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Positional cloning of the *parthenocarpic fruit* (*pat*) mutant gene and identification of new parthenocarpic sources in tomato (*Solanum lycopersicum*, L.)

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Al mio Professore di vita…

Con la speranza che questo mio lavoro

tu lo possa recepire come un piccolo

GRAZIE

per tutto quello

che mi hai dato

ed insegnato.

Buona lettura, papà

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Abstract

In this work we characterized two parthenocarpic sources in tomato by positionally cloning the gene responsible of the *parthenocarpic fruit* (*pat*) phenotype and by TILLING the *SlIAA9* gene, a major member of the ovary repressor machinery before pollination.

The importance of parthenocarpic mutations is due to their possible use in breeding programs, as well as in studies aimed to the comprehension of mechanisms underlying the fruit set. An understanding of the molecular events underlying parthenocarpy, in fact, would provide information on factors regulating fruit and seed formation, and thus open new perspectives for yield improvements by biotechnological means.

The *pat* mutation (Bianchi and Soressi, 1969), induces parthenocarpy with strong expressivity along with other pleiotropic effects such as short anthers and aberrant ovules (Mazzucato *et al.,* 1998). Through Bulk Segregant Analysis the *Pat* gene was mapped, by using two segregating populations, on the long arm of chromosome 3 between two conserved ortholog set (COS) markers (COSes; Fulton *et al.,* 2002), T0796 and T1143 (Beraldi *et al.,* 2004), previously anchored on the genetic tomato map (EXPEN 2000, www.sgn.cornell.edu).

We developed and mapped novel PCR-derived COS markers inside the target window for the *Pat* gene by pursuing the microsynteny between tomato and Arabidopsis (Fulton *et al.,* 2002). Through genetic and physical mapping, the genetic region spanning 1.2 cM between COSes T0796 and T1143, was refined with new anchor-points and the target interval for the *Pat* locus was restricted to less than 0.2 cM between markers named T17 and T20. The small size of the new target region and the recent publication of the tomato genome sequence (SGN, www.sgn.cornell.edu) allowed us to carry out a candidate gene approach with the aim to clone the gene responsible for the *pat* phenotype. Four of the nine potential candidate genes mapping in the T17-T20 genomic window, were amplified and sequenced from WT (Chico III) and *pat* lines. A point mutation in the *SlHB15* gene, a transcriptor factor belonging to the HD-Zip III family, was found and proposed as responsible for the *pat* phenotype. In order to confirm this hypothesis a complementation experiment with the *HB15* WT gene will be necessary. *HD-Zip III* and *KANADI*, two antagonistic gene families, were included in a new model that would explain the *pat* phenotype and the molecular pathway that triggers the fruit set.

In the second part of this work, a TILLING approach has been undertaken in order to identify tomato genotypes carrying mutations in the *SlIAA9* coding sequence. As demonstrated in recent publications, TILLING shows promise as a non-transgenic tool to improve domesticated crops by introducing and identifying novel genetic variation in genes that affect key traits. *SlIAA9*, a member of the Aux/IAA family of transcription factors in tomato, has been described as playing a major role in the ovary repressor machinery and plants silenced by antisense showed several IAA-related developmental defects and a parthenocarpic behavior (Wang *et al.,* 2005). The analysis of M3 families yielded three lines carrying a genetic lesion in the coding sequence of *SlIAA9*, two showing a point mutation leading to amino acidic substitution and the third showing a single-base deletion leading to a frame-shift and a premature stop codon. Characterization of the former lines, showed some of the expected phenotypes, albeit with low penetrance and expressivity (occurrence of polycots, abnormal growth of axillary shoots, low number of seeds per fruit or seedlessness). Characterization of the latter line showed severe phenotypes, in agreement with those expected, that mainly consisted in an obvious loss of leaf compoundness and parthenocarpy. This observation suggest the interest of this line in studying the role of the IAA9 transcription factor in reproductive development, although its partial sterility may hamper its employment in breeding parthenocarpic tomato varieties.

Riassunto

In questo lavoro sono state caratterizzate due fonti di partenocarpia in pomodoro attraverso il clonaggio per mappatura del gene responsabile del fenotipo del mutante *partenocarpic fruit-1 (pat)* e attraverso il TILLING del gene *SlIAA9*, uno dei principali membri del sistema di repressione dell'ovario prima dell'impollinazione.

L'importanza delle mutazioni di partenocarpia è dovuta al loro possibile ruolo nei programmi di breeding ma anche in studi il cui scopo è quello di comprendere i meccanismi molecolari che portano alla formazione del frutto. Infatti, la comprensione dei meccanismi molecolari che inducono la partenocarpia fornirebbe informazioni anche sui fattori che regolano la formazione del seme e del frutto, e così aprirebbe nuove prospettive per il miglioramento genetico attraverso mezzi biotecnologici.

La mutazione *pat* (Bianchi and Soressi, 1969) induce partenocarpia con forte espressività ma causa anche altri effetti pleiotropici quali la formazione di antere corte e

ovuli aberranti (Mazzucato *et al.,* 1998). Attraverso l'analisi dei gruppi segreganti (BSA) il locus *Pat* è stato precedentemente mappato sul braccio lungo del cromosoma 3 di pomodoro tra due marcatori ortologhi conservati (COS; Fulton *et al.,* 2002), T0796 e T1143 (Beraldi *et al.,* 2004), ancorati sulla mappa genetica pubblicata all'SGN (*Sol* Geomics Network; EXPEN 2000, www.sgn.cornell.edu).

In questo studio, tramite l'utilizzo della microsintenia tra Arabidopsis e pomodoro (Fulton *et al.,* 2002), sono stati sviluppati e mappati all'interno della regione target per il gene *Pat* nuovi marcatori COS PCR-derivati. Attraverso un lavoro di mappatura genetica e fisica, la regione genetica di 1,2 cM, tra i marcatori T0796 e T1143, è stata rifinita con nuovi marcatori e la regione target per il locus *Pat* è stata ristretta a circa 0,2 cM, tra due marcatori denominati T17 e T20. Le ridotte dimensioni della nuova regione e la recente pubblicazione del genoma di pomodoro (SGN; www.sgn.cornell.edu) hanno quindi permesso di attuare l'analisi di potenziali geni candidati al fine di isolare quello responsabile del fenotipo *pat*. Quattro di nove geni, localizzati nella regione genomica tra i marcatori T17 e T20, sono stati sequenziati nella linea *pat* e nel corrispettivo WT (Chico III). In questo modo, una mutazione puntiforme sul gene *SlHB15*, un fattore di trascrizione appartenente alla famiglia genica *HD-Zip III*, è stata identificata e proposta come responsabile del fenotipo *pat*. Al fine di confermare questa ipotesi, sarà necessario un esperimento di complementazione con il gene *HB15* WT sulla linea *pat*. Due famiglie geniche antagoniste, *HD-Zip III e KANADI*, sono state incluse in un nuovo modello che spiegherebbe il fenotipo *pat* e il pathway molecolare che porta alla allegagione del frutto.

Nella seconda parte di questo lavoro, al fine di identificare nuove fonti di partenocarpia in pomodoro, il gene *SlIAA9* è stato saggiato su due popolazioni di TILLING (cultivar Red Setter; Minoia *et al.,* 2010) disponibili presso l' Istituto di Ricerca Metapontum Agrobios. Infatti, come recentemente dimostrato in diverse pubblicazioni, il TILLING può rappresentare una metodologia che non ricorre al transgenico per migliorare le specie di interesse agrario attraverso l'induzione e l'identificazione di nuove forme alleliche in geni che controllano tratti chiave per il miglioramento genetico. Il gene *SlIAA9*, un membro della famiglia di fattori di trascrizione Aux/IAA in pomodoro, è uno dei componenti chiave del complesso molecolare responsabile della repressione dell'ovario prima dell'impollinazione; il silenziamento di questo gene provoca difetti di sviluppo correlati all' IAA e la formazione di frutti partenocarpici (Wang *et al.,* 2005). L'analisi di circa 5000 famiglie M3 ha portato all'identificazione di tre linee con una lesione sulla sequenza codificante il gene *SlIAA9*: due di esse mostrano una mutazione puntiforme che causa una sostituzione

amminoacidica, la terza mostra una delezione di una base che causa un errore di lettura ed un prematuro codon di stop.

La caratterizzazione delle prime due linee ha mostrato alcuni dei tratti fenotipici attesi, anche se con bassa penetranza ed espressività (formazione di più cotiledoni, crescita anormale di germogli ascellari, basso numero o assenza di semi nel frutto).

La caratterizzazione della terza linea ha mostrato caratteri fenotipici più forti, in accordo con quelli attesi, che consistono soprattutto in una perdita di complessità delle foglie e partenocarpia. Queste osservazioni mettono in risalto l'interesse di questa linea nello studio del ruolo che il fattore di trascrizione IAA9 ha nello sviluppo riproduttivo; la sua maggiore caratterizzazione relativa alla capacità di produrre frutti e semi ne indicherà le prospettive reali di utilizzo nel miglioramento genetico di pomodoro.

1. Introduction

The fruit is the result of the development of the ovary. His role is to provide a suitable environment for the development of seeds and often to support efficient dispersal of mature seeds. In normal fruit development, the initiation of fruit set depends on the successful completion of pollination and fertilisation. Pollination occurs after formation of pollen grain in the anther and its release. Fertilization occurs in the female *sporangium*, the ovule, located within the carpel of the flower. Fertilization requires pollen germination, penetration and growth of the pollen tube in the stylar tissue towards the embryo sac in order to fuse with the egg cell (Dumas and Mogensen, 1993). Fertilization of the ovule generally triggers the development of the ovary into a fruit (Gillaspy *et al.,* 1993).

The normal process of fruit development may occasionally be independent of pollination and fertilization . Two main phenomena, apomixis and parthenocarpy, can led to the development of fruits without pollination and fertilization. Apomixis is defined as asexual reproduction through seeds, leading to the production of clonal progeny (Koltunow and Grossniklaus, 2003). Parthenocarpy is the growth of the ovary into a seedless fruit in the absence of pollination and/or fertilization (Gustafson, 1942). Parthenocarpy may occur naturally or can be induced artificially with the application of various hormones (Gustafson, 1936, 1942; Nitsch, 1952; Osborne and Went, 1953). The expression of genetic parthenocarpy is correlated with the accumulation of auxins and giberellins in the ovaries, which is autonomus and precocious compared to the respective wild-type (George *et al.,* 1984).

The importance of parthenocarpic mutations is due to their possible use in breeding programs, as well as in studies aimed to the comprehension of mechanisms underlying the fruit set. An understanding of the molecular events underlying parthenocarpy, in fact, would provide information on factors regulating fruit and seed formation, and thus open new perspectives for yield improvements by biotechnological means.

In the recent years, parthenocarpy was supposed to have a role in the species evolution, this would explain why this phenomena is so taxonomically widespread in nature. In the following paragraphs we first describe the recent hypothesis about an adaptive role of parthenocarpy in wild species than the molecular pathway that lead to fruit set and parthenocarpy, to conclude with the description of parthenocarpy in tomato (*Solanum lycopersicum*, L.), the species object of this research.

1.1 Adaptive role of parthenocarpy

Seedless fruits can develop in one of two ways: either the fruit develops without fertilization (parthenocarpy), or pollination triggers fruit development but the ovules or embryos abort without producing mature seeds (stenoparthenocarpy). Parthenocarpy (the production of seedless fruits) and abortion of reproductive structures at different developmental stages are important processes limiting female fecundity in a lot of plant species.

Many plants in fact produce far more fruits than eventually mature, with a large proportion of developing fruits being aborted midway through the development process. Whether this is due to the parthenocarpy, or it's a maternally controlled late-acting self-incompatibility mechanism, or an expression of inbreeding depression, is often difficult to determine.

The production of seedless fruits is intriguing because empty fruits do not contribute directly to the production of offspring and their maturation is presumably costly to the maternal plant (Willson and Burley, 1983). In 1890, Sturtevant remarked: "If we see in nature an occurrence which is distinctly prejudicial to the continuance and distribution of species, we found an illustration in seedless fruits". Darwin (1876) referred frequently to the production of fruit without seed and attributed the condition to infertility. Willson and Burley (1983) have considered the phenomenon to be either a "mistake", which suggest that there is no resource limitation during fruit development or the result of a developmental error. When there's failure of seed to set, the abscission of the young fruitlet should be of advantage to the parent plant, avoiding the waste of resources in growing structures not fulfilling their biological purpose; however in many species, this doesn't occur suggesting the possibility of adaptive reasons for retaining empty fruits.

Tough appearing taxonomically widespread, parthenocarpy has been stated as more frequent in species that produce fruits with several to many while it represents relatively a rare event in species having single seeded-fruits (Roth, 1977). Recently the occurrence of parthenocarpy in the plant kingdom has been carefully investigated through bibliographic sources, referring to the relevant review by Gustafson (1942) and a number of more recent studies (Selleri *et al.,* 2010). The data regard the number of parthenocarpic taxa grouped in species and subdivided into two categories, one with plants producing single/two-seeded ("monospermic") fruits, the second with plants having multi-seeded ("plurispermic")

fruits. Seventy-five per cent of the species scored for this trait belong to the plurispermic fruit category. In addition the plants have been distinguished in wild, cultivated as ornamental, cultivated for fruit or seed. These three categories have been introduced to evidence a possible different selective pressure on the parthenocarpy trait. Parthenocarpy has been observed mostly among dicots (98 out of 100 species scored) in both wild and cultivated taxa. The number of parthenocarpic variants in the cultivated species with plurispermic fruits is about six-fold that of the wild species of the plurispermic wild species, suggesting a selective pressure for parthenocarpy during their domestication/breeding. This was not evident in the monospermic fruit category where the number of wild and cultivated species is approximately the same.

Despite 75% of about 100 species scored for parthenocarpy belonged to the plurispermic fruit category (Selleri *et al.,* 2010), clear evidence of an adaptive role of parthenocarpy has recently been proposed only in taxa with one single/two ovules per flower. In this plant group such a role is based mainly on different mechanisms by which the empty fruits would reduce seed predation. First of all, Willson and Burley (1983) suggested that sterile but well developed fruits dilute the depredations of seed predators. However the possibility that plants may benefit from the production of fruits without seeds or with empty seeds has only recently begun to be explored.

For instance, wild parsnips (*Pastinaca sativa* L.) produce parthenocarpic fruits in remarkable abundance; as much as twenty per cent of the fruit crop can be seedless. Parthenocarpic fruits are indistinguishable from normal fruits but if they are viewed with lighting from the back they are without the large endosperm that creates a shadow in the normal fruit. Zangerl *et al.* (1991) showed that the persistence of parthenocarpic fruits in parsnip is related to their defensive value against their most destructive enemy the parsnip webworm. Given a choice between parthenocarpic and normal fruit, webworms overwhelmingly prefer those that are parthenocarpic even when rare. This preference is unaffected by the relative frequency of normal and parthenocarpic fruit; even when rare, the parthenocarpic fruits are preferentially eaten. However, on parthenocarpic fruits, insects fed less efficiently and grew more slowly than insects fed normal fruits; this is not surprising because seedless fruits contain less nitrogen and calories than normal fruits. Furthermore, they also contain only half of the toxic furanocoumarins molecules that are known to enhance the resistance of parsnips to webworms. The benefit of such fruit to the parsnip is clear: parthenocarpic fruits, in which few resources are invested, are sacrificed for fruits containing viable offspring (Zangerl *et al.,* 1991). Also A. Traveset (1993)

presents a case in which parthenocarpy reduces the incidence of seed predation by insects. At least the first generation of chalcidoid wasps that oviposit in the fruits of *Pistacia terebinthus* L. (Anacardiaceae) cannot discriminate among viable and not-viable fruits, allocating energy and time to oviposition on fruits that are not suitable for larval development. She found a negative correlation between the number of parthenocarpic fruits and the number of wasp-damaged seeds. Verdù and Garcia-Fayos (1998) found that the presence of aborted seeds in *Pistacia lentiscus* shrubs, reduced seed predation rates by chalcidoid wasps in the same way that parthenocarpic fruits did in *Pistaca terebinthus* (Traveset,1993).

In *Bursera morelensis* parthenocarpic fruits, in the first stages of development, are characterized from an unusual spread of internal walls that invade the ovary locules and prevent ovule development. In the parthenocarpic fruit when the mesocarp spreads and the cells became more elongated, the endocarp gets fragmented forming unprotected sites. Furthermore, unlike fruits with seeds, parthenocarpic fruits don't have calcium oxalate crystals in the ovary wall (Ramos-Ordonez *et al.,* 2008). These crystals provide reinforcement, giving additional strength to the tissues and minimize predation (Molano-Flores *et al.,* 2001). The absence of insects in the fruits with crystal of calcium oxalate of *B. morelensis* suggests that the plant can produce fruits armed mechanically and chemically to ensure seed development and defence as well as unprotected seedless fruits as a deceit low-cost strategy to reduce the probability of seed predation (Molano-Flores *et al.,* 2001).

Fuentes and Schupp (1998) has been the first to demonstrate that empty seed can reduce predation by vertebrate seed eaters and the first to demonstrate discrimination based on seed filling at the level of whole plant. They considered the role of deceptive fruits on *Parus inornatus* predators choice of *Juniperus osteosperma* plants and documented that the trees suffering high levels of seed predation had also a higher levels of filled seed. The time and effort a seed predator requires to found a rewarding (filled) seed is likely to increase as the proportion of empty seeds on a plant increases. As a consequence, foraging seed predators would benefit from choosing plants with higher proportions of filled seeds, and plants producing more empty seeds would enjoy reduced levels of visitation and seed predation. Plant choice may be especially important when fruits containing empty seeds are visually indistinguishable from those containing filled ones, and seed predators have to invest substantial time and effort handling fruits or seeds to assess whether or not they contain food (Fuentes and Schupp, 1998). Similarly it has been

suggested that in *Pistacia lentiscus* deceptive fruits can reduce seed predation by birds under particular conditions that increase fruit handling costs to seed predation (Verdu and Garcia-Fayos, 2001).

Thus empty seeds may be maintained by natural selection because they reduce seed predation; for this to be true, the benefit of increased lifetime reproduction due to greater seed survival in the presence of empty seed must exceed the cost of reduced lifetime reproduction due to investment in empty seed. It's evident that in the plurispermic fruit species the energy that the plant invest on each fruit is bigger compared to the mono-seeded fruit species, so a such adaptive role it's difficult to speculate. It has been proposed that plants have evolved flowers with a great number of ovules as a response to habitats where pollination is more uncertain (e.g. due to asynchronism between flowering and the appearance of pollinators or critical climate variation (Verdu *et al*., 1998). In this case, a plant with several to many ovules per flower very often experiences a variable seed/ovule ratio (Burd, 1994). As a source-sink component of the reproductive system, the forming seeds supply the ovary with the hormones necessary for triggering fruit-set and development. In these species, under low pollination rates, the plant produces very few embryos, possibly not enough to support fruit growth, thus causing fruitlet abscission. Under these circumstances, it has been proposed that parthenocarpy could offer the opportunity to accomplish the production and dispersal of few seeds that otherwise would be lost (Selleri *et al.,* 2010). When the seed/ovule ratio is extremely low, for instance in limiting environmental conditions for pollination and fertilization, it is likely that the possibility of the ovary to develop into a fruit represents a selective advantage. In other words, a weak source of hormones triggered by a scant seed-set, unable to stimulate the fruit growth, would far more benefit from the natural fixation of major or minor genes acting for parthenocarpy, leading to a boost of specific fruit setting-related hormones such as auxin and gibberellins (Selleri *et al.,* 2010).

A further hypothesis for an adaptive role of parthenocarpy, that concerns both the mono and the plurispermic species, is that parthenocarpic fruits develop because resources are not limited (Willson and Burley, 1983). In Mediterranean habitats water is a major limiting resource (Mooney and Dunn, 1970; Aerts, 1995; Hobbs *et al.,* 1995), and thus, increased water availability should result in increased rates of parthenocarpy. Jordano (1988) studied rates of parthenocarpy in a *Pistacia lentiscus* population during two consecutive years, and found that the higher rate corresponded to a wet year (47,9 %) and the lower to a dry year (35,8 %). Different results have been found in *Lavandhula latifolia*, where irrigation

increased both the number of flowers per inflorescence and the fruit-set, but not the number of seeds for fruit (Herrera 1990, 1992). Herrera (1992) suggested that fruit-set is an inheritance characteristic feature of individual plants, independent of current resource levels. Similarly, Zahoueh *et al.* (1991) concluded that fruit-set in *Pistacia lentiscus* can be "adjusted" in different ways depending on the life history of each individual because uncertainty about water availability is produced along the whole life of each individual. In agreement with this hypothesis, Verdu and Garcia Fayos (1997) didn't find an immediate response to irrigation, either reproductive or vegetative, but a delayed effect at the following reproductive season: *Pistacia lentiscus* irrigated females increased fruit-set from one year to the next in 21.3 percentage points and seed viability in 32.3, whereas non-irrigated females only increased fruit-set in 12.2 percentage points and seed viability in 15.2. The adjustment of progeny to the available water resources *in Pistacia lentiscus* is probably an individual feature inherent to the history of each individual, and therefore not exclusively dependent on the current resource level. This adjustment may have adaptive value in relation to uncertainty about resource availability, because water resources are distributed along the life of each individual depending on their necessity, as suggests the increase of seed set in the next year after experiment (Verdu and Garcia Fayos 1997).

Alternatively, empty seed may be due to events and/or selection occurring during fertilization and embryo development, and positive effects on seed survival are simply fortuitous (Jordano, 1989; Traveset, 1993). Many gymnosperms and some angiosperms have "delayed fertilization" taking place long after pollination and the onset of fruit development (Willson and Burley, 1983), so fertilization failure or embryo abortion can result in empty seeds of fruits (e.g. Dogra, 1967; Fechner, 1967). Willson and Burley (1983) offered several adaptive hypotheses of delayed fertilization, including mate choice and assessment of offspring quality by females, and/or completion among males. A further possibility reconciling the different scenarios is that the production of empty seed is under the control of a variety of selective pressures acting at different reproductive stages (Fuentes and Schupp, 1998).

1.2 Fruit set and parthenocarpy

Although the influence of phytohormones, such auxin and gibberellin, over fruit development was already acknowledged back in the early 20th century (Gustafson, 1937, 1939; Wittwer *et al.,* 1957), the molecular mechanisms that underlie fruit set are still largely unknown and are now starting to be unravelled.

Gustafson (1936) was the first to demonstrate that the application of substances closely related to auxin onto stigmas of tomato and several other species caused the ovary to develop into a parthenocarpic fruit. Auxin supplied either exogenously or genetically conferred parthenocarpy to both fleshy and dry fruits (Schwabe and Mills 1981, Vivian-Smith and Koltunow 1999, Spena *et al.* 2001. Wittwer *et al.* (1957) showed that a second type of growth regulator, gibberellins (Gas), can also stimulate parthenocarpic fruit set. The application of gibberellin can induce an increase in auxin content (Sastry and Mur 1963), but, in turn, auxin seems to be able to stimulate gibberellin biosynthesis (Koshioka *et al.,* 1994), which indicates that there is a tight regulation between these two hormones during the early stages of fruit development.

Also the application of pollen extracts to the outside of the ovary causes the ovary to develop into a parthenocarpic fruit, which led to the hypothesis that pollen grains contain plant hormones similar to the growth substances. Sastry *et al.* (1963) showed that at the stage of anthesis, no auxin was present in flowers of tomato. However, auxin concentrations increased within 28 h after gibberellin treatment, suggesting that, it is not auxin, but gibberellin that is transferred from the germinating pollen to the ovary. Consistently, the concentrations of both growth regulators rapidly increase during the first 10 days after anthesis, probably after pollination and fertilization, which occur between 2 d and 6 d after anthesis (Mapelli *et al.,* 1978). In natural parthenocarpic varieties, these hormones might already have reached a threshold concentration prior to pollination, resulting in the formation of seedless fruits (Gustafson, 1939; Nitsch *et al.,* 1960; Mapelli *et al.,* 1978, 1979).

The current model of fruit set implies that ovary growth is blocked before pollination and that auxin and gibberellins are key regulators of ovary growth de-repression at fruits set. Anyway a large body of evidence supports the notion that also other phytohormones (e.g. cytokinin, brassinosteroid, ethylene and abscisic acid) either effect and/or are involved in fruit initiation and/or development (Shwabe and Mills, 1981; Vriezen *et al.,* 2008).

1.2.1 Auxin biosynthesis

Even if many natural and synthetic compounds exhibit auxin-like activity in bioassays, indole-3-acetic acid (IAA) is recognized as the key auxin in most plants (Woodward and Bartel, 2005).

The IAA biosynthesis via tryptophan (TrpIAA) is synthesized both from tryptophan using Trp-dependent pathways and from an indolic Trp precursor via Trp-independent pathways; none of thes epathways is fully elucidated. Plants can also obtain IAA by β-oxidation of indole-3-butyric acid (IBA), a second endogenous auxin, or by hydrolysing IAA conjugates, in which IAA is linked to amino acids, sugars or peptides. To permanently inactivate IAA, plants can employ conjugation and direct oxidation.

The auxin biosynthesis pathways are non-redundant, each pathway acts in a tissue-specific and developmental stage-specific manner (Woodward and Bartel, 2005; Zhao, 2008).

Tomato plants, homozygous for the *sulfurea* mutation, suffer from auxin deficiency, which is probably due to defects in the Trp-independent pathway. Auxin deficiency was largely confined to the shoot meristems, but also frequently resulted in abnormalities in floral morphology, including missing floral organs, fused organs, and homeotically transformed organs (Ehlert et al., 2008). However, fruit development in the *sulfurea* mutant was unaffected, indicating that auxin synthesis in developing fruits occurs via the Trp-dependent pathway. These findings correspond to the results of Epstein et al. (2002), that showed that there is a switch from the Trp-dependent to the Trp-independent auxin production, occurring between the mature green and the red-ripe stages of tomato fruit development.

During fruit development, two peaks of auxin are observed. The first peak reaches its maximum 8 d after pollination at the end of active cell division, and the second peak reaches its maximum at 30 d. The latter was not found in parthenocarpic fruit (Mapelli et al., 1978), suggesting that, at least during the later stages of fruit development, the embryo supplies the auxin necessary for continued fruit growth. Lemaire-Chamley et al. (2005) showed that candidate key genes for auxin biosynthesis, transport, signalling, and responses were already expressed in the locular tissue during the early stages of fruit growth. More detailed analysis of genes differentially expressed between the locular tissue and the outer pericarp, revealed that the expressions of these genes follow a gradient from the central part of the fruit (placenta and locular tissue) to the outer part of the fruit

(Lemaire-Chamley et al., 2005). It is possible that, in response to pollination and fertilization, the auxin is newly synthesized or hydrolysed from its conjugates in the central parts of the tomato fruit, the developing ovule and/or its surrounding tissues, respectively, and subsequently transported to the outer layers. This transport leads to the formation of the auxin gradient in the fruit tissues, which is translated into auxin responses, such as cell division, cell expansion, and into cross-talk with other hormones, such as newly synthesized gibberellins (Lemaire-Chamley et al., 2005).

1.2.2 Auxin signaling pathway in fruit set

The plant hormone auxin influences virtually every developmental program in plants. Auxin acts by regulating transcription through the action of at least three protein families called the TIR1/AFB F-box proteins (Dharmasiri et al., 2005; Mockaitis et al., 2008), the Aux/IAA transcriptional repressors (Overvoorde et al., 2005; Remington et al., 2004), and the ARF transcription factors (Guilfoyle et al., 2007; Okushima et al., 2005).

The TIR1 F-box protein acts as an auxin receptor and directly links auxin perception to degradation of the Aux/IAA proteins (Dhamarsiri et al., 2005; Kepinski et al., 2005). Structural studies recently revealed that auxin acts as a “molecular glue” to increase the strength of the interaction between TIR1 and the Aux/IAA protein, thus promoting their ubiquitination and degradation (Tan et al., 2007). In Arabidopsis there are 29 Aux/IAA genes (Overvood et al., 2005). The only known function of Aux/IAA proteins is to repress transcription of the auxin-regulated genes. According to the current model, Aux/IAAs recruit the co-repressor TPL to promoter through interactions with both TPL and ARF proteins (Szemenyei et al., 2008; Tiwari et al., 2001, 2004; Weijers et al., 2005). The presence of auxin, promotes Aux/IAA protein ubiquitination through the TIR1 complex (Gray et al., 2001). As a result, the Aux/IAA protein is degraded by the 26S proteasome and the ARF is released from the repressive effect of the Aux/IAA protein, leading to the activation of the auxin response genes (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

IAA9, a member of the tomato *Aux/IAA* gene family, has shown to be involved in fruit set (Kim et al., 1997; Ulmasov et al., 1997). The reduction of *IAA9* transcript levels in tomato plants resulted in a pleiotropic phenotype. The transgenic lines formed simple leaves instead of wild-type compound leaves, and fruit development was initiated prior to

pollination and fertilization. These phenotypes together with auxin dose-response assays showed that down-regulation of *IAA9* leads to auxin hypersensitivity, confirming that *IAA9* acts as a transcriptional repressor of auxin signalling (Wang *et al.*, 2005). In turn, some of the auxin response genes encoded by Aux/IAAs, such as *IAA2* and *IAA14* and also *IAA9* are induced by auxin treatment of the unpollinated ovary (Serrani *et al.*, 2008).The mRNAs levels of *IAA2* and *IAA14* were also found to increase in the pollinated ovary, specifically in the placenta and ovular tissues (Vriezen et al., 2008). It seems likely that, in the presence of auxin, either after pollination or auxin application, de novo synthesized Aux/IAA proteins are rapidly degraded due to SCFTIR1-mediated ubiquitination. However, despite their rapid turnover, the transcriptional activation of these Aux/IAAs suggests that a minimum level of Aux/IAAs is required in order to create a negative feedback loop in the auxin signal transduction pathway, which enables the plant to fine-tune the strength of the auxin response (Gray et al., 2001).

Recently, a new member of the tomato ARF gene family, *SlARF7*, the homologue of Arabidopsis ARF7, has been characterized. *SlARF7* mRNA levels are high in the placental tissues of the mature flower, and rapidly decrease after pollination. Decreasing these levels by using an RNAi approach resulted in parthenocarpic fruit development, suggesting that SlARF7 may act as a negative regulator of fruit set. The parthenocarpic fruits displayed characteristics typically related to high levels of auxin and gibberellin, which indicate that SlARF7 might be involved in the crosstalk between these two hormones (de Jong et al., 2008). In Arabidopsis siliques, *ARF8/FRUIT WITHOUT FERTILIZATION* (*FWF*) shows a similar expression pattern as *SlARF7* (Goetz et al., 2006), and the mutated *fwf* allele triggers the formation of parthenocarpic siliques (Vivian- Smith et al., 2001). These similarities suggest that SlARF7 is the functional equivalent of AtARF8/FWF. Interestingly, the *fwf* allele contains a mutation in the putative translation initiation codon, but is still transcribed and probably also translated (Goetz et al., 2006), resulting in a truncated protein, which is missing at least part of its DNA binding domain. However, the exact nature of the mutant protein is still unclear (Goetz et al., 2007). Introduction of the *fwf* allele in wild-type plants also induced the formation of parthenocarpic siliques, even though endogenous *AtARF8* transcript levels were not reduced (Goetz et al., 2007). These findings suggest that the aberrant form of *AtARF8* may compete with the endogenous AtARF8 protein in the formation of protein complexes. Introduction of the Arabidopsis fwf mutant allele in tomato also results in parthenocarpic fruit set, indicating that not only *SlARF7*, but also the tomato homologue of *AtARF8/FWF*, *SlARF8*, plays a role in

regulating tomato fruit set (Goetz et al., 2007). This hypothesis is supported by the findings of Gorguet et al. (2008), who identified *SlARF8* as a candidate gene for two parthenocarpy QTLs. However, instead of being down-regulated after auxin-treatment or pollination, SlARF8 transcript levels were found to increase after auxin treatment (Serrani et al., 2008), suggesting that although *SlARF8* might have a function in tomato fruit set, it probably functions in a different manner than *SlARF7* or *AtARF8*. The introduction of the aberrant form of AtARF8 in tomato may interfere with the protein complex formation of SlARF7 with other factors that might be involved in tomato fruit set, resulting in parthenocarpic fruit growth.

The mechanism of action in auxin signal transduction of the two recently identified *AUCSIA* genes is unknown, but reduction of *AUCSIA* transcript levels by an RNA interference approach led to a pleiotropic phenotype that could be related to auxin, such as alterations in leaf development and reduced polar auxin transport in the roots. Interestingly, the *AUCSIA*-silenced plants formed parthenocarpic fruit when flowers were emasculated. The total IAA content in these flower buds was 100 times higher than in the wild-type, which seems likely to be the cause of the parthenocarpic fruit growth. However, it is unknown whether the auxin accumulation is the consequence of an increased auxin synthesis, decreased auxin degradation, or altered auxin transport (Molesini et al., 2009).

1.2.3 Gibberellin biosynthesis

GAs constitute a group of plant hormones that control developmental processes such as germination, shoot elongation, tuber formation, flowering, and fruit set, and growth in diverse species (Hedden and Kamiya, 1997; Olszewski *et al.,* 2002). The metabolism of GA has been deeply investigated and is quite well understood (Sonsel and Hedden, 2004). In summary, ent-kaurene, synthesized from geranylgeranyl diphosphate by the action of two cyclases, is metabolized by the action of P450-dependent monoxygenases to GA12 and/or GA53, which in turn are metabolized by GA 20-oxidases and GA 3-oxidases, acting consecutively, to active GAs through two parallel pathways: the non-13-hydroxylation (leading to GA4) and the early 13-hydroxylation one (leading to GA1 and GA3 in some cases).Active GAs and their precursors can be irreversibly inactivated by GA 2-oxidases introducing a hydroxyl at the 2β position (Sponsel and Hedden, 2004). The existence of genes encoding GA deactivating enzymes catalyzing 16α,17- epoxidation in rice (*Oryza*

sativa; Zhu *et al.,* 2006) and formation of GA methyl esters in Arabidopsis (*Arabidopsis thaliana*; Varbanova *et al.,* 2007) has been reported, although the importance of these reactions for GA homeostasis in other species is unknown.

The young tomato flower bud contains metabolites of both the non-13-hydroxylation and 13-hydroxylation pathways, which levels decrease progressively during ovary development (Fos *et al.,* 2000). After pollination, the total gibberellin content within the ovary increases again, although low levels of the metabolites of the GA4 biosynthetic pathway are detected (Bohner *et al.,* 1988; Koshioka *et al.,* 1994; Serrani *et al.,* 2007b). These findings suggest that the 13-hydroxylation pathway is mostly used in the growing ovary (Fos *et al.,* 2000).

Most of the genes encoding all those enzymes have been cloned in many plant species (Hedden and Kamiya, 1997; Hedden and Phillips, 2000; Sponsel and Hedden, 2004) and their expression is regulated by endogenous and environmental factors (Yamaguchi and Kamiya, 2000; Garcìa-Martìnez and Gil, 2001). GA 20-oxidases, GA 3-oxidases, and GA 2-oxidases are 2-oxoglutarate dependent dioxygenases that have been found to be encoded by small gene families (e.g. in the case of Arabidopsis five GA20ox, four GA3ox, and seven GA2ox), whose expression is temporarily and developmentally regulated (Hedden and Phillips, 2000). The GA2ox family is particularly complex since it is composed of two classes differing in their substrate specificity, C19-Gas and C20-GAs, respectively (Schomburg *et al.,* 2003). In addition, some GA2ox enzymes using C19-GAs as substrates have multicatalytic activity, converting the GAs successively to 2b-hydroxylated metabolites and to GA catabolites (Serrani *et al.,* 2007a; Ubeda-Tomàs *et al.,* 2006).

Along with GA biosynthesis, enzymes involved in the final part of the metabolic chain have been reported as being rate limiting. In particular, the transcription of the GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) gene families, is developmentally regulated (García-Martínez *et al.,* 1997; Kang *et al.,* 1999; Rebers *et al.,* 1999; Ozga *et al.,* 2003b) and finely tuned by feedback (García-Martínez *et al.,* 1997), light (Kamiya and García-Martínez 1999; Hisamatsu *et al.,* 2005) and IAA-sensitive mechanisms (Ross *et al.,* 2000). An additional role in the regulation of GA biosynthesis has been shown for the KNOTTED-type homeobox (KNOX) transcription factors that directly suppress the transcription of GA20ox genes (Tanaka-Ueguchi *et al.,* 1998; *Hay et al.,* 2002).

1.2.4 Gibberellin signaling pathway in fruit set

The role of hormones in fruit set and development has mainly studied using biosynthetic and hormone perception mutants of *Arabidopsis*. More than 15 years ago, the *Arabidopsis spindly* (*spy*) mutant which confers hypersensitivity to GA was very helpful in understanding fruit set and development. This mutant was first identified by virtue of his resistance to both germination inhibiting and dwarfing effects of paclobutrazol, a gibberellin (GA) biosynthesis inhibitor (Jacobsen and Olsezewski, 1993). The Arabidopsis SPY protein negatively regulates the gibberellin signalling pathway. Compared to wild type, the *spy* mutant exhibits parthenocarpy and showed that the control of GA sensitivity can mimic the role of pollination and fertilization in fruit set and development. Recently Silverstone *et al.* (2007) showed that SPY is an O-linked N-acetylglucosamine (GlcNAc) transferase (OGT) with a protein-protein interaction domain consisting of 10 tetratricopeptide repeats (TPR).

Also the *Spindly* homologue from tomato has been isolated (Greb *et al.,* 2002) and it has been shown that in the tomato wild type its transcript level increases at anthesis and decreases again after pollination while in the tomato parthenocarpic mutant (*pat*) this increase did not occur (Olimpieri *et al.,* 2007). The KNOTTED-like homeobox (KNOX) genes, which might be direct repressors of *GA20ox* expression (Hay *et al.,* 2002), are also highly expressed at anthesis in wild-type plants and transcript levels decrease after pollination. In the tomato *parthenocarpic fruit-1* (*pat*) mutant, KNOX transcript levels already decrease prior to anthesis (Olimpieri *et al.,* 2007). These findings indicate that SPY and members of the KNOX-gene family might also act as negative regulators of fruit growth, directly repressing gibberellin signalling and biosynthesis, respectively, in unpollinated ovaries (Olimpieri *et al.,* 2007)

In addition to SPY, the DELLA proteins (REPRESSOR OF ga1-3 [RGA], GA INSENSITIVE [GAI], RGA-LIKE1 [RGL1], and RGL2) are also negative regulators of GA signalling in Arabidopsis (Olszewski *et al.,* 2002; Peng and Harberd, 2002). The DELLA proteins are thought to be nuclear transcriptional regulators. RGA and GAI play a major role in repressing stem elongation and floral induction (Dill and Sun, 2001; King *et al.,* 2001) and RGL2 is important in inhibiting seed germination (Lee *et al.,* 2002), whereas a combination of RGA, RGL1, and RGL2 modulates floral development (Cheng *et al.,* 2004; Tyler *et al.,* 2004; Yu *et al.,* 2004). It has been demonstrated that GA de-represses its signaling pathway by inactivating the DELLA proteins, and the conserved DELLA motif, which is

located near the N terminus of these proteins, is required for GA-dependent proteolysis of these repressors (Sun and Gluber, 2004). GA has been shown to induce degradation of RGA, GAI, and RGL2 (Silvestone *et al.,* 2001; Dill *et al.,* 2004; Tyler *et al.,* 2004), and mutant alleles that encode DELLA-motif-deleted DELLA proteins confer a GA-insensitive dwarf phenotype (Peng *et al.,* 1997; Dill *et al.,* 2001; Wen and Chang, 2002). This proteolysis event is likely to be target by an ubiquitin E3 ligase complex SCF^{SLY1} to the 26S proteasome (McGinnis *et al.,* 2003; Dill *et al.,* 2004; Fu *et al.,* 2004; Tyler *et al.,* 2004). The DELLA proteins and their function in GA signaling are conserved in plants (Thomas and Sun, 2004). For example, the DELLA proteins in barley (*Hordeum vulgare*; SLN1) and in rice (*Oryza sativa*; SRL1) are also GA signaling repressors and are responsive to GA-induced degradation by the ubiquitin-proteasome pathway (Fu *et al.,* 2002; Itoh *et al.,* 2002; Sasaki *et al.,* 2003). Mutations in the DELLA motif of SLN1 and SLR1 also confer GA-insensitive dwarf phenotype.

In tomato the reduction of *SlDELLA* mRNA levels induced the formation of parthenocarpic fruit (Dill *et al.,* 2001; Marti *et al.,* 2007). This fruit was smaller and had a more elongated shape than wild-type fruit. The pericarp contained fewer but bigger cells than wild type, which is similar to gibberellin-induced parthenocarpy fruit (Bunger-Kibler and Bangerth, 1983). Thus the reduced *SlDELLA* mRNA levels in the antisense lines may allow the release of repression of downstream proliferating factors involved in the gibberellin signaling pathway, which are normally induced after successful pollination and fertilization (Marti *et al.,* 2007). Thus, also in tomato, SlDELLA appears to be a negative regulator of fruit set by restraining the gibberellin signal and thereby preventing ovary growth prior to pollination and fertilization (de Jong *et al.,* 2009).

1.2.5 The fruit set at transcriptome level

The fruit set and early fruit development have also been studied at the transcriptome level. To identify the key steps and processes in tomato carpel development and fruit set, Pascual *et al.* (2009) analyzed the carpel transcriptome at four different stages (bud, bud to pre-anthesis, anthesis and 3 DPA) in a control and a facultative parthenocarpic line (*pat-3/pat-4*). Comparison between array experiments determined that anthesis was the most different stage during flower development and the key point at which most of the genes were modulated. 758 genes were differently regulated in parthenocarpic fruit set. The most

significant differences were found in cell cycle related genes. Cell cycle was not stopped at anthesis in the parthenocarpic line, contrary to normal development where carpels remain in a state of temporary dormancy, waiting for pollination signals. GA and ethylene synthesis key genes were activated in the parthenocarpic , and some aux/IAA gene expression was also altered despite the lack of differences in the auxin metabolism (Nuez *et al.,* 2009).

In light of the fact that plant hormone action and fruit set are tightly linked, transcriptomes were compared also from tomato pollinated ovaries and GA-treated ovaries (Vriezen *et al.,* 2008). Gibberellin induces fruit set without seeds, which makes it possible to identify a set of genes that can induce fruit growth independently of pollination or fertilization. As it could be expected, pollination triggered genes that were not all triggered after the application of GA_3 and vice versa. GA_3 induced genes involved in the cell cycle more rapidly than the pollination. The reason of this difference can be explained by the time that pollen tubes require to reach the ovules, what would suggest that the induction of fruit growth does not occur prior to fertilization and that the substances, which are present in the pollen, are only released after the pollen tubes have reached the ovules (Vriezen *et al.,* 2008). The pollination led to the induction of GA biosynthesis, whereas GA_3 application did not (Vriezen *et al.,* 2008). This can be explained by the negative feedback of GA in many plants on its own biosynthesis (Hedden and Kamiya, 1997). Pollination exerted a profound effect on GA biosynthesis, possibly via auxin signaling, conversely the expression of auxin signaling genes seemed not be greatly influenced by GA treatment. In fact two ARFs genes were modulated during fruit set after pollination but not after GA_3 treatment. Similarly the AUX/IAA genes such as the tomato *IAA2* and *IAA14* appeared only to be induced after pollination and specifically in the placenta with ovule samples (Vriezen *et al.,* 2008). In contrast with previous model for the fruit set, the expression of these genes indicate that pollination induces auxin signaling. Auxin signaling as a consequence of the pollination event might induce GA biosynthesis but GA, in turn, does not modulate the auxin signal (Vriezen *et al.,* 2008).

Furthermore the gene expression profiles obtained suggest that, in addition to auxin and gibberellin, ethylene and abscisic acid (ABA) are involved in regulating fruit set. Before fruit development, many genes involved in biotic and abiotic responses are active in the ovary. In addition, genes involved in ethylene and ABA biosynthesis were strongly expressed, suggesting relatively high ethylene and ABA concentrations before fruit set. Induction of fruit development, either by pollination or by gibberellin application, attenuated expression

of all ethylene and ABA biosynthesis and response genes within 24 h. It is proposed that the function of ABA and ethylene in fruit set might be antagonistic to that of auxin and gibberellin in order to keep the ovary in a temporally protected and dormant state; either to protect the ovary tissue or to prevent fruit development before pollination and fertilization occur (Vriezen *et al.,* 2008).

1.3 Parthenocarpy in tomato

Tomato (*Solanum lycopersicum* L.),has a self-fertilizing mode of reproduction with wind as the primary pollination stimulus. Normally, the tomato plant produces seeded fruits after pollination and fertilization. However, these processes depend on narrow environmental constrains (Picken 1984). Good pollen production is permitted by night temperatures ranging between 15 and 21°C, and air circulation is necessary to ensure pollen shedding. Such conditions are not often met in unheated greenhouses or tunnels during winter or early spring cultivations. Parthenocarpy offers a method of dealing with the problem of poor fruit set by circumventing the temperature and wind-dependent fertilization process. Parthenocarpy is mainly used for crops where seedless fruits are desired by consumers, such as bananas, citrus, grapes, etc. (reviewed by Varoquaux *et al.,* 2000). The shelf life of seedless fruits is sometimes longer than seeded fruits because seeds produce hormones that triggers senescence. In spite of the advantages, parthenocarpy is still of limited use in agriculture. Mutations causing parthenocarpic fruits often have pleiotropic effects and are often associated with unfavourable characteristics, such as male of female sterility or smaller and misshapen fruits (Varoquaux *et al.,* 2000)

In tomato, parthenocarpy can be genetic or can be induced artificially with the application of various hormones. Genetic parthenocarpy can be artificially achieved with exogenous genes, which, through ovary and/or ovule specific promoters, drive the over-sensitisation to or the accumulation of auxins in carpel tissues before anthesis or different tomato lines carrying mutations for parthenocarpy have been discovered or selected (reviewed by Lukyanenko 1991; Gorguet *et al.,* 2005).

1.3.1 Artificial parthenocarpy in tomato

Growth regulators, such as synthetic auxins, are used by tomato growers to induce parthenocarpic fruit set in some production regions. However, these agricultural practices add extra costs to the production, and fruit defects may be a problem when auxins or other growth regulators are applied (Schwabe and Mills 1981).

Gustafson (1936) was the first to demonstrate that the application of substances closely related to auxins onto the stigma of tomato causes the ovary to develop into parthenocarpy fruit. The application of pollen extracts to the outside the ovary showed similar results, which led to the hypothesis that pollen grains contain plant hormones similar to the growth substance auxin. Wittwer *et al.* (1957) showed that a second type of growth substance, gibberellins (Gas), can also stimulate parthenocarpy fruit set.

However, the application of GA and auxin induces different morphological and histological development of tissue ovaries. For instance, whereas parthenocarpic growth induced by auxin is associated with more cell divisions in the mesocarp, GA-induced fruits have much larger mesocarp cells (Serrani *et al.,* 2007a). Also, the presence of pseudo-embryos with unknown function in auxin- but not in GA-induced fruits has been reported (Serrani *et al.,* 2007a).

1.3.2 Genetic engineering of parthenocarpy in tomato

The large number of patents on genetic engineering of parthenocarpy clearly shows that seedless fruits are commercially very interesting.

The first success has been obtained in tobacco and eggplant with the introduction of the *iaaM* gene under control of the placenta and ovule specific DefH9 promoter from *Anthirrhum majus* (Rotino *et al.,* 1997). The *iaaM* gene codes for a tryptophan monoxigenase that converts tryptophan to indolacetamide. Indolacetamide is converted, either chemically or by plants hydrolases to IAA, the main form of auxin in plants. In the same way, Ficcadenti *et al.* (1999) obtained parthenocarpic plants in two different tomato genotypes; in general, these plants didn't significantly differ from the control for all the characteristics considered (fruit weight, pH, skin colour and flesh consistency) , except for the parthenocarpic trait (Ficcadenti *et al.,* 1999). However, the expression of the transgene can be too high in tomato cultivars which are more sensitive to high levels of auxins. As a

consequence, malformations of the fruits can occur, comparable with the effects of excess exogenous auxin or by higher sensitivity to hormonal treatment (Pandolfini *et al.,* 2002). If necessary, the expression of the transgene can be reduced by modifying the 5' ULR (Untraslated Leader Region) of the insert. The 53 nucleotides upstream of the AUG initiation codon were replaced by an 87 nucleotide-long sequence derived from the *rolA* intron, that reduced *rolA* gene action in *Arabidopsis* and tobacco (Magrelli *et al.,* 1994; Spena and Langekemper, 1997) and originates from *A.rhizogenes*. After this substitution, the *iaaM* mRNA was translated three to four times less efficiently, and the flower buds of the new trasformant contained five time less auxin than the original trasformant. This reduction resulted in a normal fruit shape and showed that is possible to produce high-quality parthenocarpic tomato fruits cultivars with a high sensitivity to hormonal treatment, such as field-grown tomatoes (Pandolfini *et al.,* 2002).

An alternative approach for the induction of parthenocarpy is based on ovary-specific expression of the *Agrobacterium rhizogenes*-derived gene *rolB* (Carmi *et al.,* 2003). Although it's not clear the *rolB* function it was chosen because *rolB* transgenic plants manifest several syndromes characteristic of auxin treatment. Tomato plants transformed with a chimeric construct containing the *rolB* gene fused to the ovary and young-fruit specific promoter TPRP-F1 developed parthenocarpic fruits. Fruit size and morphology, including jelly fill in the locules of the seedless fruits, were comparable to those of seeded fruits of the parental line. However, the reduction of flower development and the substantial decrease in the number of fruits per truss resulted in a decrease in yield (Carmi *et al.,* 2003). Although it is not known whether ROLB signals for the same cassette of genes involved in fertilization-dependent fruit development, it clearly activates a battery of genes that enable successful completion of seedless fruit development. This lets suppose that this gene can also be used in other species where synthetic auxin is used to increase fruits set.

The rolB-based parthenocarpy differs from the iaaM-controlled one in two aspects. First, biosynthesis of optimal levels of IAA depends on adequate spatial and temporal expression of endogenous hydrolases for which indolacetamide is a substrate, while the effect of ROLB does not seem to depend on any special endogenous gene-expression. Second, because ROLB acts autonomously in the cells in which it is expressed there is less risk of spreading of the auxin-like effects to neighboring organs (Carmi *et al.,* 2003). Indeed high expression of *iaaM* in the ovules led to development of malformed fruit (Pandolfini *et al.,* 2002).

Developing parthenocarpic cultivars, with the help of genetic engineering, seems to have more potential than traditional breeding. This is due to the presence of numerous possible approaches with combinations of genes and promoters, while traditional breeding relies on a few parthenocarpic genes with deleterious pleiotropic effects (Gorguet *et al.,* 2005).

1.3.3 Main sources of natural parthenocarpy in tomato

Different tomato lines carrying mutations for parthenocarpy have been discovered or selected (reviewed by Lukyanenko 1991 and Gorguet *et al.,* 2005); among these genes, the most interesting for breeding purposes are:

1) *parthenocarpic fruit* (*pat*; Soressi and Salamini 1975)
2) *parthenocarpic fruit-2* (*pat-2*; Philouze and Maisonneuve 1978)
3) *parthenocarpic fruit-3/4 (pat 3/4*; Philouze, 1983b, 1985)

Pat gene

The *pat* gene, the object of part of this research, was obtained by mutagenesis with ethyl methanesulfonate. This mutant is characterized by a parthenocarpic phenotype with high penetrance and expressivity, that also entails earlier ripening and enhanced fruit quality (Falavigna *et al.,* 1978). Anyway, the expressivity of the mutation can be reduced by high night temperatures and by certain genetic backgrounds (Mazzucato *et al.,* 1999).

In the *pat* mutant, the ovary growth is triggered before anthesis while in WT ovaries it starts only two days after anthesis, pollination has occurred and fertilization has taken place (Mazzucato *et al.,* 1998). In fact 1-3 days before anthesis, *pat* ovaries are significantly bigger than WT ovaries of the same stage.

As a pleiotropic effect, *pat* flowers have aberrant androecia and ovules, and therefore reduced male and female fertility The aberrations described for male *pat* organs show similarities with those found in some male-sterile tomato mutants (*sl*, *sl-2*, *ms15*, *ms33*) which produce shrunken stamens, bearing carpel-like structures and often external ovules. *pat* is the first defective ovule mutant described in tomato: it seems to be dependent on disruption of the normal integument growth (Mazzucato *et al.,* 1998). Most of the ovule mutants described in literature appear to also involve disruptions of the integuments however none of them showed parthenocarpy.

However these don't seem the only reasons for seed infertility because hand-pollination fails to restore seed set on *pat* ovaries where a fraction of the ovules are still morphologically normal. Inside the ovary of *pat* plants, pollen tubes appeared disoriented; they wandered about in the ovarian cavity and often lost their adherence to the placental surface. So also "normal" ovules cannot guide pollen tubes to their micropyle in the altered *pat* ovary because adhesion molecules are not properly arrayed on a placenta that is already preparing for cell division or, alternatively, chemotropic signals in the *pat* ovary may be altered by the presence of aberrant ovules, which are not simply devoid of attractiveness, but disrupt pollen tube guidance overall. Therefore, even in conditions favourable for seed production, *pat* genotypes give a very low seed set (Mazzucato *et al.,* 2003).

Successively, other phenotypic characteristics have been described in the *pat* mutant that differ from the WT (defects in cotyledon number and morphology, higher frequency of compound inflorescences, earlier flowering time, lower number of flowers per inflorescence and increased number of carpels per ovary) but that are in common to GA-overdosed plants. Also the retained responsiveness to the GA-bosynthesis inhibitor PAC indicates that the *pat* phenotype is, at least in part, mediated by an increased GA signal. Expression analysis of genes encoding key enzymes involved in GA biosynthesis shows that transcriptional regulation of *GA20ox1* mediates pollination-induced fruit set in tomato and that parthenocarpy in *pat* results from the miss-regulation of this mechanism. As genes involved in the control of GA synthesis (KNOX transcription factors *LeT6*, *LeT12* and *LeCUC2*) and response (*SPY*) are also altered in the *pat* ovary, it is suggested that the *pat* mutation affects a regulatory gene located upstream of the control of fruit set exerted by GAs (Olimpieri *et al.,* 2007).

The temporal succession of abnormalities in the generative development of *pat* plants suggests that parthenocarpy could be an induced, secondary effect of a mutated gene, whose primary function is to regulate floral organ development (Mazzucato *et al.,* 1998). Anther aberrancy, and particularly the occurrence of adaxial carpel-like structures bearing external ovules, seem to indicate that *pat* is a mutation of a putative gene with homeotic functions. The *Pat* gene has been mapped on the long arm of chromosome 3 (Beraldi *et al.,* 2004) allowing its discrimination whit mapped genes that confer similar phenotype and opening the way towards cloning the gene. Genetic analysis and mapping information showed in fact that the gene underlying the *pat* mutation is not allelic to either of the two tomato mutations putatively involved in B function, *stamless (sl)-2* (allelic with *sl-1* and

ortholog of *DEFICIENS*; Hafen and Stevenson 1958, Gomez *et al.,* 1999; Mazzucato *et al.,* 2008) and *pistillate* (Goto et al *al*., 1994) or to genes encoding class B transcription factors. (Mazzucato *et al.,* 2008).

It has been suggested (Mazzucato *et al.,* 2008) that the PAT protein positively regulates the activity of members of the repressing machinery such as homeobox (KNOX; Olimpieri *et al.,* 2007) and class B MADS box (DEF; Mazzucato *et al.,* 2008) transcription factors. The relationship between PAT and other characterized repressors of the ovary, such as members of the auxin/IAA9 (Wang *et al.,* 2005) and ARF8 (Goetz *et al.,* 2007) gene families or other as yet unknown. Pollination and pollination-induced signals as well as mutations located upstream (as *pat*), destabilize the repressing system and give rise to fruit initiation (Mazzucato *et al.,* 2008). Whereas this model could be extended to the control of fruit set in other species, it seems evident that species specific mechanisms have evolved based on functional differentiation of class B genes (Zahn *et al.,* 2005).

Pat-2

Another source of parthenocarpy has been found in the tomato cultivar “Severianin”. Philouze and Maisonneuve (1978) and Nuez *et al.,* (1986) showed that a single recessive *pat-2* gene was responsible for parthenocarpy in “Severianin”. It is of particular interest because of its strong expressivity, its facultative character, and its simple genetic control (Philouze *et al.,* 1978). The capability of cv Severianin to set seedless fruits with complete locule fill under unfavorable environmental conditions is mainly due to the *pat-2* (Philouze and Maisonneuve, 1978; Nuez *et al.,* 1986; Vardy *et al.,* 1989), which induces a different protein pattern in the ovary after anthesis (Barg *et al.,* 1990). The analysis by two-dimensional PAGE of *in vitro* translation products of RNAs from flowers and ovaries before anthesis also shows a differential expression associated to *pat-2*-induced parthenocarpy. This suggests that the molecular events observed in *pat-2* ovaries before anthesis, which are associated with parthenocarpic fruit growth, may modify the hormone content of the ovary before pollination. (Fos and Nuez,1996; Fos and Nuez, 1997).

The inhibition of fruit-set by paclobutrazol and its reversal by GA3 suggested that fruit growth of both seeded wild-type (wt) and parthenocarpic *pat-2* tomato depends on GAs. The quantification of endogenous GA levels in developing tomato ovaries showed the accumulation of very large amounts of GA20 in *pat-2* ovaries before anthesis. However, GA20 may not be active per se, because the application of an inhibitor of 2-oxoglutarate-dependent dioxygenases decreased parthenocarpic fruit development. These results

suggest that the parthenocarpic capability of *pat-2* ovaries may be the result of the accumulation of GA20, leading to an early higher synthesis of active GA in the absence of pollination and fertilization (Fos *et al.,* 2000). Accordingly, *SlGA20ox1* was found to be expressed at high levels throughout ovary development and fruit growth in the *pat* and *pat-2* mutant (Olimpieri *et al.,* 2007; Serrani *et al*., 2007a).

Pat3-4

The *pat3/pat4* system (RP75/59) was described in a progeny from a cross between *Atom x Bubjekosko.* Studies of RP75/59 have finally led to the acceptance of a genetic model with two genes, *pat3* and *pat4* (Nuez *et al.,* 1986).

The comparison of in vitro translation products of mRNA from flowers and unpollinated ovaries before anthesis of *pat-2* and *pat3/pat-4* near isogenic lines shows similar differential expression patterns compared with wild type (Fos and Nuez 1996, 1997). This suggests that both parthenocarpic genetic systems may have a common or confluent molecular and physiological basis associated with the parthenocarpic fruit-set in tomato (Fos *et al.,* 2001).

Fos *et al.* (2001) investigated the role of gibberellins (GAs) in the induction of parthenocarpic fruit-set using wild type (Cuarenteno) and near-isogenic line derived form the German lone RP75/59 (the source of *pat-3/pat-4* parthenocarpy) The inhibition of fruit-set by paclobutrazol and its reversal by GA3 suggested that fruit growth of the parthenocarpic *pat-3/pat-4* tomato depends on GAs. The quantification of endogenous GAs showed that *pat-3/pat-4* developing ovaries contained higher content of GA_1 and GA_3 in the ovaries before anthesis. These results suggest that the parthenocarpic capability of pat-3 :pat-4 ovaries is the result of higher content of active GAs in the absence of pollination and fertilization (Fos *et al.,* 2001). The change of GA metabolism produced by *pat-3/pat-4* is different to that found in *pat-2* that is associated to an increase of GA_{20} and GA_1 (Fos *et al.,* 2000) and *pat* that is associated to GA_{20} (Olimpieri *et al.,* 2007).

So also the parthenocarpic phenotype of the *pat-3/pat-4* tomato mutant depends on gibberellin, but in contrast to the *pat* and *pat-2* mutants, the entire 13- hydroxylation pathway is enhanced, resulting in a high content of GA1 and GA3 in the ovary before pollination (Fos *et al.,* 2001).

1.4 Aim of the work

Tomato, *Solanum lycopersicum* L., has a self-fertilizing mode of reproduction with wind as the primary pollination stimulus. Normally, the tomato plant produces seeded fruits after pollination and fertilization. However, these processes depend on narrow environmental constrains (Picken, 1984). Good pollen production is permitted by night temperatures ranging between 15 and 21_C, and air circulation is necessary to ensure pollen shedding. Such conditions are not often met in unheated greenhouses or tunnels during winter or early spring cultivations. Parthenocarpy, which is the formation of seedless fruits in the absence of functional pollination or other stimuli (Gustafson 1942), offers a method of dealing with the problem of poor fruit set by circumventing the temperature and wind-dependent fertilization process.

The importance of parthenocarpic mutations is due to their possible use in breeding programs, but also in studies aimed to the comprehension of mechanisms underlying the fruit set, in fact, it would provide information on factors regulating fruit and seed formation, and thus open new perspectives for yield improvements by biotechnological means.

The goals of this research are:

- to identify the gene responsible of the *parthenocarpic fruit* (*pat;* Bianchi and Soressi 1969) phenotype through positional cloning: the *pat* mutation induces parthenocarpy with strong expressivity along with other pleiotropic effects, such as short anthers and aberrant ovules (Mazzucato *et al.,* 1998). This will allow an improvement in the understanding of the molecular events underlying the fruit set as well as the possibility to easily transfer the parthenocarpic trait, through non-transgenic tools, in different tomato genetic backgrounds.
- to identify tomato genotypes carrying mutations in the *SlIAA9* (Wang *et al.,* 2005) coding sequence through TILLING. *SlIAA9* gene, a member of the Aux/IAA family of transcription factors in tomato, has been described as playing a major role in the ovary repressor machinery and plants silenced by antisense showed a parthenocarpic behaviour (Wang *et al.,* 2005). TILLING is used to develop new parthenocarpic lines by introducing and identifying novel genetic variation in the *SlIAA9* gene.

2. Genetic and physical mapping of the tomato *parthenocarpic fruit (pat)* mutation

Different genes able to confer parthenocarpy have been described in tomato, even though none of them has been molecularly characterized yet. The *pat* mutation (Bianchi & Soressi, 1969), induces parthenocarpy with strong expressivity along with other pleiotropic effects such as short, narrow anthers and aberrant ovules (Mazzucato *et al.,* 1998). The importance of the *pat* mutation is due to its possible use in breeding programs, as well as in studies aimed to the comprehension of mechanisms underlying the fruit set. An understanding of the molecular events underlying parthenocarpy, in fact, would provide information on factors regulating fruit and seed formation, and thus open new perspectives for yield improvements by biotechnological means.

In order to map the *Pat* locus, two populations segregating from the interspecific cross *S. lycopersicum x S. pennellii* were grown and, and the progeny plants were classified as parthenocarpic or wild-type by taking into account some characteristic aberrations affecting mutant anthers and ovules. Through Bulk Segregant Analysis, the *Pat* gene was mapped on the long arm of chromosome 3, between two conserved ortholog set (COS) markers (COSes; Fulton *et al.,* 2002), T0796 and T1143 (Beraldi *et al*., 2004; Fig. 2), previously anchored on the genetic tomato map (EXPEN 2000, www.sgn.cornell.edu).

The present chapter describes how new molecular markers linked to the tomato *parthenocarpic fruit* (*pat*) gene were developed in order to refine its map position and to isolate it through positional cloning.

2.1 Materials & Methods

2.1.1 Plant materials: mapping population

Two populations, a BC_1F_1 and a F_2 respectively of 625 and 664 individuals, derived from the interspecific cross between *Solanum lycopersicum* L. line homozygous for the *pat* mutation (cv. Chico III genetic background) and a *Solanum pennellii* L. (TGRC accession number LA716) plant (homozygous for the wild type allele, WT), previously built to locate

Pat gene on chromosome 3 (Beraldi *et al.,* 2004) were used to map the new molecular markers.

To validate the map position of markers putatively linked to the target locus a set of *S. pennellii* alien substitution lines (ASLs) for chromosome 1, 2, 3, 4, 6, 8 and 11 (accession number LA2091, LA1639, LA1640, LA3469, LA3142, LA1642 and LA1643, respectively; Rick 1969) and a set of *S. pennellii* introgressioin lines (ILs; Eshed and Zamir, 1991) were used.

2.1.2 DNA extraction and molecular marker development through microsynteny with Arabidopsis

Total DNA was extracted from the parental lines of the mapping population, *S. lycopersicum and S. pennellii*, according to Doyle and Doyle (1990), using about 200 mg of fresh tissue (leaves and inflorescences) collected in a 1.5-ml Eppendorf tube.

By exploiting the microsynteny existing between Arabidopsis an the tomato genomes, a total of 54 Arabidopsis gene sequences, 23 surrounding COS marker T1143 on the BAC T2O4 and 31 surrounding COS marker T0796 on the BAC F19K16, were identified. They were named from T2O4-1 to T2O4-23 and from F19K16-1 to F19K16-31 considering their position respectively on BAC T2O4 and F19K16 (Fig. 2-1).

The 47 Arabidopsis sequences (The Arabidopsis Information resource; www.arabidopsis.org), were compared with those of tomato at *Sol* Genomics Network (SGN; www.sgn.cornell.edu) using the BLAST2 package and the BLASTX and BLASTN algorithms (Altschul *et al.,* 1990). Additional search was done in the GenBank/ EMBL/DDBJ EST Division and in the TIGR Tomato Gene Index (www.tigr.org). In this way, 21 tomato Tentative Consensus or Expressed Tagged Sequences were identified (Tab1)

DNA of *S. lycopersicum (*cv.M82) and *S. pennellii* (TGRC accession number LA716) were amplified with primers specific for each of the 21 tomato Tentative Consensus (TCs) or Expressed Tagged Sequences highlighted (Tab.2-1 a and b). Amplifications were made in a total volume of 25 ml, using 50 ng of DNA, 2.5 ml of 10x polymerase chain reaction (PCR) buffer, 2 ml of 100 mM dNTPs, 1.5 ml of 25 mM MgCl2, 50 pmol of each of the two primers and 1 U Taq DNA polymerase (Pharmacia Biotech, San Francisco, CA). After a denaturation step of 95°C for 4 min, amplification was carried out for 30 cycles of 95°C for

1 min, 60°C for 1 min and 72°C for 2 min, followed by 72°C for 7 min. Primer sequences are reported in Tab.2-1 a and b.

The PCR products were resolved by electrophoresis on 1% (w/v) agarose gel and stained with ethidiumbromide. If a length (in/del) polymorphism between S. *lycopersicum* (cv. Chico III) and *S. pennellii* was not found, cleaved amplified polymorphic sequences (CAPS) polymorphisms were searched using several 4- and 6-cutter restriction enzymes. The CAPS technique was performed by digesting 10 ml of the PCR reaction with 5 U of the appropriate enzyme (see Tab. 2-1a and b). Restriction fragments were resolved on 2.5% (w/v) agarose gels.

Once a polymorphism was detected, its map position was addressed by screening a set of alien substitution lines (ASLs) for chromosome 1, 2, 3, 4, 6, 8 and 11 and a set of *S. pennellii* introgression lines (ILs) covering the whole tomato genome (Eshed and Zamir 1995). The map position of the markers located on the long arm of chromosome 3 was refined by testing the recombination rate in the mapping populations for the *Pat* gene.

Tab. 2-1. Primer sequences and PCR reaction parameters for tomato orthologue TC to the Arabidopsis genes on BAC T2O4 (a) and on BAC F19K16 (b).

(a)

Name	At Unigenes	Tomato orthologue TC	Primer sequences (5' – 3')	PCR product length (bp)	Annealing temperature (°C)	No. of cycles
T2O4-2	At3g16490.1	TC118813	5'CACCTCTTGGAACGCATG3' 5'TTGTTGCCTGTTTAGCACGG3'	916	58.0	32
T2O4-3	At3g16480.1	TC123797	5'CATTTACGGTGATGGACTGGG3' 5'AAAGCCTACAATCTGCCCACC3'	512	58.4	32
T2O4-5	At3g16460.1	TC126926	5'GGTGGAAGTGGAGGGAATGTC3' 5'TTTGGCGGAGCTAAAGATGG3'	691	58.7	32
T2O4-10	At3g16410.1	TC129481	5'GAAGGCAAATGGATCAAGCTG3' 5'CATCAAGCTCATCACCGGAAA3'	585	58.7	32
T2O4-13	At3g16380.1	TC116029	5'TTTCGGTTAGGGTTTGCAGG3' 5'TTTCCATTCATCTCTGCGAGC3'	955	55.0	32
T2O4-14	At3g16370.1	TC123815	5'GGAGAATGGGCATCTACAA3' 5'CCTTTAGGATGGATTCCG3'	1100/900	57.0	32
T2O4-15	At3g16360	TC227544	5'GAGAAGCCTCTTGGATCAGGG3' 5'TGATGGGCTTGTTCACTTCG3'	427	60.5	32
T2O4-16	At3g16350.1	TC225793	5'CCAGTCGAAGTGGAGGTGG3' 5'AACGGATTGTTGCTTTGCATC3'	642	56	32
T2O4-17	At3g16340.1	TC117501	5'TTAGGCCAGGCGTATTGACAG3' 5'CCATTGTTTCCAGAAGCAGGA3'	902	56.9	32
T2O4-18	At3g16330.1	TC117493	5'ATGAGGCAATCTTCACCTGG3' 5'TTCAGCTGATAGTTCCTGCG3'	172/179	57.0	32
T2O4-19	At3g16320.1	TC117278	5'GTTGAGCTATCTGGCGCAGGTG3' 5'GCCTGGTATGGGCTTGGAATG3'	594	59.1	32
T2O4-20	At3g16310.1	TC174773	5'CTGTTTCTGCTTTATGGCGTGA3' 5'CTGACGTTGTGTTGGATCCACT3'	677	59.0	32
T2O4-21	At3g16290.1	TC235589	5'CCAGACATATTGGACCCAGCA3' 5'ACCAGAGCTCATCAGCAGCA3'	550	59.7	32
T2O4-22	At3g16280.1	TC240810	5'TGGATTCAAGCAGGAGGGC3' 5'TCCACCGGAGGTTGCAATA3'	766	61.4	32

(b)

Name	At Unigenes	Tomato orthologue TC	Primer sequences (5' – 3') Forward and reverse	PCR product length (bp)	Annealing temperature (°C)	No. of cycles
F19K16-1	AT1G80030.1	TC127369	5'GCTGCTTGCCTTTCTGACTGG3' 5'CGTGGATCCATTACAACGGC3'	653	59,0	32
F19K16-5	AT1G79990.1	TC226741	5'TCTATGGAACGGCAGTGTGG3' 5'AACAATGCCATCACCGCA3'	385	57,0	32
F19K16-8	AT1G79960.1	TC245138	5'TTTGCTCGCGTTGTACCAGA3' 5'ATGACGAATGACACGGCCC3'	515	59,5	32
F19K16-9	AT1G79950.1	TC119374	5'GGGAAATAATGTCAGGCGTGA3' 5'GGGTCATGTCCAGGAGGTTCT3'	598	59,3	32
F19K16-10	AT1G79940.1	TC116675	5'TTGCTGGCTTCAAGAAAGGC3' 5'AGTTTGGCCCTTTCACCCTT3'	805	58,0	32
F19K16-16	AT1G79880.1	TC116946	5'TCGGAGTATTGATGGCCTGTC3' 5'TCAACATGAGGACCACGGC3'	701	57,0	32
F19K16-19	AT1G79850.1	TC22118	5'GCCCAATGGCTACAGCAAA3' 5'GAGAATCCCGAAGGAAGCAAA3'	723	55,0	32
F19K16-20	AT1G79840.1	TC28688	5'TGCTCTCACCAAAGAAAGGGA3' 5'TCCTCACATCGACGTTCACTG3'	588	58,8	32
F19K16-26	AT1G79770.1	TC116115	5'TGGTGAGGATAGTGCCACAGA3' 5'AGTTTAATGCGGCCATCAGC3'	878	58,6	32

2.1.3 Genetic mapping

The software package JoinMap 3.0 (van Ooijen and Voorrips, 2001) was used to perform the linkage analysis between the molecular markers and the *Pat* locus, and to integrate the map distances of the two mapping populations: a LOD score of 3.0 or above was specified. The Kosambi mapping function was used to convert recombination frequencies into map distances.

2.1.4 Physical mapping: BAC DNA extraction and molecular marker analysis

Contigs 6859 and 1042, used in the "Tomato Genome Project sequencing chromosomes", were identified at published physic map (SGN; www.sgn.cornell.edu) because containing Bacterial Artificial Chromosomes (BAC) matching respectively with COS markers T0796 and T1143.

BAC singletons whose end sequences had a high identity value (>95%) with either the end sequences or the complete sequences of the BACs belonging to the contigs 6859 or 1042 were also identified using the BLAST2 package.

All the highlighted BACs were requested at SGN as stabs. The recombinant BAC clones, constructed with the Hind III/MboI/EcoRI-pBeloBAC11 vector and hosted in the bacterial strain DH10B, were grown in 10 ml of LB (Luria-Bertani) medium with 12 µg/ml of chloramphenicol as selective antibiotic. The strains were stored at –80° C after adding ½ vol of sterile glycerol. The BAC clones DNA was extracted and purified using the QIAGEN Plasmid Midi Kit (QIAGEN Inc., Valencia, CA, USA). Bacterial lysates were cleared by centrifugation. The cleared lysates were then loaded onto a anion-exchange tip where BAC clone DNA selectively bound under appropriate low-salt and pH conditions. RNA, proteins, metabolites, and other low-molecular-weight impurities were removed by a medium-salt wash, and ultrapure plasmid DNA was eluted in high-salt buffer. The DNA was concentrated and desalted by isopropanol precipitation and collected by centrifugation. After a wash with 500 µl of 70% ethanol, the vacuum dried DNA pellets were dissolved in 50 µl of sterile water. DNA concentration was determined by spectrophotometric measuring at OD260 and final concentration was adjusted at about 200 ng/µl. Samples were stored at –20° C. Amplifications on the BAC clones were conducted in the same way as for the plant DNA (see above).

2.1.5 Physical vs genetic mapping

End sequences of the BACs, confirmed to be located on the *Pat* locus region through markers matching, have been used to develop new molecular markers.

Specific primers have be designed based on the published sequences at SGN (Tab. 2-2 www.sgn.cornell.edu). The PCR products were resolved by electrophoresis on 1% (w/v) agarose gel and stained with ethidium bromide. If a length (in/del) polymorphism between *S. lycopersicum (cv*. Chico III) and *S. pennellii* was not found, cleaved amplified polymorphic sequences (CAPS) polymorphisms were searched using several 4- and 6-cutter restriction enzymes. The CAPS technique was performed by digesting 10 ml of the PCR reaction with 5 U of the appropriate enzyme (Table 2-2). Restriction fragments were resolved on 2.5% (w/v) agarose gels.

Once a polymorphism was detected, its map position was addressed by screening a set of *S. pennellii* introgression lines (ILs) covering the whole tomato genome (Eshed and Zamir, 1995).

Tab. 2-2. Primer sequences and PCR reaction parameters for BAC_end sequences

Arabidopsis BAC name	End sequences	Primer sequences (5' – 3')	PCR product length (bp)	Annealing temperature (°C)	No. of cycles
Hba004D04	T7	5'ACTTCTGCCTTCCACATGTTG3' 5'CACATGTTGCGACAATATAGCAC3'	328	58	32
Hba210F21	Sp6	5'TGTTTGGGAGACATCACGG3' 5'CCACAGCCAATATGTTTCCTG3'	477	55	32
MboI0001K18	T7	5'GATTATATGAATCAATCACC3' 5'TATCATGTCTGATGGCCAGC3'	266	55	32
Hba0228N05	T7	5'TAATGACTTTGATATAGAGC3' 5'ACTACAAACTCTTACACCAAC3'	620	56	32
EcoRI0092J03	Sp6	5'TATTAGCAGATGAATGAGC3' 5'ACAATATTACTTGCTAGGACG3'	424	55	32
Hba0025G09	Sp6	5'TATGCAGGCCCATTAGGTCG3' 5'GTTATAGTCTGGACTTCTCC3'	524	58	32

2.2 Results & Discussion

2.2.1 Tomato-Arabidopsis microsynteny: development of new COS markers

Novel PCR-derived COS markers have been developed and mapped inside the target window for the *Pat* gene by pursuing the microsynteny between tomato and Arabidopsis (Fulton *et al*., 2002).

Two unlinked Arabidopsis BACs, T2O4 on chromosome 3 and F19K16 on chromosome 1, matching respectively with COS markers T1143 and T0796, previously associated to the *Pat* locus (Beraldi *et al.,* 2004), were selected at TAIR (The Arabidopsis Information Resources; www.arabidopsis.org). A total of 47 gene sequences, 24 surrounding COS marker T1143 on the BAC T2O4 and 23 surrounding COS marker T0796 on the BAC F19K16, were used to identify orthologue genes of tomato. Arabidopsis genes were named from T2O4-1 to T2O4-24 and from F19K16-1 to F19K16-31 considering their position respectively on BAC T2O4 and F19K16 (Fig. 2-1; Tab. 2-3a and b).

This procedure yielded a total of 21 tomato Tentative Consensus (TC) or Expressed Tag Sequences (EST) with high identity values (threshold: I>70%, P<42) with the Arabidopsis genes (Tab. 2-3).

PCR reactions gave a good amplification product on both the parental lines used to build the map populations, S. *lycopersicum* and *S. pennellii,* only for 15 of the 21 tomato TCs or EST sequences.

If a length (in/del) polymorphism between *S. lycopersicum* (cv. Chico III) and *S. pennellii* was not found, cleaved amplified polymorphic sequences (CAPS) polymorphisms were searched using several 4- and 6-cutter restriction enzymes.

T18 and T21 sequences showed a polymorphism in/del between the parental lines. Cleaved amplified polymorphic sequences (CAPS) polymorphisms were found on F19K16-1,5,10,16, 19, 20, 21 (=26) and T2O4-13, 14, 16, 17, 18, 19, 20, 21, 23 using several 4- and 6-cutter restriction enzymes. Such polymorphisms were confirmed in the mapping population by screening a small number of individuals.

The newly developed COS markers were named from T1 to T24 and from F1 to F21 depending on their position on respectively Arabidopsis BAC T2O4 and F19K16 (Tab. 2-3 a and b).

Marker position on tomato genome was first roughly tested by using a set of S*. pennellii* alien substitution lines (ASLs) for chromosome 1, 2, 3, 4, 6, 8 and 11 and a set of S*. pennellii* introgression lines (ILs; Eshed and Zamir, 1991). This way a total of six markers, F5, T17, T18, T19, T20 and T21 were positioned on the long arm of chromosomes 3 (Tab. 3 a and b).

Tab. 2-3. Arabidopsis genes (TAIR; www.arabidopsis.org) on BAC clone T2O4 (a) and F19K16 (b) and molecular markers developed from the orthologue tomato tentative consensus sequences. In red COS marker T1143 and T7096 (Beraldi *et al.,* 2004).

(a)

Genes on At T2O4 BA C	At unigenes	Tomato TC with identity ≥70%	Marker type	Marker name	Marker positioned on long arm of ch. 3
T2O4-1	At3g16500.1	-	-	-	-
T2O4-2	At3g16490.1	TC118813	-	-	-
T2O4-3	At3g16480.1	TC123797	-	-	-
T2O4-4	At3g16470.1	-	-	-	-
T2O4-5	At3g16460.1	TC126926	-	-	-
T2O4-6	At3g16450.1	-	-	-	-
T2O4-7	At3g16440.1	-	-	-	-
T2O4-8	At3g16430.1	-	-	-	-
T2O4-9	At3g16420.1	-	-	-	-
T2O4-10	At3g16410.1	TC129481	-	-	-
T2O4-11	At3g16400.1	-	-	-	-
T2O4-12	At3g16390.1	-	-	-	-
T2O4-13	At3g16380.1	TC116029	In/Del	T13	-
T2O4-14	At3g16370.1	TC123815	In/Del	T14	+
T2O4-15	At3g16360.1	TC227544	-	-	-
T2O4-16	At3g16350.1	TC225793	In/Del	T16	-
T2O4-17	At3g16340.1	TC117501	CAPS (*AluI*)	T17	+
T2O4-18	At3g16330.1	TC117493	SSR	T18	+
T2O4-19	At3g16320.1	TC125845	CAPS (*RsaI*)	T19	+
T2O4-20	At3g16310.1	TC117278	CAPS (*RsaI*)	T20	+
T2O4-21	At3g16290.1	TC119915	In/Del	T21	+
T2O4-22	At3g16280.1	TC240810	-	-	-
T2O4-23	At3g16270.1	-	-	-	-

(b)

Genes on At F19K16 BAC	At Unigenes	Tomato TC With identity ≥70%	Marker type	Marker name	Marker positioned on long arm of ch. 3
F19K16-1	At1g80030.1	TC127369	CAPS (*EcoRI*)	F1	-
F19K16-2	At1g80020.1	-	-	-	-
F19K16-3	At1g80010.1	-	-	-	-
F19K16-4	At1g80000.1	-	-	-	-
F19K16-5	At1g79990.1	TC226741	CAPS (*Eco RI*)	F5	+
F19K16-6	At1g79980.1	-	-	-	-
F19K16-7	At1g79970.1	-	-	-	-
F19K16-8	At1g79960.1	BI931700	-	-	-
F19K16-9	At1g79950.1	TC119374	-	-	-
F19K16-10	At1g79940.1	TC181762	CAPS (*Alu I*)	F10	-
F19K16-11	At1g79930.1	-	-	-	-
F19K16-12	At1g79920.1	-	-	-	-
F19K16-13	At1g79910.1	-	-	-	-
F19K16-14	At1g79900.1	-	-	-	-
F19K16-15	At1g79890.1	-	-	-	-
F19K16-16	At1g79880.1	TC116946	CAPS (*EcoRI*)	F16 = COS T0796	+
F19K16-17	At1g79870.1	-	-	-	-
F19K16-18	At1g79860.1	-	-	-	--
F19K16-19	At1g79840.1	TC22118	CAPS (EcoRI)	F19	-
F19K16-20	At1g79830.1	TC128688	-	-	-
F19K16-21	At1g79820.1	-	-	-	-
F19K16-22	At1g79810.1	-	-	-	-
F19K16-23	At1g79800.1	-	-	-	-
F19K16-24	At1g79800.1	-	-	-	-
F19K16-25	At1g79790.1	-	-	-	-
F19K16-26	At1g79780.1	TC116115	Bad presence/absence	F26	-
F19K16-27	At1g79770.1	-	-	-	-
F19K16-28	At1g79760.1	-	-	-	-
F19K16-29	At1g79750.1	-	-	-	-
F19K16-30	At1g79740.1	-	-	-	-
F19K16-31	At1g79730.1	-	-	-	-

2.2.2 Genetic linkage map construction

The new developed markers together with COS markers T1143 and T0796 were used to build a genetic linkage map of the *Pat* gene target region by testing their rate of recombination on the BC_1 and F_2 populations.

A microsatellite maker (SSR320), a RFLP marker (TG214), and two COSII markers (C2_At1g55170 and C2_At2g42110), mapped between T1143 and T0796 on the EXPEN

2000 linkage map (www.sgn.cornell.edu; Mueller *et al.,* 2005), have been also used. The genetic linkage map was constructed with JoinMap 3,0 (Van Ooijen and Voorrips 2001).
The order of the markers on the genetic target window is presented in Fig. 2-2.
The published markers SSR320 and C2_Atg42110 (below referred to as C2-42110) were confirmed to be located inside the target region between T0976 and T1143. On the contrary, markers TG214 and C2_At1g55170 mapped outside the target region (Fig 2-2).
Markers F5, T17, T18, T20 and T21 were mapped inside the target region between T0796 and T1143. Markers T19 was external to T0796 at distance of 0.07 cM. The polymorphism used to build in/del marker T18 is the same used in the published microsatellite marker SSR320 (Fig 2-2).
The whole data obtained allowed us to refine with new anchor-points the genetic region spanning 1.2 cM between COSes T0796 and T1143, and to restrict the target interval to 0.19 cM (Fig. 2-2) between marker T17 and T20. Markers T18 and C2-42110 co-segregate with the *pat* mutation in all the individuals of the mapping populations.
Such a procedure yielded 6 new markers starting from 47 genes of Arabidopsis that allowed us to find out 21 tomato orthologue genes (flow chart reported in Fig. 2-3a). The efficiency, in detecting such COS markers on the long arm of chromosome 3 was about 13% (Fig. 2-3b).
Besides, the new tomato COS markers developed from T2O4 Arabidopsis BAC clone showed a strict order conservation with those present in At. The microsynteny, the colinearity and similar expression in the ovary (data not shown) of these markers, suggest that the genes may share a common evolution pathway and functional patterning (Fig. 2-4)

2.2.3 Genetic vs physical mapping

COSes T0796 and T1143 display a clear hit with a number of tomato BACs belonging to two plausible unlinked contigs at the *HindIII*, *MboI* and *EcoRI* physical maps (www.sgn.cornell.edu; Mueller *et al.,* 2005).
Hence we have verified the occurrence of T0796 and T1143 on contigs 6859 and 1042 respectively and we carried out a matching test of the new COS markers on the two contigs. COS T5 was found to hit on BACs Hba210F21, Hba043F15 and Hba0177L08 pertaining to contig c6859 (Fig. 2-5). Since COSes T17, T18, T20 and T21 have matched

none of the tomato BACs, it was plausible that they reside in the gap comprised between the two contigs (Fig. 2-5).

With the aim to fill the gap between the two contigs 6859 and 1042, we searched for new BACs by blasting at SGN physical map both the end sequences and the eventual full sequences of the BACs contained in the contigs. Particularly, among different false positives, 4 interesting singletons BACs have been identified, three on the side of contig 6859 and one on the side of contig 10242 (Fig. 2-6).

The Sp6 end sequences of BACs EcoRI0092J03 (hereafter referred to as J03) and Hba0228N05 (referred as to N05) matched with BAC Hba043F15, a fully sequenced BAC belonging to contig 6859 (identity 100%). Furthermore, T7 end sequence of BAC MboI0001K18 (referred to as K18) overlapped with Sp6 sequence of BAC Hba210F21 (identity 100%), another BAC of same contig (Fig. 2-6).

On the other side, the Sp6 end of BAC Hba0025G09 (referred as G09) overlapped with the T7 sequence of BAC Hba166B19 (100% identity) (Fig. 2-6).

Hence we have verified through PCR analysis the occurrence of markers T17, T18 and T20 and T21 on the new retrieved BAC clones: T20 and T21 were identified on BACs K18, N05 and J03 whereas T17 on BAC G09 (Fig. 2-7); T18 did not report any matches (Fig 2-7).

The new BACs allowed us to narrow the physical map of about 200Kb in the whole, considering the estimated length at SGN of the added BACs K18, G09, N05 J03 and the two contigs (Fig. 2-6 and 2-7). The lack of a match of marker T18 on the new internal BACs demonstrates the permanence of a physical gap in the *Pat* target region.

The physical/genetic distance ratio in a genomic region of chromosome 3 proximal to the *Pat* locus, was estimated to be 123 Kb/cM on average (Mesbah *et al.,* 1999), six time lower than the mean calculated for the tomato genome (750 Kb/cM; Tanskley *et al.,* 1992). In our case we show that the estimated 1.2 cM *Pat* locus interval (Beraldi *et al.,* 2004) corresponds to at least 250 Kb (about 210 Kb/cM).

2.2.4 Physical vs genetic mapping

In order to confirm their map position, end sequences of some BACs pertaining to the contigs 6859 and 10242 were used to develop new markers inside the target window for the *Pat* gene. Two CAPS markers, called D04-T7 and F21-Sp6, derived from T7 end

sequence of BAC Hba004D04 (contig 10242) and Sp6 end sequence of BAC Hba210F21 (contig 6859) respectively, were integrated into the target window by testing their recombination in the BC_1 and F_2 populations for the *Pat* locus (Fig. 2-7). They both confirmed the expected map position: marker F21_Sp6 was between marker T5 and T21, whereas marker D04-T7 between marker T1143 and T17 (Fig. 2-7).

Also the end sequences of the singletone BACs (K18_T7, N05_T7, J03_Sp6, G09_Sp6), confirmed to be located on the target region for the *Pat* locus through markers matching, were used to develop new molecular markers. Three CAPS markers were derived from K18_T7, N05_T7 and G09_Sp6 end sequences. No polymorphism was found in the J03_T7 end sequence between *S. pennellii* and *S. lycopersicum.*

Marker G09-Sp6 co-segregates always with marker T17 in the mapping populations (Fig. 2-7). Markers N05 and K18 map in the genetic region between marker T17 and marker T20; they co-segregate with the *Pat* mutation, marker T18 and marker C2_At2g42110 in the mapping populations so they did not allow to restrict the target window for the *Pat* locus (Fig. 2-7).

Tab. 2-4. Molecular markers developed from BAC end sequences confirmed to be located internally to the target region for the *Pat* locus.

BAC name	BAC end	Marker type	Marker name	Marker positioned on long arm of ch. 3	Marker positioned between COS marker T1143 and T0796
Hba004D04	T7	CAPS	D04-T7	+	+
Hba210F21	Sp6	CAPS	F21-Sp6	+	+
MboI0001K18	T7	CAPS	K18-T7	+	+
Hba0228N05	T7	CAPS	N05-T7	+	+
EcoRI0092J03	Sp6	No polymorphism	J03-Sp6	-	-
Hba0025G09	Sp6	CAPS	G09-Sp6	+	+

2.3 Conclusion

The whole data obtained through a microsynteny approach with Arabidopsis allowed us to refine with new anchor-points the genetic region spanning 1.2 cM between COSes T0796 and T1143, and to restrict the target interval to about 0.2 cM between marker T17 and T20. Besides, the new tomato COS markers developed from T2O4 Arabidopsis BAC clone showed a strict order conservation with those present in Arabidopsis. Nonetheless the data from the physical map show that T17-T20 chromosome interval in tomato is longer than in Arabidopsis, likely due to a difference of gene number.

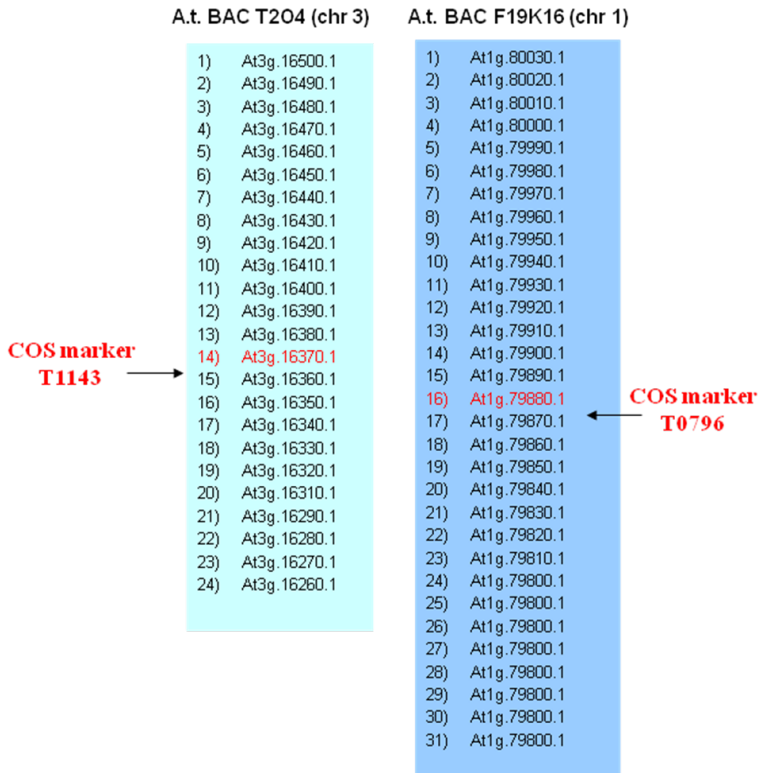


Fig. 2-1. Arabidopsis BACs T2O4 (chr 3) and F19K16 (chr 1) and their gene annotation at TAIR (The Arabidopsis Information Resource). COS marker T1143 matches with At3g16370 on BAC T2O4 (see narrow) instead COS marker T0796 matches with At1g.79870 on BAC F19K16.

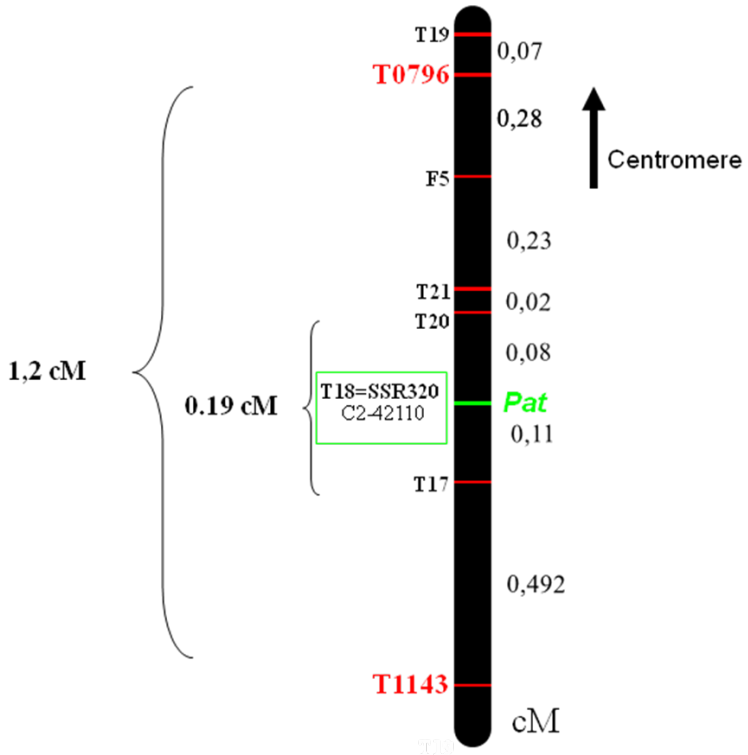


Fig. 2-2. Genetic linkage map surrounding the *Pat* locus on the long arm of chromosome 3. The new COS markers, particularly T17 and T20, restrict the target region for the *Pat* locus from 1,2 cM (T1143-T0796; Beraldi *et al.,* 2004) to about 0,19 cM. The COS marker C2_Atg42110 (EXPEN2000 genetic map; SGN, www.sgn.cornell.edu) and the in/del marker T18 co-segregate with the *pat* mutation in the BC_1 and F_2 mapping populations for the *Pat* locus.

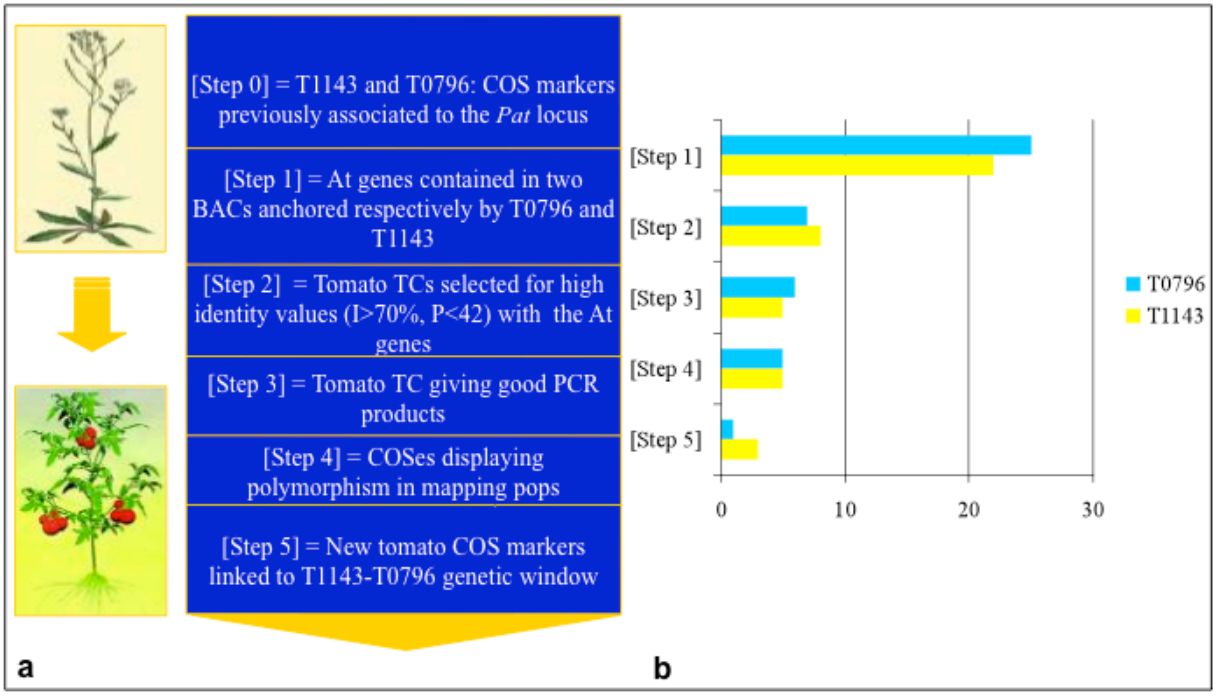


Fig. 2-3. The procedure followed for generating new COS markers (a); efficiency of developing COS markers in the *Pat* locus genomic region (b).

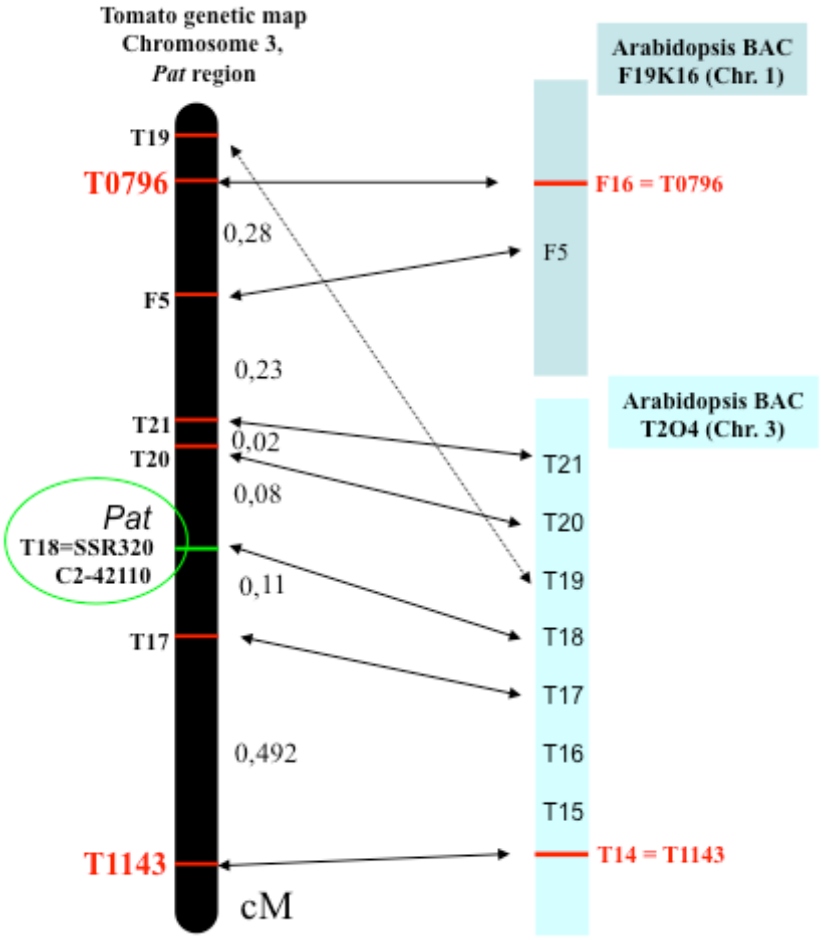


Fig. 2-4. Arabidopsis-tomato microsynteny in the *Pat* locus genomic region. Arabidopsis genes on T2O4 BAC clone show a strict order conservation with those ones present in tomato *Pat* locus region.

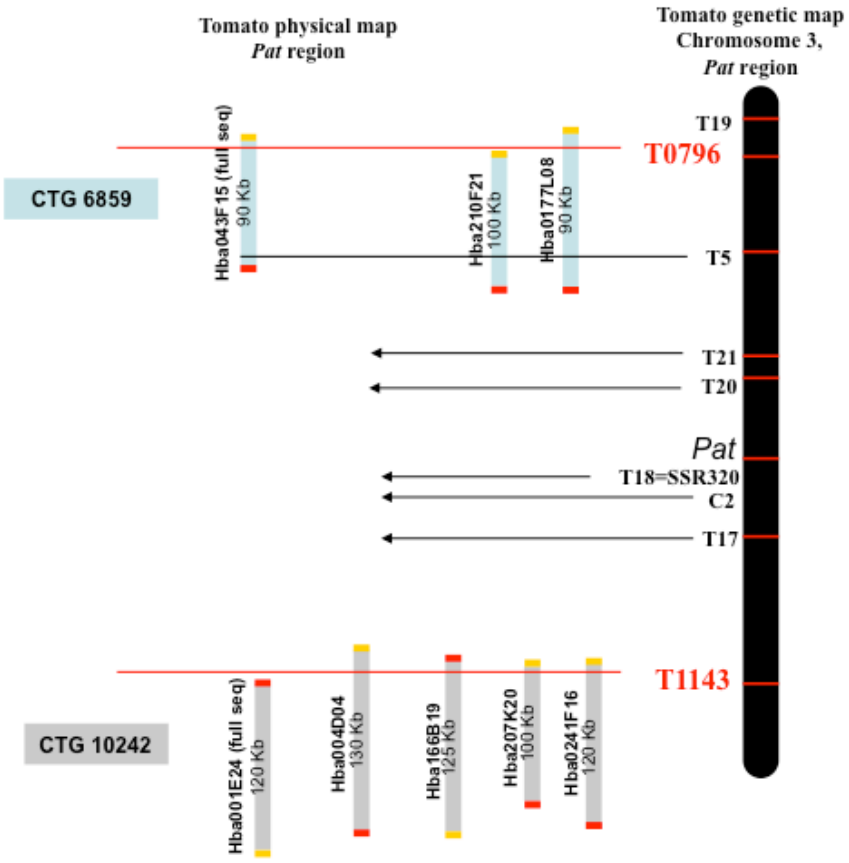


Fig. 2.5. Integration of the physical map on the genetic map. COSes T0796 and T1143 display a clear hit with a number of tomato BACs belonging to two plausible unlinked contigs at the *Hind*III, *Mbo*I and *Eco*RI physical maps (www.sgn.cornell.edu; Mueller et al., 2005). COS T5 matches on BACs Hba210F21, Hba043F15 and Hba0177L08 pertaining contig c6859. COSes T17, T18, T20 and T21 don't match any BACs, they reside in the gap comprised between the two contigs.

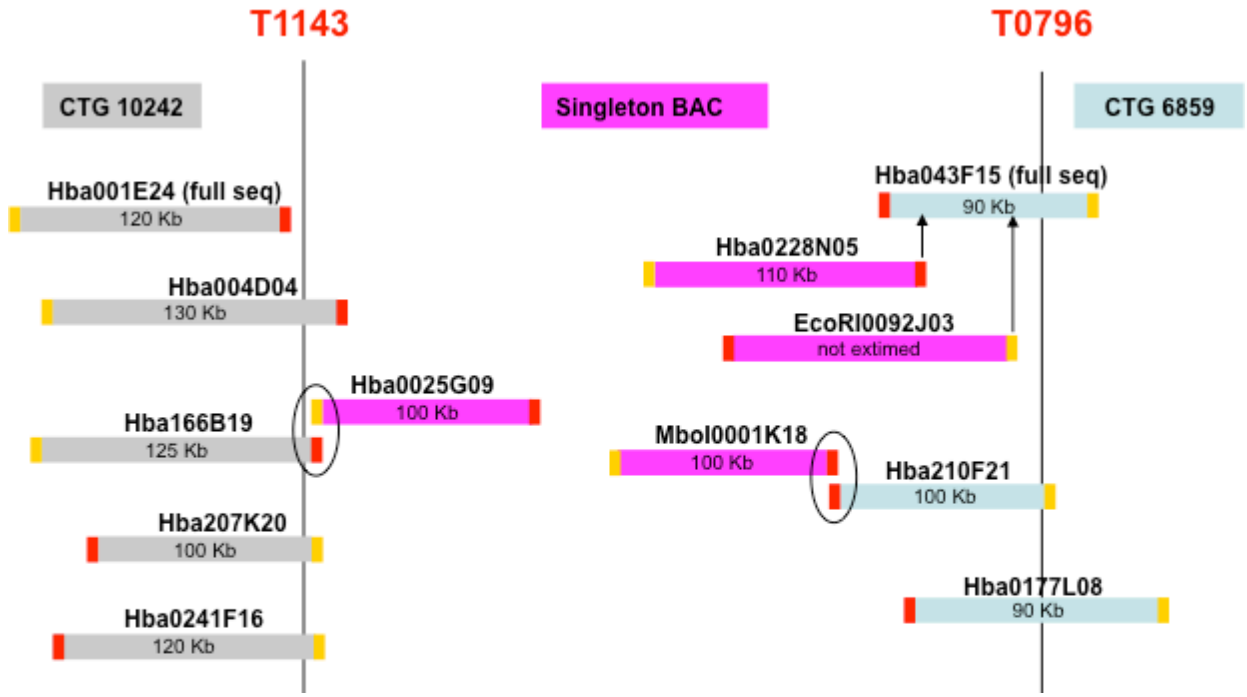


Fig. 2-6. Tomato physical map in the *Pat* locus region. New singleton BACs (in violet) were added in the T1143-T0796 genetic window by blasting at SGN physical map both the end sequences and the eventual full sequences of the BACs contained in the contigs 10242 (in grey) and 6859 (blue). The Sp6 end sequences of BACs EcoRI0092J03 and Hba0228N05 matched with BAC Hba043F15, a fully sequenced BAC belonging to contig 6859. Furthermore T7 end sequence of BAC MboI0001K18 overlapped with Sp6 sequence of BAC Hba210F21 of another BAC of same contig. On the other side, the Sp6 end of BAC Hba0025G09 (referred as G09) overlapped with the T7 sequence of BAC Hba166B19. All the BACs have the Sp6 end sequence in red and the T7 end sequence in yellow.

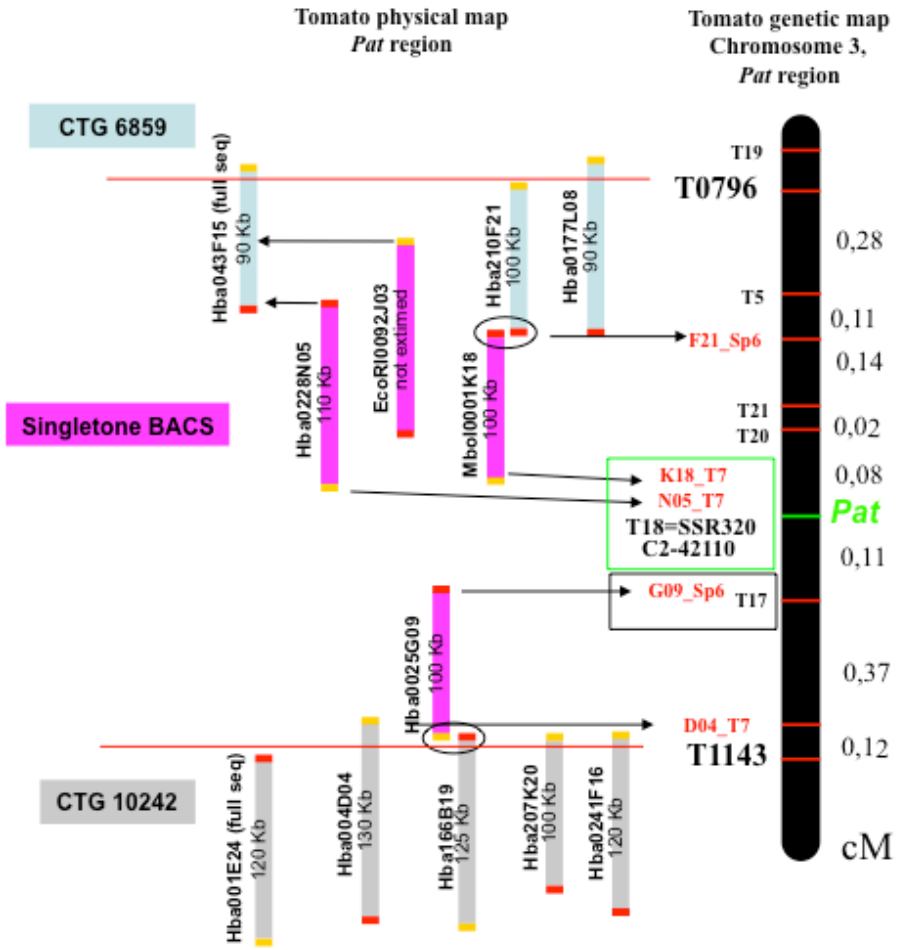


Fig. 2-7. Physical vs genetic mapping in the *Pat* locus region. The end sequences of the BACs, confirmed to be located internally to the target region for the *Pat* locus, were used to develop new molecular markers. In this way five CAPS markers (in red) were derived from F21_Sp6, D04_T7, K18_T7, N05_T7 and G09_Sp6 end sequences. All the BACs have the Sp6 end sequence in red and the T7 end sequence in yellow.

3. Positional cloning of the *parthenocarpic fruit* (*pat*) mutation in tomato by candidate gene approach

Chapter 2 describes how the *Pat* locus has been localised by genetic and physical mapping in a genetic window (long arm of chromosome 3) of about 0.2 cM on the long arm of chromosome 3, a distance that was physically estimated in at least 100 Kb. The small size of the target region and the recent publication of the tomato genome sequence (Sol Genomics Network; SGN, www.sgn.cornell.edu) allowed us to carry out a candidate gene approach with the aim to isolate the gene responsible for the *pat* phenotype.

The temporal succession of abnormalities in the generative development of *pat* plants suggests that parthenocarpy could be an induced, secondary effect of a mutated gene, whose primary function is to regulate floral organ development (Mazzucato *et al.,* 1998). Anther aberrancy, and particularly the occurrence of adaxial carpel-like structures bearing external ovules, seem to indicate that *pat* is a mutation of a putative gene with homeotic functions.

Genetic analysis and mapping information showed that the gene underlying the *pat* mutation is not allelic to either of the two tomato mutations putatively involved in B function, *stamless (sl)-2* (allelic with *sl-1* and orthologue of *DEFICIENS*; Hafen and Stevenson 1958, Gomez *et al.,* 1999; Mazzucato *et al.,* 2008) and *pistillate* or to genes encoding class B transcription factors (Mazzucato *et al.,* 2008). Even if *SlDEFICIENS* is not allelic with the *pat* locus, the developmental regulation of this gene in the wild-type (WT) at anthesis as well as its differential transcription in the *pat* ovary suggest that it plays a role in the control of ovary growth. Accordingly, when compared with the WT, the gene was also differentially expressed in the *parthenocarpic fruit-2* (*pat-2*) mutant, that is not allelic to *pat* and has normal ovule development. Thus, *Sl*DEF could play a role in the control of ovary growth and that the *pat* mutation would be located upstream of this regulatory cascade. (Mazzucato *et al.,* 2008).

The retained responsiveness to the GA-biosynthesis inhibitor PAC indicates that the *pat* phenotype is, at least in part, mediated by an increased GA signal. Expression analysis of genes encoding key enzymes involved in GA biosynthesis shows that transcriptional regulation of GA20ox1 mediates pollination-induced fruit set in tomato and that parthenocarpy in *pat* results from the miss-regulation of this mechanism. As genes involved in the control of GA synthesis (KNOX transcription factors LeT6, LeT12 and

LeCUC2) and response (SPY) are also altered in the *pat* ovary, it was suggested that the *pat* mutation affects a regulatory gene located upstream of the control of fruit set exerted by GAs (Olimpieri *et al.,* 2007).

The relationship between PAT and other characterized repressors of the ovary, such as members of the auxin/IAA9 (Wang *et al.,* 2005) and ARF8 (Goetz *et al.,* 2007) gene families or other as yet unknown (Fig. 3-1). Pollination and pollination-induced signals as well as mutations located upstream (as *pat*), destabilize the repressing system and give rise to fruit initiation (Mazzucato *et al.,* 2008; fig. 3-1).

All this information allowed us to select four of the nine potential candidate genes located in the T17-T20 target region. They were amplified and sequenced from WT (Chico III) and *pat* lines. A significant mutation in one of the four genes was highlighted and proposed as responsible for the *pat* phenotype.

3.1 Materials & Methods

3.1.1 Candidate genes identification

COS markers T0796, T1143, T17, T18, T20 and CAPS markers N05_T7, K18_T7, G09_Sp6 (chapter 2) were blasted at SGN scaffold section (Tomato whole-genome-shotgun scaffolds from version 1.03 of the WUR assembly; www.sgn.cornell.edu). They were all physically positioned on the scaffold SL2.31sc03701 localised, as expected, on the long arm of chromosome 3 .

The sequence comprised between markers T17 and T20, showed to define a genetic window of 0,2 cM around the *Pat* locus, was blasted at SGN unigene section (Unigene and Genbank sequences related associated with SGN loci; www.sgn.cornell.edu) using the BLAST2 package and the BLASTX and BLASTN algorithms (Altschul *et al.,* 1990). Additional search was done in the GenBank/EMBL/DDBJ EST Division and in the TIGR Tomato Gene Index (www.tigr.org). A total of 23 unigenes were retrieved that later were assembled in 9 genes.

3.1.2 Candidate genes validation

Plants material

The tomato (*Solanum lycopersicum* L.) *cv.* Chico III (referred to as wild type, WT) was used as control for comparison with a near-isogenic line (NIL) homozygous for the *parthenocarpic fruit (pat)* allele. WT seed was obtained from Petoseed Co. (Woodland, CA, USA) and the *pat* line was developed from the original mutant stock through backcrossing. All plants were grown in an unheated tunnel and under ambient light conditions of Viterbo, Italy, in late spring summer with standard culture practices.

DNA extraction

Total DNA was extracted according to Doyle and Doyle (1990) using about 200 mg of fresh tissue (leaves or inflorescences) collected in a 1,5 ml Eppendorf tube from WT and *pat* line.

RNA extraction and reverse transcription

Total RNA was isolated from 100 mg of ovary or fruits tissue with TRIzol (Invitrogen, Carlsbad, CA,USA) following the manufacturer's instructions. After extraction, RNA was dissolved in diethylpyrocarbonate-treated water and 5 μg used to make cDNA using 1 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and a 3'-oligo(dT) primer in a final volume of 20 μl.

DNA cloning, sequencing and sequence analysis of the candidate genes

In order to obtain the genomic and the coding sequences of the candidate genes, *Stig1*, *Avr9-Cf-9*, *G20-βox* and *HB15,* specific primers, based on the published sequence (SGN), was designed. PCR amplification reactions were performed both on DNA and cDNA (from WT and *pat* line) previously extracted using the following cycling conditions: 94°C, annealing temp. reported in Tab. 3-1 and 72°C for 1 min each, plus a final extension time of 72°C for 7 min.

Selected amplicons were cloned into TA site of pGEM-T Easy (Promega) and recombinant plasmid used to transform competent *Escherichia coli* DH5α cells. DNA sequencing analyses were performed on both strands using an ABI PRISM 310 automatic sequencer (PerknElmer Biosystems). The nucleotide and deduced amino acids sequences from WT and *pat* line were compared between them and with those in GenBank/EMBL/DDBJ/PDB

non-redundant sequence databases using the BLAST2 package and the BLASN and BLASTX algorithms (Altschul *et al.,* 1990).

Tab. 3-1. Primer sequences and PCR reaction parameters used on cDNA / DNA of the candidate genes

Gene name	Primer sequences (5'– 3')	PCR product length (bp)	Annealing temp. (°C)	No. of cycles
Stig1	5'CTTCTCATCGCCATCCTTGC3' 5'TTAGGCACTGACACAAAGGC3'	450	60	32
Avr9/Cf9	5'TGGAACATAGTGCGAATAGC3' 5'GCTCCCAATCTTCTTCATCCA3'	773	60	32
G20-βox	5'TTAAAAGCGAAGGGACATGC3' 5'TCCTCTGCACCAAACAAACTC3'	627	60	32
	5'GGGATGGAATCTTTGGGATT3' 5'CAGCCCTTCCTATCACATGC3'	405	60	32
	G'GGTCTCTTTTCAGCCTGGT3' 5'TTGACAAAACATATCCGAAAACC3'	503	60	32
HB15	5'TGAAGAAGATCTGTTTCTGGG3' 5'GGTATACACCTGAGCAGGTT3'	555	60	32
	5'ATGGCTTCCTGCAAGGATGG3' 5'GGCTACTGGAACTGCTGTTGA3'	533	62	32
	5'AGTAGTGACTAGTGGTCAGC3' 5'GGTCCAAGTATGCCACCTGT3'	504	60	32
	5'TGGTGGAACCATTGAACTCC3' 5'CCATCCTTGTGAACTCTTCT3'	550	60	32
	5'AATGGTATAGCTGATGAGGG3' 5'GCGATAATGCCACGAGATAT3'	419	58	32
	5'AAGTTGGAAGGTCATTCTCC3' 5'GGAGGTCTTCGTTTGCCATT3'	473	60	32
	5'CTCGACAGTATGTCCGAAGC3' 5'GATACTGCTCATTGCATCTGT3'	527	60	32

In silico protein analysis

Protein structure analysis were performed using different tools such as SIFT (Ng PC and Henikoff S. 2001 and 2003), ELM (Puntervoll *et al.,* 2003) and I-TASSER (Zhang *et al.,* 2008a and 2008b).

3.2 Results & Discussion

3.2.1 Candidate genes identification

Integration of the genetic and physic map on the scaffold SL2-3103701

As described in Chapter 2 of this work, through genetic and physical mapping, the genetic region spanning 1.2 cM between COSes T0796 and T1143, was refined with new anchor-points. Consequently, the target interval for the *Pat* locus was restricted to less than 0.2 cM between T17 and T20 markers.

The recent publication of the tomato genome sequence (SGN; www.sgn.cornell.edu) allowed us to finely analyze this target region. In particular, through *in silico* analysis, markers T0796 and T1143 were located on the scaffold SL2.31sc03701 (previous scaffold 04007) mapped, as expected, on the long *arm of chromosome* 3 at SGN (www.sgn.cornell.edu) defining a region of 370 Kb on it (Fig. 3-2).

The physical/genetic distance ratio in a genomic region of chromosome 3 proximal to the *Pat* locus, was estimated to be 123Kb/cM on average (Mesbah *et al.,* 1999), six time lower than the mean calculated for the tomato genome (750 Kb/cM; Tanskley *et al.,* 1992). In our case, we show that the estimated 1,2 cM *Pat* locus interval (Beraldi *et al.,* 2004) corresponds to about 310 Kb/cM (Fig. 3-2).

Hence we have both verified the occurrence of the new molecular markers tightly linked to the *Pat* gene (T17, T18, T20, N05_T7, K18_T7, G09_Sp6 and C2-42110) and calculated their relative physical distance on the scaffold SL2-3103701 of tomato. We showed that COS markers T17 and T20 define a interval of about 0,2 cM surrounding the *Pat* locus, this distance corresponds physically to about 103 Kb on scaffold SL2-3103701 (Fig. 3-2). The same interval in the Arabidopsis collinear region is almost 8 times smaller (13.5 Kb) and contains 4 genes.

Markers T18, K18_T7, N05_T7 and C2-42110, that co-segregate with the locus *Pat* in the mapping population, have been confirmed on the scaffold SL2.31sc03701 between markers T17 and T20 (Fig. 3-2). Marker T18 is about 52 and 51 Kb far from T20 and T17 respectively; markers K18_T7, N05_T7 and C2-42110 are really closed among them and about 79 and 22 kb far from T20 and T17 respectively. Marker G09_Sp6, as expected from the genetic map, is tightly linked to marker T17 (0.4 Kb; Fig. 3-2).

Genes identification in the T17-T20 genetic region

By blasting the genomic sequence spanning markers T17 and T20 at SGN "Unigene and Genebank sequences related" section, 23 unigenes were identified. According with the last SGN gene annotation release (SL2.31), they were assembled into 11 complete gene sequences and they were named from *Solyc03g120880* to *Solyc03g120980*. Their position in the genetic interval T20-T17 is showed in Fig 3-3.

Nine of them, according to the map position, are candidate as responsible for the *pat* phenotype; the two outer genes in the genetic window, *Solyc03g120880* and *Solyc03g120980*, can be excluded because they segregate with the *Pat* locus since they match with marker T20 and T17 respectively (Fig. 3-3).

Three of the nine candidate genes (*Solyc03g120920*, *Solyc03g120940*, *Solyc03g120950*) encode for a protein of unknown function. Two of them are transcription factors: Solyc03g120890 encodes for a GATA whereas *Solyc03g120910* for a HDIII zip transcription factor. *Solyc03g120930* (*Avr9/Cf9*) and *Solyc03g120960* (*STIG*) encode for two small proteins involved in the host-parassite and pollen-pistill interaction respectively The *Solyc03g120900* gene encodes a SEC13-like protein transport. Finely, *Solyc03g120970* encodes a "Gibberellin 2-beta-dioxygenase" a downstream target of AGL15 (Zhu et al., 2005)., a member of the MIKC subfamily of MADS genes (Fig. 3-3).

3.2.2 Candidate genes validation

HB15, AVR-Cf9, STIG1, GA2-βox gene sequence analysis in WT and *pat* lines

By pursuing the candidate gene approach, 4 of the 9 genes (Solyc03g120910, Solyc03g120930, Solyc03g120960 and Solyc03g120970) located in the target region for the *Pat* locus, were selected. Potential candidate genes were chosen by considering their possible correlation with the *pat* mutant phenotype.

Normally, pollination and fertilization of the ovule triggers the development of the ovary into a fruit whereas *pat* line produces parthenocarpic fruit in absence of pollination. A mutation in a gene that mediates the signaling between sporophyte and gametophyte could be responsible of the *pat* phenotype. *Solyc03g120960* encodes *Le*STIG1, a cysteine-rich protein involved in pollen-pistill interactions (Goldman *et al.,* 2004). *STIG1* has been identified in tobacco as a gene developmentally regulated and expressed specifically in the stigmatic secretory zone. Tang *et al.* (2004) showed that tomato LeSTIG1 binds the

extracellular domains of both LePRK1 and LePRK2, two pollen-specific receptor kinases that participate in signaling during pollen tube growth.

Solyc03g120930 is an *AVR-Cf9* gene; it's involved in host-parassite interaction (Rowland *et al.,* 2005), but also an involvement in pollen-pistill interaction can't be excluded.

Solyc03g120970 encodes a "gibberellin 2-beta-dioxygenas" (*GA2ox*) regulated by AGAMAUS-like 15, a member of the MIKC subfamily of MADS domain transcription factors that specifies both the carpel and ovule identity in Arabidopsis (Zhu *et al.,* 2005). The retained responsiveness to the GA-biosynthesis inhibitor PAC indicates that the *pat* phenotype is, at least in part, mediated by an increased GA signal. Expression analysis of genes encoding key enzymes involved in GA biosynthesis shows that transcriptional regulation of *GA20ox1* mediates pollination-induced fruit set in tomato and that parthenocarpy in *pat* results from the miss-regulation of this mechanism.

HB15 is a HD-ZIP III transcription factor. This class of homeodomain-leucine zipper proteins regulate critical aspects of plant development such as ovule polarity, apical and lateral meristem formation, and vascular development (Kim et al., 2005; Ochando et al., 2006;; Kelley et al., 2009).

Genomic and coding sequences length, number of exons and protein sequence length are indicated in Tab. 3-2 for each one of the four candidate genes. Their cDNA and DNA were sequenced in the WT (Chico III line) and in the *pat* mutant.

SlHB15, AVR-Cf9 and *GA2-βox* sequences did not show any difference between Chico III (WT) and *pat* lines (data no shown). On the contrary, a significant difference was found in *SlHB15* coding sequence between the two genotypes: a G to A substitution at position 1747 in the *pat*. *SlHB15* has a genomic sequence of 6464 bp and a coding sequence of 2511 bp (Fig. 3-4). Structurally, the gene is composed of 18 exons and 17 introns and the mutation is located on the exon 14 (SGN; Fig. 3-5).

This kind of mutation is in agreement with the action of ethyl methanesulfonate (EMS), the mutagen used by Soressi (1975) to induce the *pat* line. EMS, in fact, commonly causes transitions by alkylation of G residues that result in mispairing and base changes. The alkylation of guanine (G) causes the formation of O6-ethylguanine which can pair with thymine (T) but not with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be replaced with A /T (Pastink *et al.,* 1991).

Tab. 3-2. Position on the chromosome 3, genomic and coding sequences length, number of exons and protein sequence length of the candidate genes responsible of the *pat* phenotype.

Gene name (SGN)	Gene description	Chromosome 3 position (bp)	Gene lenght (bp)	Exon number	Coding sequence lenght (bp)	Protein lenght (aa)
Solyc03g120910	HB15; class III homeodomain-leucine zipper; contains Interpro domain(s) IPR013978 MEKHLA	63196426-63202889	6464	18	2508	836
Solyc03g120930	Avr9/Cf-9 rapidly elicited protein 146	63216540-63217007	468	1	468	156
Solyc03g120960	STIG1 contains Interpro domain(s) IPR001368 TNFR/CD27/30/40/95 cysteine-rich region	63243987-63244418	432	1	432	144
Solyc03g120970	Gibberellin 2-beta-dioxygenase; downstream gene of AGL15. It contains Interpro domain(s) IPR005123 Oxoglutarate and iron-dependent oxygenase	63244710-63246761	2052	3	1140	380

HB15 protein characterization in WT and *pat* lines

G1747A substitution in *pat HB15* allele is located in position I of the codon 583 causing a substitution of a Glicine (Gly, WT line) with an Arginine (Arg) on the primary sequence of the *Sl*HB15 protein (Fig. 3-6).

Homeodomain leucine zipper (HD-Zip) proteins are transcription factors classified into four different groups based on gene structure, presence of unique domains and function (reviewed by Elhiti and Stasolla, 2009). Unique features of all HD-Zip members are the presence of a homeodomain (HD) and a leucine zipper motif (Zip). The HD domain is involved in DNA binding whereas the Zip domain is involved in protein homo heterodimerization (Elhiti and Stasolla, 2009). Functional studies using truncated proteins have shown the requirement of the Zip motif for the DNA binding ability of the HD domain (Elhiti and Stasolla, 2009). Members of the class III group share three additional domains: a MEKHLA domain possibly involved in oxygen redox and light signaling, a START domain motif with putative lipid binding capability and a SAD domain which is a transcriptional activation domain (Elhiti and Stasolla, 2009).

Even if the mutated amino acid is not located on one of the four typical conserved domain of the HD-Zip III protein family, it belongs to a sequence of 4 amino acids conserved on 55 characterized HD-Zip III proteins (Fig. 3-7; ELM server; Punervoll *et al.,* 2003).
Bioinformatics studies on the effect of a mutation on protein function, based on sequence homology and the physical properties of amino acids, predicted the substitution of Gly 583 to Arg to be not tolerated (SIFT: Sorting, Intolerant From Tolerant; Ng PC and Henikoff S. 2001, 2003). Computational analysis on the protein tertiary structure, showed the incapability of the mutated protein to correctly fold and to form the homeodomain binding site (Fig.3-8; I-TASSER; Zhang, 2008a, b). The combination of these data is a first step in demonstrating a loss of function of the *Sl*HB15 protein in *pat* mutant and allows us to suppose its involvement in causing *pat* phenotype.

*Sl*HB15 protein in the *pat* mutant and fruit set

The *HB15* is a member of the class III homeodomain-leucine zipper family. Only five members belong to this family in Arabidopsis: *ATHB8, PHAVOLUTA/ATHB9* (PHV*), PHABULOSA/ATHB14* (*PHB*), *CORONA/ATHB15* (CAN) and *REVOLUTA/IFL1* (REV). In tomato is not clear how many genes belong to this family, whereas *HD-Zip III* genes are highly conserved in land plants (Sakakibara *et al.,* 2001).
This gene family regulates apical embryo patterning, embryonic shoot meristem formation, organ formation, vascular development and meristem function (Prigge *et al.,* 2005). Genetic analysis revealed a complex pattern of overlapping functions. Phylogenetic relationship and/or by gene expression patterns do not help to understand the precise role of the members of this gene family (Prigge *et al.,* 2005).
It is interesting to note that when the Arabidopsis *men-1,* a mutant in which a MIR166a gene is activated by the insertion of the CaMV 35S enhancer leading to a drastic reduction of *ATHB15* mRNA level, was pollinated with wild-type pollen, fruits with no seeds were produced (parthenocarpy). Furthermore *men1*, similarly to *pat* mutant, exhibits pleiotropic alterations in the floral structure and leaf morphologies (Kim *et al.,* 2005). Prigge *et al.* (2005) did not isolate a *CORONA/ATHB15* single mutant but they showed that the Arabidopsis *phb phv can* triple mutant, similarly to the *pat* mutant, had extra cotyledons and reduced fertility assignable to defects in ovule development. Many ovules of *phb phv cna* plants had short integuments.
CORONA/ATHB15 was also identified in as screen for mutations modifying the *clavata-1* (*clv-1*) mutant phenotype; *CLAVATA (CLV)* gene family promotes cell differentiation and

organogenesis. It has been proposed that CNA protein acts in parallel with the CLV pathway to promote organ formation (Clark *et al.,* 2005). Recently Kelley *et al.* (2009) showed that HD-Zip III genes such as *CNA* (*HB15*), *PHB* and *PHV* act in concert with *ABERRANT TEST SHAPE* (*ATS*, also known as *KANADI 4*) to control ovule integument morphogenesis. Arabidopsis triple mutant for HD-Zip III genes parallel *pat* phenotype showing ovules with short integuments (actually two integuments in Arabidopsis vs only one in tomato; Fig. 3-9) and a significant reduced seed set (Kelley *et al.,* 2009; Mazzucato *et al.,* 1998). It is interesting to note that also *kan4* mutant displays a phenotype with a single integument consisting of fused outer and inner integuments (McAbee *et al.,* 2006).

Izhai *et al.* (2007) showed that in Arabidopsis auxin signaling during embryogenesis is mediated by KAN and HD-Zip III activity via *PIN-FORMED (PIN)* gene family localization. PIN is a family of proteins that controls the dynamic changes in auxin flux and maxima. One consequence of auxin maxima created by convergent auxin flow is the activation of AUXIN RESPONSE FACTORS (ARFs), transcription that activate and/or repress target genes. ARF proteins are negatively regulated by AUXIN/INDOLE-3ACETIC ACID (AUX/IAA) proteins, which are targeted for degradation in response to auxin (Bowman *et al.,* 2007). Mutations in one member of the *ARF* gene family in Arabidopsis and tomato, *ARF8* (referred to as *FRUIT WITHOUT FERTILIZATION*, *FWF*), result in fruit set in the absence of pollination and fertilization (Goetz *et al.,* 2007). Gorguet *et al.* (2008) identified *SlARF8* as a candidate gene for two parthenocarpy QTLs. Also transgenic plants with decreased *SlARF7* mRNA levels formed parthenocarpic fruits (de Jong *et al.,* 2009). It also interesting to note that either Arabidopsis *arf8*-mutated line or *pat* line present defects in the floral organs, in particular aberrant ovules and short anthers (Goetz *et al.,* 2007; Mazzucato *et al.,* 1998; Fig. 3-10). These data strengthen *HB15* as putative responsible of the *pat* phenotype and allow us to hypothesize that the PAT (HB15) protein acts upstream to the ovary regulating machinery members, such as *ARF* and *Aux/IAA* genes.

Consistently with these findings, we purpose a model that explains the function of PAT protein in the molecular pathway of fruit set. It supports the hypothesis of de Jong *et al.* (2009) who asserted that in the unpollinated ovary, SlARF7 acts as a transcriptional activator of “auxin response attenuating genes”, repressing the auxin and gibberellin signaling pathways that are necessary to initiate tomato fruit development. In this model, PAT (HD15) protein acts upstream to this cascade possibly regulating positively the *ARF7* gene. After pollination, the *KANADI* gene family, acting antagonistically with the class III HD-Zip III, would down regulate *Pat* and consequently *ARF7* genes giving rise to the fruit

set (Fig. 3-11a). This will explain the down-regulation of *AtARF8* mRNA after pollination (de Jong *et al.,* 2009). Although the mechanistic nature of the mutually antagonistic activities of KANADI and Class III HD-Zip genes is not known, expression patterns and mutant phenotypes suggest an association with auxin. For instance, leaf phenotypes of *kan1 kan2* plants are strikingly similar to those of *ettin* (*arf3*) *arf4* plants (Pekker *et al.,* 2005), and lateral root initiation, known to require auxina maxima, is altered by both *KANADI* loss-of-function and *REV* gain-of-function alleles (Hawker and Bowman, 2004). Another possibility is that *Pat* (*HB15*) is modulated directly from the auxin in analogy with *ATHB8* gene, the most phylogenetically closed HD-Zip gene to *HB15* (Baima *et al.,* 2005). Furthermore, the auxin flow in the ovary inhibits the ARF7 protein that are still present by auxin-induced Aux/IAAs, such as IAA9 (Fig. 3-11b).
In *pat* mutant the HD15 loss function doesn't allow the transcription of *ARF7* gene. The absence of ARF7 protein doesn't allow the activation of the "auxin response attenuating genes" and consequently the ovary repression (Fig. 3-11c).

3.3 Conclusion

The small size of the genomic region in which we previously mapped the locus *Pat* and the recent publication of the tomato genome sequence (SGN, www.sgn.cornell.edu) allowed us to carry out a candidate gene approach with the aim to clone the gene responsible for the *pat* phenotype. Four of the nine potential candidate genes mapping in the T17-T20 genomic window, were amplified and sequenced from WT (Chico III) and *pat* lines. A point mutation in the *pat HB15* gene, coding for a transcriptor factor belonging to the HD-Zip III family, was found and proposed as responsible for the *pat* phenotype. In order to confirm this hypothesis a complementation experiment with *SlHB15* WT gene in the *pat* line is ongoing. *HD-Zip III* and *KANADI*, two antagonist gene families, were included in a new model that explain the molecular pathway that triggers the fruit set.

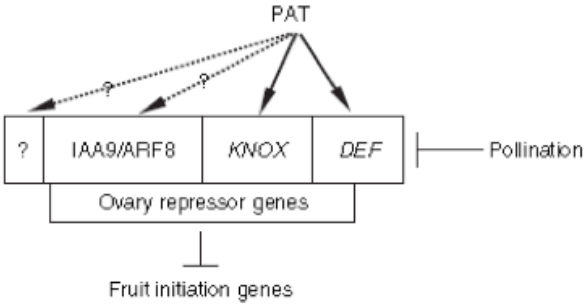


Fig. 3-1. A model for the role of the *Pat* gene in controlling ovary growth and fruit set in tomato (Mazzucato *et al*., 2008). The PAT protein positively regulates the activity of members of the repressing machinery such as homeobox (KNOX; Olimpieri *et al.,* 2007) and class B MADS box (DEF, Mazzucato *et al*., 2008) transcription factors. The relationship between PAT and other characterized repressors of the ovary, such as members of the auxin/IAA9 (Wang *et al.,* 2005) and ARF8 (Goetz *et al.,* 2007) gene families or other as yet unknown proteins (?) deserves further investigation. Pollination and pollination-induced signals as well as mutations located upstream (as *pat*), destabilize the repressing system and give rise to fruit initiation.

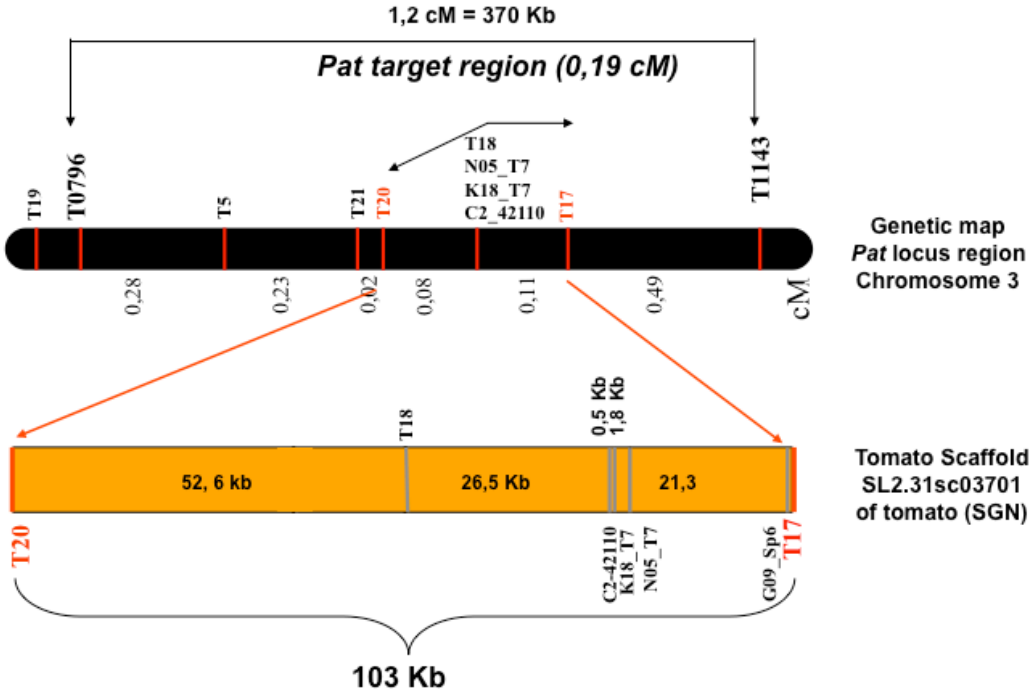
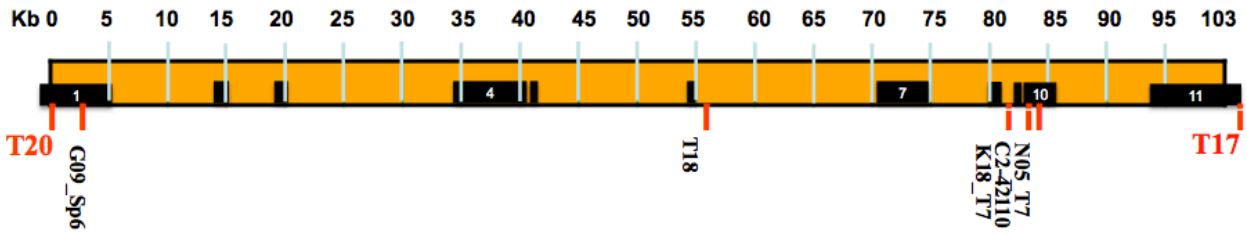


Fig. 3-2. Integration of the target region for the *Pat* locus on the scaffold SL2-3103701 (at SGN) of tomato: through genetic and physical mapping, *Pat* gene was located on the long arm of chromosome 3 between two markers named T17 and T20 that define a window of 0,19 cM. With the tomato genome sequencing it has been possible to locate markers T17 and T20 on the scaffold SL2-3103701 at SGN and to calculate their physical distance. They define a window of 103 Kb, T18, K18_T7 and N05_T7, three markers that co-segregate with the locus *Pat* in the mapping population, have been confirmed on the scaffold SL2-3103701 between markers aT17 and T20.



Position in the T17-T20 genetic window	Gene name (SGN)	Gene description	Marker match
1	Solyc03g120880	Nucleoporin NUP53	G09_Sp6
2	Solyc03g120890	GATA transcription factor 9	
3	Solyc03g120900	Protein transport SEC13-like protein	
4	Solyc03g120910	Class III homeodomain-leucine zipper III	
5	Solyc03g120920	Unknown Protein	
6	Solyc03g120930	Avr9/Cf-9 rapidly elicited protein 146	T18
7	Solyc03g120940	Os06g0483900 protein (Fragment)	
8	Solyc03g120950	Unknown Protein	
9	Solyc03g120960	STIG1	K18_T7
10	Solyc03g120970	Gibberellin 2-beta-dioxygenase 2	
11	Solyc03g120980	ATP-binding cassette transporter	

Fig. 3-3. Gene annotation and their relative position on the target window for the locus *Pat* spanning T17-T20 markers (103Kb). In the last column on the right of the table and on the picture are indicated the map position of the markers previously located between marker T17 and T20.

***Sl*HB15 coding sequence**

```
1     ATGGCTTCCT GCAAGGATGG TAAGTCGGTG GTGTTGGATA ACGGAAAGTA TGTCCGGTAT
61    ACACCTGAGC AGGTTGAAGC CCTTGAGAGG CTTTATCATG ATTGTCCTAA ACCAAGTTCA
121   ATGCGCCGTC AGCAGCTTAT CAGAGAATGT CCTATCCTCT CTAACATTGA ACCTAAGCAA
181   ATCAAAGTTT GGTTCCAGAA TCGAAGGTGC AGGGAGAAAC AGAGGAAAGA GGCGTCAAGG
241   CTTCAGTCTG TGAATAGGAA GCTGACGGCA ATGAACAAGC TATTAATGGA AGAAAATGAT
301   AGACTACAGA AGCAAGTTTC TCAACTGGTG TATGAAAATG GCTACTTTCG CAGGCAAAGT
361   CACCAGACTC CACTTGCTAC GAAAGATACC AGTTGTGATT CAGTAGTGAC TAGTGGTCAG
421   CACCACTTGA CATCTCAGCA TCCGCCGAGG GATGCTAGTC CTGCAGGGCT TTTGTCCATT
481   GCAGAAGAAA CTTTAACAGA GTTTCTTTCA AAGGCTACTG GAACTGCTGT TGAGTGGGTC
541   CAAATGCCTG GAATGAAGCC TGGTCCGGAT TCCATTGGAA TCATTGCTAT TTCTCATGGT
601   TGCACTGGCG TGGCAGCAAG AGCTTGTGGC CTGGTTGGTC TCGAGCCAAC GAGGGTAAGC
661   GAAATCCTTA AGGATCGACC TTCTTGGTAT CGTGACTGCC GGGTTGTTGA AGTTCTCAAT
721   GTGCTGCCTA CTGCCAATGG TGGAACCATT GAACTCCTTT ACATGCAGCT TTATGCACCA
781   ACAACGTTGG CACCTGCCCG TGATTTCTGG CTCTTACGTT ATACAACTGT TATGGATGAT
841   GGCAGTCTCG TGGTGTGTGA AAGGTCACTT GGAAATACTC AAAATGGTCC AAGTATGCCA
901   CCTGTTCAGA ATTTTGTGAG AGCAGAAATC CTACCTAGTG GATATCTGAT TAGACCTTGT
961   GAGGGGGGTG GTTCAATTAT CCACATTGTT GATCATATGA ATTTAGAGGC ATGGAGTGTG
1021  CCTGAAGTCT TACGCCCACT TTATGAGTCA TCAGCAGTGC TAGCTCAGAA GACGACAGTG
1081  GCTGCACTAC GCTACCTCCG GCAGATTGCA CAGGAGGTTT CACAGACTAA TGTTACTAAC
1141  TGGGGAAGAC GACCCGCAGC TCTACGTGCA TTAAGCCAGA GGCTGAGCAG GGGCTTCAAT
1201  GAGGCTCTTA ATGGTATAGC TGATGAGGGC TGGTCAATGC TGGATAGTGA TGGAATGGAT
1261  GATGTTACCA TCCTTGTGAA CTCTTCTCCT GACAAGTTGA TGGGCTTAAA CCTTCCTTTT
1321  GCGAATGGAT TTTCACCTAT GAGCAACGCG GTTATGTGTG CAAAAGCATC AATGCTTCTA
1381  CAGAATGTGC CTCCGGCTAT TCTTCTCAGG TTCCTACGTG AACATCGATC TGAATGGGCA
1441  GACAACAATA TAGATGCTTA TGCTGCTGCA GCCATTAAAG TTGGTCCCTG CAGCTTACCT
1501  GGGGCTCGAG TTGGTAACTT CGGGGGTCAA GTGATACTTC CACTCGCTCA CACTGTTGAG
1561  CATGAATTGC TGGAGGTCAT TAAGTTGGAA GGTCATTCTC CTGAGGACGC GATAATGCCA
1621  CGAGATATGT TTCTATTGCA AATTCTATGC AGTGGAATGG ATGAAAATGC TGTTGGAACT
1681  TGTGCCGAAC TCGTGTTTGC TCCTATTGAT GCCTCTTTTG CTGATGATGC ACCATTGCTT
1741  CCGTCTGGGT TTCGCATTAT TTCACTCGAG TCTGGAAAGG AAGCGTCCAG TCCAAATCGT
1801  ACCCTTGATC TTACTTCTGC TCTTGAGACT GGCCCAGCGG AAAACAAAGC AGCCAATGAT
1861  CTTCACACTA GTGGTGGCTC ATCAAGATCT GTCATGACAA TTGCTTTTCA ATTTGCTTTT
1921  GAAAGCCACA TGCAAGAGAG TGTTGCTTCA ATGGCTCGAC AGTATGTCCG AAGCATTATT
1981  TCATCTGTTC AAAGAGTTGC ATTAGCACTT TCACCATCTC ATTTGGGTTC TCATGGAGGT
2041  CTTCGTTTGC CATTGGGGAC TCCTGAAGCA CATACATTAG CTCGTTGGAT CTGCCAAAGT
2101  TACAGGTGCT TTTTGGGCGT GGAGCTTCTC AAATTGAATA CTGATCAAGG AAGTGAATCA
2161  ATCCTCAAAA GCCTATGGCA TCACTCAGAT GCTATTATCT GCTGCTCCCT GCAGGCTTTG
2221  CCAGTTTTCA CATTTGCAAA TCAGGCTGGT CTTGACATGC TCGAGACAAC CTTGGTTGCA
2281  CTTCAAGATA TTACCTTGGA GAAAATATTT GATGATCATG GAAAGAAGAA CCTCTGCACT
2341  GAGTTCCCCC AGATAATGCA ACAGGGTTTT GCATGTCTAC AAGGGGGAAT TTGCTTGTCA
2401  AGCATGAGCA GACCTATATC TTATGAGAGA GCAGTAGCAT GGAAAGTTAT GAATGAAGAA
2461  GATACTGCTC ATTGCATCTG TTTCATGTTT GTCAACTGGT CATTTGTCTA A
```

Fig. 3-4. *SlHB15* coding sequence: in red the start and stop codon; in grey the consensus sequence for the miRNA 166. In green the codon 583 “GGG” that is mutated to “AGG” in the *pat* line.

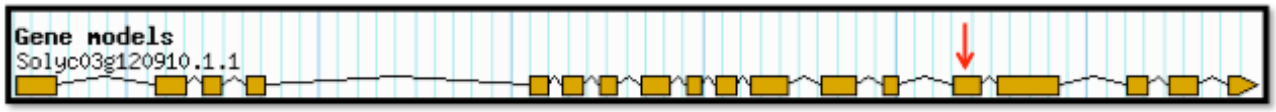


Fig. 3-5. *SlHB15* gene model (SGN; www.sgn.cornell.edu). It's composed by 18 exons and 17 introns. The arrow indicates the exon (14) affected by the mutation in the *pat* line.

Alignment of WT and *pat* SlHD15 protein

```
HB15 WT    MASCKDGKSVVLDNGKYVRYTPEQVEALERLYHDCPKPSSMRRQQLIREC     50
HB15 pat   MASCKDGKSVVLDNGKYVRYTPEQVEALERLYHDCPKPSSMRRQQLIREC     50

HB15 WT    PILSNIEPKQIKVWFQNRRCREKQRKEASRLQSVNRKLTAMNKLLMEEND    100
HB15 pat   PILSNIEPKQIKVWFQNRRCREKQRKEASRLQSVNRKLTAMNKLLMEEND    100

HB15 WT    RLQKQVSQLVYENGYFRRQSHQTPLATKDTSCDSVVTSGQHHLTSQHPPR    150
HB15 pat   RLQKQVSQLVYENGYFRRQSHQTPLATKDTSCDSVVTSGQHHLTSQHPPR    150

HB15 WT    DASPAGLLSIAEETLTEFLSKATGTAVEWVQMPGMKPGPDSIGIIAISHG    200
HB15 pat   DASPAGLLSIAEETLTEFLSKATGTAVEWVQMPGMKPGPDSIGIIAISHG    200

HB15 WT    CTGVAARACGLVGLEPTRVSEILKDRPSWYRDCRVVEVLNVLPTANGGTI    250
HB15 pat   CTGVAARACGLVGLEPTRVSEILKDRPSWYRDCRVVEVLNVLPTANGGTI    250

HB15 WT    ELLYMQLYAPTTLAPARDFWLLRYTTVMDDGSLVVCERSLGNTQNGPSMP    300
HB15 pat   ELLYMQLYAPTTLAPARDFWLLRYTTVMDDGSLVVCERSLGNTQNGPSMP    300

HB15 WT    PVQNFVRAEILPSGYLIRPCEGGGSIIHIVDHMNLEAWSVPEVLRPLYES    350
HB15 pat   PVQNFVRAEILPSGYLIRPCEGGGSIIHIVDHMNLEAWSVPEVLRPLYES    350

HB15 WT    SAVLAQKTTVAALRYLRQIAQEVSQTNVTNWGRRPAALRALSQRLSRGFN    400
HB15 pat   SAVLAQKTTVAALRYLRQIAQEVSQTNVTNWGRRPAALRALSQRLSRGFN    400

HB15 WT    EALNGIADEGWSMLDSDGMDDVTILVNSSPDKLMGLNLPFANGFSPMSNA    450
HB15 pat   EALNGIADEGWSMLDSDGMDDVTILVNSSPDKLMGLNLPFANGFSPMSNA    450

HB15 WT    VMCAKASMLLQNVPPAILLRFLREHRSEWADNNIDAYAAAAIKVGPCSLP    500
HB15 pat   VMCAKASMLLQNVPPAILLRFLREHRSEWADNNIDAYAAAAIKVGPCSLP    500

HB15 WT    GARVGNFGGQVILPLAHTVEHELLEVIKLEGHSPEDAIMPRDMFLLQILC    550
HB15 pat   GARVGNFGGQVILPLAHTVEHELLEVIKLEGHSPEDAIMPRDMFLLQILC    550

HB15 WT    SGMDENAVGTCAELVFAPIDASFADDAPLLPSGFRIISLESGKEASSPNR    600
HB15 pat   SGMDENAVGTCAELVFAPIDASFADDAPLLPSRFRIISLESGKEASSPNR    600

HB15 WT    TLDLTSALETGPAENKAANDLHTSGGSSRSVMTIAFQFAFESHMQESVAS    650
HB15 pat   TLDLTSALETGPAENKAANDLHTSGGSSRSVMTIAFQFAFESHMQESVAS    650

HB15 WT    MARQYVRSIISSVQRVALALSPSHLGSHGGLRLPLGTPEAHTLARWICQS    700
HB15 pat   MARQYVRSIISSVQRVALALSPSHLGSHGGLRLPLGTPEAHTLARWICQS    700

HB15 WT    YRCFLGVELLKLNTDQGSESILKSLWHHSDAIICCSLQALPVFTFANQAG    750
HB15 pat   YRCFLGVELLKLNTDQGSESILKSLWHHSDAIICCSLQALPVFTFANQAG    750

HB15 WT    LDMLETTLVALQDITLEKIFDDHGKKNLCTEFPQIMQQGFACLQGGICLS    800
HB15 pat   LDMLETTLVALQDITLEKIFDDHGKKNLCTEFPQIMQQGFACLQGGICLS    800

HB15 WT    SMSRPISYERAVAWKVMNEEDTAHCICFMFVNWSFV                  836
HB15 pat   SMSRPISYERAVAWKVMNEEDTAHCICFMFVNWSFV                  836
```

Fig. 3-6. Alignment of WT (Chico III line) and *pat Sl*HB15 protein: in yellow the Glycine (G) 583 that is mutated in Arginine (R) in the *pat* line.

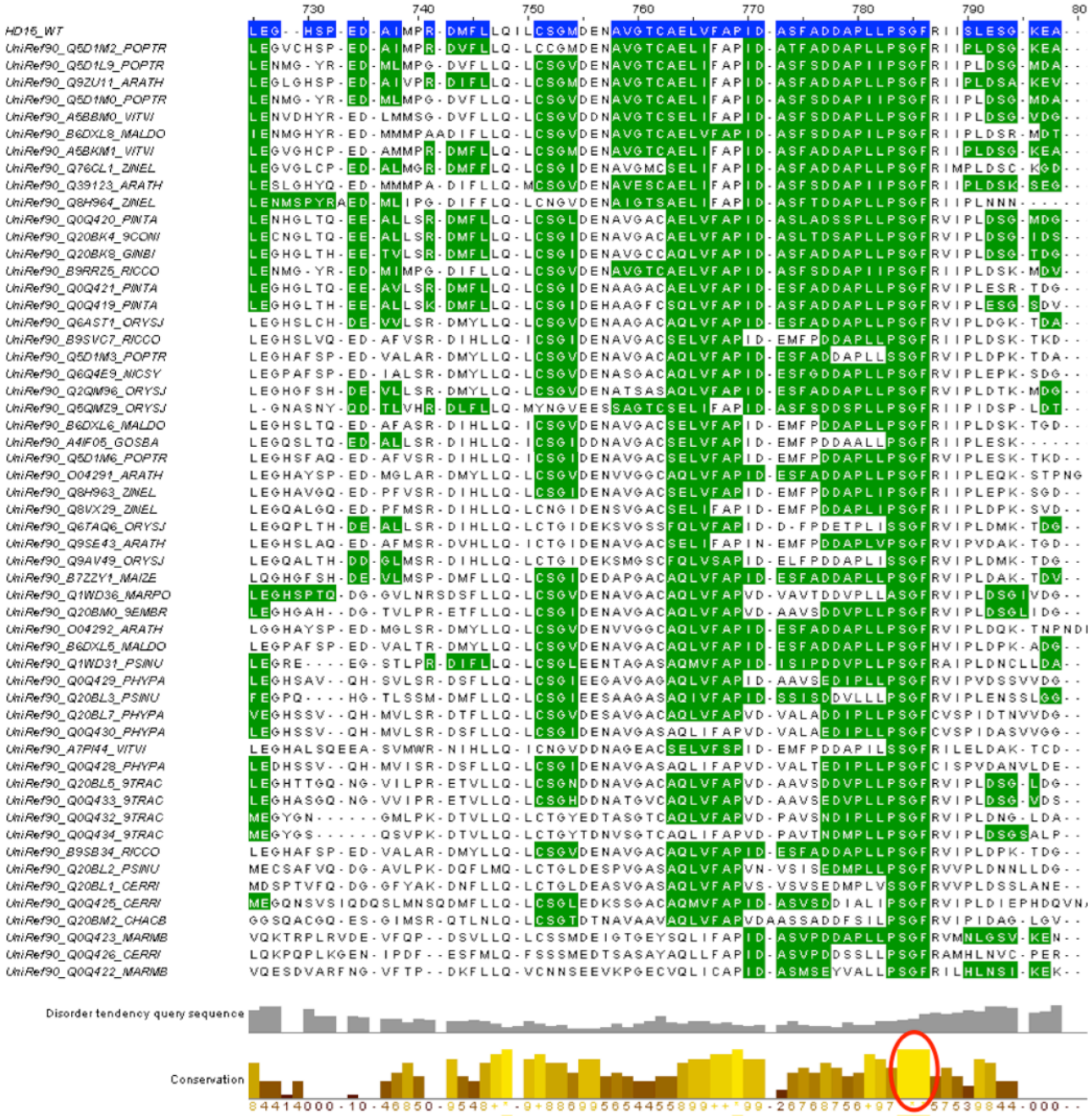


Fig. 3-7. Alignment of the mutated region of *SlHB15* (in blue) with 55 HD-Zip III proteins present in gene bank (ELM server; Punervoll *et al.,* 2003). Circled is a cluster of 4 amino-acid (containing the Gly 583) that is conserved in all the 55 analyzed proteins belonging to the HD-Zip III family.

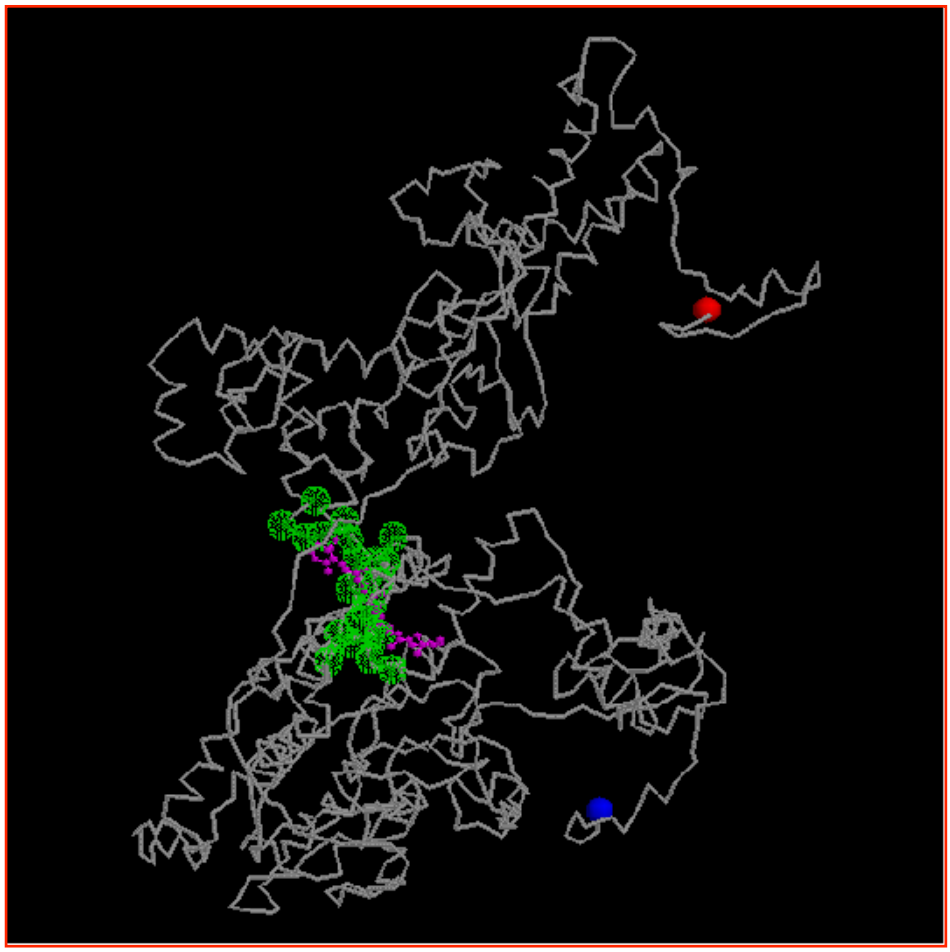


Fig. 3-8. Binding site prediction of the WT *Sl*HB15 protein made with (I-TASSER; Zhang, 2008a, b). In green the homeodomain DNA binding site that is not predicted from the same program in the *pat* HB15 mutated protein.

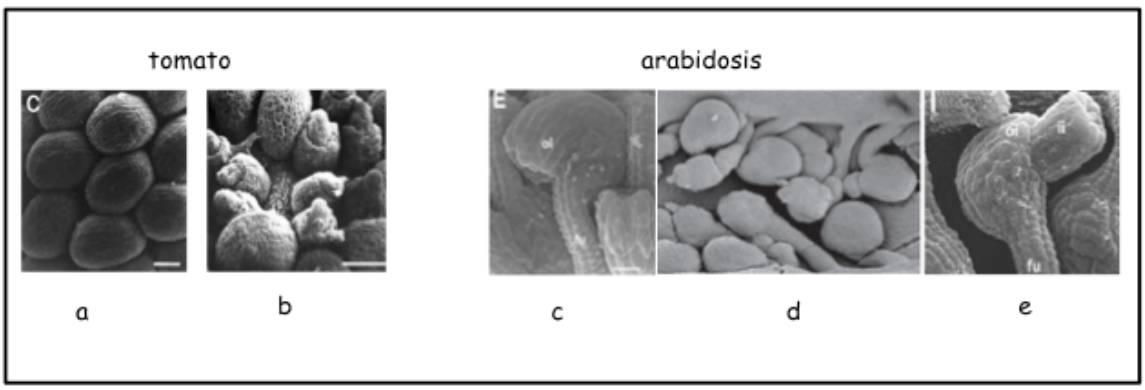


Fig. 3-9. *pat* ovule phenotype (b; Mazzucato *et al.,* 1998) parallels Arabidopsis HD-Zip III *cna phb phv* triple mutant (d; Kelley *et al.,* 2009) and *arf6-8* mutant (e; Goetz *at al.,* 2007). They all show short integuments compared to the respective WT (a and c).

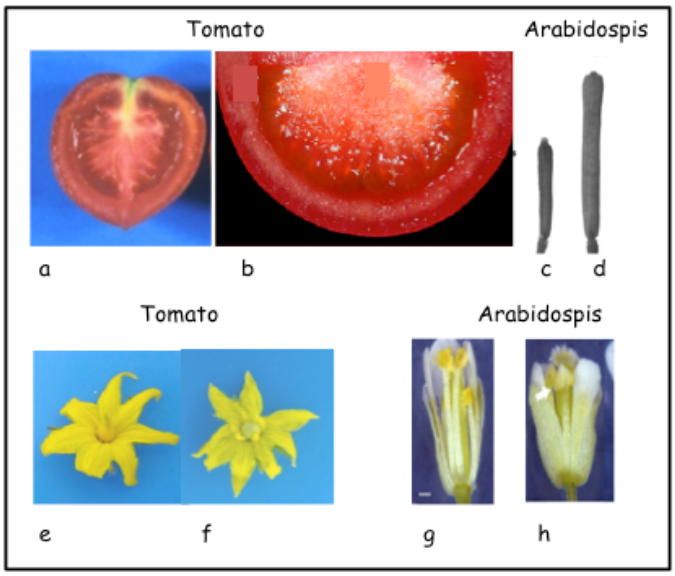


Fig. 3-10. *pat* fruit and flower phenotypes parallel tomato and Arabidopsis *Arf-8* loss-function lines: *pat* seedless fruit (a) ; tomato *Arf-8* loss of function line produces parthenocarpic fruit (b; Goetz *et al.,* 2007) ; Arabidopsis *Arf-8* loss of function line (c) shows a bigger pistil (parthenocarpy) compared with the WT (d) line at 7 days after emasculation (c; Goetz *et al.,* 2007); *pat* flower (f) exhibits short anthers compared to the near isogenic WT line (e; Mazzucato *et al.,* 1998); Arabidopsis *Arf-6/Arf-8* loss of function line (h) exhibits shorter anther compared to the WT (g; Goetz *et al.,* 2007).

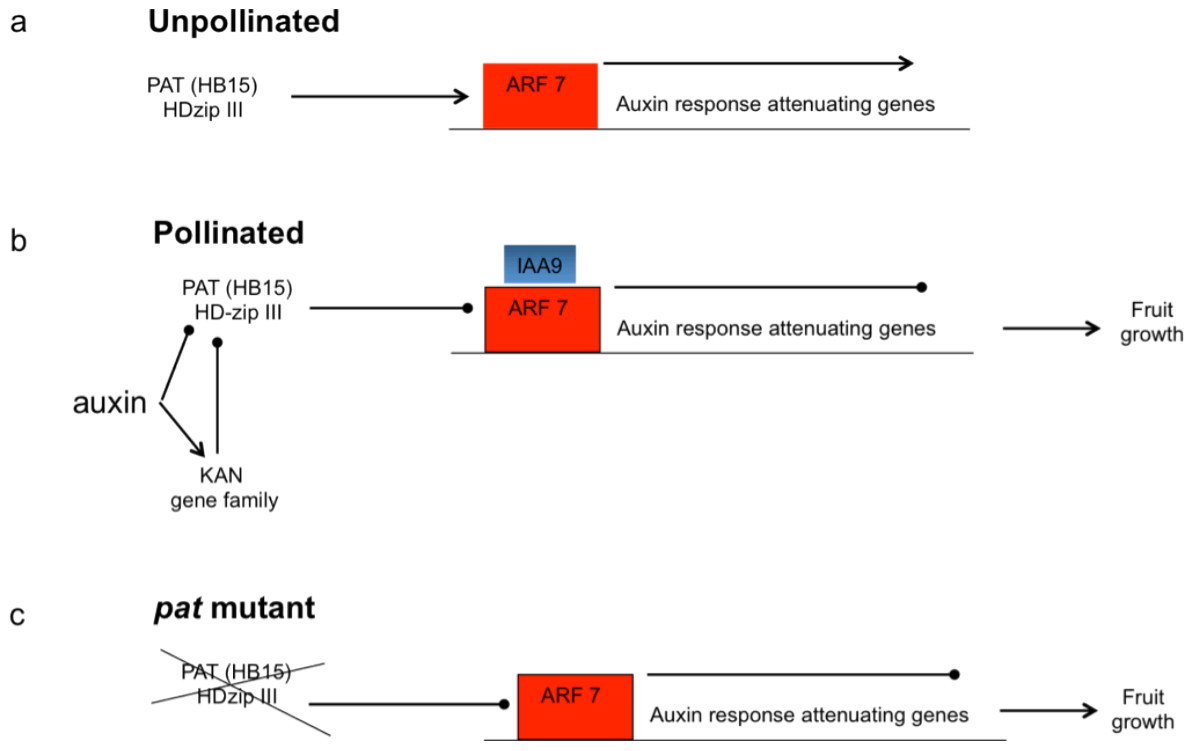


Fig. 3-11. Model about PAT (HB15) protein function in the fruit set. It supports the hypothesis of de Jong *et al.* (2009) who asserted that in the unpollinated ovary, SlARF7 acts as a transcriptional activator of "auxin response attenuating genes", repressing the auxin and gibberellin signaling pathways that are necessary to initiate tomato fruit development. In this model, PAT (HD15) protein acts upstream to this cascade regulating positively the *ARF7* gene (a). After pollination, the *KANADI* gene family, acting antagonistically with the class III HD-Zip, down regulate PAT and consequently *ARF7* gene giving rise to the fruit set. Another possibility is that *Pat* (*HB15*) gene is modulated directly from the auxin that inhibits also the *ARF7* protein that are still present in the ovary by auxin-induced Aux/IAAs, such as IAA9 (b). In *pat* mutant the *HD15* loss function does not allow the transcription of *ARF7* gene. The absence of ARF7 protein does not allow the activation of the "auxin response attenuating genes" and consequently the ovary repression (c).

4. Harnessing novel parthenocarpic mutants in tomato by TILLING a hormone response-related gene

The parthenocarpic growth of the ovary into a seedless fruit without pollination and/or fertilization is a very attractive trait for the breeders and has been extensively studied in tomato, where natural, facultative parthenocarpy sources are known. The recent light shed on molecular genetic mechanisms controlling fruit set in tomato paved the way to harness new mutation for parthenocarpy. The characterization of existing mutants and the extensive reverse genetics researches indicated that a network of growth repressors is established in the ovary of mature fruits.

Among these, AUX/IAA are short-lived transcription factors known to repress the transcription of the auxin-regulated genes. These proteins share four highly conserved domains. Domain I contains a functionally characterized transcriptional repressor motif (Tiwari *et al.*, 2004), while domain II interacts with a component of the ubiquitin-proteasome protein degradation pathway shown to be essential for auxin signaling. Domains III and IV act as C-terminal dimerization domains, mediating homodimerization and heterodimerization among Aux/IAA family members and dimerization with similar domains found in ARFs (Kim *et al.*, 1997; Ulmasov *et al.*, 1997; Ouellet *et al.*, 2001).

According to the current model, Aux/IAAs recruit the corepressor TPL to promoter through interactions with both TPL and ARF proteins (Szemenyei *et al.*, 2008; Tiwari *et al.*, 2001, 2004; Weijers *et al.*, 2005). The presence of auxin, promotes Aux/IAA protein ubiquitination through the TIR1 complex (Gray *et al.*, 2001). As a result, the Aux/IAA protein is degraded by the 26S proteasome and the ARF is released from the repressive effect of the Aux/IAA protein, leading to the activation of the auxin response genes (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Goetz *et al.*, 2007) (Fig. 4-1).

Aux/IAA genes are expressed in distinct spatial and temporal patterns, contributing to the diversity of auxin responses in different in different plant tissues, organs, and developmental stages (Abel *et al.*, 1995). Screens for *Arabidopsis thaliana* mutants with altered auxin response or morphology have identified at least 20 Aux/IAA genes; they presented mutations manly in the highly conserved domain II, which is responsible for protein degradation. The mutations stabilize the proteins, resulting in gain-of function phenotypes.

The *aux/iaa* mutants exhibit a variety of auxin-related developmental phenotypes, including altered phototropism /gravitropism, root formation, apical dominance, stem/hypocotyl elongation, leaf expansion, and leaf formation in the dark (Wang *et al.*, 2005).

Silencing of these repressors can release also the autonomous development of the ovary, that results into parthenocarpy. A member of the Aux/IAA family of transcription factors in tomato, *SlIAA9*, has been described as playing a major role in this machinery, because plants silenced by antisense showed several IAA-related developmental defects and a parthenocarpic behaviour (Wang *et al.*, 2005). Aux/IAA proteins can be grouped into four subfamilies. IAA9 falls into subfamily IV along with sequences from grape (*Vitis vinifera*), cucumber (*Cucumis sativus*), *Zinnia elegans*, and *Arabidopsis*. Subfamily IV is clearly distinguishable as its members are longer (typically >300 amino acids) than members of all other subfamilies (about 200 aa) and typically contain 40 to 60 additional amino acids N-terminal to domain I and >50 amino acids between domains I and II (Wang *et al.*, 2005).

RT-PCR showed that constitutive expression of the *IAA9* gene was high in roots, stems, leaves, flowers and fruits. IAA9 expression also showed constitutive expression throughout leaf and fruit onotogeny. In contrast with the majority of Aux/IAA gene family members, including IAA2, IAA3 and IAA8, that show rapid and strong induction by auxin, IAA9 mRNA levels did not alter markedly after 30 min of auxin treatment, but increased after 3h (Wang *et al.*, 2005).

IAA9-inhibited lines exhibited a wide range of phenotypic effects, suggesting an important role for IAA9 in a number of developmental processes. The most readily visible phenotype was related to leaf morphology. Wild type tomato leaves are unipinnately compound with a terminal leaflet and three pairs of lobed major lateral leaflets with pinnate venation. By contrast, the leaves of *IAA9*-inhibited lines were characterized by minimally lobed simple leaves varying from perfect entire-margined simple leaves to compound leaves depending on the level of downregulation displayed by the different transgenic lines (Wang *et al.,* 2005). Downregulation of IAA9 resulted in a dramatic alteration of early fruit development, with all IAA9 inhibited-lines exhibiting precocious fruit set prior to anthesis, resulting in parallel fruit and flower development. Despite their parthenocarpic character, these fruits were similar in appearance to wild-type tomato in terms of size, skin colour and flesh consistency as well as ripening-associated ethylene production (Wang *et al.*, 2005). This indicates that IAA9 is a key mediator of leaf morphogenesis and fruit set. *In situ* hybridization experiments revealed that a tissue-specific gradient of IAA9 expression is

established during flower development, the release of which upon pollination triggers the initiation of fruit development (Wang *et al.*, 2009). In addition, antisense plants displayed auxin-related growth alterations, including enhanced hypocotyl/stem elongation, increased leaf vascularisation and reduced apical dominance. Auxin dose-response assays revealed that IAA9 downregulated lines were hypersensitive to auxin (Wang *et al.*, 2005).

Recently, a method called Targeting Induced Local Lesions IN Genomes (TILLING) was developed to take advantage of the numerous DNA sequence information and to investigate the functions of specific genes. As demonstrated in a recent publication (Slade *et al.*, 2005), TILLING also shows promise as a non transgenic tool to improve domesticated crops by introducing and identifying novel genetic variation in genes that affect key traits.

In this work, a TILLING approach has been undertaken in order to identify tomato genotypes carrying mutations in the *SlIAA9* coding sequence. *IAA9* "naturally" mutated lines, for their expected parthenocarpic phenotype, can be of value in tomato breeding and help the elucidation of *Iaa9* gene role in the fruit set machinery.

4.1 Materials & Methods

4.1.1 Identification of IAA9 mutated plants

Plant material

To identify tomato genotypes carrying mutations in the SlIAA9 coding sequence, a TILLING approach has been undertaken using the Red Setter (RS) tomato mutant collection and the methods described (Minoia *et al.*, 2010). A population of 5.200 M3 families, available at the Metaponto Agrobios research institute, have been analyzed. It is composed of 3924 and 1297 M3 families obtained by tratment with 0.7% and 1% EMS respectively. The mutation density estimated is 1/322 kb in the 1% EMS and 1/574 Kb in the 0.7% EMS red setter population (Minoia *et al.*, 2010).

DNA extraction and sample pooling

For each M3 family, the genomic DNA was extracted from four young leaves collected from four different plants of the same family. The leaf samples were collected in 96-well

plates and the DNA was isolated by using DNeasy 96 Plant Kit (Quiagen, Hilden, Gemany). The quantification of extracted DNA was carried out on 0.8% agarose gel using λ DNA (Invitrogen, Carlsbad, CA, USA) as a concentration reference. Genomic DNA samples were then diluted tenfold and pooled eightfold to obtain the working material (Minoia *et al.*, 2010).

PCR amplification, mutation detection

PCR amplification was based on nested-PCR and was carried out using one couple of target-specific primers tgggtctatctgattgttcgtc + cataccaggagctatcttctgctc that amplify a region of 654.

4 ng of pooled genomic DNA was used for the first PCR and forward-strand primers and reverse-strand primers 5'-end labeled with IRDye 700 and IRDye 800 dye (LI-COR, Lincoln, NE, USA) respectively were employed for the second PCR. Mutatin detection was performed as previously described by Triques *et al.* (2007).

After discovery, mutations were validated by sequence analysis.

4.1.2 Validation of the mutation in the subsequent generations

Seeds of the positive M3 families have been germinated and plantlets genotyped by CAPS markers to assess the status of the mutation.

Total DNA was extracted according to Doyle and Doyle (1990) using about 200 mg of fresh tissue (leaves) collected in a 1.5 ml Eppendorf tube from M3 positive plants.

Pairs of specific primers flanking mutation A, B and C have been designed and PCR amplification reactions were performed on DNA previously extracted using the following cycling conditions: 94°C, 55°C and 72° C for 1 min each, plus a final extension time of 72°C for 7 min.

4.1.3 Phenotyping of the IAA9 mutated plants

Genotyped plants were grown in an unheated tunnel and under ambient light conditions of Viterbo, Italy, in late spring summer with standard cultural practices. Positive and control plants have been analysed for the expected phenotypes. In the M3 generation 33 mutants, 6 heterozygous and 4 nullisegregants have been observed for the A mutation, 22 mutants, 4 heterozygotes and 3 nullisegregants for the B mutation and 4 mutants, 4 heterozygotes and 5 nullisegregant for the C mutation. In M4, nine progenies (6 plants each) from A mutants and one each from a heterozygote and a nullisegregant were grown. For the B mutation, three progenies were grown from mutants, and one each from a heterozygote and a nullisegregant. In M5, five progenies (6 plants each) from A mutants and one each from a heterozygote and a nullisegregant were grown. For the B mutation, two progenies were grown from mutants.
The advanced generations have been obtained by selfing those plants showing the most penetrant phenotype, which have also been backcrossed to Red Setter.

Cotyledon phenotype. Aberrations in cotyledon phenotype were counted in all the generations and the progenies analysed and reported as the percentage over the total number of seedlings.

Vegetative development. Defects in phyllotaxis and the pattern of axillary shoot development have been observed in all the M3 plants in comparison with the control RS. Leaf compoundness has been evaluated on M5 plants for the A and B mutations and on M3 plants for the C mutation by counting the number of simple and compound leaflets of the fourth leaf in 6 to 12 plants per progeny. Heterozygotes and nullisegregants have also been counted in progenies segregating the A or the B mutation after backcrossing (BC_1F_2 generation).

Reproductive development. For the A and B mutations the reproductive behaviour was analysed in the M3 (single plant basis), M4 and M5 (progeny basis) generations. All plants were controlled for the phenotype of ovary enlargement before true anthesis. Later, the reproductive behaviour was observed by measuring the unitary weight of fruits collected on the first four trusses and the unitary number of seeds per fruit. Results have been pooled according to the mutation and its status.

In M4, ovaries detached from RS and A and B mutant plants have been surface sterilised with sodium hypochlorite and placed in MS growth medium in 24-well plates. Four ovaries were collected from a RS plant and from six plants homozygous for each the A and B mutation. Ovaries were collected and weighted after 15 days of in vitro growth.
For the C mutation, reproductive behaviour was only observed on M3 generation plants. Due to the low number of plants available and to the rate of inflorescence abortion, only preliminary observations could be done on the fruit size and number of seeds. Plants heterozygous for the mutation were selfed and backcrossed to RS to obtain increase of segregating seed and cleaning the background from other undesired mutations.

4.2 Results & Discussion

4.2.1 Identification of IAA9 mutated plants

The analysis of the DNA from 5.200 M3 families yielded three positives carrying a genetic lesion in the *IAA9* coding sequence: two consisted in a point mutation leading to amino acidic substitution (hereafter referred as mutations A and B) and the third in a single-base deletion leading to a frame-shift and a premature stop codon (hereafter referred as mutation C; fig. 4-2).

4.2.2 Phenotyping of the IAA9 mutated lines

Cotyledon phenotype. Aberrations in cotyledon morphology were never found in RS nor in nullisegregants. Differently they were evident in plants homozygous for the three mutations with a penetrance of about 5-10%. In the M3 families segreganting the A mutation, aberrations on cotyledons were found also on heterozygotes. Cotyledon aberrations consisted of “leafy” cotyledons and tricot (Fig. 4-3) and were likely linked to the IAA9 mutations. Polycots were frequently found in ASIAA9 plants (Wang *et al.*, 2005) as well as in natural mutants for parthenocarpy such as *parthenocarpic fruit* (*pat*, Olimpieri *et al.*, 2007).

Vegetative development. Several defects in vegetative developments were observed in M3 mutant plants, among which aberrant phyllotaxis (Fig. 4-4b), increased axillary shoot development (Fig. 4-4c) and absence of axillary shoots (Fig. 4-4d). Although some phenotypes are not likely linked to the mutation, the acropetal growth pattern of the lateral shoots (first lateral shoot arising from the first leaf node, Fig. 4-4c) has been described in AS-IAA9 plants (Wang *et al.*, 2005).

Leaf compoundness was evaluated in the M5 generation for the A and B mutations and in M3 for the C mutation. Whereas nulli-segregants and heterozygotes showed leaves comparable to RS, plants carrying the mutations in the IAA9 gene showed a wide variability in this character with both more complex and simpler leaves (Fig. 4-5). Notably, plants carrying the C mutation showed a drastic reduction in leaf compoundness and a minimally lobed leaf margin (Fig. 4-6) in parallel with the phenotype of the *entire* mutant (Zhang *et al.*, 2007) and of the AS-IAA9 plants (Wang *et al.*, 2005).

Reproductive development. The reproductive development of M3, M4 and M5 mutant plants was first evaluated as the capability of emasculated flowers to develop in absence of fertilization and later as mean fruit weight and number of seeds per fruit. Only in rare cases emasculated flowers developed without fertilization; however the plants carrying the mutations showed fruits with a size comparable with the control but a significantly lower number of seeds (Fig. 4-7). Whereas RS and nulli-segregants generally did not show fruits bigger than 10 g without seed, their frequency was higher in the mutants. Surprisingly this phenotye was penetrant also in heterozygotes (Fig. 4-8).

The attitude to parthenocarpy of IAA9 mutant plants, was also assessed in the M4 generation by evaluating the capacity of ovary growth after *in vitro* culture of whole flowers for 15 d. Whereas RS ovaries showed symptoms of senescence, ovaries in selected plants carrying the A and B mutations showed significant growth (Fig. 4-9). This phenotype is likely the consequence of different hormone levels in mutant flowers and was proposed as a screening method in the study of parthenocarpic tomato mutants (Young *et al.*, 1990). Also in this generation fruits without seeds were found in the mutants (Fig. 4-10). For the C mutation only few homozygous plants were available and could be observed in M3. Such plants showed a tendency to abort inflorescences at the lower tusses, but they were fruitful in older inflorescences. The few fruits obtained showed normal size but they were completely seedless (Fig. 4-11).

4.4 Conclusion

The analysis of M3 families yielded three lines carrying a genetic lesion in the coding sequence of *SlIAA9*, two showing a point mutation leading to amino acidic substitution and the third showing a single-base deletion leading to a frame-shift and a premature stop codon.

Characterization of the former lines, showed some of the expected phenotypes, albeit with low penetrance and expressivity (occurrence of polycots, abnormal growth of axillary shoots, low number of seeds per fruit or seedlessness). Characterization of the latter line showed severe phenotypes, in agreement with those expected, that mainly consisted in an obvious loss of leaf compoundness and parthenocarpy.

In the plants carrying the missense mutations such genotype were variably penetrant. This could be due either to a leaky effect of the mutation or to the interaction of other mutations in the genetic background. The final characterization and the assessment of the value of these mutants in breeding tomato parthenocarpic varieties will be thoroughly assessed when the genetic load of these genotypes will be substantially reduced through backcrossing.

Characterization of the latter mutation, that results in a premature stop codon, put in evidence, as expected, more severe vegetative phenotypes that mainly consisted in a loss of leaf compoundness. The reproductive behaviour of this line appears more severely affected, because early inflorescences aborted in the mutants and only the latest could develop functional flowers, last inflorescences gave parthenocarpic fruits. This observation suggest the interest of this line in studying the role of the IAA9 transcription factor in reproductive development, although its partial sterility may hamper its employment in breeding parthenocarpic tomato varieties.

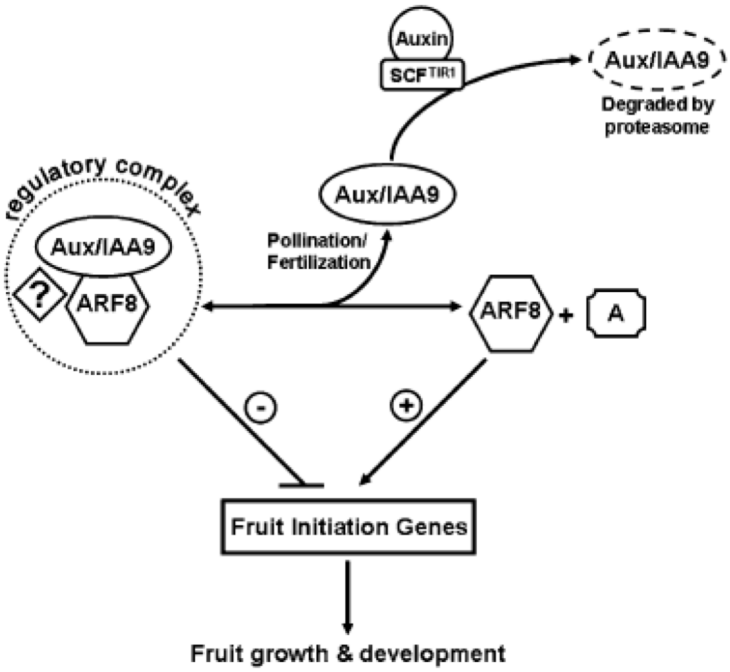


Fig. 4.1. A model for the role of Aux/IAA9 proteins in the control of fruit initiation and growth (Goetz *et al.* 2007). ARF8 and Aux/IAA9 proteins, together with potentially other as yet unknown proteins, form a regulatory complex that can either directly block transcription of target(fruit initiation) genes, or act indirectly by preventing ARF8 from functioning as a transcriptional activator. After pollination and fertilization occur, auxin acts by binding to its receptor, TIR1, promoting degradation of Aux/IAA9 proteins via the SCFTIR1 ubiquitin ligase complex. In the absence of Aux/IAA9, ARF8 together with additional signals and activators (5A) stimulate expression of early auxin responsive genes, initiating fruit growth and development. Destabilization of the regulatory complex or reduction of its functionality by aberrant ARF8 transcripts and possible products can lead to a reduction or loss of the inhibition of transcription of the fruit initiation genes, resulting in parthenocarpic fruit growth.

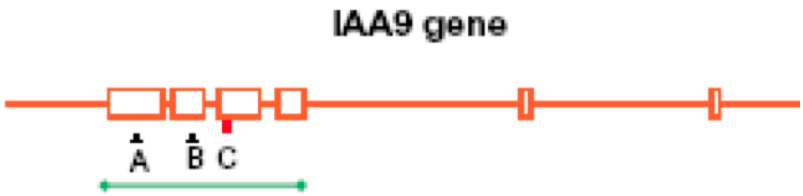


Fig. 4.-2. Graphic representation of mutations in the IAA9 gene. Drawing made with PARSESNP (www.proweb.org/parsesnp/). Exons are represented by boxes and introns by lines. The green arrow indicates the IAA9 gene region screened by TILLING. Black triangles indicate missense mutations A and B while the red square indicates the frameshift mutation C.

Fig. 4-3. Cotyledon phenotype in the RS control (a) and in two seedlings of the IAA9 mutant families (b, c).

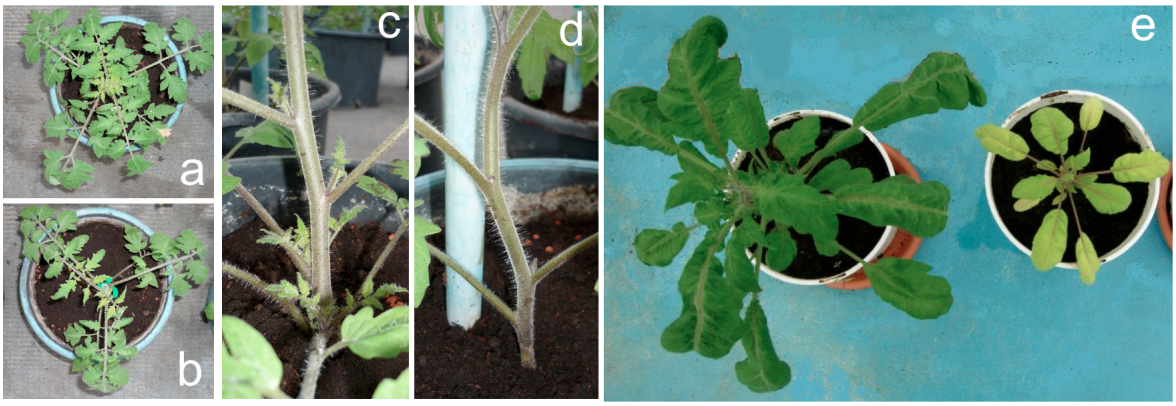


Fig. 4-4. Normal (a) and aberrant (b) phyllotaxis, enhanced (c) and reduced (d) axillary shoot growth and "entire" leaf phenotype in the C mutants (e).

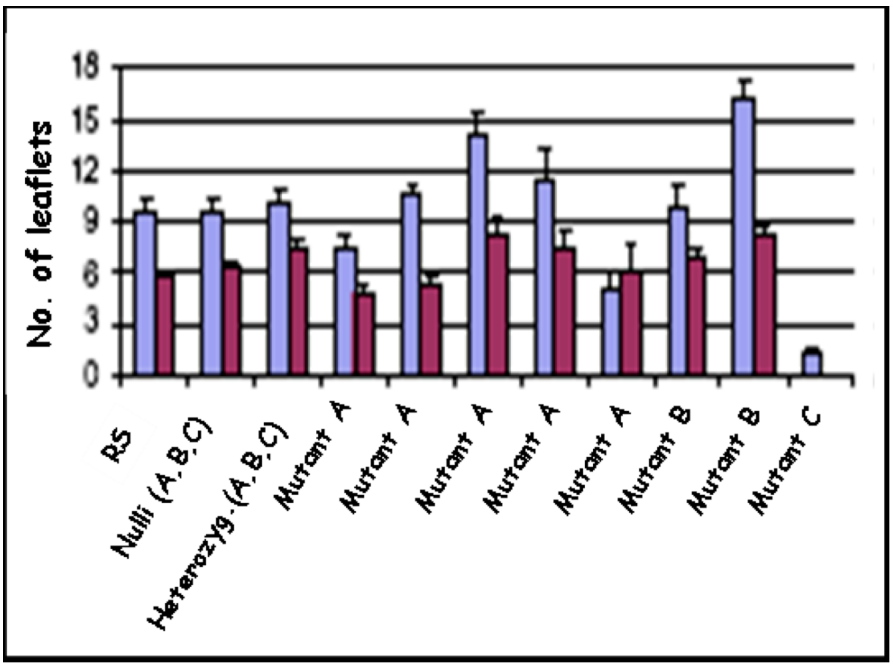


Fig 4-5. Number of simple (blue bars) and compound (purple bars) leaflets counted on the 4th leaf (n= 6-12) of the RS control, nulli-segregant plants, heterozygotes and homozygotes for the A, B and C mutations. Bars represent mean values ± SEM.

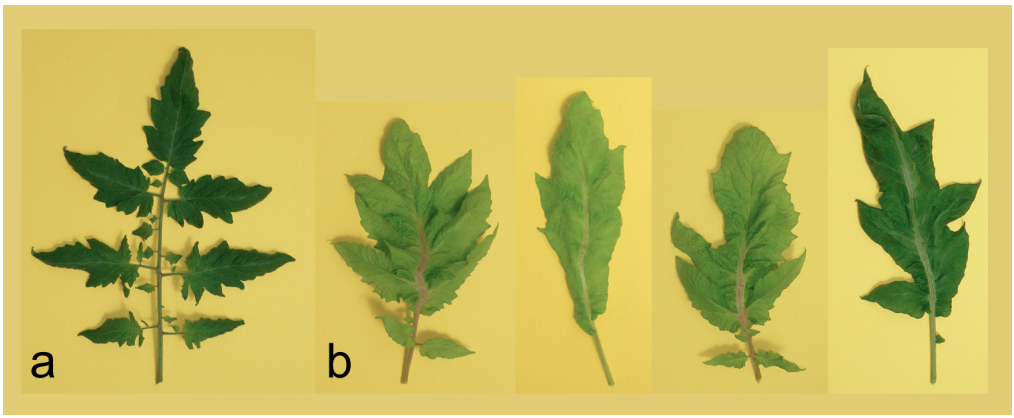


Fig. 4-6. Phenotype of a Red Setter control leaf (a) and of the four mutants homozygotes for the C.

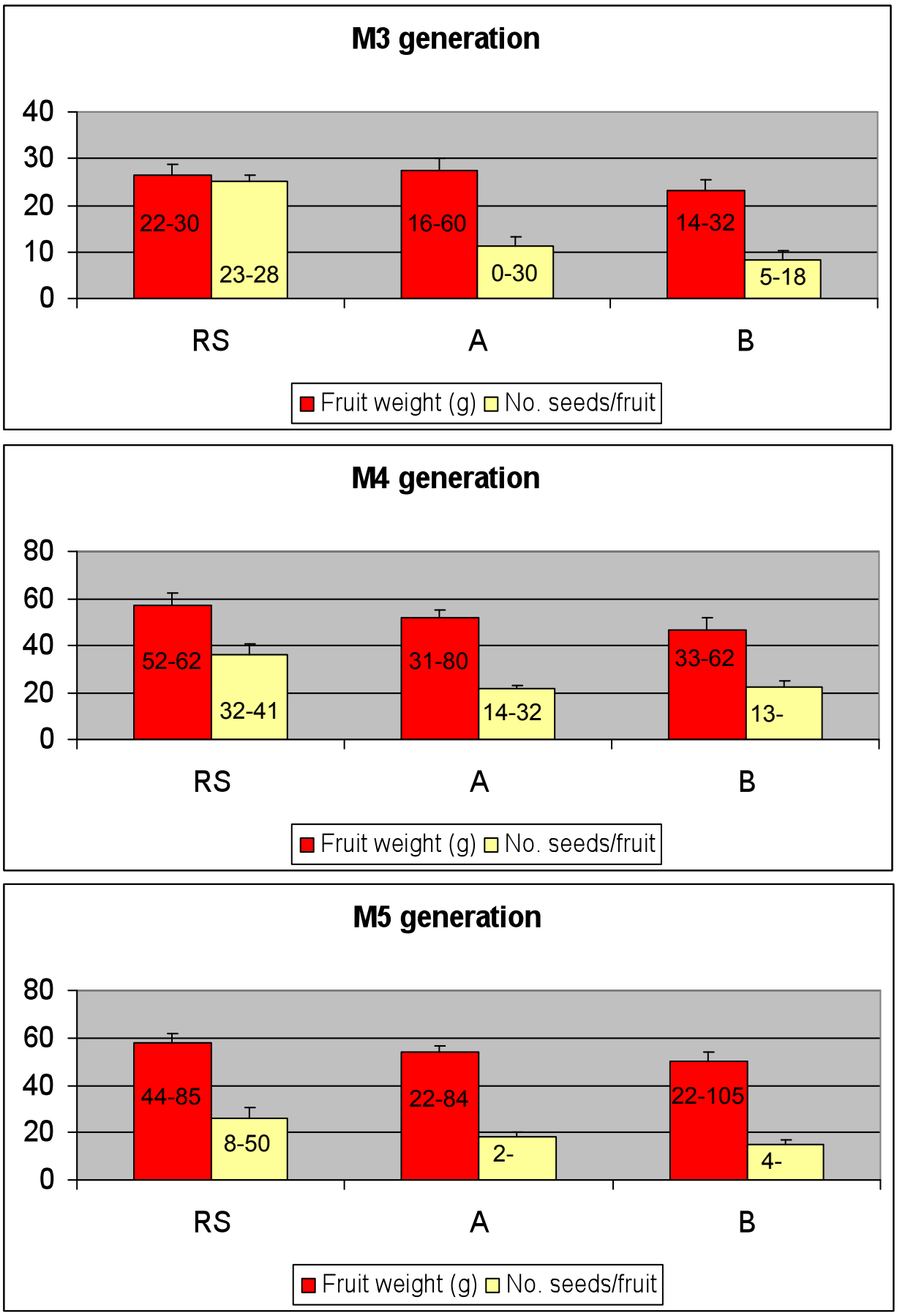


Fig. 4-7. Mean fruit weight (g) and number of seeds per fruit in plants of the Red Setter control (RS) and mutants homozygous for the A and B mutations in the M3 (a), M4 (b) and M5 (c) generation. Bars represent mean values ± SEM; ranges are reported inside each bar.

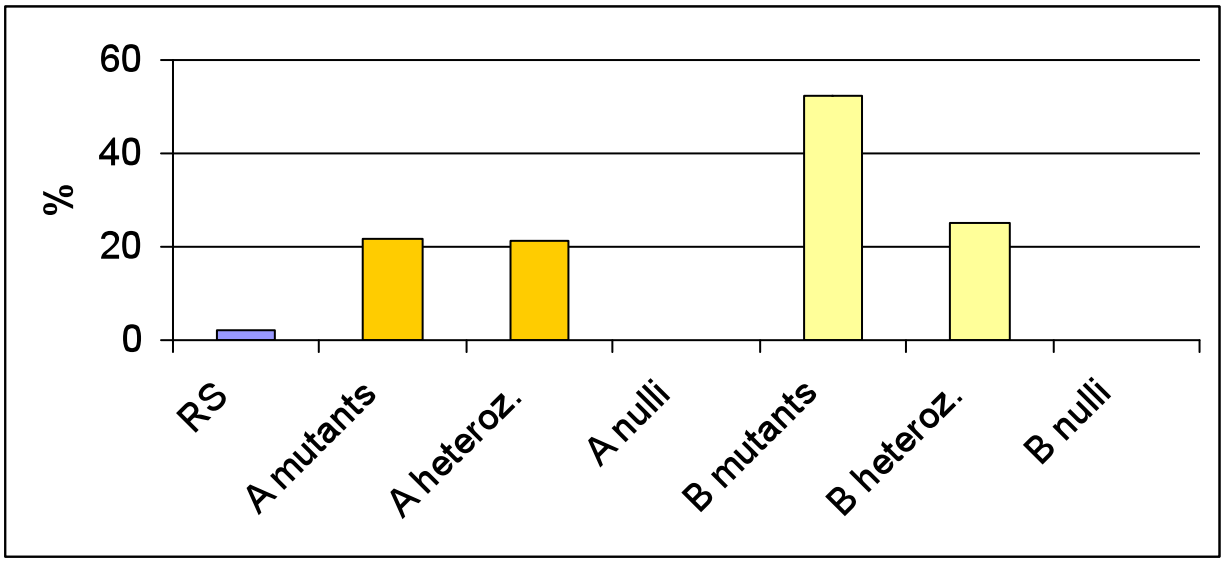


Fig. 4-8. Percentage of berries heavier than 10 g without seed in plants of the Red Setter control (RS) and mutants homozygous for the A and B mutations, heterozygotes and nullisegregants.

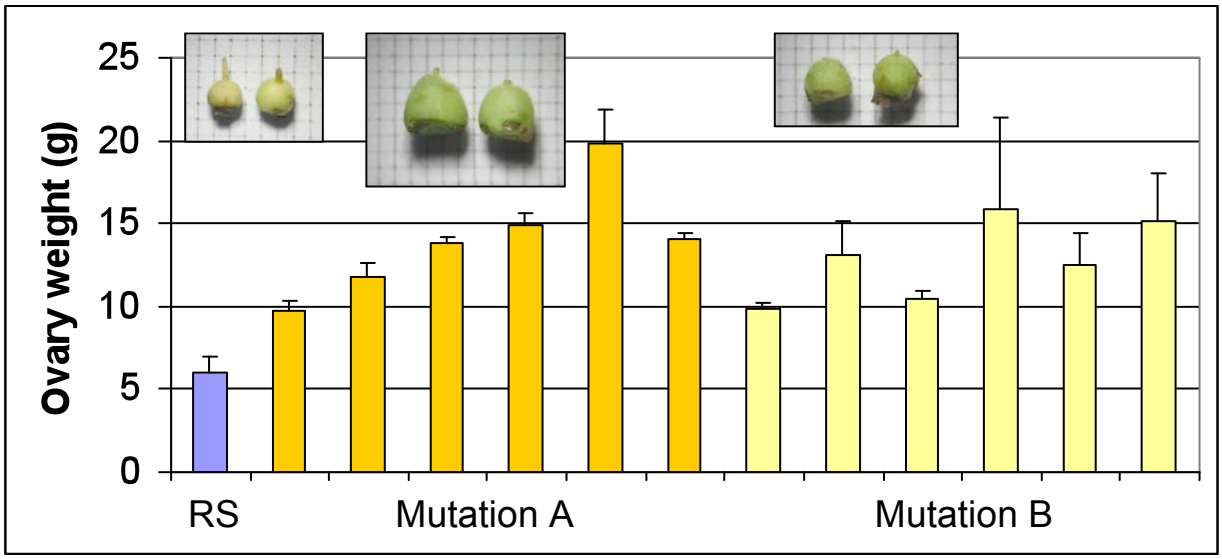


Fig. 4-9. Ovary weight after 15 days of *in vitro* growth in plants of the control (RS) and of selected mutants homozygous for the A and B mutation. Bars represent means±SEM; representative ovaries are reported in inserts.

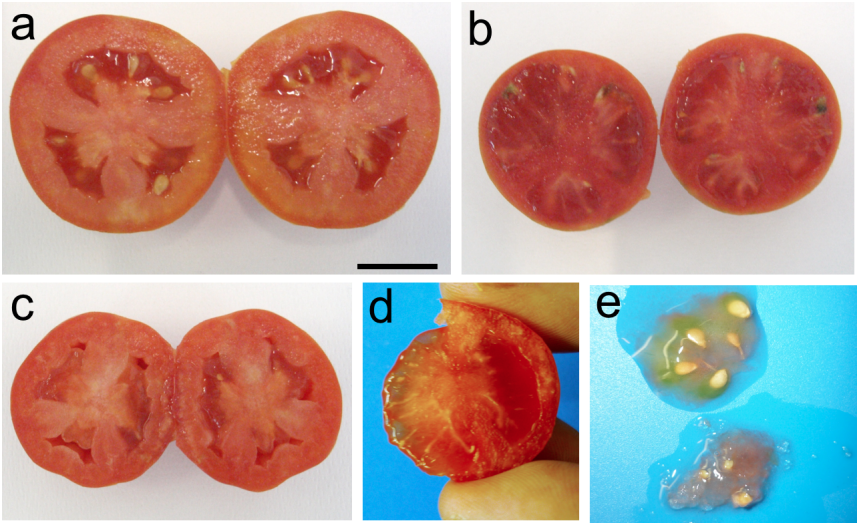


Fig. 4-10. Seeded (a) and seedless (b,c) fruits in mutants of the M4 generation; details of placenta without seeds (d) and of pseudoseeds compared with normal seeds (e).

Fig. 4-11. The early inflorescences (a) abort (arrow) in the plants homozygous for the C mutation and only the latest produce functional flowers and parthenocarpic fruits (entire b, sectioned c).

5. Final conclusion

The whole data obtained through a microsynteny approach with Arabidopsis allowed us to refine with new anchor-points the tomato genomic region spanning 1.2 cM between COSes T0796 and T1143, and to restrict the target interval for the locus *Pat* to about 0.2 cM between markers T17 and T20.

The new developed tomato COS markers showed a strict order conservation in the *Pat* locus region with those present in Arabidopsis. Nonetheless the data from the physical map show that T17-T20 chromosome interval in tomato (103 Kb) is about 8 times longer than in Arabidopsis (13.5 Kb) and it contains 11 genes instead of 4. This made more difficult than expected the positional cloning of the *Pat* gene.

The recent publication of the tomato genome sequence (SGN, www.sgn.cornell.edu) allowed us to finely analyze the new target region of about 103 Kb and to find out 4 candidate genes for the *pat* phenotype. HB15, a transcriptor factor belonging to HD-Zip III gene family, showed a nonsynonymus point mutation on the coding sequence of *pat* line. A loss of function of this gene has been proposed as responsible for the *pat* phenotype. Structural analysis of the mutated protein and similarities of *pat* with Arabidopsis *men1*, a mutant in which a MIR166a gene is activated by the insertion of the CaMV 35S enhancer leading to a drastic reduction of *ATHB15* mRNA level, strengthen this hypothesis. Consistently with these findings, we propose a model that explains the function of PAT (SlHD15) protein in the fruit set molecular pathway: it acts upstream to the ovary repressing machinery members, such as *ARF* and *Aux/IAA* genes. In order to strengthen this hypothesis, *in vivo* and *in vitro* functional analysis, are ongoing. The confirmation of this hypothesis will allow a non-transgenic transfer of *pat* phenotype in different tomato genetic background by molecular assisted backcrossing. Considering that HD-zip III genes are highly conserved in land plants, it will be also plausible to transfer the parthenocarpic trait to other species by silencing the *HD15* orthologue gene.

The screening of *SlIAA9* gene in two TILLING populations yielded mutants with the expected phenotypes. In the plants carrying the missense mutations such genotype were variably penetrant. This could be due either to an intrinsec low penetrance of the mutation or to the interaction of other mutations in the genetic background. Characterization of the latter mutation, that results in a premature stop codon, put in evidence, as expected, more severe vegetative phenotypes that mainly consisted in a loss of leaf compoundness. The

reproductive behavior of this line appears more severely affected, because early inflorescences aborted in the mutants and only the latest could develop functional flowers; higher order inflorescences gave parthenocarpic fruits. The final characterization and the assessment of the value of these mutants in breeding tomato parthenocarpic varieties will be thoroughly assessed when the genetic load of these genotypes will be substantially reduced.

Taken together, the results show that both *HB15* loss-function and *Aux/IAA9* loss-of-function (Wang *et al.,* 2005; this work) are able to confer parthenocarpy and that they take part to the same molecular pathway. Our model suggests that, after pollination, HB15 inhibits *SlARF7* gene whereas Aux/IAA9 (Wang *et al.,* 2005) triggers the degradation of *Sl*ARF7 proteins that are still present in the ovary. *Sl*ARF7 acts as a transcriptional activator of “auxin response attenuating genes”, repressing the auxin and gibberellin signalling pathways that are necessary to initiate tomato fruit development (de Jong *et al.,* 2009).

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