800        810        820        830        840
Promoter(sgn)       CTTCATTTGA TGGATAATAG ACATTTTAAG AAAATTTCCC CTCATTGACC CTTTCAAACC
V711304             CTTCATTTGA TGGATAATAG ACATTTTAAG AAAATTTCCC CTCATTGACC CTTTCAAACC
V711305             CTTCATTTGA TGGATAATAG ACATTTTAAG AAAATTTCCC CTCATTGACC CTTTCAAACC


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            850        860        870        880        890        900
Promoter(sgn)       TATTACTATT ATTACAAAAC AAAAAACATT TTGATTTTGA TTTGATGGTT TAGATTGGAT
V711304             TATTACTATT ATTACAAAAC AAAAAACATT TTGATTTTGA TTTGATGGTT TAGATTGGAT
V711305             TATTACTATT ATTACAAAAC AAAAAACATT TTGATTTTGA TTTGATGGTT TAGATTGGAT

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            910        920        930        940        950        960
Promoter(sgn)       TAATTTCATT TATTCAAGGC ACCTAGACAA GACTCTTGCA AGGTCTACGC -TATATTCAA
V711304             TAATTTCATT TATTCAAGGC ACCTAGACAA GACTCTTGCA AGGTCTACGC -TATATTCAA
V711305             TAATTTCATT TATTCAAGGC CCCTTCACAA GACCCTTGCC AGGACTACGC -TATATTCCA


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            970        980        990       1000       1010       1020
Promoter(sgn)       AATTTATACT AAAGTTGTCG ATCAAATTCC AACTTACATA TATTCCACAA TCTTTACACT
V711304             AATTTATACT AAAGTTGTCG ATCAAATTCC AACTTACATA TATTCCACAA TCTTTACACT
V711305             AATTTATACT AAAGTTGTCG ATCATATTCC CACTTACATA TATTCCACAA TCTTTACACT


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           1030       1040       1050       1060       1070       1080
Promoter(sgn)       ATGTCCTTTT GATATGACTA GACCCTTTAA CACGTTCAAC TCAAACTAGG TTACAATATT
V711304             ATGTCCTTTT GATATGACAA GACCCTTTAA CACGTTCAAC TCAAACTAAG TTACAAAATT
V711305             ATGTCCTTTT GATATGACTA GACCCTTTAA CACGTTCAAC TCAAACTAGG TTACAATATT


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           1090       1100       1110       1120       1130       1140
Promoter(sgn)       CCTTTCGTTT CAATTTAAGT ATCTTAGTTT CATTGAACAC GATAACACGT AATTTAAAAA
V711304             CCTTTCGTTT CAATTTAAGT ATCTTAGTTT CATTGAACAC GATAACACGT AATTTAAAAA
V711305             CCTTTCGTTT CAATTTAAGT ATCTTAGTTT CATTGAACAC GATAACACGT AATTTAAAAA


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           1150       1160       1170       1180       1190       1200
Promoter(sgn)       ATAACAAAGA GATTTTATAA TCTTACAATT CTTTATTACT TTAAAATGTG TGTGTAATGT
V711304             ATAACAAAGA GATTTTATAA TCTTACAATT CTTTATTACT TTAAAATGTG TGTGTAATGT
V711305             ATAACAAAGA GATTTTATAA TCTTACAATT CTTTATTACT TTAAAATGTG TGTGTAATGT


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           1210       1220       1230       1240       1250       1260
Promoter(sgn)       AATGAAATGT TCTTTGAATC TTGTAATTTT AAACTTACAT GTAGAATGTT TGGATTGTCA
V711304             AATGAAATGT TCTTTGAATC TTGTAATTTT AAACTTACAT GTAGAATGTT TGGATTGTCA
V711305             AATGAAATGT TCTTTGAATC TTGTAATTTT AAACTTACAT GTAGAATGTT TGGATTGTCA

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           1270       1280       1290       1300       1310       1320
Promoter (sgn)      ACTTATTAAG TATAGAAAAA AAATTAAATT TTAAATGGAT AAAAAGAATA AGATGATTAA
V711304             ACTTATTAAG TATAGAAAAA AAATTAAATT TTAAATGGAT AAAAAGAATA AGATGATTAA
V711305             ACTTATTAAG TATAGAAAAA AAATTAAATT TTAAATGGAT AAAAAGAATA AGATGATTAA
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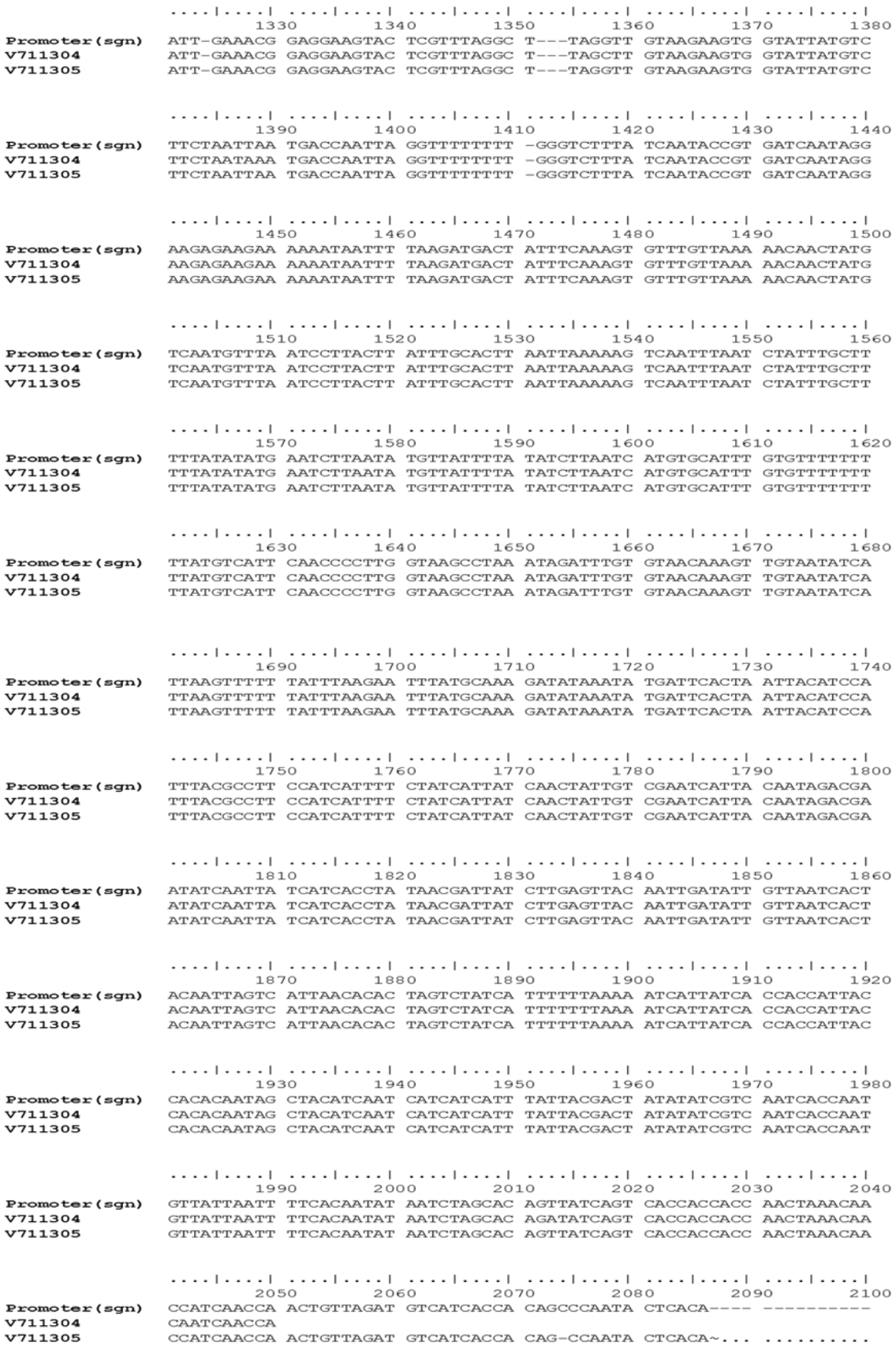
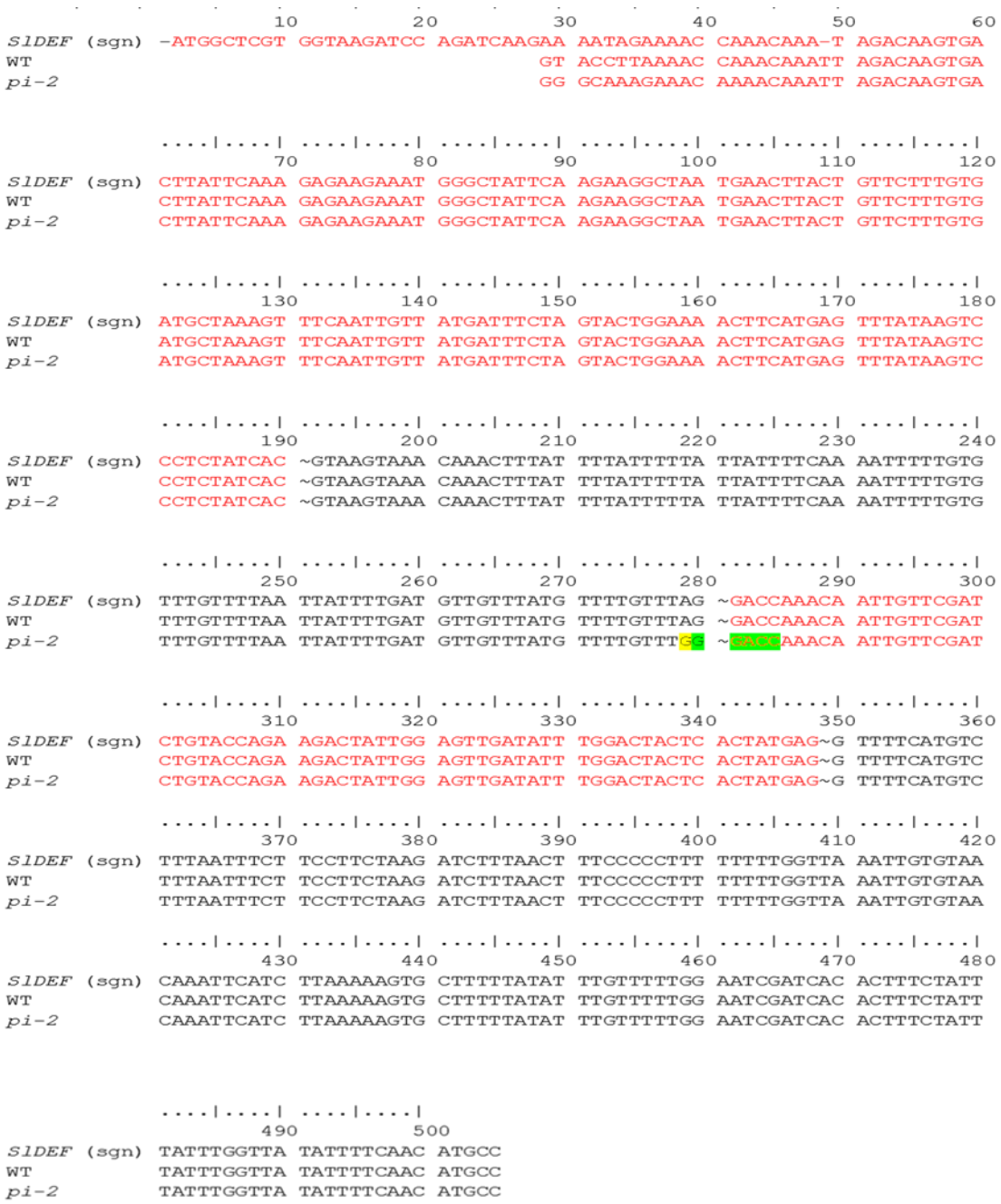
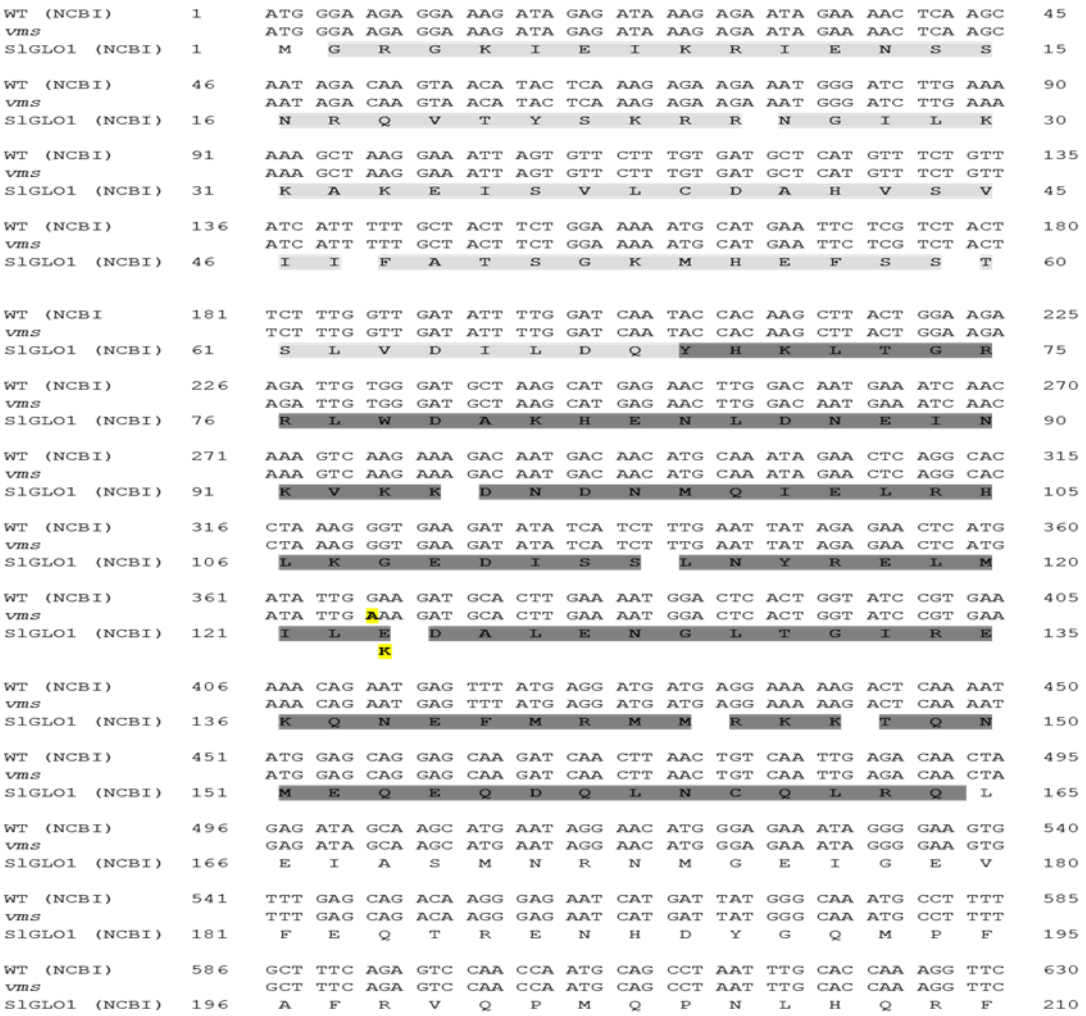


Supplementary Figure S2. Sequence of the promoter region of the *SlGLO2* gene. The first line represents the sequence from the Solanum Genomic Network website. V711304 and V711305 are respectively the WT and *7B-1* sequence.



Supplementary Figure S3. Partial genomic sequence of the *SlDEF* gene, including first and second exons and first and second introns. The first line represents the sequence from *S. lycopersicum* Genomic Network website. WT and *pi-2* represent respectively, the wild type and mutant parental genotype analyzed. The regions of the alignments that are written in red represent the exons while those written in black the introns. The letter highlighted in yellow corresponds to the nucleotidic changes in splicing site of *pi-2* with respect to the WT, while those highlighted in green are the restriction site recognized by the *NlaIV* enzyme, which is formed after the nucleotidic substitution.



Supplementary Figure S4. Mutation in the *SlGLO1* sequence of the *vms* mutant. Alignment of coding sequence of WT and *vms* genotype and respective amino acid sequence. The letters highlighted in yellow indicate the substitution G to A in position 367 of the nucleotidic sequence and the amino acids change in position 123 (E to K). The amino acids highlighted in light grey represent the MADS-box domain, while those highlighted with dark grey the K-box domain.