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Phenotypic, genetic and molecular characterization of the *parthenocarpic fruit* (*pat*) mutant and identification of the candidate gene in tomato (*Solanum lycopersicum* L.)

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Abeunt studia in mores

A mia Madre, ai miei Maestri,

SHORT ABSTRACT

Parthenocarpy is a developmental modification that entails fruit setting independently of pollination and fertilization. In parthenocarpic genotypes, fruit set can be achieved under pollen-limiting environmental conditions and in sterile genotypes. Parthenocarpy is a trait linked to fruit quality because it is a pre-requisite for seedlessness and is often associated with further positive quality aspects. Among the different sources of genetic parthenocarpy described in tomato, the *parthenocarpic fruit* (*pat*) mutation is of particular interest because of its strong expressivity, high fruit set and enhanced fruit quality. The complexity of the *pat* "syndrome" associates a strong competence for parthenocarpy with a complex floral phenotype involving stamen and ovule developmental aberrations. Previously, the *Pat* locus was mapped in a 0.19 cM window of chromosome 3, comprising nine loci. By comparative sequence analysis between the WT and the *pat* lines, a candidate gene was identified. A complementation experiment for the *pat* mutant line was carried out, by using a gene cassette consisting of the coding sequence of the candidate gene, a *class-III HD-Zip* gene (*SlHB15*), driven by a constitutive promoter (CaMV35S). Despite no WT phenotype could be scored in the T_1 generation, following post-transcriptional trans inactivation events, a number of T_1 co-suppression phenotypes were isolated showing some of the traits of the *pat* syndrome, among which anther homeotic aberrations, ovule abortion and reduction of seed number in the fruit. Further phenotypic and molecular characterization of the *pat* mutant fostered the potential role of the $SlHB15^{pat}$ allele in the *pat* syndrome.

ABSTRACT

Fruit set is a key trait for yield determination of the majority of crop species. Parthenocarpy is a developmental modification that entails fruit setting independently of pollination and fertilization. Thus, in parthenocarpic genotypes, fruit set can be achieved under pollen-limiting environmental conditions and in sterile genotypes. Parthenocarpy is a trait linked to fruit quality because it is a pre-requisite for seedlessness and is often associated with further positive quality aspects.

In order to understand the possible adaptive role of parthenocarpy, the incidence of this trait among Angiosperms was traced, by distinguishing monospermic (one/two-seed-fruited) and plurispermic (more-than-two-seed-fruited) species. Upon this classification the adaptive hypothesis of parthenocarpy for monospermic species was recalled and a hypothesis for the plurispermic species was advanced. An inventory of natural (not artificially induced) sources of parthenocarpy in the tomato proved that most of them stems from "distant" genome hybridization. This suggests that basically parthenocarpy might be the result of altered expression of modular sets of genes occurring from the inheritance of the parental genomes and caused by their heterochronicity and/or heterotopicity. Such a hypothesis, reflecting that proposed for some forms of apomixis, provides interesting similarities between these two, apparently different, phenomema.

In tomato, sources of parthenocarpy are available in natural and induced mutants. So far, this trait has also been successfully engineered by genetic transformation, but there is still a need for developing new strategies to harness seedlessness. In such a frame, a better

understanding of the mechanisms underlying fruit set and the genes that may be involved in its control represents a major objective. Among the different sources of genetic parthenocarpy described in tomato, the *parthenocarpic fruit* (*pat*) mutation is of particular interest because of its strong expressivity, high fruit set and enhanced fruit quality. Lately, the complexity of the *pat* "syndrome" has been described, which associates a strong competence for parthenocarpy with a complex floral phenotype involving stamen and ovule developmental aberrations. By a positional cloning approach the *Pat* locus was mapped in a 0.19 cM window of chromosome 3. Among the nine genes contained in the corresponding genomic interval, *SlHB15,* a *class-III HD-Zip* gene, was highlighted for the presence of a missense mutation in the *pat* genotype. To confirm the identity of *SlHB15* with the *Pat* locus, a complementation assay on the *pat* mutant line was carried out, by using a gene cassette consisting of the $SlHB15^{WT}$ coding sequence driven by the CaMV35S constitutive promoter. In the T_0 generation, no evident WT phenotype could be observed. On the contrary, from the WT line transformed with the *CaMV35S::SlHB15* construct, few putatively co-suppressed plants for *SlHB15* (CO-1 and CO-2) showed some of the traits of the *pat* syndrome, such as homeotic aberrations of anthers, ovule abortion, increased ovary growth at anthesis and a tendency to parthenocarpy (= fruits with a decreased number of seeds). Some CO-1-derived T_1 plants confirmed the phenotypes reminiscent of the *pat* syndrome and transcription analysis of *SlHB15* gave support to a co-suppression event of the gene. Further complementation experiments by using the candidate gene's own promoter are in progress which will benefit of the GoldenBraid® modular construction strategy. Further functional characterization of *SlHB15* regarded the generation of a *CRISPR/Cas9* locus targeting the third exon of *SlHB15*. After trasformation of the WT line with the *CRISPR/Cas9* construct, some of the first transformed regenerants, still in the *in vitro* growth phase, manifested induced modifications at the *CRISPR/Cas9*-targeted site. Most of the molecular and plant transformation work was carried out at Prof. Antonio Granell's Plant Genomics and Biotechnoly Laboratory (IBMCP, University of Valencia, Spain). To better characterize the candidate gene, the *SlHB15* promoter (3000 bp upstream the starting codon of the gene) was analyzed *in silico* for the presence of putative functional cis elements. The analysis was conducted on all the six members of *HD-Zip III* family. Out of 18 cis elements retrived, eight were specific to *SlHB15*. Among these, CA-box and CG-box motifs are required for regulating genes such as *NAC1, ARF19* and *IAA3/SHY2*, involved in auxin signal traansduction to promote a number of diverse processes regarding anther formation, fruit-set-driven ovary development but also root formation and root and hypocotyl development. For this reason, a functional role of *SlHB15* in processes governing root and hypocotyl formation and development was hypothesized. A comparative phenotypic analysis of the two NILs gave evidence of pleiotropic effects at the root, hypocotyl and vegetative adult plant level exherted by the *pat* lesion. Transcription analysis of key genes involved in hormone (i.e. auxin, cytokinin and gibberellins) metabolism and signal transduction pathway together with that of *SlHB15* at the seedling stage, showed alteration of their expression mainly in the hypocotyl and to a lesser extent in the root and epicotyl of the *pat* mutant when compared to the WT. Moreover, the increase of *SlHB15* transcription in the *pat* hypocotyl gave a first evidence that $SlHB15^{pat}$ allele might be functionally active. To this respect, a phenotypic analysis of an F_2 population cosegregating the *Pat* locus with *SlHB15*, allowed to reconsider the recessive

degree of the *pat* mutant, providing it with a weak dominant negative (antimorphic) effect. Such an effect could find a correspondance with the potential antimorphic effect of *SlHB15*^{pat}, as already formulated for some mutations affecting the *ICU4/CNA/HB15* gene, the *SlHB15* orthologue in Arabidopsis, thus providing more evidence of the identity between the candidate gene and the *Pat* locus.

RIASSUNTO BREVE

La partenocarpia, la formazione e lo sviluppo del frutto in assenza di fecondazione, è un carattere legato alla qualità del frutto poiché è un prerequisito per l'assenza di semi e spesso è associato ad ulteriori aspetti positivi della qualità. Tra le diverse fonti di partenocarpia genetica descritta nel pomodoro, la mutazione *parthenocarpic fruit* (*pat*) è di particolare interesse per la sua forte espressività, l'elevato numero e la migliore qualità dei frutti. La complessità della "sindrome" *pat* associa una forte competenza per la partenocarpia con un fenotipo floreale complesso che comporta l'aberrazione dello sviluppo dello stame e dell'ovulo. In precedenza, il locus *Pat* è stato mappato in una finestra 0.19 cM del cromosoma 3, comprendente nove loci. Mediante analisi comparativa di sequenza tra le due linee quasi isogeniche, WT e *pat*, è stato identificato un gene candidato. È stato condotto un esperimento di complementazione per la linea *pat*, utilizzando una cassetta genica consistente nella sequenza codificante del gene candidato, un gene *HD-Zip di classe III* (*SlHB15*), guidato da un promotore costitutivo (CaMV35S). Nonostante non sia stato possibile rinvenire un chiaro fenotipo WT nella generazione T_1, a seguito di eventi di inattivazione trans post-trascrizionale (cosoppressione) di *SlHB15*, un numero di piante T_1 della linea WT ha mostrato alcuni dei caratteri della sindrome *pat*, tra cui aberrazioni omeotiche dell'antera, aborto dell'ovulo, ingrossamento dell'ovario all'antesi e tendenza alla partenocarpia con riduzione del numero di semi nel frutto. L'ulteriore caratterizzazione fenotipica e molecolare del mutante *pat* ha confermato il ruolo potenziale dell'allele $SlHB15^{pat}$ nella sindrome *pat*.

RIASSUNTO

Caratterizzazione fenotipica e genetico-molecolare del mutante *parthenocarpic fruit* (*pat*) e identificazione del gene candidato in pomodoro (*Solanum lycopersicum* L.)

Lo studio dei meccanismi molecolari alla base del processo fisiologico della partenocarpia (formazione e sviluppo del frutto in assenza di fecondazione) si è giovato dell'impiego dei mutanti partenocarpici. Al fine di approfondire la conoscenza sui meccanismi che regolano la partenocarpia ed il suo eventuale ruolo adattativo, viene tracciata l'incidenza di questo carattere tra le Angiosperme, distinguendo quelle che presentano frutti contenenti un solo seme (specie monospermiche) e quelle con frutti che ne contengono un numero maggiore (specie plurispermiche). Sulla base di questa prima distinzione, viene ripercorsa l'ipotesi del ruolo adattativo della partenocarpia tra le specie monospermiche e proposta un'ipotesi sulla sorprendente incidenza del carattere anche tra le specie plurispermiche. L'inventario delle fonti di partenocarpia descritte in pomodoro e la constatazione che la maggior parte di esse derivi da ibridazioni "distanti", ha suggerito come alla base del processo vi possa essere un'alterata espressione e asincronia di moduli genetici che vengono ereditati dai due genomi parentali. Tale ipotesi, che ricalca quella proposta come base di alcuni fenomeni apomittici, fornisce uno spunto di riflessione sulla similitudine tra i due fenomeni (partenocarpia e apomissia *sensu lato*), apparentemente molto diversi, ma che in realtà condividono aspetti comuni.

Il mutante *parthenocarpic fruit* (*pat*) presenta elevata penetranza ed espressività per il carattere "assenza di semi" a cui si associa una serie di effetti pleiotropici quali le

alterazioni degli organi fiorali (antere dialitiche e di lunghezza ridotta, ovuli con perdita di vitalità), precocità nella maturazione del frutto, produzione di frutti qualitativamente migliori. Attraverso un approccio di clonaggio posizionale, il locus *Pat* è stato localizzato all'interno di un intervallo genetico di 0,19 cM nella porzione distale del cromosoma 3, che a livello genomico comprende nove geni. Di questi, *SlHB15*, è un gene che codifica per un fattore di trascrizione appartenente alla classe III della famiglia HD-Zip. L'analisi di sequenza di questo gene tra il mutante *pat* e la linea di controllo (WT) ha messo in evidenza la presenza di una sostituzione non sinonima che potrebbe essere all'origine della sindrome *pat*. Per dimostrare questa ipotesi, si è proceduto con la caratterizzazione funzionale di *SlHB15* attraverso test di complementazione del mutante *pat*. Parallelamente è stata trasformata la linea WT al fine di mettere in evidenza un eventuale effetto dovuto alla sovraespressione e/o cosoppressione del gene. Per la trasformazione si è impiegato un costrutto contenente la regione codificante di *SlHB15* ed il promotore costitutivo *CaMV35S*. Mentre le piante T_0 con genotipo *pat* non hanno mostrato evidente reversione del fenotipo, in due delle piante della linea WT rigenerate e contenenti il costrutto (CO1 e CO2) è stata rilevata la presenza di alterazioni di tipo omeotico (antere e ovuli aberranti), riconducibili a quelle osservabili nel mutante *pat* e che hanno fatto supporre l'avvenuta cosoppressione del gene *SlHB15*. L'analisi di espressione di *SlHB15*, eseguita con tecnica qRT-PCR su CO-1, CO-2 e relativi controlli ha messo in evidenza il silenziamento del gene nelle due piante T_0 con fenotipo alterato. La progenie T_1 di CO-1, costituita da 28 piante, ha segregato il transgene con un rapporto Mendeliano. Delle piante contenenti il transgene, 17 hanno mostrato un fenotipo cosoppresso, nuovamente confermato dall'analisi di espressione di *SlHB15*. Alla luce dell'evento di cosoppressione, è stata avviata l'attività per un nuovo test di complementazione, questa volta realizzando un costrutto contenente un promotore costituito dalla regione genomica di 2300 bp in posizione 5' rispetto all'inizio della sequenza codificante di *SlHB15*. Per la realizzazione dei tale costrutto è stato adottato il sistema di clonaggio GoldenBraid®. Per un'ulteriore caratterizzazione funzionale di *SlHB15*, è stata intrapresa la realizzazione di un costrutto di *genome editing* del gene candidato con il sistema CRISPR/Cas9. Mentre il primo costrutto è ancora in fase di realizzazione, il costrutto *CRISPR/Cas9* contenente la sequenza target per il locus *SlHB15* è stato introdotto nella linea WT con un protocollo di trasformazione sviluppato presso il laboratorio di Genomica e Biotecnologia Vegetale del prof. Antonio Granell (IBMCP, Università di Valencia, Spagna), presso cui mi sono recato per svolgere parte delle attività di clonaggio e trasformazione genetica. Alcuni dei primi rigeneranti transgenici, attualmente ancora nella fase di sviluppo *in vitro*, hanno mostrato modificazioni indotte nella sequenza di *SlHB15* in corrispondenza dell'omologia con la sequenza gRNA del costrutto *CRISPR/Cas9*. Per caratterizzare ulteriormente il gene candidato, il promotore di *SlHB15* è stato analizzato *in silico* con un software per la ricerca di motivi cis con ruolo funzionale documentato. L'analisi è stata eseguita a carico dei promotori (3000 bp) dei sei membri della famiglia *HD-Zip III* di pomodoro. Dei 18 motivi trovati sul promotore di *SlHB15*, otto risultano gene specifici. Tra questi, gli elementi regolatori CA-box e CG-box sono richiesti per la regolazione di geni come *NAC1, ARF19* e *IAA3/SHY2*, coinvolti nella trasduzione del segnale ormonale dell'auxina per promuovere un numero elevato e diversificato di processi come la formazione delle antere, della progressione dello sviluppo dell'ovario una volta avvenuto il fruit-set ma anche in processi come la formazione e sviluppo delle radici laterali e lo sviluppo dell'ipocotile. Quindi, è stato ipotizzato che *SlHB15* potesse avere un ruolo anche a livello della formazione e sviluppo delle radici e dell'ipocotile. Un approfondimento sullo studio di possibili effetti pleiotropici della mutazione *pat* a carico anche di questi organi, ha messo in evidenza come nel mutante *pat* la radice primaria sia più lunga rispetto alla linea WT e presenti un numero più elevato di radici secondarie ed avventizie, anch'esse di lunghezza maggiore rispetto al

controllo mentre l'ipocotile del mutante risulta più corto. L'analisi di espressione di geni chiave coinvolti nel metabolismo e nella trasduzione del segnale di ormoni come auxina, citochinine e gibberelline ha potuto mettere in evidenza come nelle radici e nell'ipocotile del mutante *pat,* l'espressione di tali geni sia alterata e come i loro pattern di espressione si possano mettere in relazione con la manifestazione dei caratteri pleiotropici osservabili a livello vegetativo nel mutante. Inoltre, l'aumento del livello di trascrizione di *SlHB15* nell'ipocotile ha fornito l'indicazione che *SlHB15^{pat}* possa raprresentare un allele funzionale fornendo un interessante spunto di riflessione sulla relazione di identità tra *pat* e *SlHB15*. A tal fine l'osservazione fenotipica di una popolazione segregante per il locus *pat,* unitamente al locus associato *SlHB15*, ha permesso di riconsiderare il grado di recessività del mutante *pat*, attribuendo un potenziale effetto antimorfico alla mutazione *pat*. Tale effetto potrebbe trovare una chiara corrispondenza (identità) con il potenziale effetto di tipo dominante negativo (antimorfico) dell'allele *SlHB15^{pat}*, al pari di quanto avviene in alcuni mutanti di Arabidopsis per il gene ortologo di *SlHB15*.

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Chapter 1

Introduction

Fruit set and parthenocarpy

The fruit forms through an intimate developmental collaboration between ovules (the developmental precursors to seeds) and carpels (the precursors to the body of the fruit). In tomato, the ovary, which develops in concert with the rest of the flower organs (growth phase I, according to Gillaspy *et al*., 1993), ceases to undergo cell divisions shortly (1–2 days) before anthesis and enters an 'ovary arrest' state. Pollination occurs after formation of pollen grain in the anther and its release. Subsequently, fertilization, the fusion of the two gametes, requires pollen germination, penetration and growth of the pollen tube in the stylar tissue towards the ovule, a structure containing the embryo sac, in order to fuse with the egg cell (Dumas and Mogensen, 1993). Only if fertilization is successfully completed, a signal believed to be produced by the young embryo provokes the ovary to resume growth. This growth involves initially a ph
ase of rapid cell division and expansion (designated phase II or 'fruit set') for 5–10 days (Bohner *et al*., 1988; Varga and Bruinsma, 1986), and subsequently (during phase III) growth is driven mainly by cell enlargement concomitant with nuclear polyploidization (Chevalier *et al*., 2011, and references therein). Once reached full size, the ripening processes initiates. As such, the normal process of fruit development is the result of a highly orchestrated series of molecular events and may occasionally be independent of pollination and fertilization.

The similarity between fruit seedlessness and parthenogenesis led Noll (1902) to introduce the term "Partenokarpie" to denote the seedless condition of the fruit. To date, two main mechanisms are responsible for the formation of seedless fruits (Varoquaux *et al*., 2000): (a) parthenocarpy, where the fruit develops in the absence of fertilization, as in cultivated pineapples, some *Citrus* cultivars, and bananas; and (b) stenospermocarpy, where pollination and fertilization are required, but embryos either do not form or they abort before completion of seed formation, as in seedless watermelons and many seedless grapes (Bouquet and Danglot, 1996). In both cases, the plant must have an inherent or acquired ability to sustain fruit development in the absence of seed formation.

Parthenocarpy may occur naturally but, for a long time, humans have attempted to impair the "ovary arrest" state to develop seedless fruits to make them easier to consume. Parthenocarpy can be induced artificially with the application of various hormones (Gustafson, 1936, 1942; Nitsch, 1952; Osborne and Went, 1953; Schwabe and Mills, 1981). 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).

Parthenocarpy in tomato

Tomato is an important crop in the fresh vegetable and food processing industries. Due to its relatively small genomic size and to the fact that it exhibits the same haploid chromosome number and conserved genome organization as other solanaceous plants, tomato is recognized as a representative species of Solanaceae for studies of fruit development. Although yield is the most important breeding trait in tomato, relatively few genes involved in early fruit development have been isolated, and many of these are associated with cell division and the cell cycle (Tanksley, 2004). However, recent advances in genetic and molecular tools and the associated bioinformatics platforms, coupled with the availability of increased sequence information have accelerated the identification of other components that participate in fruit development, leading to an improved understanding of the molecular framework that regulates fruit development intomato. In particular, there is considerable evidence that fruit set is under a complex hormonal control and that plant hormones such as auxin, gibberellin (GA), and cytokinin (CK) are main components of such a process (Gillaspy *et al*., 1993; Ruan *et al*., 2012; Ariizumi *et al*., 2013; McAtee *et al*., 2013). Application of these phytohormones to unpollinated tomato ovaries can induce parthenocarpic fruit set (Mapelli *et al*., 1978; Serrani *et al*., 2007b; Matsuo *et al*., 2012). In addition, several parthenocarpic tomato varieties have been developed, but most of the genes responsible for the traits remain unknown.

Identification of genes conferring parthenocarpy by forward genetics approaches

Despite the plenty of natural genetic sources of parthenocarpy, few are the genes that have been isolated so far. Parthenocarpy has been found in apple cultivars that produce flowers

lacking petals and stamens, and presenting two whorls of sepals and up to 15 styles (Tobutt, 1994). In *Malus domestica* L., the Arabidopsis orthologue of the class-B MADS-box gene *PISTILLATA* (*MdPI*) has been isolated as the responsible for the presence of the parthenocarpic trait (Yao *et al.,* 2001). *MdPI* is required for both petal and stamen identity specification (Goto and Meyerowitz, 1994). In tomato, parthenocarpy has been observed in male sterile mutants displaying deficiencies in stamen formation and/or anther dehiscence which result in the failure of fertilization. The mutation of genes involved in stamen identity specification was responsible for occasional parthenocarpy in male sterile mutants, such as *stamenless* (Gomez *et al.*, 1999) and *pistillate* (Olimpieri and Mazzucato, 2008). These observations suggest that parthenocarpy can be triggered by the absence of the normal synchronized development of male and female organs.

In Arabidopsis, a member of the *Auxin Response Factor* (*ARF*) gene family, *ARF8/FRUIT WITHOUT FERTILIZATION* (*FWF*), has been characterized (Goetz *et al*., 2006). mRNA levels of this gene are high in the placental tissues of the mature flower, and rapidly decrease after pollination. The mutated *fwf* allele triggers the formation of parthenocarpic siliques (Vivian- Smith *et al*., 2001). 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* allele in tomato also results in parthenocarpic fruit set, indicating that likely 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 *AtARF8*. The third case of a gene identified as responsible for seedless fruit production was found in a mutant of *Annona squamosa* L. (sugar apple) which fails to form seeds due to a defect in ovule development (Lora *et al*., 2011). In this case the ovules of the mutant lack the outer of two normal integuments, a

phenocopy of the *inner no outer* (*ino*) mutant of Arabidopsis, displaying seedless siliques. Through a candidate-gene approach, the INO gene orthologue from *A. squamosa* confirmed conservation of the outer integument-specific expression pattern of this gene between the two species but no expression could be observed in the mutant, indicating deletion of the *INO* locus (Lora *et al.,* 2011).

Induction of parthenocarpy in tomato by hormone-related reverse genetics approaches

Even though parthenocarpy represents a potentially valuable trait for many fruit crops, parthenocarpic varieties have been widely cultivated for only a few species (e.g., cucumber and banana) because high-quality fruits and a high yield are seldom combined with the parthenocarpic trait (Varoquaux *et al*. 2000; Pandolfini *et al*. 2002). This limitation is also true for tomato. In addition, the environment in which the fruits are cultivated has a major influence on parthenocarpy, and so the consistent control of the phenotypes in a commercial setting has proven difficult. These barriers have made it difficult to develop parthenocarpic varieties that stably produce high-quality fruits. In tomato, different tomato lines carrying mutations for parthenocarpy have been discovered or selected (see Chapter 2; reviewed by Philouze, 1983; Lukyanenko, 1991 and Gorguet *et al.,* 2005) but their genetic basis has not been disclosed so far. However, the induction of fruit set by modifying the expression level of mainly plant hormone-related genes has greatly been demonstrated in tomato. Auxin and GA are the most common phytohormones used to trigger artificial parthenocarpy in crop plants. Recent studies on genetic parthenocarpy have contributed to the mechanisms underlying the biological effects triggered by auxin and GA but also by other hormones.

Auxin-related genes

The action of auxin-related compounds in triggering fruit set was first described in the early twentieth century (Gustafson 1936, 1937, 1939), and several natural and synthetic auxins were subsequently shown to induce parthenocarpic fruit set (Ho and Hewitt, 1986). More recently, several reports have described the induction of tomato parthenocarpy by altering the expression of several genes related to auxin metabolism and signalling (de Jong *et al.* 2009; Ariizumi *et al*. 2013). For example, a chimeric gene, DefH9-iaaM, was used in

early trials to produce transgenic parthenocarpic fruits in *Solanaceae*. This chimeric gene was formed of two components: the promoter of *Deficiens homolog* (*DefH9*), which is an *Antirrhinum majus* MADS-box gene that is expressed specifically in the ovule, and *Tryptophan-2-monooxygenase* (*iaaM*), derived from *Pseudomonas syringae*, which can induce auxin biosynthesis via synthesis of the indole acetic acid (IAA) precursor indol acetamide. The expression of *DefH9-iaaM* was detected in the developing buds of the transgenic plants (Ficcadenti *et al.* 1999), which showed normal vegetative growth and developed fruits from emasculated flowers similarly to what happened in WT flowers after pollination from non-transformed plants. The fresh weight of individual transgenic fruits was lower, but it was compensated for by an increased fruit number, resulting in a yield comparable with that of parental non-transgenic lines.

Expression of *iaaM* under the promoter of the ovule-specific *INO* gene, which is expressed only in the ovule outer integument, has also been shown to induce parthenocarpy (Martinelli *et al*. 2009), but with fruits showing no significant morphological abnormalities.

Another chimeric gene, comprising *Agrobacterium rhizogenes rolB* fused to the ovary- and young-fruit-specific promoter, *TPRP-F1*, has also been used to generate parthenocarpic fruits (Carmi *et al*. 2003). Tomato plants transformed with *rolB* showed auxin-responsive phenotypes, although the underlying molecular mechanism is not yet known. The transgenic plants developed seedless fruits with a size and morphology comparable to those of seeded fruits from the parental lines, but fruit yield and several other qualities were different in greenhouse-cultivated plants.

AUXIN CUM SILENCING ACTION (*AUCSIA*) was identified as a gene that was repressed in parthenocarpic flower buds of *DefH9-iaaM* transgenic plants (Molesini *et al*. 2009). The tomato genome has two *AUCSIA* genes (*AUCSIA1* and *AUCSIA2*) that encode small polypeptides. RNA interference (RNAi)-mediated simultaneous suppression of *AUCSIA1* and *AUCSIA2* has been shown to result in parthenocarpy and an approximately 100-fold increase in total IAA content in the buds. The parthenocarpic fruit size and weight were smaller than those of wild-type fertilized fruits. The role of *AUCSIA* during fruit set is still unclear, but it might be involved in either auxin synthesis or transport*PIN-FORMED* (*PIN*) auxin efflux transporters play important roles in fruit set by controlling polar auxin transport (PAT) between ovules and nearby tissues. Of the ten *PIN* genes (*SlPIN1–SlPIN10*) identified in tomato (Pattison and Català, 2012), *SlPIN4* has been shown to participate in fruit set (Mounet *et al*. 2012). *SlPIN4* is predominantly expressed in flower buds and young developing fruits, where the expression level is higher in the placenta than in the locular

tissue and pericarp. Specific silencing of *SlPIN4* using an RNAi approach resulted in parthenocarpy, suggesting a negative role in fruit set (Mounet *et al*., 2012).

Auxin signalling is mediated by both transcription-dependent and –independent pathways, but only a few molecular components underlying the latter pathway have been identified (Mockaitis and Estelle, 2008; Hayashi, 2012). In the transcription-dependent pathway, protein–protein interactions among several key components lead to auxin responses. At low auxin levels, auxin/IAA (Aux/IAA) transcriptional repressors interact with auxin response factor (ARF) transcription factors, which repress the transcriptional expression of auxin-responsive genes (Tiwari *et al*., 2004; Guilfoyle and Hagen, 2007). At high auxin levels, auxin promotes the degradation of the Aux/IAA proteins via a ubiquitin–proteasome system (Gray *et al*., 1999; Dharmasiri and Estelle, 2002). Auxin is perceived by the TIR1/AFB family of F-box proteins acting as auxin receptors, which form SCFE3 ubiquitin ligase complexes, leading to the ubiquitination of Aux/IAA (Gray *et al*., 2001; Kepinski and Leyser, 2005; Dharmasiri et al., 2005; Maraschin *et al*., 2009). Auxin-dependent proteolysis of Aux/IAA leads to the induction of auxin-responsive gene expression via activation of ARF transcription factors. A total of 26 Aux/IAA repressor family genes (*SlIAA1–SlIAA26*) have been found in tomato (Wu *et al*., 2012). *SlIAA9*, which is highly expressed throughout the plant, has been shown to play a regulatory role in fruit development, and antisense lines of *SlIAA9* show a wide range of auxin-related growth alterations, including reduced leaf complexity and the production of parthenocarpic fruit with size, color and flesh consistency that are similar to those of wild-type fruits (Wang *et al*., 2005). The accumulation of *SlIAA9* transcripts at anthesis forms a gradient, where the transcript levels are higher in the ovule, placenta, and funiculus but lower in the ovary wall and columella (Wang *et al*., 2009). Rapid dissipation of the signal gradient occurs approximately one day after pollination, suggesting an important role for *SlIAA9* in the early stages of fertilization-induced fruit set. Three independent mutants of *SlIAA9* (*iaa9-3, iaa9-4,* and *iaa9-5*) exhibiting altered vegetative phenotypes and parthenocarpy have been identified in ethyl methylsulfonate (EMS)-mutagenized or γ-ray-irradiated populations (Saito *et al*., 2011). Another allele, iaa9-618, isolated by TILLING strategy displayed similar phenotypes (Mazzucato *et al*., 2015). The rates of parthenocarpy and seedless fruit expansion vary among the mutants, suggesting that the functional activity and extent to which parthenocarpy is conferred vary for the different SlIAA9 alleles.

The tomato ARF family comprises at least 17 members (*SlARF1–SlARF17*; Kumar *et al*., 2011). *SlARF7* is predominantly expressed in unpollinated tomato ovaries and its

expression rapidly decreases after pollination (de Jong *et al*. 2009). RNAi transgenic tomato lines with reduced *SlARF7* mRNA levels produce parthenocarpic fruits, suggesting that this ARF gene acts as a negative regulator of fruit set. Furthermore, the parthenocarpic fruits showed GA-related phenotypes, such as a thick pericarp due to extensive cell expansion, in addition to auxin-related phenotypes, specifically a heart-shaped fruit and the formation of seed-like structures resembling pseudoembryos. These findings suggest that *SlARF7* could be involved in the cross talk between auxin and GA during fruit set. To this respect, a model suggests that *SlARF7* activates auxin response-attenuating genes (such as Aux/IAAs) in unpollinated ovaries, while downregulation of SlARF7 after pollination results in an activation of both auxin and GA signalling that is required for fruit set (de Jong *et al.,* 2011).
In Arabidopsis, *arf8* mutants produce parthenocarpic fruits (siliques) without fertilization, suggesting that *AtARF8* is a negative regulator of fruit set (Goetz *et al*., 2006). Goetz *et al*. (2007) further showed that the introduction of aberrant forms of *AtARF8* led to parthenocarpy in Arabidopsis and tomato. Since the expression of *AtARF8* was not reduced in the transgenic plants, the mutated form of the AtARF8 protein may have functionally competed with endogenous AtARF8 protein and its tomato homolog. A model was proposed by the authors in which ARF8 forms a regulatory complex with Aux/IAA, and this complex directly or indirectly represses transcription of fruit set-regulating genes (Goetz *et al*., 2006, 2007).
A putative tomato auxin receptor, *SlTIR1*, plays an important role in the early stage of fruit set (Ren *et al.*, 2011). *SlTIR1* is highly expressed in the ovary and sepal at anthesis, but its expression decreases after pollination. Overexpression of *SlTIR1* results in an auxin-responsive phenotype, including altered vegetative morphology, sterility and parthenocarpy. *SlTIR1* has been suggested to positively regulate the auxin response via the 26S proteasome-mediated signalling pathway (Ren *et al.,* 2011).

GA-related genes

The regulatory effect of GAs on fruit development has been well documented (Wittwer *et al*., 1957; Sastry and Muir, 1963; Serrani *et al*., 2007a). Expression of genes in the tomato GA20ox family (*SlGA20ox2*–*SlGA20ox3*) that mediate bioactive GA synthesis increases in the ovaries after pollination, suggesting a central role for GA20ox genes in GA synthesis during fruit set (Olimpieri *et al*., 2007; Serrani *et al.*, 2007b). Additionally, overexpression of a citrus *GA20ox* gene (*CcGA20ox1*) in tomato resulted in pleiotropic phenotypes similar to those of GA-treated plants, including parthenocarpy (Garcià-Hurtado *et al*., 2012). DELLA

family proteins act as key repressors of GA signalling. GA induces the degradation of DELLA proteins via the ubiquitin–proteasome system, leading to GA responses (Dill *et al*., 2001; Mcginnis *et al*., 2003; Sun, 2010). Tomato has a single *DELLA* gene (*SlDELLA*); antisense-mediated silencing of this gene results in constitutive GA-responsive phenotypes, such as elongated plant shape and parthenocarpy (Martì *et al*., 2007). In addition to being smaller and elongated, the parthenocarpic fruit had a reduced number of cells, which were elongated in the pericarp. These features are similar to those seen in GA-induced fruits (Serrani *et al*., 2007a), suggesting that parthenocarpic fruit development in *SlDELLA* antisense bypasses auxin-regulated cell division (Martì *et al*., 2007). A loss-of-function mutant of *SlDELLA*, *procera* (*pro*), also exhibits a constitutive GA-responsive phenotype, including parthenocarpy (Bassel *et al*., 2008). The *pro* mutation influences auxin signalling with a reduction of *SlARF7* expression during fruit set (Carrera *et al*., 2012), supposing a role of *SlARF7* in the cross talk between GA and auxin signalling during fruit set.

Cytokinin-related genes

Cytokinin (CK), a growth-promoting hormone, induces parthenocarpy in many agricultural species. CK and GA act antagonistically in various developmental processes (Harberd *et al.,* 2009; Weiss and Ori, 2007). In tomato, it is likely that GA inhibits CK responses, while CK inhibits GA responses. For example, anthocyanin accumulation, hypocotyl length and leaf complexity in tomato are strongly associated with the ratio of GA to CK, rather than with the absolute levels of these hormones (Fleishon *et al.,* 2011). The inhibitory effect of GA on CK-induced phenotypes (e.g., anthocyanin accumulation and leaf complexity) occurs likely via both a DELLA-independent pathway and a DELLA dependent pathway. CK may inhibit the downstream steps of the GA signalling pathway, whereas GA may inhibit the early steps of CK signalling (Fleishon *et al*., 2011). The level of CK is up-regulated five days after anthesis, at a time when cell division is active, suggesting that there is a positive correlation between CK and cell division (Bohner *et al.*, 1988). In fact, CK is believed to be secreted from the developing seeds and may activate cell division in the tissue ssurrounding seeds (Bohner and Bangerth, 1988). Recent evidence demonstrated that the application of synthetic CK to pre-anthesis tomato ovaries resulted in parthenocarpic fruit formation by activating cell division (Matsuo *et al*., 2012); thus, CK acts as a positive regulator of fruit growth.

Ethylene-related genes

Ethylene plays a critical role in many developmental processes, such as senescence and abscission of leaves (Lim *et al*., 2007) and flowers (van Doorn and Woltering, 2008) and fruit ripening (Barry and Giovannoni, 2007). Llop-Tous *et al*. (2000) showed that pollination induces transient increases in the production of ethylene in tomato pistils for several hours, although this apparently does not induce ovary senescence (Vriezen *et al*., 2008) and ethylene production decreases after 12 h of pollination (Llop-Tous *et al*., 2000). The expression of various genes related to the biosynthesis and signalling of ethylene has been observed to change during the early development of both pollinated and parthenocarpic tomato fruits (Vriezen *et al*., 2008; Pascual *et al*., 2009; Wang *et al*., 2009), all suggesting that ethylene also plays a regulatory role in tomato fruit set; however, further studies are required to elucidate the exact mechanisms. Overexpression of a tomato component of ethylene signalling, the *TETRATRICOPEPTIDE REPEAT PROTEIN 1* (*SlTPR1*) that interacts with the ethylene receptors *NEVER RIPE* (*NR*) and *ETHYLENE RECEPTOR1* (*SlETR1/LeETR1*) results in ethylene-related pleiotropic effects and parthenocarpic fruit set (Lin *et al*., 2008). The upregulation of an auxin-responsive gene in the buds of *SlTPR1*-overexpressing plants suggests that *SlTPR1* is directly or indirectly involved in auxin signalling, while downregulation of *SlIAA9* in the ovaries of *SlTPR1* transgenic plants may contribute to parthenocarpic fruit set. The application of ethylene inhibitors has been found to induce parthenocarpy in zucchini (*Cucurbita pepo*), indicating that ethylene negatively regulates its fruit set (Martìnez *et al*., 2013). In Arabidopsis, ethylene is involved in ovule senescence and negatively regulates parthenocarpic fruit set induced by GA (Carbonell-Bejerano *et al*., 2011).

Flower development-related genes

MADS-box proteins are multifunctional TFs found in a wide range of eukaryotic organisms; they function in the regulation of organ and cell differentiation in flower development (Theissen and Saedler, 2001). Tomato has at least 36 *MADS-box* genes (Hileman *et al*., 2006), and several studies have indicated a relationship between tomato parthenocarpic fruit development and MADS-box proteins. In Arabidopsis, three *MADS-box* genes, *SEPALLATA1–SEPALLATA3* (*SEP1–SEP3*) are required for normal floral organ development (Pelaz *et al.*, 2000). Studies with tomato have shown that tomato *MADS-box 29* (*TM29*), an ortholog of Arabidopsis *SEP1*, is continuously expressed in developing flowers and preferentially in the peripheral region of well differentiated ovaries and fruits (Ampomah-Dwamena *et al*., 2002). Transgenic tomato plants constitutively expressing an antisense

construct of *TM29* produce morphologically altered flowers and parthenocarpic fruits, suggesting that TM29 not only is required for normal flower development but may also function as a negative regulator of fruit set. In apple (*Malus domestica*), antisense suppression of *MADS8* and *MADS9* (homologs of *SEP1* and *SEP2*, respectively) resulted in an increased expression of auxin biosynthetic genes, a reduced expression of the *GH3 auxin-conjugating enzyme* genes, and a high accumulation of free auxin in the fruits during the early ripening stage (Schaffer *et al.,* 2013). Although auxin concentration in the *TM29*-suppressed ovaries had not been measured, production of parthenocarpic fruits by silencing of these *SEP* family genes might be a consequence of the elevated auxin concentration. *TM5*, a tomato orthologue of Arabidopsis *SEP3*, is known to function in both flower and fruit development (Pnueli *et al*., 1994). *TM5* is continuously expressed in the central apical zone of the floral meristem throughout differentiation in the tissues of petals, stamens, and pistils. Antisense suppression of *TM5* expression resulted in parthenocarpy and altered identities of floral organs (Pnueli *et al*., 1994).

In addition, parthenocarpic fruit development resulted from a mutation in, or silencing of, the duplicated *MADS-box* genes, tomato *APETALA3* (*TAP3*)/*SlDEF* and *TM6* (de Martino *et al*., 2006). These genes belong to the *AP3* group, a subfamily of B class *MADS-box* genes that are required for specification of petals and stamens. *TAP3* is expressed predominantly in developing petals and stamen primordia until the late stage of flower differentiation. A *tap3* null mutant was shown to have sepaloid petals and carpel-like anthers, and occasionally exhibited parthenocarpy. Recently, *two stamenless* (*sl*) mutants, which exhibit floral homeotic conversion to different degrees, were found to develop parthenocarpic fruits and their phenotypes most likely resulted from mutation(s) in the coding sequence and promoter region, respectively (Quinet *et al*., 2014). *TAP3*-antisense plants exhibited similar floral homeotic conversion to *tap3* mutant, developing parthenocarpic fruits. Similarly, *TM6* is weakly but constitutively expressed in the primordia of petals, stamens, and carpels (de Martino *et al*., 2006), and *TM6*-RNAi transgenic plants are defective in stamen development and exhibit occasional parthenocarpy.

Flavonoid-related genes

Genetic engineering of the flavonoid biosynthesis pathway represents yet another method to obtain parthenocarpic fruit. RNAi-mediated suppression of *chalcone synthase* (*CHS*), which encodes an important enzyme in flavonoid biosynthesis, has been reported to reduce total flavonoid levels and induce parthenocarpy (Schijlen *et al*., 2007). Transgenic

plants showed normal vegetative growth, but their fruits were seedless and smaller than those of the seeded control fruits. Flavonoids play an essential role in reproductive processes such as pollen development and pollen tube growth. The parthenocarpic fruit development in the *CHS*-RNAi plants appeared to be pollination-associated, as pollen tube growth was impaired, and so fertilization was prevented. These findings suggest that pollination is required and sufficient to trigger fruit set and that fertilization leads to subsequent normal fruit development and expansion. Seedless fruits with reduced flavonoid levels were also generated by introduction of the grape (*Vitis vinifera*) *stilbene synthase* (*StSy*) gene, encoding a key enzyme in the synthesis of the antioxidant resveratrol (Giovinazzo *et al*., 2005; Ingrosso *et al*., 2011). It was suggested that the altered flavonoid metabolism in *StSy* transformed plants was caused by the competition between the biosynthetic pathways of resveratrol and chalcone and the parthenocarpic fruits contained high concentrations of soluble antioxidants, ascorbate and glutathione (Giovinazzo *et al.*, 2005). Flowers of *StSy* transformed plants displayed an open anther structure and were disturbed in pollen development, resulting in reduced seed set. The possibility that male sterility in the transgenic plants may be associated with its parthenocarpy was also suggested (Ingrosso *et al*., 2011; Medina *et al*., 2013).

The *pat* mutant and its candidate gene

The *pat* mutant phenotype was firstly ascribed to the action of two tightly linked genes, named *sha* for '*short anthers*' and *pat* for '*parthenocarpic fruit*' (Fig. 1.1; Soressi and Salamini, 1975). The discovery of a spontaneous *sha-pat* mutation in the line "Montfavet 191" (Pecaut and Philouze, 1978) proved that the previously described phenotype was caused by a single recessive mutation with pleiotropic effects and the mutation was finally named *pat* (Philouze and Pecaut, 1986). The *Pat* locus was mapped on the long arm of chromosome 3 and its position located in a genetic window spanning 1.2 centiMorgan (cM) between the conserved orthologous set (COS) sequences T0796 and T1143 (Beraldi *et al*., 2004) of the EXPEN2000 genetic map (SGN, http://www.sgn.genomics.net/; Fulton *et al*., 2002). Integration with the tomato physical map, allowed to refine with new anchor-points the *Pat* locus, narrowing the genomic interval to a region of about 0.19 cM between two internal BAC-developed markers named T17 and T20 (Selleri, 2010), corresponding respectively to the *Solyc03g120880* and *Solyc03g120980* loci (SGN nomenclature).

Figure 1.1. Ripe tomato fruit setion of cv. Chico III (left) and its near-isogenic-line for the *pat* mutation (right) wich is distinguished by its complete seedlessness and smaller size. Bar corresponds to 1 cm lenght.

Nine predicted genes are contained in this region and their putative function is reported in Chapter 4. Sequence comparison of four of these genes, putatively involved in tomato reproductive processes, were performed between the WT (cv Chico III) and the *pat* NIL (Selleri, 2010). In particular, the coding region of *Solyc03g120910*, *Solyc03g120930*, *Solyc03g120960* and *Solyc03g120970* were analysed. A single point mutation (G1747A) in the coding DNA sequence (CDS) of the *Solyc03g120910* was found. The nucleotide substitution is in agreement with the action of the EMS used by Soressi to generate the *pat* line (Bianchi and Soressi, 1969). This gene is composed by 18 exons and the G1747A nucleotide substitution in the *pat* allele is located in the exon 14th (Selleri, 2010). This lesion affects the first position of the codon 583 and is responsible of an amino acid change from glycine (G) to arginine (R) at the protein level (Selleri, 2010). By *in silico* analysis, the G583R substitution was predicted to be deleterious for the protein activity and 3D structure comparison highlighted structural differences in terms of protein folding between the WT and *pat* alleles (Ruiu, 2013). Another feature of the mutated protein regards the lack of a GlcNHglycan motif (also named SLiM) and the formation of three additional SLiMs. These motifs are present in arabinogalactan proteins and are hypotezised to be involved in plant reproductive development, pattern formation, and somatic embryogenesis, as well as in the underlying processes of cell division, cell expansion, and cell death (Nothnagel, 1997; Estévez *et al.*, 2006). *Solyc03g120910* corresponds to the Arabidopsis *ATHB15/CNA/ICU4* orthologue, subsequently named *SlHB15* (Ruiu, 2013). *ATHB15/CNA/ICU4* belongs to the class III of the *Homeodomain-Leucine Zipper* (*HD-Zip III*) family of transcription factors (TFs) that comprises other four members named *ATHB8*, *ATHB9*/*PHAVOLUTA (PHV)*, *ATHB14*/*PHABULOSA* (*PHB*) and *INTERFASCICULAR FIBERLESS1 (IFL1*)/*REVOLUTA* (*REV*).

The HD-Zip family is characterized by the presence of two functional domains (Fig. 1.2): the homeodomain (HD), responsible for DNA binding and the leucine-rich zipper (LZ), located in C-terminal position to the HD, acting in protein-protein interaction. These two domains are also present in TFs of species belonging to other eukaryotic kingdoms, but their association in a single protein is unique to plants (Schena and Davis, 1992). HD-Zip proteins bind to DNA as homo- or heterodimers, and the absence of LZ abolishes their protein binding ability, which indicates that the relative orientation of the monomers, driven by this motif, is crucial for an efficient recognition of DNA (Ariel *et al.*, 2007). HD-Zip III members present an additional loop formed by four amino acids between the HD and LZ domains. Among these TFs, more than a half of the amino acids are conserved and exhibit a common steroidogenic acute regulatory protein related lipid transfer (START) domain followed by an adjacent conserved region called START-adjacent domain (SAD). Although many START-containing proteins found in the animal kingdom have been well characterized, no lipid ligands have been identified in plants (Schrick *et al.*, 2004).

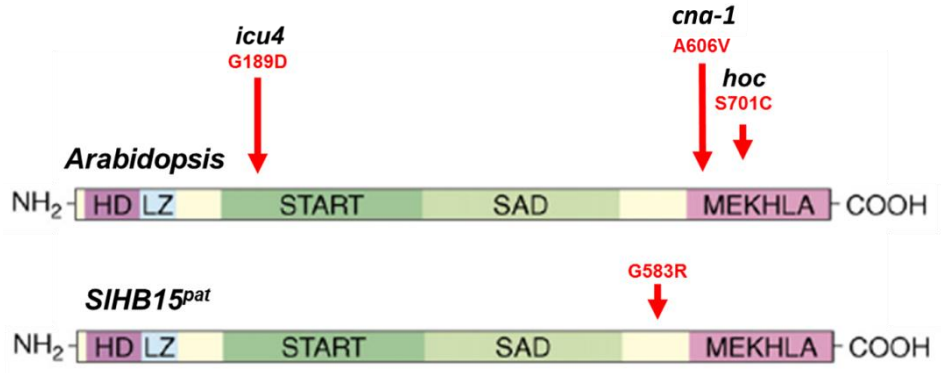


Figure 1.2. Representative model of the HD-Zip III HB15 protein showing the functional domains, the amino acid substitutions of *cna, hoc* and the *miR165/166*-derived gain-of-function *icu4* mutants generated in Arabidopsis and the EMS-induced G583R modification in the tomato *pat* line.

However, the high conservation of this motif achieved throughout evolution indicates it as a likely player in the regulatory activity (Ariel *et al.*, 2007). Additionally, all HD-Zip III members have an extra conserved domaim at the C-terminus, called MEKHLA, a member of the Per-ARNT-Sim (PAS) domain superfamily (Magnani and Barton,2011). PAS domains are signal sensors that regulate a wide range of signal transduction pathways in all kingdoms (Mukherjee and Bürglin, 2006). They respond to a variety of chemical and physical stimuli and regulate the activity of covalently linked effector domains, such as kinases, cyclases, ion channels and transcription factors (Magnani and Barton, 2011). The interaction between HD-Zip III and DNA is less studied than in the case of the other three

HD-Zip subfamilies (I, II and IV). Nonetheless, GTAAT(G/C)ATTAC was determined to be the extended sequence for which ATHB9 has the highest affinity *in vitro* (Elhiti and Stasolla, 2009).

HD-Zip III genes found in the tomato genome were named according to their degree of homology with Arabidopsis members (Table 1.1). Differently from Arabidopsis, in tomato, *Solyc03g120910* (*SlHB15*) presents a parologous gene with a different chromosomal location, named S*lHB15*-like (*Solyc12g044410*) (Ruiu, 2013). HD-Zip III proteins are known to play a role in many processes, such as meristem formation and regulation, embryonic shoot and root development, leaf polarity, vascular tissue establishment, plant architecture, lateral root primordia initiation, auxin transport and signalling (Zhong and Ye, 2001; Eshed *et al*., 2001; Emery *et al*., 2003; Prigge *et al*., 2005; Ochando *et al*., 2006; Scarpella *et al*., 2006; Izhaki and Bowman, 2007). Functional characterization of HD-Zip III family members (*ATHB15/CNA/ICU4, PHB*, *PHV* and *REV)* in Arabidopsis has provided evidence for their fundamental involvement in the regulation of the vegetative as well as the reproductive organ (i.e. ovule integuments) development (Kelley *et al*., 2009). Different induced *ATHB15/CNA/ICU4* single mutants have been phenotypically characterized. These are: *corona-1* (*cna-1*) loss-of-function mutant (Green *et al*., 2005), *cna-2* null mutant (Prigge *et al*., 2005), *incurvata4-1* (*icu4-1*) gain-of-function mutant (Ochando *et al*., 2006) and *hoc* loss-of-function mutant (Duclercq *et al*., 2011). The lesions carried by these mutants and the G583R amino acid substitution observed in the SlHB15 protein sequence of the *pat* genotype are represented in Figure 1.2.

Table 1.1. Arabidopsis and tomato *HD-Zip III* gene correspondance according to their protein homology (adapted from Ruiu, 2013) .

Arabidopsis		**Tomato**	
Gene name	**TAIR locus ID**	**Gene name**	**SGN locus ID**
ATHB8	*At4g32880*	*SlHB8*	*Solyc08g066500*
ATHB9/PHV	*At1g30490*	*SlHB9*	*Solyc02g069830*
ATHB14/PHB	*At2g34710*	*SlHB14*	*Solyc02g024070*
ATHB15/CNA/ICU4	*At1g52150*	*SlHB15**	*Solyc03g120910*
		SlHB15-like	*Solyc12g044410*
IFL1/REV	*At5g60690*	*SlIFL1*	*Solyc11g069470*

Among the vegetative phenotypes displayed by the Arabidopsis mutants, *icu4-1* presented a shorter primary roots but more secondary roots, a higher number of root hairs, altered phyllotaxis with curved leaves, supernumerary rosette leaves and axillary shoots. The vascular system was also affected as xylem overgrowth in the stem was observed when compared to its WT counterpart (Ochando *et al.*, 2006). A notable trait put in evidence also in the *pat* mutant (Olimpieri *et al*., 2007) was the presence of seedlings forming tricotyledons in both the *icu4-1* mutant (Ochando *et al*., 2006). This trait has recently been observed in the *cna* mutant (Ruiu, 2013). At the vegetative level, this mutant exherts its effect when combined with other *HD-Zip III* mutants as in the triple *phb, phv, cna* mutant or in the quadruple *phb, phv, athb8, cna* mutant, where an enlarged shoot apical meristem and a reduced plant size are observed (Prigge *et al*., 2005). An enhancement of the shoot meristem size was recorded also when *cna* was combined with c*lavata* loss-of-function mutants; a gene regulating the shoot meristem activity (Green *et al*., 2005). The *hoc* mutant displays an increase of the primary root lenght, shorter hypocotyl, reduced apical dominance and a higher number of leaves (Catterou *et al*., 2002).

Objectives and outline of the thesis

The importance of natural parthenocarpic mutations is due to their possible use in breeding programs but also they represent a model system for understanding the mechanisms underlying fruit set.

Fruit seedlessness represents an apparent paradox in nature but there is an undefined evidence of its occurrence among plant species. This thesis gives the opportunity to investigate on the impact of parthenocarpy and provide hypotheses on its potential adaptive role.

The main objective of the present thesis is to confirm the identity between the *Pat* locus and *SlHB15*, the best candidate according to a number of evidences cumulated during the last few years. For this reason, a suitable transformation strategy will be applied through i) complementation test of the mutant, ii) overexpression of the WT allele of the candidate gene (*SlHB15*^{WT}) in the WT line, iii) silencing of the candidate gene by cosuppression and iv) its knock out by genome editing. According to the description from literature of the *SlHB15* orthlog and its respective mutants in Arabidopsis, a second objective of the thesis is to expand the phenotypic and molecular characterization of the *pat* mutant to the vegetative plant level in order to provide robustness of the candidate gene.

To further functionally characterize *SlHB15,* which codes for a transcription factor, an *in silico* analysis of the gene promoter will be carried out in order to detect motifs indicating putative interactors.

Confirmation of the identity of *SlHB15* with the *Pat* locus will represent the first case of characterization by forward genetics of a gene conferring parthenocarpy in tomato.

Chapter 2

Inventory of parthenocarpic species: an "adaptive" role for parthenocarpy

Introduction

"That some plants produce fruits without seeds is a fact observed and recorded by the ancients, according to Sturtevant (1890)" is the first introductory statement reported in Gustafson's comphrenesive work regarding the subject of parthenocarpy (Gustafson, 1942). This sentence is soon after linked to the reasons for such an interest as "seedless fruits are thought to be better and also because many varieties are self sterile, necessitating the planting of more than one variety in an orchard to insure a profitable crop".

Parthenocarpy may occur naturally but, for a long time, humans have attempted to impair the "ovary arrest" state to develop seedless fruits to make them easier to consume. Parthenocarpy can be induced artificially with the application of various hormones (Gustafson, 1936, 1942; Nitsch, 1952; Osborne and Went, 1953).

As already reported, seedless fruits can develop in one of two ways: either the fruit develops directly without fertilization (parthenocarpy) or pollination triggers fruit development but the ovule or embryo aborts without producing mature seed (stenospermocarpy). Parthenocarpy and abortion of reproductive structures at different developmental stages are important and drastic processes limiting female fecundity in many plant species. The production of seedless fruits is an intriguing phenomenon because empty fruits do not contribute directly to the production of offspring and their maturation is presumably costly to the maternal plant. When there is failure of seed set, the abscission of the young fruitlet would be of advantage to the seed-bearing plant, avoiding the waste of resources in growing structures not fulfilling their biological purpose; however, in some circumstances this may not occur suggesting the possibility of adaptive reasons in retaining empty fruits. An extensive bibliographic investigation of the occurrence of parthenocarpy in the plant kingdom allowed us to better feature such a trait, having been observed mostly among dicots in both wild and cultivated taxa.

Materials and Methods

Bibliographic search of data and classification criteria adopted

Data search of parthenocarpic species was carried out through the literature. The main source was represented by the comprehensive report by Gustafson (1942), and single publications found also through interrogation of the following bibliographic search engines: Google Scholar (http://www.google.com), Pubmed (www.ncbi.nlm.nih.gov/pubmed) and ScienceDirect (http://www.sciencedirect.com), by using "parthenocarpy", "parhenocarpic fruit", "seedless" and "seedless fruit" as entry keywords. For each species, the number of ovule per ovary was considered to distinguishing "monospermic" (one- or two-seeded) and plurispermic (more-than-two-seeded) species. The reason of the inclusion of species with two-seeded fruits in the "monospermic" category stems from the assumption that these fruits derive from an ovary containing two carpels, which give origin to one seed each. This is what happens, for example, in the *Apiaceae* family where the resulting dry fruit is a diachene, that normally splits into two indipendent monoseeded achenes. Following this first classification, species were distinguished according to their occurrence as wild, cultivated for ornamental or consumption in order to evidence a possible different selective pressure on the parthenocarpic fruit trait. (see Table 2.1). Secondarily, the botanical family, systematic clade, according to the flowering plant classification of the Angiosperm Phylogeny Group III (Bremer *et al*., 2009), number of ovule integuments, type of fruit, mating system, reproductive structure were also considered. All data are reported in a supplementary Table (Table S1).

Results and discussion

Distribution of parthenocarpy in wild and cultivated species

Roth (1977) proved that, even if parthenocarpy appears taxonomically widespread, it is "not uncommon" in species producing fruits with several to many seeds while it represents a relatively rare event in species having "monospermic" fruits. Accordingly, starting from a previous preliminary bibliographic investigation (Selleri, 2010), the occurrence and

distribution of natural parthenocarpy in flowering plants were assessed (Tab. 1). Sixty-five per cent of 96 species scored for this trait belong to the plurispermic fruit category (Table 2.1). In addition, parthenocarpy was observed mostly among dicot taxa in both the wild and cultivated categories (Table 2.1). The number of parthenocarpic cultivated species with plurispermic fruits was about six-fold that of the wild plurispermic 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 was approximately (1.2 ratio) the same (Table 2.1). A domestication-driven selective pressure in the parthenocarpic plurispermic species can be based on a number of reasons: fruit set and production are less affected by environmental factors adverse for pollination and fertilization, seedlessness is advantageous for fruit processing (e.g. tomato paste), may improve fruit quality or is a feature appreciated by consumers. Seedless fruits often have a longer shelf life than seed-bearing fruits; fruit set and development often start before anthesis, consequently, parthenocarpy might allow early fruit production and harvest (Pandolfini, 2009). Selected varieties of watermelons, grapes, *Citrus*, pineapples and bananas are clear examples of plurispermic species producing seedless fruits.

Table 2.1. Classification of ninety-six parthenocarpic species grouped according to "monospermy" (one single/two seeded fruits) and plurispermy (several to many seeded fruits). In each category, the same species are subcategorized in wild, cultivated as ornamental and cultivated for the fruit/seed consumption.

Category	"Monospermic" species			"Plurispermic" species		
Subcategory	Wild	Cultivated as ornamental	Cultivated for fruit/seed	Wild	Cultivated as ornamental	Cultivated for fruit/seed
N° (%)	13 (38)	4 (12)	17 (50)	5 (8)	13 (21)	43 (71)
Total n° (%)	34 (35)			62 (65)		

The mission of a seedless fruit: an adaptive role of parthenocarpy

The production of seedless fruits represent an apparent biological paradox because empty fruits do not contribute directly to the production of offspring. Fruits form through an intimate developmental collaboration between ovules (the developmental precursors to seeds) and carpels (the precursors to the body of the fruit). For centuries, humans have attempted to interrupt this association to develop seedless fruits to make them easier to

consume (Lora *et al*., 2011). This is a difficult task, because fruit development is usually arrested in the absence of seed formation (Varoquaux *et al*., 2000). In addition, most mutations resulting in seedless fruits disappear unnoticed because sexual reproduction is precluded and intentional vegetative propagation is required to maintain seedless genotypes (Darwin, 1876). Darwin (1876) frequently reported the production of fruits without seeds 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 seed set fails, 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 spontaneous species this phenomenon occurs, suggesting the possibility of adaptive reasons for retaining empty fruits.In the "monospermic" plant category such a role is mainly based on different mechanisms by which parthenocarpic fruits would reduce seed predation. In this sense, a functional role of seedless fruits has been proposed for wild parsnip (*Pastinaca sativa* L.) by Zangerl *et al.* (1991), who related the persistance of parthenocarpic fruits to their defensive value against their most destructive enemy, the parsnip webworms (*Depressaria pastinacella*). In this case, given a choice between parthenocarpic and normal fruits, webworm prefers seedless fruits because of the lower concentration of the deterrent furanocoumarins they contain. In terebinth (*Pistacia terebinthus* L.), parthenocarpic fruits appear to reduce seed predation because predators cannot discriminate between seeded and parthenocarpic (deceptive) fruits, as ovaries are not yet enlarged at the time of oviposition and larvae soon die because in the parthenocarpic fruits there is no endosperm available (Traveset, 1993). A similar explanatory hypothesis of an adaptive role of parthenocarpy has also been proposed for *Juniperus osteosperma* Torr. Little (Fuentes and Schupp, 1998), *Pistacia lentiscus* L. (Verdù and Garcia-Fayos, 1998) and *Bursera morelensis* L. (Molano-Flores *et al.,* 2001). All these cases share the role of seedless (deceptive) fruits in reducing, in species specific ways, the probability of seed predation by their natural enemies (mainly insects or birds).

If this explains an adaptive role of parthenocarpy in "monospermic" species, it more difficultly applies to multispermic taxa. Within the latter, an hypothesis relies on the observation that parthenocarpy, as a trait, may display a variable expressivity, in the sense that partial seedlessness (fruits with few seeds) may arise in some circumstances. Plants have evolved flowers with a great number of ovules as a response to habitats where pollination is more uncertain, for example, due to asynchrony between flowering and the

presence of pollinators or critical climate variation (Verdù and Garcia-Fayos, 1998). In these cases, 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 (Sotelo *et al*., 2014). It has been proposed that differences in seed number per fruit alter the cost of the fruit for the mother plant: plants invest fewer resources per seed in multi-seeded fruits than in few-seeded fruits (Obeso, 2002). Accordingly, as a strategy to optimize resources, mother plants avoid the development of fruits with few seeds (Obeso, 2002). However, plants can still exhibit positively skewed frequency distributions for seed number per fruit, so that many fruits are usually few-seeded in several species (Uma Shaanker *et al.* 1988). In the case of plurispermic 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, parthenocarpy could offer the opportunity to accomplish the production and dispersal of few seeds that otherwise would be lost (Selleri, 2010). When the seed/ovule ratio is extremely low, for instance in limiting environmental conditions for pollination and fertilization, the possibility of the ovary to develop into a fruit may represent a selective advantage. This kind of control of parthenocarpy would provide an adaptive role for this trait in wild multispermic species and could be particularly evident in cultivated species as in tomato. The *parthenocarpic-2* (*pat-2)* mutation allows the production of completely seedless berries only in unfavourable conditions for seed production (relatively low night temperatures and increasing photoperiod). When the environmental conditions become progressively favourable to the pollen formation and anther dehiscence, the fruits set in the *pat-2* line contain an increasing number of seeds that however would never reach the maximum number recorded in the wild type near isogenic line (Santangelo *et al.*, 1990).
In other words, a weak source of hormones triggered by a low seed-set, unable to stimulate the fruit growth, would far more benefit from a favourable change of the genetic pattern regulating the fruit set indipendently from the one that regulates the seed formation, in the end leading to a boost of specific fruit setting-related hormones such as auxin and gibberellins (Selleri, 2010).

Inventory of tomato parthenocarpic sources: parthenocarpy as a consequence of hybridity

An informative observation to understand the genetic and molecular mechanisms controlling parthenocarpy comes from the examination of the sources of parthenocarpy described for tomato in the early and recent literature. Although recent reviews have generally focussed on reverse genetics experiments (Gorguet *et al.,* 2005; Shinozaki and Ezura, 2016), very good inventories have been compiled in the past giving a deal of information on the origin and expression of parthenocarpic lines (Philouze, 1983; George *et al.,* 1984; Lukyanenko, 1991).

With possibly the only exception of the *parthenocarpic fruit* (*pat*) mutant, that was obtained by EMS treatment (Bianchi and Soressi, 1969), almost all of the parthenocarpic lines described are derived more or less directly from crosses involving different species or conspecific taxa more or less widely distant from the cultivated forms (Table 2.2).

After the discovery of *pat*, the sources of parthenocarpy that have been better genetically characterized have been listed according to the so called "*pat* series". *pat-2* is probably the best studied parthenocarpy gene and it was first described by Dovedar (1973, cited by Philouze, 1983) as selected in the progeny of a cross involving a *L. hirsutum* (now *S. habrochaites*)parent. Line RP 75-59, which was later indicated as *pat-3*/*pat-4*, was obtained in Germany by crossing a British (Atom) and a Russian (Bubjekosoko) cultivar (Reimann-Philipp, 1968, cited by Philouze, 1983). Bubjekosoko is reported as a tomato cherry type (Mahmoud, 2014) and in the IPK seedbank is classified as a "*L. esculentum* Mill. convar. *parvibaccatum* Lehm. var. *cerasiforme* (Dunal) Alef" taxon (http://www.ipk-gatersleben.de/genbank). Two parthenocarpic lines obtained in The Netherlands (Zijlstra, 1985) were subsequently classified in the *pat* series; IVT-1 was related to a digenic control (*pat-8* and *pat-9*), whereas IVT-2 was indicated as *pat-5* (Gorguet et al. 2008). Both these lines were derived from crosses with different wild species (Table 2.2). Also IL5-1 (*pat-6*/*pat-7*) was obtained after a cross with *S. habrochaites* (Gorguet *et al.*, 2008).

The same trend is encountered in the pedigrees of those sources of parthenocarpy that were not subjected to deep genetic analysis. One of the first reports of natural parthenocarpy in tomato describes the appearance of genotypes able to set parthenocarpic fruits under adverse hot dry months from a cross between Bonny Best and Large Cherry (Hawthorn, 1937). Being a "cherry" type, the latter parent suggest the involvement of a *S.l.* var. *cerasiforme* genotype. Similarly, *S.l.* var. *cerasiforme* enters in the pedigree of the Ukrainian selection Pridneprovskij (Kraevoj, 1949, cited by Philouze, 1983) and of PI190256 (Johnson and Hall, 1954). Facultative parthenocarpy was also found after more

distant crosses of the cultivated tomato with *S. peruvianum* (Lesley and Lesley, 1953) and also in intergeneric crosses with *Cyphomandra* spp (Luneva 1957, cited by Philouze 1983).

Table 2.2. The "pat" series of tomato mutants displaying parthenocarpy. With the exception of the *pat* mutant, all the parthenocarpic lines derived from progeny selection of crosses (background) between tomato (cultivated background) and its wild relatives (taxa named according to Peralta and Spooner (2000).

Line name	Mutation/s	Cultivated background	Wild/wide-related background	Reference
Stock 2524	pat	Cv Ventura	-	Bianchi and Soressi, 1969
Severianin	pat-2	Byzon	S. habrochaites	Dovedar, 1973 (cited by Philouze, 1983) Philouze and Maisonneuve, 1978
RP 75-59	pat-3/pat-4	Atom	Bubjekosoko (S.l. var. cerasiforme)	Reimann-Philipp, 1968 (cited by Philouze, 1983)
IVT-2	pat-5	S. lycopersicum	S. peruvianum	Zijlstra, 1985
IL5-1	pat-6/pat-7	S. lycopersicum	S. habrochaites	Gorguet et al., 2008
IVT-1	pat-8/pat-9	S. lycopersicum	S. habrochaites	Zijlstra, 1985; Gorguet et al., 2008
-	-	Bonny best	Large cherry	Hawthorn, 1937
Pridneprovskij	-	S. lycopersicum	S.l. var. cerasiforme	Kraevoj, 1949 (cited by Philouze, 1983)
?	-	S. lycopersicum	L. peruvianum	Lesley and Lesley, 1953
PI190256	-	New caledonia	S.l. var. cerasiforme	Johnson and Hall, 1954
Hybrids n. 1641,1154	-	S. lycopersicum	Cyphomandra spp.	Luneva, 1957 (cited by Philouze, 1983)
Lyconorma, Lycoprea	-	Priora	Heinemänns Jubilaum	Reimann-Philipp, 1977 (cited by Philouze, 1983)
Oregon cherry	-	Farthest North	S. habrochaites, S. pimpinellifolium	Bagget and Frazier, 1978a
Oregon T 5-4	-	Farthest North	S. habrochaites	Bagget and Frazier, 1978b
Oregon 11, Gold Nugget	-	?	S. pimpinellifolium, S. habrochaites	Bagget and Frazier, 1982
?	-	S. lycopersicum	Solanum neorickii	Philouze, unpublished (cited by Philouze, 1983)
P26, P31	-	S. lycopersicum	S. pennellii	Stoeva et al., 1985 (cited by Lukyanenko, 1991)
Carobeta	-	S. lycopersicum	S. pimpinellifolium	Georgiev and Mikhailov, 1985
RG	-	S. lycopersicum	S. cheesmaniae var. minor	Mikhailov and Georgiev, 1987 (cited by Lukyanenko, 1991)

Several selections obtained in Oregon had *S. pimpinellifolium* and *S. habrochaites* in their pedigree (Baggett and Frazier, 1978a; 1978b). The accession *Heinemänns Jubilaum*, that entered in the pedigree of the parthenocarpic varieties Lyconorma and Lycoprea

(Reimann-Philipp, 1977, cited by Philouze, 1983), is classified at the IPK genebank as *L. esculentum* Mill. convar. *fruticosum* Lehm. var. *pygmaeum* Lehm., a classification that denotes a cherry type with possible introgression from *cerasiforme* types. Reports of parthenocarpic types after crosses with *L. parviflorum* and *L. pennellii* are also found in the early reviews (Philouze, 1983; Lukyanenko, 1991). Finally, the varieties Carobeta (Georgiev and Mikhailov, 1985) and RG (Mikhailov and Georgiev, 1987, cited by Lukyanenko, 1991) were bred after crossing with *L.pimpinellifolium* and *L. cheesmanii* var. *minor* respectively. Carobeta inherited from its wild parent the *B* allele responsible for the orange fruit color, but also a genetic parthenocarpic behavior.

The functional linkage between parthenocarpy and wide (intra and interspecific) hybridization was first addressed by Lesley and Lesley (1953), who found strong parthenocarpic attitudes in progenies after crossing tomato with *L. peruvianum*. These authors attributed the phenotype to an exceptional combination of genes coming from the two species that involved an excessive production of auxin. The same authors reported a similar phenotype from progenies of tomato x *L. hirsutum* (Lesley and Lesley, 1953). After that, this association was put in evidence in the review of Philouze (1983) and of Ho and Hewitt (1986), but no specific functional hypothesis was elaborated by these authors.

Hybridity as a force driving departures from normal sexual plant reproduction

Departures from a consolidated sexual process as a consequence of wide hybridization were observed and discussed in species showing apomictic behavior, that are typically derived from wide (interspecific) hybridization and polyploidization. Apomixis is a modified pathway of plant reproduction resulting in the asexual production of a seed (agamospermy, Nogler, 1984). Due to the possibility of cloning genotypes by seed, harnessing apomixis has been regarded as a very interesting perspective for plant breeders and efforts to decipher its genetic control has been strongly pursued in the last decades (Albertini *et al*., 2010).

Coupling the evidence that apomixis occurs in taxa characterized by hybridity and polyploidy (Asker and Jerling, 1992) with the modular and hierarchical structure of reproductive development in plants (Haig 1990), Carman (1997) has elaborated the "duplicate-gene asynchrony hypothesis" (or "genome collision" or "no-gene" theory) for the molecular control of apomixis.

The theory, that evolved as a revision of the Ernst hypothesis that apomixis results from hybridity (Ernst, 1918), postulates that asynchronous expression of modular sets of genes occurring from the inheritance from different species and caused by their heterochronicity (wrong expression or asynchrony in time) and/or heterotopicity (wrong expression in space) may be evoked to explain modifications of the reproductive system such as apospory, diplospory and apomixis as a whole (Carman, 1997). Thus, apomixis, polyspory and polyembryony and related abnormalities result from developmental programs that are ectopically and/or prematurely expressed due to the misregulation of duplicate genes in polyploids, mesopolyploids or paleopolyploids.

According with Carman's hypothesis, the so called "stages of evolution" of apomixis begin with weak facultative expression that has been consolidated by mutations. This is corroborated by the fact that "tendencies towards apomixis" are common in natural and synthetic polyploids (Asker and Jerling, 1992).

Experimental evidence of the soundness of the Carman's theory started to emerge from recent analysis of transcriptomes in apomicts. The occurrence of heterochronic development and gene expression has been experimentally shown in *Tripsacum* (Grimanelli *et al*., 2003; Bradley *et al*., 2007) and *Boechera* (Sharbel *et al*., 2010).

Parthenocarpy as a consequence of hybridity

By examining the series of tomato sources for parthenocarpy (Table 2.2), a similar "no-gene" (that means "no mutation") basis for parthenocarpy is intriguing. When different genomes are "colliding" after interspecific, or intraspecific wide crosses, modification of developmental programs controlling fruit set can occur by "superimposition" of regulatory signals that are asynchronous in time and/or space and thus lead to the development of the ovary before and independently of ovule fertilization. Such modification can thus become more or less fixed in genotypes and populations if an evolutive advantage is foreseen by the new developmental program as in apomicts (Carman, 1997) or if the phenotype is selected by man as in the case of parthenocarpy in crops. As for apomixis, tendencies towards parthenocarpy are common in tomato where most of the 23 cultivars subjected to emasculation exhibited some ovary growth and were able to initiate seedless fruit formation to varying degrees where the parthenocarpic fruits colored up and ripened (Goetz *et al*., 2007).

In the model reported in Figure 2.1, two sexually compatible species present different time spans for sporogenesis and gametogenesis that result in a three-days difference in timing from meiosis to anthesis. If one hybrid inherits those genes involved in sporogenesis, ovary repression at anthesis and fruit development from a wild species and those involved in gametogenesis and pollination/fertilization from the cultivated parent, it may experience the asynchrony of fruit development genes that are activated before pollination has taken place. Although it is known that a specific set of genes is activated exclusively by the pollination/fertilization events (Ruiu *et al*., 2015), it is also recognized that fruit set may be driven by fertilization-independent pathways, activation of downstream genes or removal of repressors driven by mutations or hormone treatments. In this scenario, the effect of asynchrony in hybrids may be crucial to induce fruit set positive signals before fertilization can take place (Fig. 2.1). This hypothesis is supported by other observations that emerged in a number of cases studied of natural parthenocarpy in tomato as detailed below.

1) An oligogenic control of the parthenocarpic trait has been reported in a number of cases, indicating that the trait is the results of a combination between more than one genetic determinant.
2) Parthenocarpy occurs in association with abnormal development of male or female floral organs. Defects in early ovule development have been associated with parthenocarpy in the variety Pridneprovskij (Ludnikova, 1970, cited by Philouze, 1983) and in the *pat* (Mazzucato *et al*., 1998) and the hydra (Rojas-Gracia *et al*., 2017) mutants in tomato and in pepper parthenocarpic lines (Tiwari *et al*., 2011). This indicate that autonomous development of the ovary may be a downstream or combined effect of alterations that are expressed early to drive organ development and identity, and ultimately such alterations may affect later processes.
3) Often strong parthenocarpy arose from parents without or just with weak tendencies to seedlessness. In Poland, Kubicki and Michalska (1978) crossed two Canadian varieties, “Early North” (with a limited tendency to parthenocarpy) and “Beaverlodge 6703 (with no tendency to parthenocarpy), and obtained tomato lines with a strong parthenocarpic capacity in conditions unfavourable to fertilization.
4) Evidence of parthenocarpy appears in the segregation of modern F_1 hybrid varieties (G.P. Soressi, personal communication), that are by definition obtained from the combination of distantly related genotypes that also include conspicuous introgressions of wild germplasm as a consequence of breeding for disease resistance (Lin *et al*., 2013).

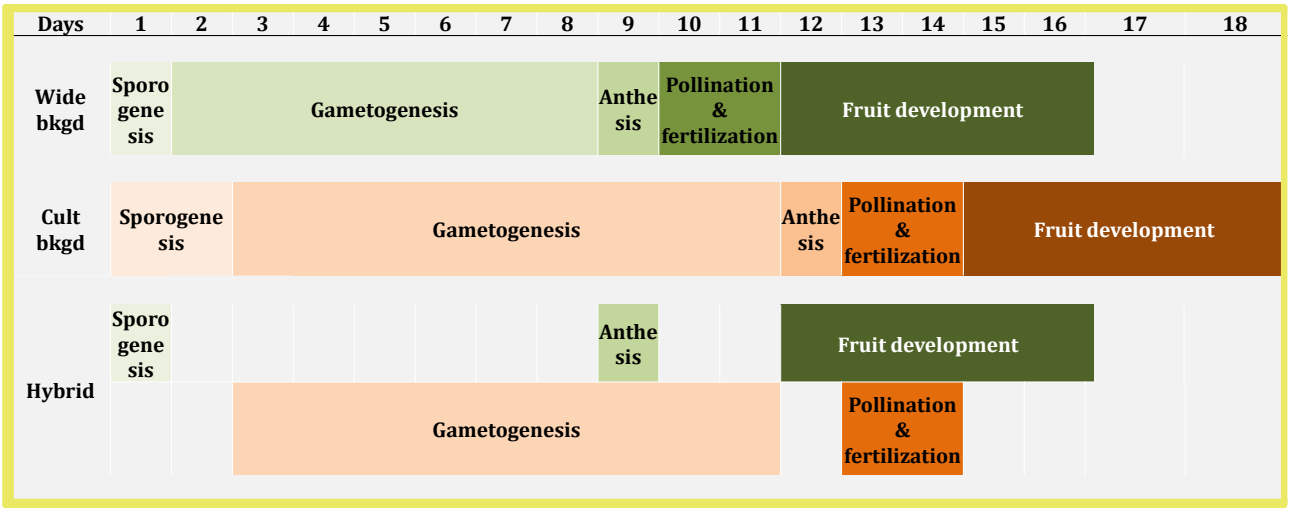


Figure 2.1. Schematic representation of the hybrid effect to drive parthenocarpy in tomato. Hypothetic wild wide-related and cultivated species show a stable and synchronous timing of reproductive events, from sporogenesis to fruit development. From meiosis to fruit set, the two species show a difference of three days. The hybrid inherits genes involved in three steps from the wild background and in the other two from the cultivated one. The asynchrony of the developmental program may drive fruit development genes to be expressed before fertilization has taken place, resulting in parthenocarpy.

Apomixis and parthenocarpy may share common genetic basis

Other events of genomic perturbation have been reported as the basis for parthenocarpy expression, that may be induced by changes in genome ploidy, trisomy, aneuploidy and translocation heterozygotes (Lukyanenko, 1991). The link between triploidy and parthenocarpy is well established and exploited in a number of species, such as watermelon and banana (Kihara, 1951; Ortiz and Vuylsteke, 1995; Varoquaux *et al*., 2000).

Accordingly, synthetic Arabidopsis allopolyploids from interspecific hybrids display new phenotypes, such as drought tolerance, apomixis, changes in flowering time, that may arise from changes in gene expression, altered regulatory interactions, and rapid genetic and epigenetic changes (Osborn *et al*., 2003). Although the tomato and its wild relatives are considered diploid species, evidences for ancient polyploidization in tomato emerged after sequencing of the whole genome (The Tomato Genome Consortium, 2012).

A similar mechanistic basis for apomixis and parthenocarpy is supported by the fact that the two phenomena seldom occur together in some taxa, as reported in birch (Bogdanov and Stukov, 1976), in subtropical species of the Asteraceae (Werpachowski *et al*., 2004), in *Citrus* (Vardi *et al*., 2008) and in *Musa* (Okoro *et al*., 2011).

Koltunow *et al*. (2002) treated apomixis and parthenocarpy as phenomena with possible common bases. These authors underlined a number of commonalities between the two

processes that appear in their consequences very different. First, they derive both from the disruption of molecular mechanisms that prevent the development of the carpel into a fruit and of the ovule into a seed in the absence of fertilization. As such, the ovule becomes a fundamental structure in the signalling of these mechanisms. The two processes are stochastic and both characterized by facultativeness, that make possible the coexistence of modified and normal processes within the same individual.

Both parthenocarpy and apomixis are processes relying on a hormone regulated program (Shinozaki and Ezura, 2016; Mordhorst *et al*., 1997). Recently, in tomato, alteration of auxin homeostasis due to deregulation of Arabidopsis *Nozzle/Sporocyteless* (*NZZ/SPL*) orthologues in tomato provides the molecular signal to the formation of parthenocarpic fruits (Rojas-Gracia *et al*., 2017). These authors identified the *hydra* mutant with the *NZZ/SPL* locus and demonstrated that fruit production in the loss-of-function mutant is linked to the absence of both male and female sporocyte development. The *HYDRA/NZZ/SPL* gene is therefore essential for the initiation of sporogenesis in tomato. In Arabidopsis (Liu *et al*., 2008) *HYDRA/NZZ/SPL* represses *YUCCA2* and *YUCCA6* expression in leaves and inflorescences. YUCCA genes in Arabidopsis are a highly redundant gene family involved in Aux biosynthesis and plant development (Cheng *et al*., 2006). In tomato, five *YUCCA-like* genes (*ToZFY2–ToZFY6*) have been characterized with *ToZFY2* being the prevalent messenger during floral stages prior to anthesis (Exposito-Rodriguez *et al*., 2011). Though Rojas-Gracia et al. (2017) reported a strong down and up regulation of, respectively, the *ToZFY2* and *ToZFY3* genes in stamens, their expression appeared reduced in the ovary along with a couple of GA biosynthetic genes. Further, auxin polar trasport-related transcription of *SlPIN-1*, *SlPIN2* and *SlPIN4* genes was differently altered, implying that auxin gradient pattern in ovule/carpel is affected. The involvement of auxin homeostasis in the control of cell fate in the female gametophyte has been postulated where auxin gradient correlates with location-specific auxin biosynthesis (Pagnussat *et al*., 2009). Both patterns, auxin biosynthesis and transport, would be pivotal for switching of gametic and nongametic cell identities in the embryo sac. In *Poa pratensis, SERK* and *APOSTART* are two loci related to apomixis as their trascription level was differentially modulated between apomictic and sexual *P. pratensis* genotypes (Albertini *et al*., 2005). According to these authors *PpSERK* activation in nucellar cells of apomictic genotypes could trigger embryo sac development and redirect signalling gene products to compartments other than their typical ones. Moreover, *SERK*-mediated signalling pathway might interact with the auxin/hormonal pathway controlled by *APOSTART* (Hecht *et al.,*

2001; Albertini *et al.,* 2005). According to Barcaccia and Albertini (2013), *APOSTART* genes in *P. pratensis* might be related to apoptosis that is involved in the non-functional megaspore and nucellar cell degeneration events that permit enlargement of maturing embryo sacs. Among the *APOSTART* members, *APOSTART6* shows a delay of expression in apomictic and parthenogenetic genotypes suggesting an involvement of *APOSTART6* in parthenogenesis (Marconi *et al*. 2013, in Barcaccia and Albertini, 2013).

Among trancripts differentially expressed between sexual and apomictic genotypes of *Hypericum perforatum* L., a number of them showed a high homology with microRNA precursors that target specific transcription factors (Galla *et al*., 2013). One of these, *miRNA166* specifically target the members of HD-Zip III TF family, involved, also, in events leading to changes of polar auxin transport (Bowman and Floyd, 2008). Recently, four of them have been regarded as inplicated in the development of somatic embryos in *Larix leptolepis* L. (Li *et al*., 2013) while one *HD-Zip III* member, *HB15/CNA/ICU4*, was highlighted for its implication in ovule fate and carpel development in tomato (Ruiu, 2013; discussed in the present thesis).

Deregulation of sex- and/or hormone-related genes, among which *HYDRA/NZZ/SPL*, *YUCCA*, *SERK* and *APOSTART*, with respect to their spatial and/or temporal expression well fits with the assumption that heterotopic/heterochronic gene expression, as a result of genome hybridity (Carman, 1997), may be the fundamental mechanism triggering the expression of parthenocarpy and the two modes of ovule-dependent asexual reproduction, apomixis and parthenogenesis.

A further specific example of a gene claimed to be involved in both apomixis and parthenocarpy is the B-class MADS-box transcription factor *APETALA3* (*AP3*) in *Arabidopsis* and *DEFICIENS* (*DEF*) in *Antirrhinum*. The example is a paradox, since B-class genes are typically expressed in the second and third floral whorl and contribute to the identity and development of petals and stamens (Weigel and Meyerowitz, 1994). Several authors reported the expression of DEF in the fourth whorl as in tomato (Mazzucato *et al*., 2008). In the apomictic *Hieracium piloselloides*, the aposporic ovule presents a downregulation of *DEFH* in a broad zone in the chalaza that coincides with the region where aposporous initials differentiate; such downregulation is not seen in sexual ovules (Guerin *et al*., 2000). In parallel, differential expression of *DEF* homologues have been reported in ovaries showing parthenocarpic behavior; in tomato, *SlDEF* show a peak of expression in ovaries at anthesis, that coincides with the signal that arrests ovary growth (Vriezen *et al*., 2008; Wang *et al*., 2009); such accumulation is absent in ovaries that develop autonomously in

the *pat* mutant (Mazzucato *et al*., 2008; Ruiu *et al*., 2015). Accordingly, the mutation of a B-class MADS box homeotic gene, the DEF paralog *PISTILLATA*, has been shown to cause parthenocarpy in apple (*Malus domestica* Borkh., Yao *et al*., 2001).
In conclusion, the inventory of the species showing parthenocarpic behaviour and of the sources of parthenocarpy in the specific case of tomato offered novel insights into the role that autonomous ovary development may have played in natural evolution and in the man-driven activity of selection and breeding. As for apomixis, such insights pave the way to novel investigation and consequently to new opportunities to harness seedlessness in tomato and in other fruit crops.

In summary, the ovary to fruit and ovule to seed development represent two important indipendent processes for the final pursue of seed production and dispersion. They are genetically regulated and timely coordinated and reflect the homeotic and modular nature (phytomer theory) of the plant reproductive structures (Mathews *et al*., 2012). Accordingly, master regulatory genes (e.g. homeotic genes) have undergone duplication and the resulting paralogs have acquired sub/neofunctualization which is found to be structure-specific (i.e. carpel-specific and ovule specific), allowing the developmental program of these structures to evolve in a sort of evident "related-indipendence" (Mathews *et al*., 2012). Disruption (by mutation or hybridity) of such an orchestrated program may lead to an asynchronous gene expression which in turns may give rise to a complete dissociation between the two reproductive structures. Such an hypothesis is supported by Carman (1997) who formulated that asynchronously-expressed duplicate genes that control female development are responsible of apomixis. The two distinct sets of genes from these hybrids would be asynchronously expressed, which might lead to precocious embryo sac initiation and parthenogenesis (Carman, 1997). If hybridization may play a substantial role in triggering an autonomous development of a reproductive structure (i.e. the female gamethophyte), an hybridization-triggered authonomous growth of the ovary (a reproductive structure) is not far from a realistic scenario.

Chapter 3

Phenotypic and molecular characterization of the *pat* mutant: pleiotropic traits at the vegetative level

Introduction

The main objective of the present thesis regards the demonstration of the identity of the candidate *AtHB15/CNA/ICU4* tomato orthologue, *SlHB15,* with the *pat* locus. Though Arabidopsis and tomato have a substantially different developmental program, in order to assess if the *pat* mutant phenocopies at least some of the altered traits of the induced *AtHB15/CNA/ICU4* mutants, other than those observed for the reproductive organs, phenotypic characterization of the *pat* mutant in comparison with its WT line was carried out at early vegetative (seedling stage) and adult plant level. Moreover, transcription analysis of key genes involved in hormone (auxin, GA and cytokinin) homeostasis along with the candidate *SlHB15* gene was taken into account.

Among all the *AtHB15* mutants previously described, *hoc* has the most peculiar phenotype. The *hoc* mutant was isolated in a screen for mutants that could regenerate shoots from junctions of root explants in the absence of exogenous hormone supply (Catterou *et al.* 2002). The *hoc* mutant is bushy due to extra rosettes developing from early axillary meristems and other pleiotropic effects regards the root system, which is apparently more developed in length and shows a higher number of lateral roots; plants are shorter than WT and fruits are small but the number of seeds produced per plant is approximately twofold higher than in WT. In the mutant, accumulation of cytokinins is about two- and sevenfold the cytokinin level of WT plants in its aerial parts and roots, respectively. A cytokinin overdose phenotype was never put in evidence in other *AtHB15* mutants nor in those of the other family members (Catterou *et al*., 2002). According to Duclercq *et al*. (2011), the *hoc* mutation might modify the AtHB15 conformation and affect its ability to dimerize with other transcription factors. Since the *AtHB15* mRNA levels in the *hoc* mutant and in wild type are similar, these authors concluded that the point mutation was responsible for the *hoc* phenotype. Though described as recessive, the *hoc* mutation did not appear to cause a loss-of-protein function since *athb15* T-DNA insertion knockout lines led

to a WT phenotype and F_1 plants carrying both the *hoc* and knock-out alleles showed the (gain-of-function) phenotype of the *hoc* mutant (Duclercq *et al*., 2011).

Another *AtHB15* mutant, *corona-1* (*cna-1*), was isolated after an EMS-mutated population screening and described as an enhancer of the phenotype showed by *clavata* (*clv*) mutants (Green *et al.*, 2005). The *CLV* gene regulates the size of the shoot apical meristem (SAM) and floral meristem (Clark *et al*. 1997). The *cna-1* mutation (Fig. 1.2) drops in a domain of unknown function between the SAD and MEKHLA domains (Green *et al.*, 2005). It determines subtle defects in the plant meristem development which are magnified in the *clv cna* double mutants. In this case, a crown-shaped meristem develops around the main SAM which leads to a massively enlarged apices that displays early loss of organogenesis (Green *et al*., 2005). The *cna-2* mutant differs from *cna-1* because the mutation gives rise to a null allele of *HB15* and lacks any discernable phenotype (Prigge *et al*., 2005). According to Green *et al*. (2005) *cna-1* contains the entire coding sequences and *cna-1/cna-2* heterozygotes show the same strong *cna-1* phenotype. Moreover, transformation of *clv cna-1* double mutant with a *pREV::CNA* construct containing the CNA^{WT} allele driven by the *REVOLUTA* (*REV*) promoter did not complement the mutant phenotype while the construct containing the mutated allele ($pREV::CNA^{cna-1}$), but not $pREV::CNA^{WT}$, transformed into *clv CNA* background recreated the *cna* phenotype. Therefore, the authors postulated that *cna-1* interferes with the activity of *cna* and that it is incompletely dominant and displays dominant-negative characteristics when in the *clv* background. The missense mutation of *cna-1* is located in a position analogous to the missense mutation within the *revoluta-3 mutant*, another *class-III HD-Zip* member, which itself exhibits dominant-negative characteristics (Green *et al*., 2005).

The *icu-4* mutant, due to another lesion in *AtHB15* locus, substitutes an amino acid (Fig. 1.2) in the START domain of the protein (Ochando *et al*., 2006). *icu-4* shows gain-of-function properties since the mutation changes the coding sequence in a region complementary to a *microRNA* locus (*miRNA-165*). The phenotypes of *icu-4* involves the root system, with longer root hairs and more secondary roots, curved leaves, an excess of stomata and smaller epidermal cells, development of two inflorescence at the same time, suggesting that the SAM had split (Ochando *et al*., 2006).

Recently, through an in-debt phenotypic analysis of *cna-1* and *icu-4* mutants, it was possible to put in evidence clear defects at the plant level resembling the *pat* syndrome: appearence of seedlings with supernumerary cotyledons, precocious ovary enlargement at

anthesis after emasculation, ovule abortion and parthenocarpy tendency of siliques with reduced size (Ruiu, 2013).

Materials and Methods

Plant material and growth analysis and

Seeds of the two near-isogenic WT and *pat* lines were surface sterilized with 2.5% sodium hypoclorite and sown on 9-cm diameter Petri dishes containing two disks of filter paper kept wet with deionized water. Petri dishes were maintained under light/dark cycle (16/8 hrs, 200 µmol m^{-2} s^{-1} from Sylvania F36 W/GRO fluorescent lamps). When the primary root reached 1 cm in length, each seedling was transferred into a glass tube containing Murashige & Skoog salts with sucrose (30 g l^{-1}) and agar (7 g l^{-1}), pH 5,7. Eighteen plants per each line were grown for 10 days on a 16 h light/8 h dark cycle at 22±1 °C. After 10 days of culture, the root, hypocotyl and epicotyl lengths were measured with a ruler. *In vitro* growth analysis was carried out in two replications.

Seeds of an F_2 population, obtained from the crossing of the *pat* mutant with a WT plant, were germinated in jiffy pots in controlled environment (see above). When plants were at the 4^{th}-5^{th} leaf stage, they were transplanted in field at the Experimental Station of the Tuscia University in Viterbo (42_260N, 12_040E), in spring 2017. Plants were phentyped for the *pat* syndrome traits and all data were subjected to GLM analysis and tested for significance of differences among means at the 1% level (Duncan test). The statistical analysis was performed with the SAS software package (SAS® University Edition) and graphs were elaborated with Excel (Microsoft Office 2013).

RNA isolation, cDNA synthesis and real-time PCR (qRT-PCR) analysis

Three biological replicates of root, hypocotyl and epicotyl tissue of 10-day-grown *in vitro* seedlings of the two *Pat* NILs were used for total RNA extraction, cDNA synthesis and expression analysis of *SlHB15* (*Solyc03g120910*), *SlGA20ox2* (*Solyc06g035530*), *LeT6/TKn2* (*Solyc02g081120*), *SlLOG6* (*Solyc10g084150*), *SlLAX3* (*Solyc11g013310),* *SlIAA9* (*Soly04g076850*) and *SlARF8* (*Solyc02g037530*) genes according to protocols reported in Chapter 4. qRT-PCR assays were performed in three technical replicates and using primer

pairs reported in Table 4.1. To evaluate the gene expression level, results were normalized using the house-keeping gene encoding the clathrin adaptor complexes medium subunit (CAC, Exposito-Rodrıguez *et al*., 2008). Normalized gene expression was calculated based on the comparative Ct method using CAC as internal standard. The statistical significance of gene expression differences between the two NILs was assessed by Student's *t* test using Microsoft Excel 2011.

Molecular marker (CAPS) analysis of the *SlHB15* point mutation associated to the *pat* locus in the F_2 population

Twenty-eight F_2 plants derived from a cross between the *pat* mutant and WT NIL were screened for both the pat phenotype and the allele polymorphism of the *pat*-associated *SlHB15* locus by a CAPS molecular marker. Total DNA was extracted according to Doyle and Doyle (1990) and amplified by PCR using the pair of primers, *HB15-9* and *HB15-10* (Table 4.1), which amplified a fragment of 859 bp containing the G1747A substitution in the $SlHB15^{pat}$ allele. PCR was performed in a 20-µl of total volume, containing 75 ng of genomic DNA, 10 µM of each primer, 200 µM of dNTPs, 1× DreamTaq buffer and 1 U of DreamTaq polymerase (Fermentas, ON, Canada). The PCR reaction was conducted with an initial denaturation of 4 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for1 min at 56 °C, extension for 2 min at 72 °C, plus 7 min of final extension at 72 °C. To detect CAPS polymorphism on the point mutation, amplicons were digested with *Bfa I* (New England Biolabs, Beverly, MA, USA). Ten µL of the amplification product were digested with 1 U of enzyme in a final volume of 20 µL. *Bfa I* generates one and two cuts in the amplicons of the $SlHB15^{WT}$ and $SlHB15^{pat}$ allele, respectively. The two bands of the CAPS marker with the highest molecular weight (450 and 674 bp), which identify $SlHB15^{pat}$ and $SlHB15^{WT}$, respectively, were visualized with ethidium bromide after electrophoresis on 1.5% (w/v) agarose gel.

Statistical analysis

All data were subjected to GLM analysis and tested for significance of differences among means at the 5% level (Duncan test). Preliminary assumptions of constancy of variance and normal distribution of the data have been met. The statistical analysis was performed with

the SAS software package (SAS® University Edition) and graphs were elaborated with Excel (Microsoft Office 2013).

Results and discussion

The *pat* mutant displays vegetative phenotypes at seedling stage

Besides parthenocarpy, the *pat* mutant displays pleiotropic effects regarding the reproductive organs (Mazzucato *et al.*, 1998). The *pat* mutant showed a variety of subtle vegetative growth phenotypes. At *in vitro* seedling (10-day-old) stage, *pat* displayed a longer primary root and a higher number of lateral and adventitious roots (Fig. 3.1a, b; Fig. 3.2). On the contrary, the hypocotyl in the mutant was shorter than in the WT (Fig. 3.1a; Fig. 3.2). At the flowering stage, the number of internodes and leaves was the same in the two lines (not shown) but leaves were shorter in the *pat* line, escluding the terminal leaf which was not significantly different (Fig. 3.1d). As a consequence, the plant stature of the mutant was reduced (Fig. 3.1c), its architecture resulting slightly more compact.

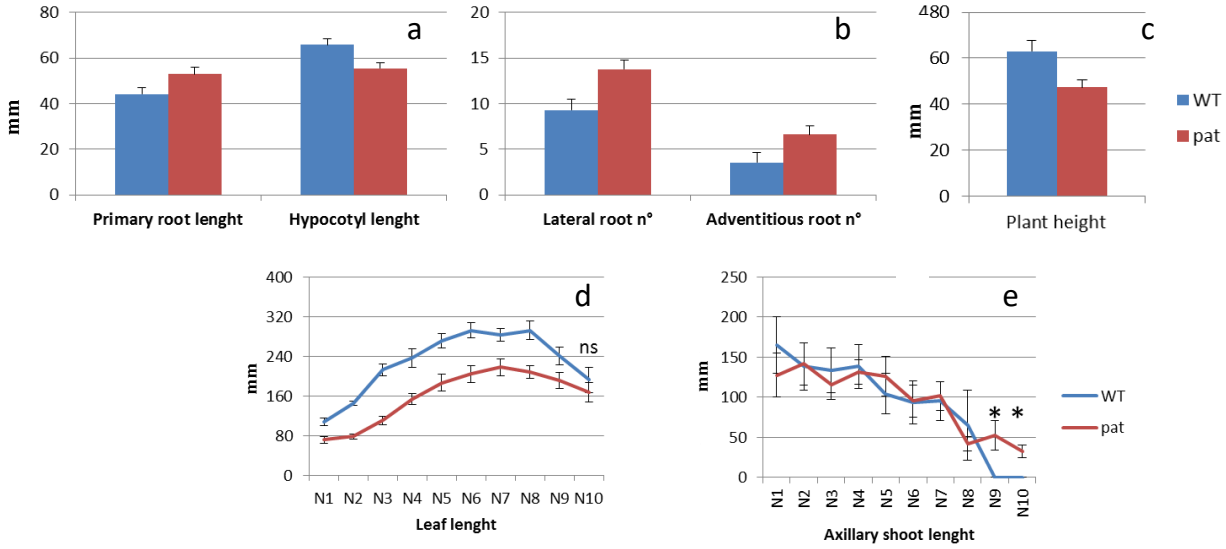


Figure 3.1. Vegetative phenotypes of WT and *pat*: a, length of primary root and hypocotyl; b, number of lateral and adventitious roots; c, plant height; d, leaf length and d, axillary shoot length were measured at flowering stage on plants grown in pot. The horizontal axis in d) and e) indicates the position of ten leaves and axillary shoots at the corresponding node (N1-N10) on the plant. In a), b), c) and d), if not stated otherwise (ns= not significant), statistical differences between pairwise data between WT and *pat* lines are significant for $P \leq 0.01$, according to Student's *t*-test; in e) the same statistical significance level is denoted by *.

Though shorter in length, the growth rate of the *pat* leaves was the same of the WT counterpart (Fig. 3.1d). In WT plants, axillary shoots (side shoots) developed from buds formed early in development in all axils of leaf primordia (=nodes), except for the upper two leaves (9th and 10th nodes). On the contrary, *pat* plants displayed side shoots in all leaf axils (Fig. 3.1e).

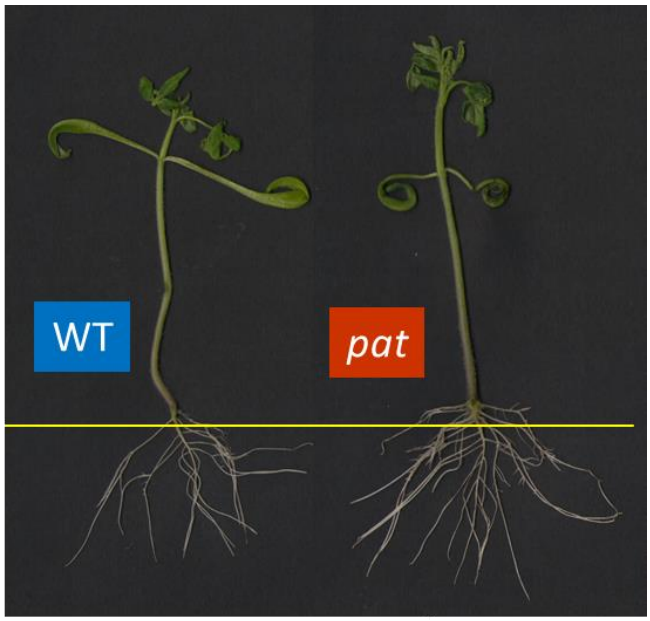


Figure 3.2. 10-day-old *in vitro* grown seedling of WT and *pat* in comparison for primary root length, number of lateral roots and hypocotyl length. The yellow line marks the emission point of adventitious roots at the base of the hypocotyl (above) and that of lateral roots from the primary root (below).

Plant morphogenesis is governed by coordinated cell division and cell expansion. A large body of evidence suggests that plant hormones are involved in regulating these events, mainly auxin, citokinins and GA. HD-Zip III proteins are involved in a number of processes, such as meristem formation and regulation, embryonic shoot and root development, lateral root primordia initiation, leaf polarity, vascular tissue establishment, plant architecture, auxin transport and signalling and cytokinin signalling (Zhong and Ye, 2001; Eshed *et al*., 2001; Emery *et al*., 2003; Prigge *et al*., 2005; Ochando *et al*., 2006; Scarpella *et al*., 2006; Izhaki and Bowman, 2007; Dello Ioio *et al*., 2012).

In order to gain a preliminary insight into a possible role of both *SlHB15* and hormones in the manifestation of the phenotypes observed in the *pat* mutant at seedling level (Fig. 3.2), RT-PCR analysis of selected genes involved in hormone biosynthesis, signalling and response was undertaken in the root, hypocotyl and epicotyl of the *pat* mutant in comparison with the WT line. In particular, one TF regulating cytokinin synthesis,

SlLONELY GUY-6 (*SlLOG6*), two genes implicated in auxin transport and signalling (*SlLAX3, SlIAA9* and *SlARF8*) and two in GA synthesis (*LeT6/TKn2* and *SlGA20ox2*) were analyzed along with the expression of *SlHB15* in the three different organs (Fig. 3.3).

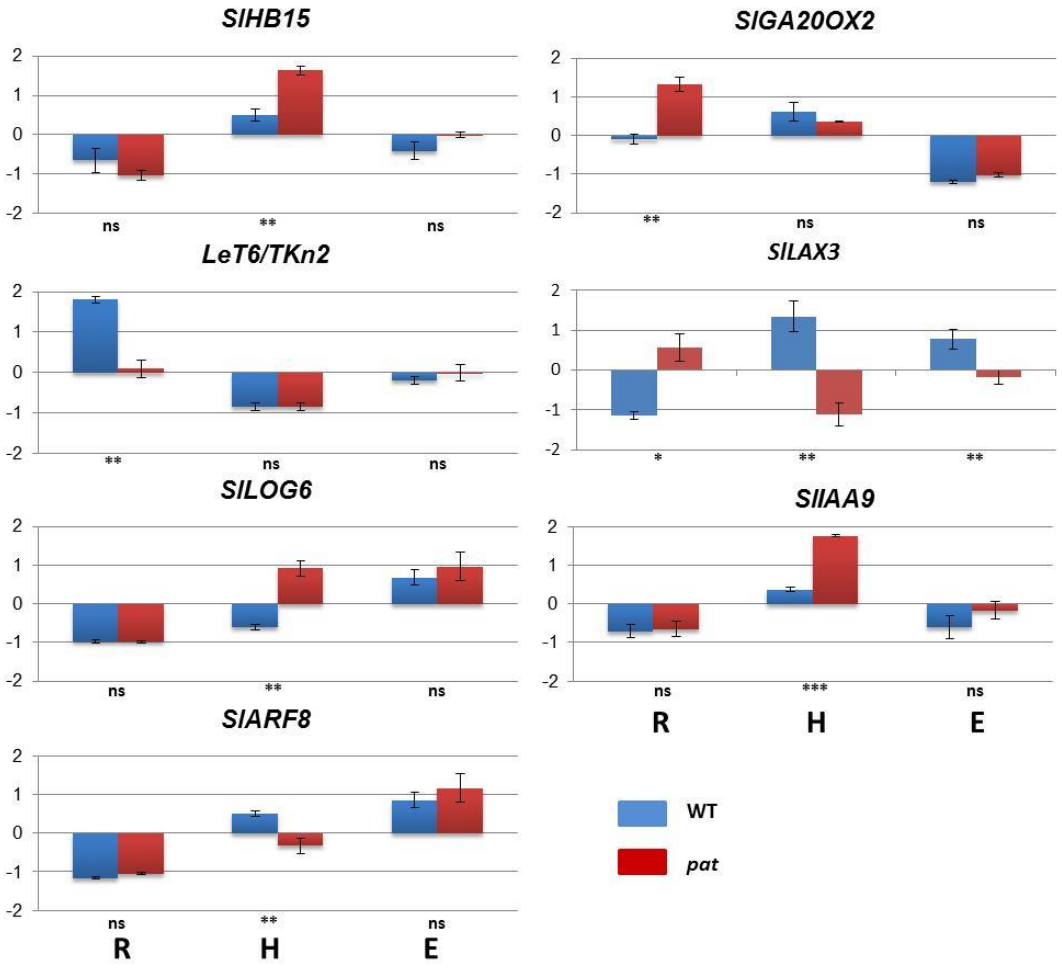


Figure 3.3. Normalized gene expression of seven genes involved in hormone homeostasis in WT and *pat* root (R), hypocotyl (H) and epicotyl (E) of 12-day-grown seedlings. Transcript levels were normalized using the house-keeping gene CAC and standardized as described in ''Materials and methods''. Data represent the mean ± SEM of three independent biological replicates. Students' *t* test between genotypes within stage: ns not significant; *≤ 0.05; **≤ 0.01; ***≤ 0.001.

According to a recent transcription analysis of a number of TFs involved in the regulatory network of the fruit set in the *pat* mutant (Ruiu *et al*., 2015), *SlLOG6*, *SlLAX3*, *SlIAA9* and *SlARF8* expression either did not differ between NILs or was repressed in the flower at the studied steps of fruit set. In the root system, *SlGA20ox2*, *LeT6/TKn2* and *SlLAX3* were differentially expressed, with the two GA-related genes being up- and downregulated, respectively. This is in line with the repressing activity of *LeT6/TKn2* on the GA biosynthesis pathway (Bolduc and Hake, 2009). No differential transcripts of these two

genes was detected in the hypocotyl and epicotyl between the two NILs. Heterologous overexpression of *SlGA20ox2* in tomato (Garcìa-Hurtado *et al*., 2012) produced the characteristic phenotypes observed in plants from diverse species overexpressing *GA20ox* genes (Eriksson *et al*., 2000; Vidal *et al*., 2001; Phillips, 2004): longer roots and hypocotyls, taller plants with longer and thinner internodes compared with controls. The promoting effect of GA on root formation and growth resides on its ability to destabilize DELLA growth repressors, such as RGA and GAI (Hardtke *et al*., 2003). Furthermore, auxin is important for GA mediated control of root growth and attenuation of polar auxin transport or auxin signalling delays GA induced disappearance of RGA from root (Fu *et al*., 2003).

In Arabidopsis, ARF7 and ARF19 positively regulate the expression of many auxin-responsive genes (Okushima *et al*. 2005). Among the direct targets of ARF7 and ARF19 are *LATERAL ORGAN BOUNDARIES-DOMAIN*(*LBD*)/*ASYMMETRIC LEAVES2-like* (*ASL*) genes, *LBD16/ASL18* and *LBD29/ASL16*, which encode plant specific nuclear proteins that are important for lateral and adventitious root development (Okushima *et al*. 2007; Bellini *et al*., 2014). The involvement of auxin-mediated gene expression stresses the importance of auxin signalling and transport during the process of root formation as exogenous application of auxin increases the number of lateral roots (Blakely *et al*., 1988; Laskowski *et al*., 1995). Preventing auxin transport from the shoot to the root inhibits lateral root development in *Arabidopsis* (Reed *et al*., 1998), and it has been shown that both acropetal and basipetal transportation are required for lateral root formation (Casimiro *et al*. 2001, Marchant 2002, Reed *et al*. 1998).

Another downstream target of *ARF7* is *LAX3*, which has recently been demonstrated to be important for lateral root emergence in Arabidopsis (Swarup *et al*., 2008). Since *SlLAX3* expression was increased in the root of the *pat* mutant, it is possible that either an increase of auxin influx in the root tissue or an alteration of polar transport is sufficient to activate GA-regulated TF in promoting root growth (Hardtke *et al.,* 2003). An auxin signal, like a local auxin accumulation, might account for the release of *SlGA20ox2* expression by deregulating *LeT6/ KTn2* TF (Hay and Tsiantis, 2012).

RT-PCR analysis showed an upregulation of *SlHB15, SlLOG6 and SlIAA9* in the *pat* hypocotyl while *SlLAX3* downregulation was observed here and in the epicotyl of the mutant (Fig. 3.3). An increase of *SlHB15* transcriptional actitvity represents a novel phenotype in the *pat* system (Fig. 3.3).

Hypocotyl elongation is very plastic and is influenced strongly by factors that regulate cell elongation in the adult plant such as light, plant hormones, temperature and touch. There

are mutants in Arabidopsis and to a lesser extent in the tomato with altered hormone biosynthesis or signalling and many of these mutations affect hypocotyl elongation.

Cytokinins (CKs) are chemical signals that control numerous developmental processes throughout the plant life cycle, including gametogenesis, root meristem specification, vascular development, shoot and root growth, meristem homeostasis and more (Zurcher and Muller, 2016). Endogenous CK content is known to be spatially and temporally regulated by a fine balance between synthesis and catabolism (Hirose *et al.*, 2007; Matsuo *et al*., 2012). To become biologically active, CK nucleotides, produced by *IPT* and *CYP735A* biosynthetic genes, must be converted to the free-base forms*. LOG* genes are CK-activating enzymes, which directly converts CK nucleotides to the active nucleobases (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009). The expression patterns of *LOG* genes are spatially and quantitatively differentiated but overlap in various tissues during plant development suggesting redundant functions of members of the *LOG* gene family (Kuroha *et al*., 2009). In Arabidopsis, single or double T-DNA insertion (loss-of-function) mutants for *LOG* genes do not show any difference in term of primary root length but higher order (quadruple, quintuple) mutants have severe retardation of the primary root growth (Tokunaga *et al*., 2012) and adventitious root formation and flower number are affected (Kuroha *et al*., 2009). Instead, constitutive overexpression of different *LOG* genes shows a semidwarf phenotype with shorter leaves and more axillary stems, reminiscent of a reduced shoot apical dominance in the transgenic lines (Kuroha *et al*., 2009). There is an evidence that *LOG* genes, at least *LOG4*, are transcriptionally promoted by HD-Zip III TF in Arabidopsis (Muller *et al*., 2016). Traditionally, cytokinins are thought to be synthezised in the root and transported to the shoot through the xylem (Letham and Palmi, 1983; Beveridge *et al*., 1997). Recent studies have demonstrated that cytokinins are produced not only in roots, but also in various sites within the aerial parts of the plant as activation of the *IPT* and *LOG* genes takes place in numerous organs including leaves, stems, flowers and fruits (Miyawaki *et al*., 2004; Takei *et al*, 2004; Kurakawa *et al*., 2007; Kuroha *et al.*, 2009). Moreover, long-distance transport of cytokinins is accomplished through xylem and phloem sap (Kudo *et al*., 2010) and acropetal transport of cytokinin (toward the shoot apex) has been implicated in the control of shoot branching (Shimizu-Sato *et al*., 2009). Moreover, long-distance basipetal transport of cytokinin is relevant for controlling polar auxin transport and maintaining the vascular pattern in the root meristem (Bishopp *et al*., 2016).

According to the transcriptional increase of *SlLOG6* in the hypocotyl of the *pat* mutant (Fig. 3.3), it is possible to infer that an increase of *SlLOG6* stimulates cytokinin biosynthesis in this organ which could lead to an increase in other parts of the plant.

In silico analysis of *SlLOG6* promoter

To analyze *SlLOG6* putative promoter, a sequence of 2300 bp upstream the starting codon of the gene was analyzed through the Plant Promoter Analysis Navigator 2.0 (PlantPan) predictive software (http://plantpan2.itps.ncku.edu.tw) in order to retrive functional *cis*-regulatory elements within the conserved regions of homologous genes. A putative short motif shared homology with that residing in the *AtLOG6* promoter, a functional motif regarded as a binding site for AtHB15 (Fig. 3.4).

Two interesting hypotheses can be formulated: 1) the simultaneous higher transcription of *SlHB15* and *SlLOG6* in the hypocotyl might find an explanation in case the *SlHB15* allele determines a structural modification of the protein leading to an increase of its activity (gain-of-function); if so, *SlHB15* might induce *SlLOG6* trasncription; 2) upregulation of *SlHB15* transcription might be the result of a positive feedback loop like that operating in Arabidopsis in a molecular network providing stable levels of cytokinin in the root (Dello

Figure 3.4. Motif sequence Logo of AtHB15 TF transcription factor binding site sharing homology (q-value score: 0.92) with one position (at -393) of the 2300-bp-long promoter of *SlLOG6.*

Ioio *et al*. 2012). Within this network, a feed-forward loop regulates the activity of the *HD-Zip III PHABULOSA* (*PHB*) member. Such a loop comprises three components: the first component, the *Arabidopsis Response Regulator 1* (*ARR1*) TF, regulates the activity of the second component (*PHB*), ARR1 represses the transcription of *PHB* and the expression of a third component, *microRNA165/166*, that exerts post-transcriptional repression of *PHB*. PHB promotes the biosynthesis of cytokinin (via *IPT7*), which activates *ARR1*. This provides

a molecular circuit whereby cytokinin both represses *PHB* and prevents the repression of *PHB*, which in turn feeds back on the synthesis of the original signalling molecule, cytokinin.

In Arabidopsis, the cytokinin signal, mediated by *ARR1*, activates IAA3/SHY2, an auxin response inhibitor (Dello Ioio *et al*., 2008; Moubaudin *et al*., 2010). By its inhibiting action, IAA3/SHY2 regulates the ARF7- and ARF19-dependent auxin signalling cascade, which promotes the root system (Okushima *et al*., 2007; Goh *et al.,* 2012). The loss-of-function *iaa3/shy2-24* mutant exhibits a longer primary root while the gain-of-function *iaa3/shy2-2* allele has an inhibitory effect on root lengthening (Tian *et al*., 2002). In tomato, antisense *SlIAA3/SHY2* plants retain triple cotyledons, longer roots, short hypocotyls and the plants have an increased number of lateral shoots (Chaabouni *et al*., 2009).

SlIAA9 plays a major role in the fruit set because plants silenced by antisense technology showed strong parthenocarpic behaviour (Wang *et al*., 2005). Moreover, antisense (AS) *SlIAA9* plants have several IAA-related developmental defects such as dramatic reduction of leaf compoundness, enhanced hypocotyl/stem elongation, increased leaf vascularization and reduced apical dominance (Wang *et al*., 2005). These authors investigated the effect of downregulation of the *SlIAA9* gene on the expression of a number of early/primary auxin responsive genes and observed that this gene downregulates *SlIAA3*, hence acting as a transcriptional repressor of auxin signalling (Wang *et al*., 2005).

We do not have information on *SlIAA3/SHY2* expression in the *pat* mutant but the observed increase of *SlIAA9* transcripts in the *pat* hypocotyl (Fig. 3.3) might provide an inhibition switch to *SlIAA3/SHY2*, leading to a reduced hypocotyl growth, as observed in the *AS-IAA3* plants.

In Arabidopsis, the balance of shoot/root auxin appears to be essential for coordinated development of the seedling. A high shoot/root auxin ratio directs resources to shoot elongation at the expense of cotyledon and root growth, while shifting the auxin balance towards the root favours cotyledon and root development (Halliday *et al*., 2009). The polarized transport of auxin is crucial for maintaining such a coordination (Petrasek and Friml, 2009). Polar auxin transport is driven and steered by influx and efflux carrier proteins: auxin is actively taken into the cytosol by the AUX/LAX influx carriers and leaves the cell by auxin efflux carriers such as PIN proteins and P-glycoproteins of the ATP-binding cassette family B transporter family (Halliday *et al*., 2009). AUX/LAX proteins mainly generate auxin sinks and control auxin levels in the auxin channels (Petrasek and Friml, 2009). Also in tomato, AUX/*LAX* genes regulate auxin influx into the cell, and

maintain auxin sink-strength necessary to induce auxin signalling pathways in the cell (Pattison and Catala, 2012). Auxin biosynthesis and signalling control hypocotyl elongation (Hornitschek *et al*., 2012; Procko *et al*., 2016) and regulation of *AUX/IAA* gene stability is critical for auxin signalling (Kepinski and Leyser, 2002). Downregulation of *SlLAX3* in the *pat* hypocotyl (Fig. 3.3) may account for a decreased auxin influx in the cell which may account for the transcriptional activation of *SlIAA9* (Fig. 3.3), decreasing its short half-life and consequentially affecting the downstream auxin response pathway necessary for cell elongation in the hypocotyl.

The *AUXIN RESISTANT3/IAA17 (AXR3/IAA17)* gene is a member of the *Aux/IAA* gene family (Rouse *et al*., 1998). As already reported (Chapter 1), these genes inhibit the activity of the DNA-binding auxin response factors (ARFs) whereas their degradation leads to the activation of ARFs and to subsequent auxin-responsive gene expression (Tiwari *et al.*, 2001; Zenser *et al.*, 2001; Hagen and Guilfoyle, 2002). AXR3/IAA17 protein participates in the auxin-induced growth of the hypocotyl (Leyser *et al*., 1996;). The *axr3/iaa17*-1 (gain-of-function) mutation results in a stabilization of the AXR3/IAA17 repressor and the persistence of the protein in the seedling was hypothesized (Collett *et al*., 2000) and subsequently confirmed (Fendrych *et al*., 2016) as the signal affecting hypocotyl growth.

Transgenic downregulation of *IAA9* and *ARF8* in both tomato and Arabidopsis results in uncoupling fruit set from pollination and fertilization, giving rise to parthenocarpy, thus suggesting that these genes encode negative regulators of fruit set (Wang *et al.*, 2005; Goetz *et al.*, 2007; de Jong *et al.*, 2009). Although the interaction between ARF and Aux/IAA proteins plays a pivotal role in regulating auxin responses (Dharmasiri and Estelle, 2004; Guilfoyle and Hagen, 2007), it is still not well known whether *IAA9* and *ARF8* control fruit set through common or distinct pathways (Ruan *et al.*, 2012). *SlARF8* transcription was downregulated in the *pat* hypocotyl (Fig. 3.3); if this might be the result of IAA9-induced post-translational degradation deserves a specific investigation. By the way, it is relevant to observe that constitutive expression of the microRNA, *miR167a*, targeting the *SlARF8* gene in tomato, led to plants displaying shortening of vegetative (internodes, leaves) and reproductive organs (flower organs) (Liu *et al*., 2014), thus demonstrating that ARF8 plays a role in promoting stem elongation and leaf expansion.

Some of the phenotypes of the tomato lines with altered *SlIAA9* and *SlARF8* expression configure the possibility of an antagonistic interaction between these two gene functions in plant development. Investigation of *SlLAX3, SLIAA9* and *SlARF8* expression level in the adult plant of the *pat* mutant might further put in relation auxin transport/homeostasis

mediated by *SlLAX3* and auxin signalling activated by *SlIAA9* and *SlARF8* with reduction of plant growth in terms of reduced plant height and leaf size, as observed in the *pat* mutant (Fig. 3.1).

Verification of the recessive inheritance of the *pat* mutation

To test the degree of recessivity of the *pat* mutation, an F_2 population of 28 plants, derived from crossing the *pat* mutant with a WT plant, was phenotypically analyzed, together with the segregation of a CAPS marker designed on the *SlHB15* mutation. According to Figure 3.5a, six plants carried a WT phenotype (*PatPat*), that cosegregated with the $SlHB15^{WT}$ (A, fig. 3.5d) allele and did not show any appreciable homeotic alteration at the flower level, reminiscent of the *pat* syndrome. WT plants showed the highest values in term of mean fruit weight (Fig. 3.5b) and mean number of seeds per fruit (Fig. 3.5c). Seven plants showed a typical aberrant flower phenotype (Fig. 3.5a), indicative of the *pat* phenotype (*patpat*), that cosegregated with the $SlHB15^{pat}$ allele (B, fig. 3.5d). They displayed seedless fruits (Fig. 3.5c) and the mean fruit weight was lower than the WT as expected for the *pat* mutation (Fig. 3.5b).

Seventeen plants (*Patpat*) bore both CAPS alleles (H, fig. 3.5d) and showed a leaky expression of the traits regarding the flower (Fig. 3.5a) while values of the phenotypes for the seeds and fruit were approximately intermediate between the two homozygous groups of plants (Fig.3.5b and c).

Phenotypic and preliminary expression analysis of key genes involved in hormone (auxin, GA and cytokinin) biosynthesis, signalling or response allowed to expand the knowledge on the pleiotropic effects of the *pat* mutation at the morphological and molecular level, operating already at an early developmental stage.

Analysis of an F_2 population cosegregating *pat* and *SlHB15*, by means of a CAPS marker identifying the $SlHB15^{pat}$ mutation, allowed to highlight a lower expressivity of the *pat* phenotypes among the plants which turned to be hetorozygous for *SlHB15* and full or absence of expressivity of such traits in the homozygous plants bearing either the $SlHB15^{pat}$ or the $SlHB15^{WT}$ allele, respectively. Despite the relatively little size of the F_2 population, the leaky phenotype showed by the heterozygotes persuaded us to reconsider the complete recessivity of the *pat* mutation and address it with an activity that might be evoked by a hypofunctional dominant negative (antimorphic) mutation in the $SlHB15^{pat}$. This issue

corroborates the hypothesis of identity between the *pat* syndrome and the predicted structural alteration of the HD-Zip III SlHB15/CNA/ICU4 protein.

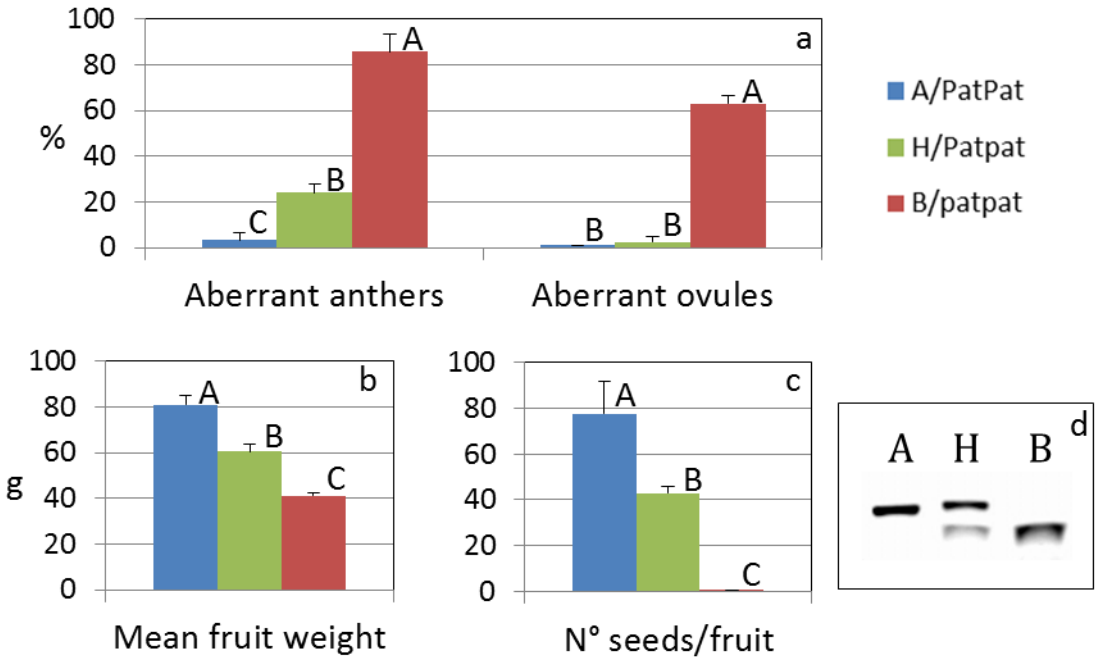


Figure 3.5. Phenotypic characterization of 28 plants segregating the *pat* allele in association with the CAPS marker designed on the mutation of *SlHB15*. Pleiotropic traits of the *pat* mutant regarded the flower (a), as percentage of aberrant anthers and ovules, and the fruit in terms of mean fruit weight (b) and mean number of seeds per fruit (c). Cosegregation of the *pat* phenotype and the CAPS marker (d) allowed to easily identify three "Mendelian" phenotypic classes for the *pat* phenotype: one class with six WT (*PatPat*) plants and homozygous for one marker allele, band A of the CAPS; a second class with seven *pat* (*patpat*) plants homozygous for the other marker allele, band B and a third class with 17 plants, putatively heterozygous for the *pat* mutation (*Patpat*), displaying a weak expression of the pleiotropic phenotypes analyzed and heterozygous for the marker, bands H. Different block letters on top of bars indicate means significantly different beetween plant categories within each variable for $P \leq 0.001$ after Duncan multiple range test.

There is evidence that the MEKHLA domain of the REVOLUTA protein acts as a negative regulatory domain that might prevent dimerization of this transcription factor (Magnani and Barton, 2011). According to these authors, the MEKHLA domain sterically inhibits heterodimerization, perhaps by blocking access to the leucine zipper domain, believed to be the site of dimerization. The missense mutation in the *SlHB15*^{pat} allele, located immediately upstream the MEKHLA domain, might account for a modification of the ability of this domain to inhibit protein dimerization, as hypothesized for the *hoc* mutant (Duclercq *et al*., 2011).

Chapter 4

Functional validation of the *SlHB15* candidate gene for the *Pat* locus

Introduction

Fruit set is an essential process to ensure successful sexual plant reproduction. The development of the ovary into a fruit is actively repressed in the absence of pollination. However, some cultivars from a number of species are able to develop seedless fruits overcoming the standard restriction of unpollinated ovaries to growth. We report here the functional strategies adopted for the identification of the tomato *parthenocarpic fruit* (*pat*) mutant that produces seedless fruits. Such a trait is linked to homeotic modification of anthers and abortion of ovules coupled with a precocious growth of the ovary prior to anthesis. Moreover, the *pat* mutant entails other pleiotropic phenotypes that regard the development of both the seedling and the adult plant (Olimpieri *et al*., 2007*;* this thesis). Using positional cloning, the *pat* locus was confined in a genetic window of chromosome 3, containing nine predicted genes (Selleri, 2010). Among them, *SlHB15* was proposed as a candidate for identification of the *Pat* locus. Eligibility of the candidate benefited from phenotypic analogies with Arabidopsis mutants orthologous to *SlHB15*. Different functional approaches were adopted: complementation of the *pat* mutation with the WT allele of *SlHB15* and gene function suppression in the WT line by the same allele used for complementation (gene knock-out by cosuppression) and through the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) system (gene knock-out by genome editing). In recent years, the gene editing technology has been intensively developed, featured by the application of sequence specific nucleases, including the RNA-guided CRISPR-ASSOCIATED9 (Cas9) nuclease. The target sequence needs to be complementary to the guide sequence and an adjacent NGG motif which is called protospacer adjacent motif (PAM) is required to be present (Wiedenheft *et al*., 2012; Sorek *et al*., 2013). The system combines the guide sequence and the chimeric tracrRNA (trans activating CRISPR RNA)/crRNA unit into a single guide RNA (sgRNA) and additionally contains a *Cas9* expression cassette. The efficacy of the CRISPR/Cas9-mediated

DNA interference has been proven in both model and crop plants, such as rice, sorghum, wheat, maize, sweet orange, soybean, potato, apple, and tomato (Seth, 2016). CRISPR/Cas9 technology, also, would enable fast integration of the induced *SlHB15* mutation into any elite cultivar of interest.

Both transgenic approaches were carried out by using *Agrobacterium tumefaciens* (*A.t.*) mediated transformation. To further explore the functional relation of *SlHB15* with the *pat* syndrome, *in silico* analysis of the putative regulatory sequence (promoter) upstream the gene starting codon was performed.

Materials and Methods

Preparation of *SlHB15* complementation/cosuppression and genome editing constructs

A gene construct containing the CDS of the WT allele of *SlHB15 (SlHB15^{WT}*) driven by a constitutive promoter (*CaMV35S*), previously obtained (Ruiu, 2013), was used for the complementation assay (Fig. 4.1a). The full-length CDS of the *SlHB15* gene (2511 bp) was amplified from the cDNA of WT ovaries at opening flower stage (Stage 3), combined into *pBI121* vector containing the *CaMV35S* enhancer and the *nopaline synthase* (*NOS*) terminator (Chen *et al.*, 2003). Afterwards, the obtained final construct (*pBI12::CaMV35S::SlHB15*) was sequence-verified. Later, a new gene construct was pursued by including the putative promoter region (2300 bp) and the 5' and 3' UTR regions of the gene as regulatory elements (Fig. 4.1b).

A CRISPR/Cas9 construct was designed to create a deletion/insertion in *SlHB15* and potentially generate knock-out versions of the gene (Fig. 4.1c). The construction of the gene cassettes took advantage of the GoldenBraid® (GB) gene assembly method (Sarrion-Perdigones *et al.*, 2013). GB3.0 relies on the use of Type IIS restriction enzymes for DNA assembly in an entry vector (*pUPD2*) and proposes a modular cloning scheme with positional notation. Gene domestication (GBpart domestication) is the first step in GB3.0 in order to clone the single gene elements which are combined into a construct. Subsequently, gene assembly (pDGB construction) is accomplished by destination binary vectors (pDGB3s). Each pDGB3 vector contains two restriction/recognition sites corresponding to two different type IIS enzymes, *BsMBI* and *BsaI*. The special orientation and arrangement of the restriction sites define two levels of pDGB3s, the alpha-level and omega-level plasmids,

which are used for the *BsaI* and *BsmBI* reactions, respectively. Plasmids also differ in the resistance marker that is associated with each level (kanamycin for level alpha and spectinomycin for level omega). To ensure an endless cloning design, a minimum set of four pDGBs is required (*pDGB3alpha1*, *pDGB3alpha2*, *pDGB3omega1* and *pDGB3omega2*).

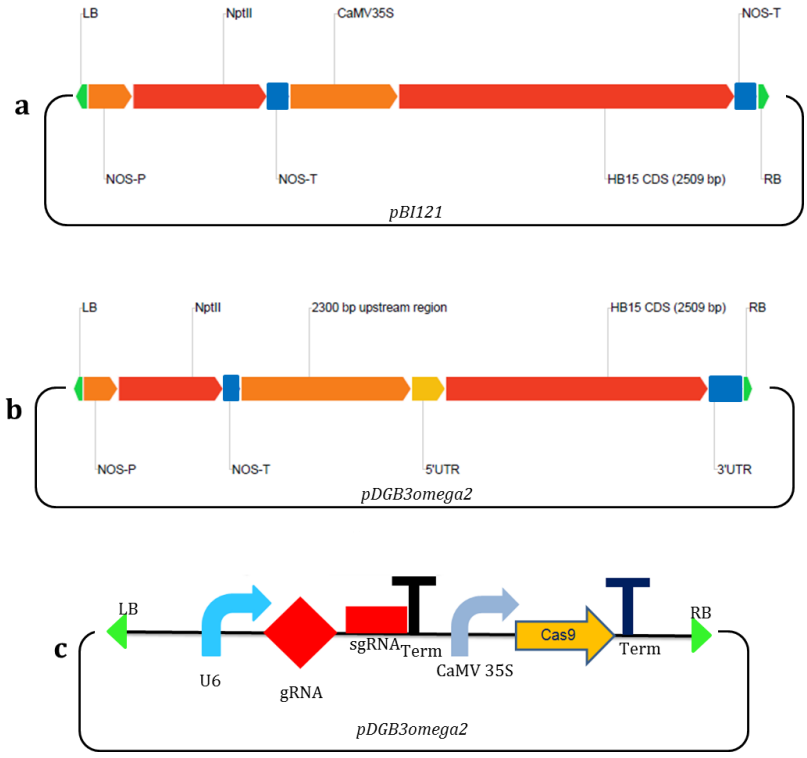


Figure 4.1. Gene constructs designed for *SlHB15* complementation/overexpression/cosuppression experiments with either a constitutive (a) or gene specific regulatory elements (b) and for CRISPR-Cas9-induced *SlHB15* knock-out experiments (c). Elements of the gene constructs reported in: a) LB and RB, left and right T-DNA borders; CaMV35S constitutive promoter and NOS-T terminator of *SlHB15* coding sequence (2509 bp); b) *SlHB15* promoter (2300 bp), 5'UTR (301 bp) and 3'UTR (150 bp) predicted regions; NOS-P and NOS-T. Both constructs contain the promoter and terminator of the *Nopaline phosphotrasferase II* (*NptII*) gene. In c) Elements of CRISPR/Cas9 construct for the SlHB15 locus are indicated: LB and RB, left and right T-DNA borders; RNAPolIII promoter (U6); guide RNA (gRNA, 20 bp) targeting *SlHB15*; scaffold RNA (sgRNA); terminator (Term); hCas9 coding sequence (Cas9) under the control of the CaMV35S promoter and terminator.

GB3.0 framework is facilitated by a number of web resources which include a publicly available database, tutorial and a software package (http://www.benchling.org) that provides *in silico* simulations and lab protocols for GB3.0 part domestication and multigene engineering (Sarrion-Perdigones *et al.*, 2013).

GBpart domestication

This tool allowed to adapt by PCR the different parts (promoter, 5UTR', CDS and 3'UTR) of the gene for assembly by adding to the extremities of each part an oligonucleotide adaptor containing the recognition sites of the two domestication restriction enzymes. Removal of a *BsmBI* target site in the CDS (2511 bp) by PCR generated two overlapping partial PCR segments (CDS1 and CDS2 DNA patches), each flanked by built-in restriction sites. Likewise, the same flanking nucleotides were added to the other parts to generate overlapping PCR fragments. Primer pairs and PCR amplification conditions used for sequence domestication of all *SlHB15* fragmentss are reported in Table S2. The gene construct containing the CDS of $SlHB15^{WT}$ was used as a template for generating the two domesticated CDS1 and CDS2 while the genomic DNA extracted from a WT plant was used for the amplification of the gene promoter, 5'UTR and 3'UTR regions. PCR reactions were performed in a 50 µl of total volume, containing 5 µl of ten-fold diluted cDNA or DNA, 0.4 µM of each primer, 200 µM of dNTPs, 1X Phusion High Fidelity Reaction Buffer, and 2.5 U of Phusion High Fidelity DNA Polymerase (New England Biolabs). The amplification was conducted with an initial step of 2 min at 95°C that was followed by 36 cycles of 30 s at 95°C, 30 s at a temperature according to the mean Ta of primer pairs (Table S2) and 3 min at 72°C, plus 7 min of final extension at 72°C.

GB restriction-ligation assembly reactions

With the exception of the promoter, PCR amplified products (CDS1, CDS2 and 3'UTR) were amplified, analyzed by agarose 1% (w/v) gel electrophoresis, purified using the QIAquick PCR Purification Kit (Qiagen) and quantified in the Nano Drop Spectrophotometer 2000. Then, 40 ng of each amplicon and 75 ng of the domestication vector (*pUPD2*) were mixed and incubated in a restriction-ligation assembly reaction by using *Bsm*BI, as restriction enzyme (New England Biolabs) and T4 Ligase (Promega). Reactions were set up in 25- or 50-cycle digestion/ligation reactions (2 min at 37°C, 5 min at 16°C), depending on assembly complexity. One microliter of the reaction was used to transform *E. coli* electrocompetent cells, and positive clones were selected in solid medium. Plasmid DNA was extracted using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). Plasmid DNA concentration was measured using a Nano Drop Spectrophotometer 2000 (Thermo Scientific, Rockford, USA). Construct assemblies were confirmed by restriction analysis. The resulting *pUPD2::CDS1* and *pUPD2::CDS2* clones were modularly combined in the *pDGB3alpha1* destination vector, this time using the cloning restriction enzyme *Bsa*I (New

England Biolabs) in order to reconstitute the domesticated whole CDS of *SlHB15*. This time 75 ng of the destination vector were mixed with 75 ng of *pUPD2::CDS1* and 75 ng of *pUPD2::CDS2* and incubated in a restriction-ligation assembly reaction**.** Subsequently, *pDGB3alpha1::CDS* ligation product was cloned in *E. coli* elettrocompetent cells.

Attempts to amplify the promoter sequence did not get the expected result even by adopting different amplification protocols and different brands of High Fidelity Polymerases. Alternatively, combination of a new 5' primer (*GBHB15PRO_11*) designed on an inner region of the promoter in combination with primer *GBHB15PRO_10* allowed to retrieve a faint amplicon of expected length with a high aspecific background but changing stringency parameters was unfruitful. To overcome this problem we decided to order a synthetic domesticated promoter sequence through the gBlock DNA Synthesis Service (provided by IDT Integrated DNA Technology, London, UK).

Construction of a CRISPR/Cas9 plasmid for *SlHB15*

The GB CRISPR assembler (http://goldenbraid.com) allowed to domesticate and combine a target guide RNA (gRNA) sequence with the Arabidopsis U6 promoter (GB1204) and the short guide RNA (crRNA + tracrRNA; GB0645), already cloned in pUPD2 vector (GB1204 and GB0645, respectively), in order to generate a transcriptional unit (TU) to be combined into a multipartite assembly with the hCas9 TU (GB0000). A gRNA (*gRNA18*) was selected among the 96 possible gRNAs generated through the optimized CRISPR-plant design tool of the CRISPR-P platform (http://cbi.hzau.edu.cn/crispr/; Fig. 4.2) once the *SlHB15* genomic sequence (6935 bp) was subjected to *in silico* analysis. The selection criterion took into

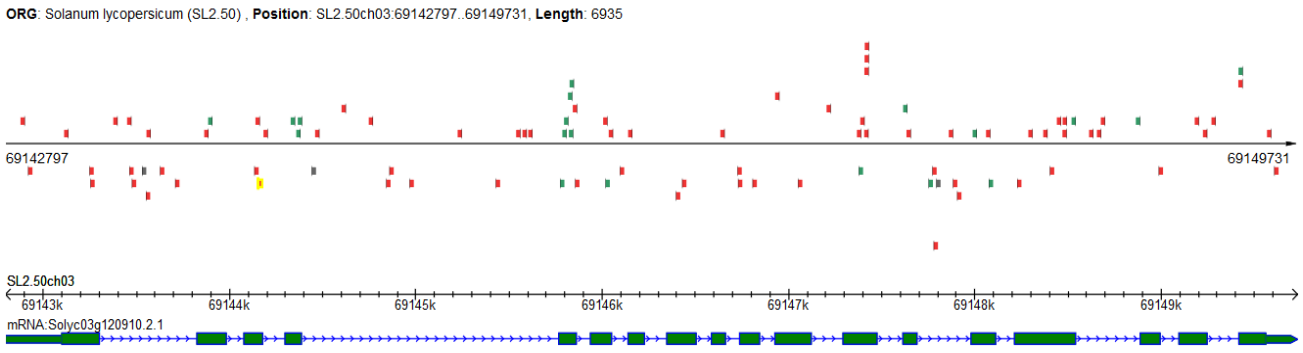


Figure 4.2 - Graphical representation of the 96 gRNA sites, with highest (in red) or medium to low(in green) on-target activity retrived along the *SlHB15* genomic sequence through CRISPR-P database. The selected gRNA (highlighted in yellow) falls in the third exon of the gene (graphically represented with exons depicted as green boxes and introns as blu arrows, below).

account the position of the gRNA in the sequence (the most proximal) and the potential highest on-target and lowest off-target activities of the gRNA. The domesticated 20 bp double strand sequence homologous to gRNA18 was generated by mixing 5 µl of both 1 µM domesticated primer *CRI-HB15-1* (5'-ATTGCAGGACTAGCATCCCTCGG-3') and *CRI-HB15-2* (5'-AAACCCGAGGGATGCTAGTCCTG-3') and letting them anneal for 30 min at room temperature. Two ng of the target sequence were added to 75 ng of GB1204, 75 ng of GB0645, 75 ng of *pDGB3alpha1* vector, 10 U of *BsaI* (New England Biolabs), 3 µl of T4 ligase (Promega), 1 µl 10 x ligase buffer, in a final volume of 10 µl. The reaction was set in a thermocycler with 25 cycles of 2 min at 37°C and 5 min at 16°C. One µl of the reaction was introduced into *E. coli* electrocompetent cells, and positive clones were selected in solid medium. Plasmid DNA was extracted using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). Plasmid DNA concentration was measured using a Nano Drop Spectrophotometer 2000. The CRISPR TU generated (*CRI-HB15*) was subsequently combined with the TU coding for the human codon optimized *hCas9* gene (GB0639, already cloned in *pDGB3alpha2*) into the *pDGB3omega2* vector. With a last cloning step, the *CRI-HB15::hCas9* module was combined with the TU for kanamycin resistance (GB0226, cloned in *pDGB3omega1R*) back into the *pDGB3alpha1* vector. All construct assemblies were confirmed by restriction analysis. Before continuing the cloning procedure, *CRI-HB15* TU was submitted to sequence quality check.

Strains and growth conditions

E. coli DH5α and Top1cells were used for the cloning procedure. *A. tumefaciens* strains GV3101 and LBA4404 were used for complementation and genome editing experiments, respectively. Both *E. coli* and *A. tumefaciens* strains were grown in LB medium under agitation (200 rpm) at 37°C and 28°C, respectively. Ampicillin (50 µg ml^{-1}), kanamycin (50 µg ml^{-1}) and spectinomycin (100 µg ml^{-1}) were used for *E. coli* selection. Rifampicin, tetracycline were also used for *A. tumefaciens* selection at 50 and 12.5 µg ml^{-1}, respectively. XGal (0.5 mM) and IPTG (40 µg ml^{-1}) were used in LB agar plates for the white/blue colony selection in the cloning process.

Generation of transgenic plants

So far, after sequence verification, only *pBI121::CaMV35S::SlHB15::NptII* and *pDGB3alpha1::CRI-HB15::NptII* constructs were used to transform *A. tumefaciens* competent cells. Positive colonies for both constructs were checked by PCR and used for plant transformation experiments. For both constructs, a single positive colony from LB agar plates kept at 4°C was picked and inoculated in 50 mL LB broth supplemented with 50 mg L^{-1} kanamycin and 10 mg L^{-1} rifampicin. The culture was placed overnight at 28°C with continuous shaking at 225 rpm in a shaker incubator. *Agrobacterium* culture was centrifuged and the pellet was resuspended in MS liquid medium (inoculation medium) containing 200 µM acetosyringone and density was set to OD_{600}=0.60. The cotyledons explants from 10-day-old aseptically-grown seedlings of the two-near-isogenic WT and *pat* lines were wounded carefully with a fine needle at multiple sites and transferred to the Petri plates having inoculation medium. A total of 120 cotyledons per line were infected for 30 min with both *A. tumefaciens* strains harbouring *rolB* gene. Later, the explants were co-cultivated for 48 h on co-cultivation medium (CCM: MS medium supplemented with 2 mg L^{-1} zeatin +0.1 mg-L^{-1} IAA) in dark and then transferred to induction medium (IM: MS medium supplemented with 2 mg L^{-1} zeatin, 0.1 mg L^{-1} IAA, 250 mg L^{-1} cefotaxime, 250 mg L^{-1} ticarcillin, 100 mg L^{-1} kanamycin) after washing with half strength MS liquid medium supplemented with 500 mg-L^{-1} cefotaxime. The plates were kept in dark for one week at 25±2°C and finally transferred to growth room.

Figure 4.3. Two *in vitro* WT explants after transformation with the *CaMV35::SlHB15^{WT}* construct grown on rooting medium (left) and other nineteen two-week-old explants rooted in potting soil (right).

The explants with emerging shoot primordia (Fig. 4.3) were carefully shifted to shoot elongation medium (EM: MS medium supplemented with 0.1 mg-L^{-1} zeatin, 0.1 mg L^{-1} IAA, 250 mg L^{-1} cefotaxime, 250 mg L^{-1} ticarcillin, 100 mg L^{-1} kanamycin). Elongated shoots (2-3 cm lenght) were cultured on rooting medium (RM: MS medium supplemented with 0.05 mg L^{-1} IBA and 100 mg L^{-1} kanamycin) for root development. Rooted shoots transformed with *CaMV35S::SlHB15^{WT}* were acclimatized in six-well Jiffy-pots containing peat and subsequently transferred to pots with potting soil under controlled growth conditions.

Plant material and phenotypic analysis

Seeds of the two NILs for the *Pat* locus were available in the tomato seed collection at the University of Tuscia (Viterbo, Italy). In spring 2015, eight *in vitro* germinated seedlings of both WT (cv Chico III) and *pat* lines and 24 *pat* and 49 WT explants, regenerating from the transformation experiments (T_0 generation) and rooted *in* MS medium supplied with 50 µg/ml kanamycin as a selective agent, were transplanted into pots and grown in controlled conditions. In 2016, a progeny of 52 T_1plants, derived from one of the two T_0 WT plants which exhibited a cosuppression phenotype (T_0-CO-1), and eight plants of both NILs were grown in the same conditions. The same procedure was adopted in 2017 with a progeny of 28 individuals (T_2 generation) from one of the T_1 plants grown in 2016 and showing the strongest cosuppression phenotype. At flowering, alterations of stamen morphology (short stamens and/or anthers with homeotic modifications), ovary weight and ovule aberrations were determined in four flowers per plant collected on the two NILs and on each of the T_0 and T_1 WT and *pat* plants at anthesis (stage 4) from the 2nd to 4th inflorescence as described in Mazzucato *et al*. (2008). Manual pollination of flowers of the T_0 and T_1 *pat* and putatively cosuppressed WT plants, before anthesis (stage 3), ensured seed production.

To assess pollen fertility, flowers collected at full anthesis were used. Two anthers per flower from four flowers of WT, *pat* and T_0 CO-1 plants were dissected, squashed on a microscope slide and the released pollen grains stained in a drop of 1% acetic orcein and 50% glacial acetic acid solution. At maturity, the number of fruits per plant, mean fruit weight and number of seeds per fruit (with the exception of fruits derived from the hand pollinated flowers) were scored in the T_1 progeny.

Plant DNA extraction and PCR amplification for selection of T_0 and T_1 transformed plants

Genomic DNA was extracted from leaflets of all the T_0, and T_1 plants by the CTAB method (Doyle and Doyle, 1987) and checked by PCR, for the stable integration of the *CaMV35S::SlHB^{WT}* construct, with *HB15-9* and *HB15-10* primers and with primers amplifying a portion of the *NptII* gene (Table S2). Amplification was conducted with an initial step of 2 min at 95°C that was followed by 36 cycles of 30 s at 95°C, 30 s at a temperature according to the mean Ta of primer pairs (Table S2) and 3 min at 72°C, plus 7 min of final extension at 72°C. Amplicons were run by 1.2 % agarose-gel electrophoresis with a proper (100bp) ladder marker.

RNA isolation, cDNA synthesis and real-time PCR (qRT-PCR) analysis

Apical young leaves and a bulk of flower buds at stage 1 and 2 (corresponding to 6-9 and 9-12 mm long flower buds, respectively 5 and 2 Days Before Anthesis, DBA; Mazzucato *et al*., 1998) from single plants of the two NILs and T_0 and T_1 transformed plants were used to form a biological replicate at two tissue level. Total RNA was extracted from 100 mg of tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA yield and purity was assessed by determination of absorbance (Abs) at 260 and 280 nm using the Agilent RNA 6000 Nano Kit with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA). RNA integrity was checked by 1% (w/v) agarose gel electrophoresis and RNA was only used when the ratio Abs260/Abs280 was higher than 1.8. Three biological replicates were used for each NIL, genotype and stage. cDNA samples were synthesized from 3 µg of three independent RNA preparations using the SuperScript II RNase-H Reverse Transcription Kit (Invitrogen, Carslbad, CA, USA) following the manifacturer's protocol. Ten-fold dilutions of the resulting first strand cDNA were used for qRT-PCR *SlHB15* expression analysis. Quantitative assays were performed in three technical replicates with SSO ADV UNIVERSAL SYBR GREEN Master Mix (Bio-Rad, USA) according to the manufacturer's instruction in the Bio-Rad CFX96 Manager system (Bio-Rad, USA), according to the manufacturer's instructions. Primers used to analyze the expression pattern of *SlHB15* by qRT-PCR are reported in Table S2. To evaluate the gene expression level, results were normalized using the house-keeping (HK) *CAC* gene (*Solyc08g006960*) recently found to provide excellent transcript normalization in tomato

developmental studies (Expósito-Rodríguez *et al*., 2008). Relative expression for each gene in each sample was calculated with the single DCt method as follow: $2^{[(\text{Ct value of target gene})-(\text{Ct value of HK gene})]}$. The statistical significance within stage was assessed by Student's *t* test using Microsoft Excel 2011.

Detection of CRISPR/Cas9-induced gene editing

To detect mutations in the gRNA18-Cas9 targeted *SlHB15* locus, genomic DNA was isolated from the first seven transformed shoots regenerated from calli grown on selective medium. To evaluate mutations introduced in the CRISPR/Cas9 transgenic plants, a region of about 800 bp surrounding the gRNA18-Cas9 targeted locus was amplified by PCR using the following specific primer pairs: 5'-ACAGAGGAAAGAGGCGTCAA-3' (*CRI-HB15-3),* and 5'-CTGCCTGACGATGCAAACTA-3' (*CRI-HB15-4)*. To detect on-target CRISPR/Cas9 events in the shoots, the PCR products were denatured and reannealed to allow heteroduplex formation between wild-type DNA and CRISPR/Cas9-mutated DNA. Two µl l 10 x NE Buffer 2 were added to 200 ng of purified PCR product and dH_2O to a total of 19 µl l in a PCR tube. Samples were run in a reannealing reaction in a PCR thermal cycler (BioRad T100): 5min, 95°C; ramp down to 85°C at -2C/s; ramp down to 25°C at -0.1C/s; hold at 4°C. T7E1 endonuclease (New England Biolabs), which recognizes and cleaves mismatched DNA, was used to digest heteroduplexes DNA that results from annealing DNA stands that have been modified after a gRNA/Cas9 mediated cut to DNA strands. One µl l (10U) of T7E1 was added to each sample and incubation was performed at 37°C for 15 min. The reaction was stopped by heating samples at 65°C for 15 min. After digestion, the samples were immediately loaded on a 1.5% agarose gel in order to put in evidence the resulting cleaved and full-length PCR products. To confirm CRISPR-Cas9-induced editing in the target region, the reannealed amplicons that showed a cleaved mismatched by E7T1 assay were analyzed in detail by deep sequencing. The sequences were analyzed by TIDE (https://tide.nki.nl), a web software which quantifies the genome editing efficacy and identifies the predominant types of insertions and deletions (indels) in the DNA sequence traces of a CRISPR-targeted plant genome locus. Similarly, for detection of gRNA18 off-target sites, one selected candidate sequence with the highest risk of off-target effects from gRNA18 (*Solyc02g074040*) was PCR amplified with a pair of primers (*CRI-HB15-5*: 5'-GGGGAAGGTATGAGCGTGAT-3' and CRI-HB15-6: 5'-GCCAACCAGTTCTAGCAAGC-3') by using athe s to generate a fragment of about 600 bp and subjected to E7T1 assay with the

same procedure as reported above. Amplification with both CRI-HB15-3/4 and CRI-HB15-5/6 primer pairs were conducted with an initial step of 2 min at 95°C that was followed by 36 cycles of 30 s at 95°C, 30 s at a temperature according to the mean Ta of primer pairs (Table S2) and 3 min at 72°C, plus 7 min of final extension at 72°C. Amplicons were analyzed by agarose 1.2 % (w/v) gel electrophoresis with a proper (100bp) ladder marker.

Molecular analysis for sequence comparison of the genes in the *Pat* window between the two NILs

For DNA sequence comparison between and the WT and *pat* NILs, pairs of primers (Table S2) were used to amplify overlapping regions of *Solyc03g120900, Solyc03g120920, Solyc03g120940* and *Solyc03g120950* genes, residing in the genomic window containing *Pat*. PCR was performed in a 20-µl of total volume, containing 75 ng of genomic DNA, 10 µM of each primer, 200 µM of dNTPs, 1× DreamTaq buffer and 1 U of DreamTaq polymerase (Fermentas, Ontario, Canada). The PCR reaction was conducted with an initial denaturation of 4 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at a temperature according to that of the primer pairs used (Table S2), extension for 2 min at 72 °C, plus 7 min of final extension at 72 °C. PCR amplified products were analyzed by agarose 1% (w/v) gel electrophoresis, purified using the QIAquick PCR Purification Kit (Qiagen) and quantified in the Nano Drop Spectrophotometer 2000. Aliquots of the purified amplicons were used for Sanger sequencing.

In silico analysis of the promoters of class *III HD-Zip* family members

To further characterize the candidate gene, its promoter sequence (2300 bp) was analyzed through a predictive software (http://www.softberry.com) order to retrive putative functional cis motifs. The analysis was also carried out over the other five members of the *HD-Zip III* family.

Results and Discussion

Initially, the *Pat* locus was mapped into a 1.2 cM interval on the long arm of chromosome 3 (Beraldi *et al*., 2004). Fine mapping and translating genetic into physical distances, the

Pat window was reduced to an interval of 0.19 cM, spanning a physical distance of about 103 Kb (Selleri, 2010). Among the five genes already sequenced, *Solyc03g120910* was the only gene containing a point mutation in the coding sequence, corresponding to the 15th exon (Selleri, 2010; Table 4.2). To validate *Solyc03g120910* as the candidate gene for *pat*, the DNA coding sequences of the remaining four genes contained in the *pat* genomic interval were analyzed. After sequencing, *Solyc03g120900* (SGN description: SEC13-like transport protein), *Solyc03g120920* (SGN description: protein of unknown function), *Solyc03g120940* (SGN description: protein of unknown function) and *Solyc03g120950* (SGN description: protein of unknown function), did not show any modification in their exonic regions upon comparison of the two NILs.

Table 4.1. Name and genomic characteristics of the nine genes contained in the *Pat* window and of the two genes harbouring the flanking markers(shaded in grey).

Gene code (SGN)	Gene description /Marker	Chr3 position (bp)	Gene lenght (bp)	Exon number	Cds lenght (bp)	Protein lenght (aa)
Solyc03g120880	Nucleoporin NUP53	63161622-63167492	6217	8	1855	502
Solyc03g120890	Transcription factor GATA9	63175833-63177054	1675	2	1506	350
Solyc03g120900	Protein transport SEC13-like protein	63180997-63181902	906	1	906	301
Solyc03g120910	Class III homeodomain-leucine zipper HB15	63196426-63202889	6464	18	2508	836
Solyc03g120920	Unknown protein	63203255-63203809	555	1	555	185
Solyc03g120930	Avr9/Cf-9 rapidly elicited protein 146	63216540-63217007	468	1	468	156
Solyc03g120940	Unknown protein (Os06g0483900 protein)	63233088-63237343	4628	9	1287	305
Solyc03g120950	Unknown protein	63241427-63242581	1408	2	646	131
Solyc03g120960	STIG1	63243987-63244418	432	1	432	144
Solyc03g120970	Gibberellin 2-beta-dioxygenase	63244710-63246761	2052	3	1140	380
Solyc03g120980	ATP-binding cassette transporter ABC-2 type transporter	63258221-63266837	9149	21	1501	5035

Functional characterization of the candidate gene: transgenic approaches

To confirm *SlHB15* as the underlying gene, the *CaMV35S::SlHB15^{WT}* construct was used to 1) perform a complementation assay on the *pat* line, 2) transform the WT line for

inducing constitutive overexpression and/or cosuppression of the transgene. Moreover, *SlHB15* gene editing through the CRISPR-Cas9 system in the WT line was performed.

Complementation of the *pat* mutant line with the *SlHB15^{WT}* allele

Only the construct driven by the constitutive promoter, *CaMV35S::SlHB15^{WT}*, could be used for the complementation assay. (Fig. 4.1). About 120 cotyledonary explants of the *pat* mutant line were employed. In Table 4.3 are summarized the data on the number of cotyledonary explants used for the transformation experiments (complementation and overexpression/cosuppression) and number of regenerant phenotypes after the molecular check.

Table 4.2. Number of *pat* and WT cotyledonary explants (CE) used for the complementation and overexpression/cosuppression experiments, respectively and indication of the T_0 transformants (TR) and false positives (FP) for the transgene after the molecular check.

T_0 explants	pat	WT
CE	120	120
TR	17	38
FP	3	7

Seventeen regenerated transgenic plants (pat4-pat25) of the *pat* mutant were analysed at the reproductive level in comparison with plants of both NILs and three regenerated untransformed (false positive) plants (Fig. 4.4).

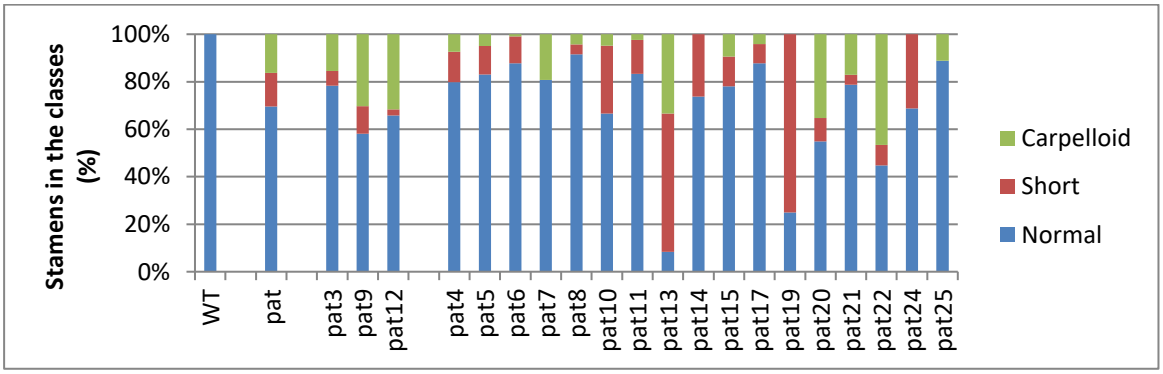


Figure 4.4. Frequency of normal (blue bars), short (red bars) and carpelloid (green bars) stamens in the original WT and *pat* lines, in three untransformed false positives (pat3, 9 and 12) and in 17 T_0 plants transformed with the *35S::SlHB15^{WT}* construct.

In particular, anthers of 4/5 flowers per plant at anthesis were classified as normal, short or carpelloid according to the type of alteration induced by the *pat* mutation. From the same flowers, ovary weight (Fig. 4.5) and the percentage of aberrant ovules (Fig. 4.6) were determined, too. Plants transformed with the construct showed a wide variety of aberrant stamen frequencies, ranging from values lower than the *pat* line, as in the case of pat6, 8, 17, 25), to values remarkably higher (e.g. pat13, 19 and 22; Fig. 4.4). WT plants showed no aberrancy of ovules while *pat* plants had on average a half of the ovules showing developmental defects (Fig. 4.5). Most of the transformed plants showed a frequency of aberrancy lower than the original mutant, with the exception of pat13 that had about 70% of aberrant ovules. Among the T_0 plants having a low frequency of aberrant stamens (Fig. 4.4), pat8 and pat17 also showed a very low frequency of aberrant ovules (Fig. 4.5).

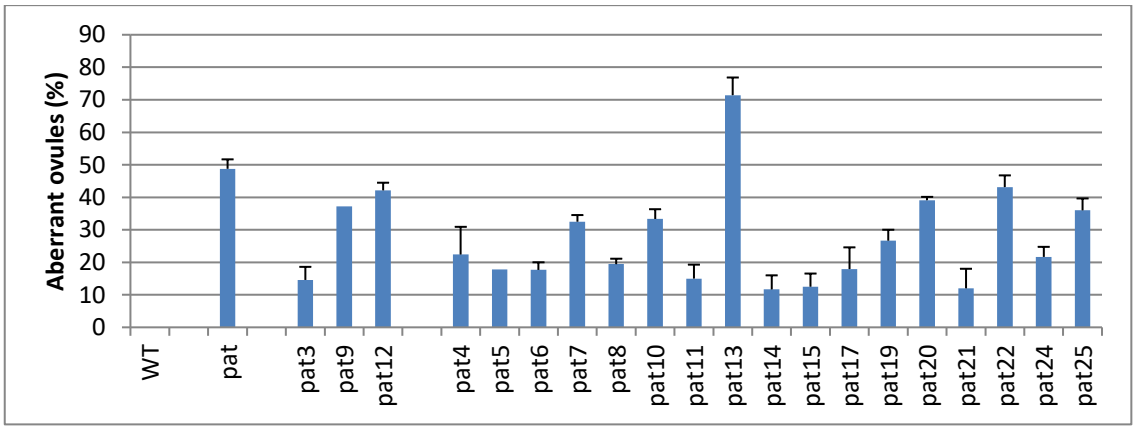


Figure 4.5. Frequency of aberrant ovules in the original WT and *pat* lines, three untransformed false positives (pat3, 9 and 12) and 17 T_0 plants transformed with the *35S::SlHB15^{WT}* construct.

Ovary weight at anthesis was finally evaluated as a direct evidence of parthenocarpic expression in the studied material. In our conditions, ovaries at anthesis weighted about 1 g more in the *pat* than in the WT line and untrasformed plants showed somehow higher values (Fig. 6). The T_0 plants showed high variation of this trait, many times accompanied by a large standard error. Of the plants mentioned before for a lower expressivity of the *pat* phenotype in stamens and ovules, only pat17 showed a significantly lower ovary weight when compared to both the original WT and *pat* and regenerated plants.

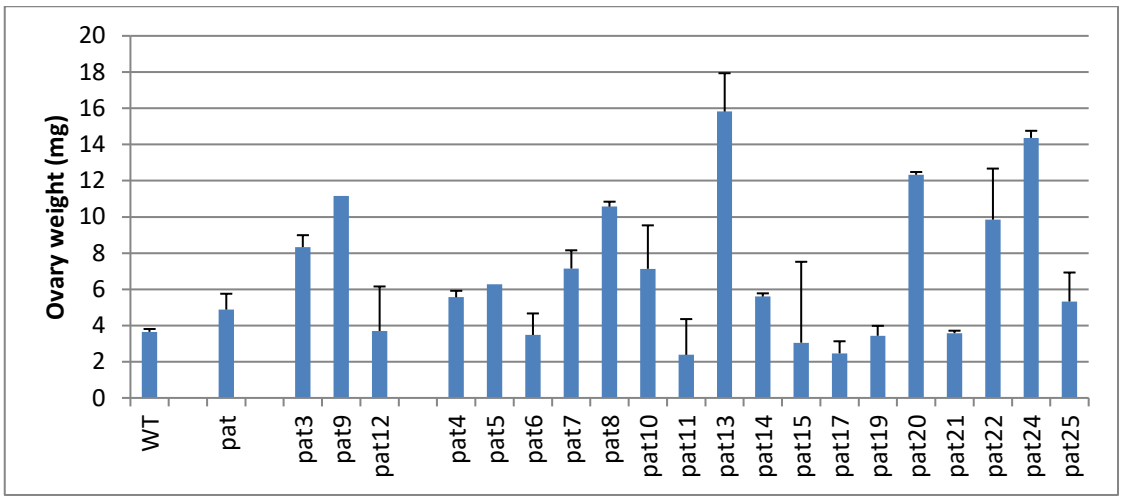


Figure 4.6. Ovary weight at anthesis in the original WT and *pat* lines, in three untransformed false positives (pat3, 9 and 12) and in 17 T_0 plants transformed with the *35S::SlHB15^{WT}* complementation construct.

Only few transformants showed a consistent decrease in expression of the three phenotypes considered. When these plants where checked by qPCR for the expression of the construct in floral organs there was no evidence that the gene was overexpressed in plant tissues (data not shown). In summary, the analysis of 17 T_0 plants transformed with the *35S::SlHB15^{WT}* complementation construct did not give a clear evidence that the mutant phenotype could be complemented by the construct. This consideration prompted us to pursue a new strategy for complementing the mutation with the *SlHB15^{WT}* allele, using the its endogenous regulatory elements (promoter and UTR regions), in order to express the gene in its own specific domains.

Putative cosuppression phenotypes of *SlHB15* in the WT line

Out of 45 WT plants regenerated after transformation (T_0 generation) with the the *CaMV35S::SlHB15^{WT}* construct, 38 were positive to the molecular check for the presence of the transgene (Fig. 4.7). Most of the plants did not display any evident phenotypic variation and resembled untransformed WT plants in vegetative (not shown) and reproductive traits. One plant showed evident vegetative defects, i.e. abnormal development, including reduced growth and abnormal phyllotaxis, but showed normal flower traits (data not shown). Two further plants (hereafter referred to as CO-1 and CO-2) showed evident reproductive defects. CO-1 and C0-2 closely paralleled the *pat* mutant for floral aberrations represented by aberrant anthers and ovules, partial pollen viability

(in terms of greater presence of morphologically irregular and less stainable tetrads) and increased ovary size at anthesis (Fig. 4.8).

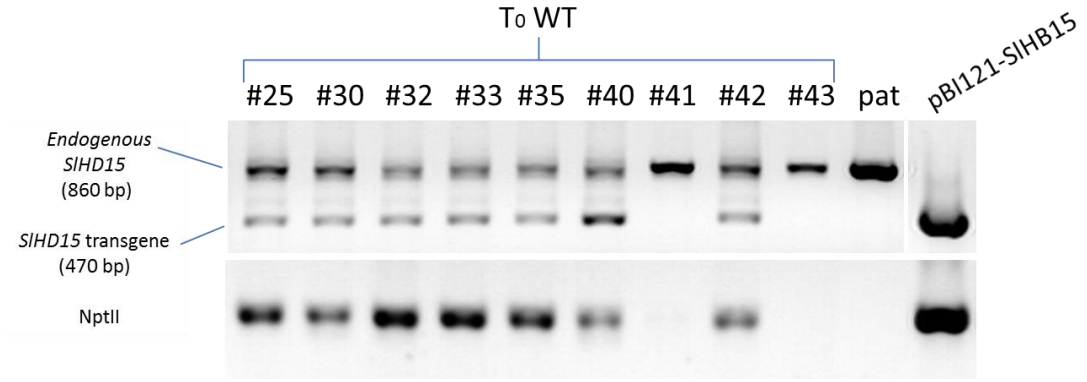


Figure 4.7. PCR-based check of nine primary trasformants of the WT line by using a pair of primers (*HB15CDS_9/10*) designed on two adiacent exons of *SlHB15*, generating a genomic fragment longer than that of the coding sequence contained in the *CaMV35S::SlHB15*^{WT} transgene. For confirmation, a second pair of primers (*NptII_1*: 5'- GAGGCTATTCGGCTATGACT-3' and *NptII_2:* 5'-ATCGGGAGCGGCGATACCGT-3') was used to amplify a fragment of the *Neomycin phosphotransfarase II* gene.

The expressivity of the parthencarpic phenotype estimated as the number of seeds per fruit could not be clearly determined in CO-1 and CO-2 plants as fruit yield was scarse though fruits had a relatively low number of seeds. Only hand-pollinated flowers of CO-1 produced very few fruits with an adequate number of seeds that were used for obtaining the T_1 progeny.

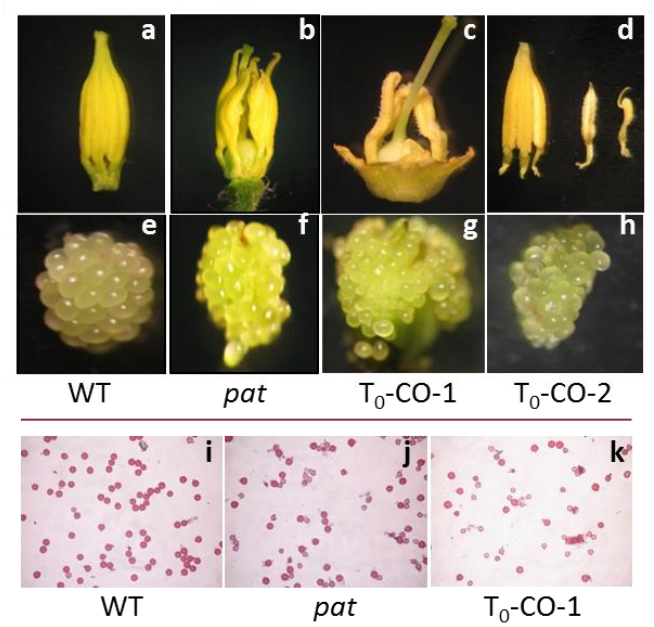


Figure 4.8. Flower phenotypes of a) WT anther in comparison with *pat* (b) and two T_0 WT transgenic plants (CO-1 and CO-2), showing a putatively cosuppression phenotype (c and d). Dissected pieces of placenta exposing ovules are from the same flowers as above (e, f, g, h). Pollen viabilityin WT (i), *pat* (j) and CO-1 (k) anthers.

The progeny of CO-1, consisting of 28 T_1 plants, was grown and phenotyped for the same traits analysed in the T_0 generation, including the presence of supernumerary cotyledons, fruit set (expressed as number of ripe fruits per plant), mean fruit weight and mean number of seeds per fruit. Also in this case all the plants were checked for the presence of the transgene. Several polycotyledonary seedlings were observed, at a frequency higher than that found in the *pat* mutant. In particular, 38.8% of T_1 seedlings showed bifid, di-, tri- or tetracotyledons (Fig. 4.9) while only a tricot phenotype (25%) was observed among *pat* plants.

Figure 4.9. Different cotyledon number (dicot, a; tricot, b; tetracot, c; found in T_1 CO-1 seedlings segregating the *CaMV35S::SlHB15*^{WT} transgene.

Eight out of 28 plants were nullisegregant (Mendelian segregation, χ^2 0.19, $P \leq 0.05$) and showed a WT phenotype while 17 out of 20 transgenic plants showed alterations at the anther and ovule level, remarkably comparable to the *pat* mutant (Fig. 4.10). Moreover, ovary weight at anthesis paralleled the situation observed in the T_0 generation (Fig. 4.10). The increase of fruit set (measured as the number of fruits per plant), another pleiotropic trait that distinguishes *pat* from its WT NIL, was slight in the 17 T_1 plants if compared to the yield of the nullisegregants but such a difference was not statistically significant (Fig. 4.10). However, even in the case of the two NILs such a difference was not appreciable at least in the specific environmental conditions. The mean fruit weight at maturity and the number of seeds per fruit of the T_1 plants showing the other *pat*-like phenotypes were lower than those observed in the WT line and in the T_1 nullisegregants, although they were significantly higher if compared to the *pat* mutant (Fig. 4.10).

In order to associate the alterations of the phenotypic traits observed to a *SlHB15* cosuprpression, the expression level of the gene was assessed by qRT-PCR at the young leaf and flower bud stages. In agreement with the expression pattern observed in the two NILs, the gene was expressed in the WT line and T_1 nullisegregants, whereas it resulted downregulated in *pat*, in the T_0 cosuppressed plants CO-1 and CO-2 and in four T_1 plants of the CO-1 progeny (Fig. 4.11). The expression profile analyzed at young leaf

and flower bud level, showed the same pattern in both tissues and was significantly higher only in the WT flower bud.

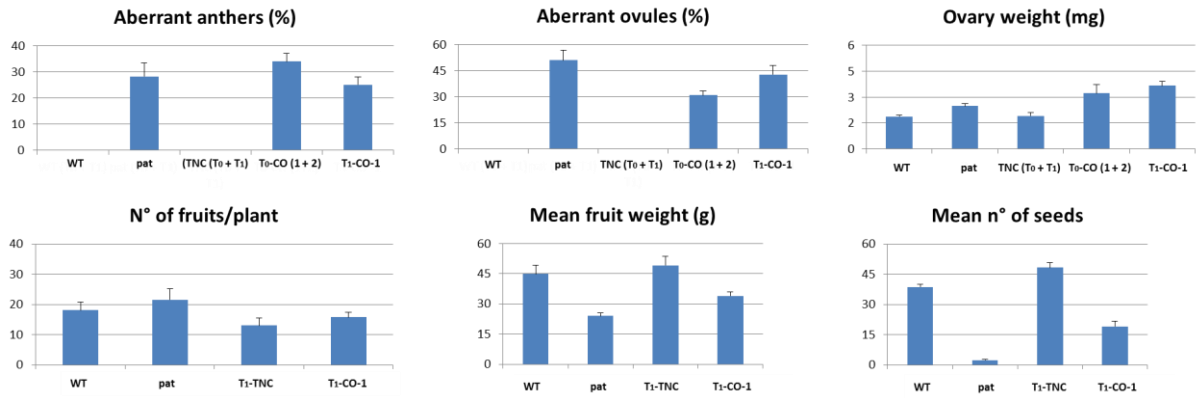


Figure 4.10. Reproductive phenotypes relative to percentage of aberrant anthers (a), ovules (b) and to ovary weight (c) of WT and *pat* plants (mean value of four plants each) in comparison with T_0 and T_1 plants from the trasformation of the WT line with the CaMV*35S::SlHB15*^{WT} construct: [TNC(T_0+T_1)], mean value of 38 T_0 and three T_1 plants with WT phenotypes; [T_0-CO(1+2)], mean value of two T_0 plants (CO-1 and CO-2); (T_1-CO-1), mean value of seventeen T_1 plants with *pat*-like phenotypes derived plant CO-1. Number of fruits per plant (d), mean fruit weight (e) and mean number of seeds per fruit (f) are referred to WT and *pat* plants in comparison with a mean value of three T_1 plants with WT phenotype.T_1 (T_1-TNC) a mean value of eight T_1 plants with pat-like phenotype (T_1-CO-1).

From the results obtained, it is possible to advance that appearance of alteration at the reproductive level by gene cosuppression event results in a lower expressivity of the typical phenotypic trait of the *pat* mutant (i.e. a quantitatively lower level of fruit seedlessness) accompanied with the manifestation of other main pleiotropic phenotypes of the *pat* mutant.

Together with cotyledonary alteration and severe homeotic malformations of stamens and ovules, *SlHB15* silencing by co-suppression indicates a role of this TF in both stamen and ovule identity and carpel development. To our knowledge, lack of evidence of a clear *SlHB15* complementation in the *pat* mutant might be attributed to a matter of reduced strenght of the CaMV35S promoter activity that restricts gene transcription. Also, we did not observe any phenotype that could be interpreted as a consequence of *SlHB15* overexpression according to what has recently been documented in tomato by Jia *et al.* (2015), though they reported overexpression phenotypes following constitutive antisense silencing of microRNAs *miRNA165/166* leading to overexpression of all the *HD-Zip III* members.

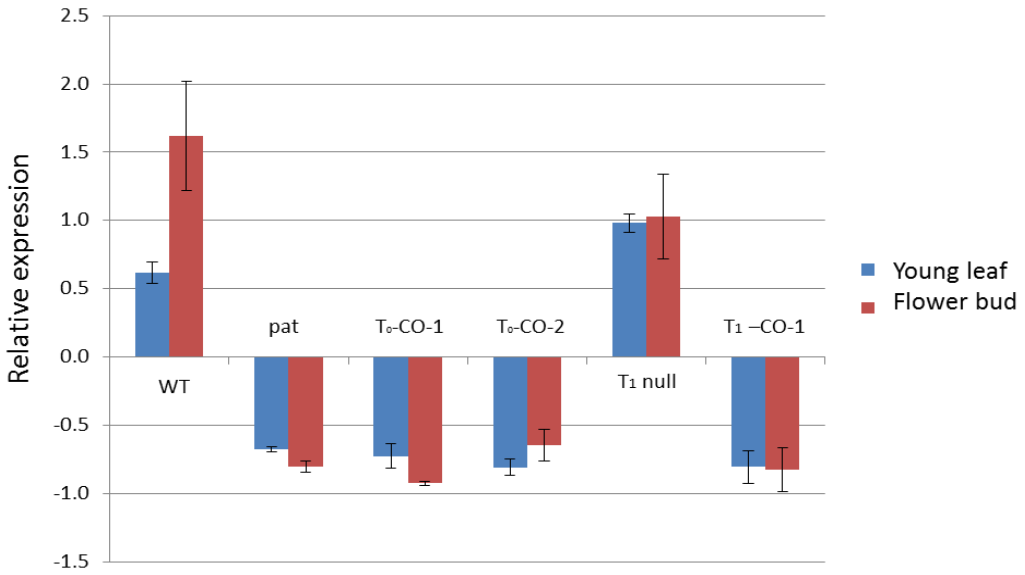


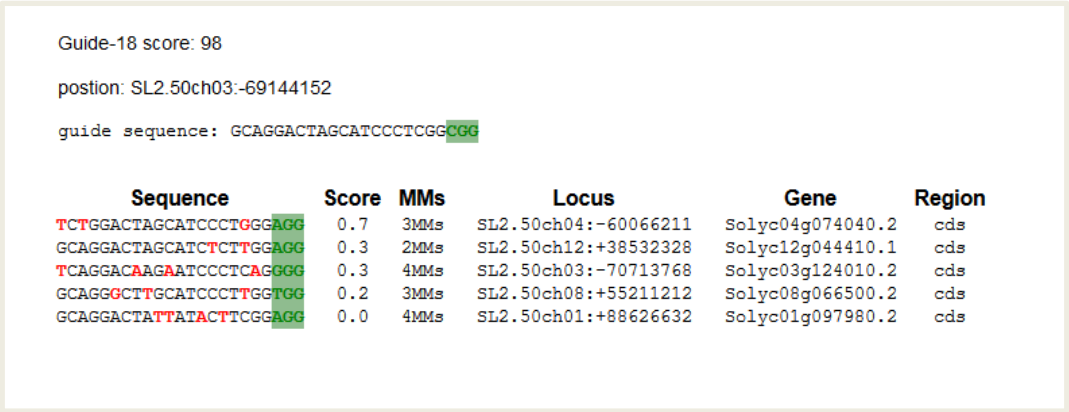
Figure 4.11. Relative expression level of *SlHB15* in vegetative tissues (blue bars) and in flower buds (red bars) in WT, *pat*, two T_0 plants (T_0-CO-1 and T_0-CO-2),T_1 nullisegregants (T_1 null), and T_1 plants (T_1-CO-1) with a *pat*-like phenotype. With the exception of both T_0 CO-1 and CO-2, mean values refer to three biological replicates for WT, *pat* and T_1 null and to four replicates in the case of T_1-CO-1.

Moreover, there is evidence that a strong transgene promoter is required for high frequency cosuppression of genes and for production of the full range of cosuppression phenotypes as in the case of Petunia *chalcone synthase* gene (Que *et al*., 1997). In most cases of gene silencing in plants, it was found that multiple copies of the transgenes were integrated into the genome (Flavell, 1994; Matzke *et al*., 1994). By the way, if only a single copy of a transgene is highly expressed it can cause gene silencing. It has been reported that single insertion of a chalcone synthase transgene driven by the CaMV35S promoter with a double enhancer triggered gene silencing, whereas the same gene driven by a less active promoter did not (De Carvalho *et al*., 1992). Further, in tomato Han *et al*. (2004) found that a threshold level of endogenous *Polygalacturonase* (*PG*) mRNA was required for the co-suppression of a truncated form of *PG* transgene and the endogenous *PG* gene or for extensive silencing of the transgene. Accordingly, Jorgensen (1995) postulated that induction of cosuppressed state is transcript-threshold dependent. As an enhancer element, the upstream activating region (UAR) of the CaMV35S promoter may vary in copy number and this greatly influences the degree of cosuppression (Que *et al*., 1997). Transgenes containing 1X UAR CaMV35 generally produce cosuppression only when the transgene is integrated into the genome as an inverted repeat (Hobbs *et al*., 1993; Van Blokland *et al*., 1994), whereas nearly all examples of single-copy sense transgenes causing cosuppression involve transgenes driven by 2X UAR CaMV35S promoters

(Vaucheret *et al.,* 1995; Jorgensen *et al*., 1996). We are not acquainted with the notion of the number and/or position of the UAR in our CaMV35S promoter. In our case, the threshold level might have been adequate, at least for the cosuppression machinery, but in consequence of *SlHB15* knock-out a compensation mechanism through functionally redundant genes, as in the case of *HD-Zip III* members, could be achieved providing for a milder expression of the *pat* syndrome in the WT line. However, the expression level of the transgene might be not enough to endure a complementation of the endogenous mutated allele in the *pat* mutant. In any way, by considering a possible involvement of promoter efficiency, we believe that generating an *SlHB15* construct driven by itsown regulatory sequences (promoter, and UTR regions) would be a preferable option.

Generation of *SlHB15* variants through gRNA18-Cas9-guided mutagenesis

To further confirm that *SlHB15* is the gene underlying parthenocarpy in the *pat* mutant, CRISPR/Cas9 technology was exploited to knockout *SlHB15*. A selected gRNA, gRNA18, was designed to target the third exon of the gene in order to generate indel mutations able to suppress *SlHB15* gene functionality with the lowest off-target rate (Fig. 4.12). Molecular detection of Cas9-mediated editing in *SlHB15* and putative off-target site presence was performed.



Guide-18 score: 98

postion: SL2.50ch03:-69144152

guide sequence: GCAGGACTAGCATCCCTCGGCGG

Sequence	Score	MMs	Locus	Gene	Region
TCTGGACTAGCATCCCTGGGAGG	0.7	3MMs	SL2.50ch04:-60066211	Solyc04g074040.2	cds
GCAGGACTAGCATCTCTTGGAGG	0.3	2MMs	SL2.50ch12:+38532328	Solyc12g044410.1	cds
TCAGGACAAGAATCCCTCAGGGG	0.3	4MMs	SL2.50ch03:-70713768	Solyc03g124010.2	cds
GCAGGGCTTGCATCCCTTGGTGG	0.2	3MMs	SL2.50ch08:+55211212	Solyc08g066500.2	cds
GCAGGACTATTATACTTCGGAGG	0.0	4MMs	SL2.50ch01:+88626632	Solyc01g097980.2	cds

Figure 4.12. CRISPR-P program description window of the gRNA selected (gRNA18) from the 96 gRNAs mapped on the *SlHB15* gene. The on-target efficiency score (98), the position in the genome and sequence of the gRNA are indicated together with the essential parameters describing the top potential off-target sites (the sequence, off-target score, the number of mismatches present in each site and highlighted in red, the position of the sites in the genome and the corresponding gene where it resides).

For detection of Cas9-induced mutations in explants regenerated from putatively transformed calli of the WT line, genomic DNA was isolated and the targeted region in the *SlHB15* gene was amplified and subjected to E7T1 enzyme assay to determine the presence of SlHB15 heteroduplex. Out of six explants, each from one transformed callus, two explants (gRNA18-4 and gRNA18-6) contained heteroduplex DNA as the 880-bp target amplicon from these two explants was partially digested into two lower bands (495 bp each), indicative of T7E1 nuclease activity (Fig. 4.13a).

To further investigate on the nature of the two Cas9-induced mutations, amplicon deep sequences and analysis with Tide software allowed to put in evidence different types of mutations induced by the Cas9 nuclease upstream of the PAM sequence.

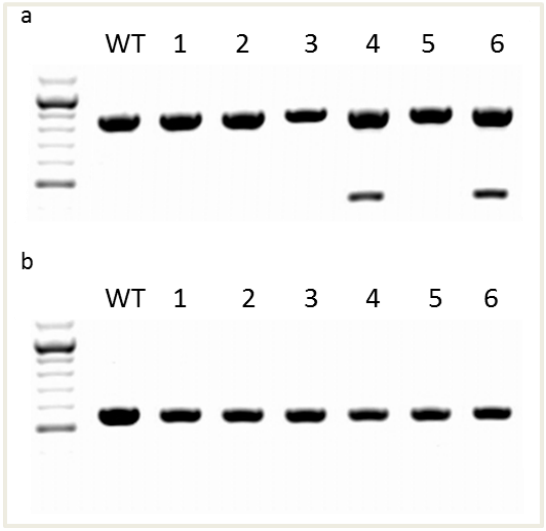


Figure 4.13. In a) gel electrophoresis of a PCR-amplified 880-bp DNA fragment containing the gRNA18 locus from a control (untrasformed) explant (WT) and six putatively CRISPR-Cas9-mutagenesis induced ones (1-6). Amplicons were treated with E7T1 assay for assessing potential heteroduplex formation following Cas9-induced indel formation. gRNA18-4 (4) and gRNA18-6 (6) explants show a lower band (440 bp) corresponding to the two fragments of equal size obtained by the digestion with E7T1 enzyme. In b) the same two reactions regarding a 550-bp DNA amplicon containing the potential off-target site with highest off-target score. The absence of lower extra bands formed after E7T1 assay was assumed as absence of off-target activity in this potential site.

In particular, according to the decomposition window of the amplicon for the six explants, a deletion of seven nucleotides and an insertion of a cytosine nucleotide were detected in explant gRNA18-4 and two different deletions (three an seven nucleotides) were detected in explant gRNA6 (Fig. 4.14).In both cases the WT allele was also present, thus indicating

that tissue of the two explants putatively is either polyploid or, more likely, chimeric. The deletion of seven nucleotides in both explants and the single nucleotide insertion in gRNA6 explant determine premature truncation of the protein sequence after 162 and 152 amino acids, respectively (data not shown).

A common issue for Cas9-based generation of mutants is the possibility of off-target cleavage of similar regions in the genome (Cho *et al*., 2014).

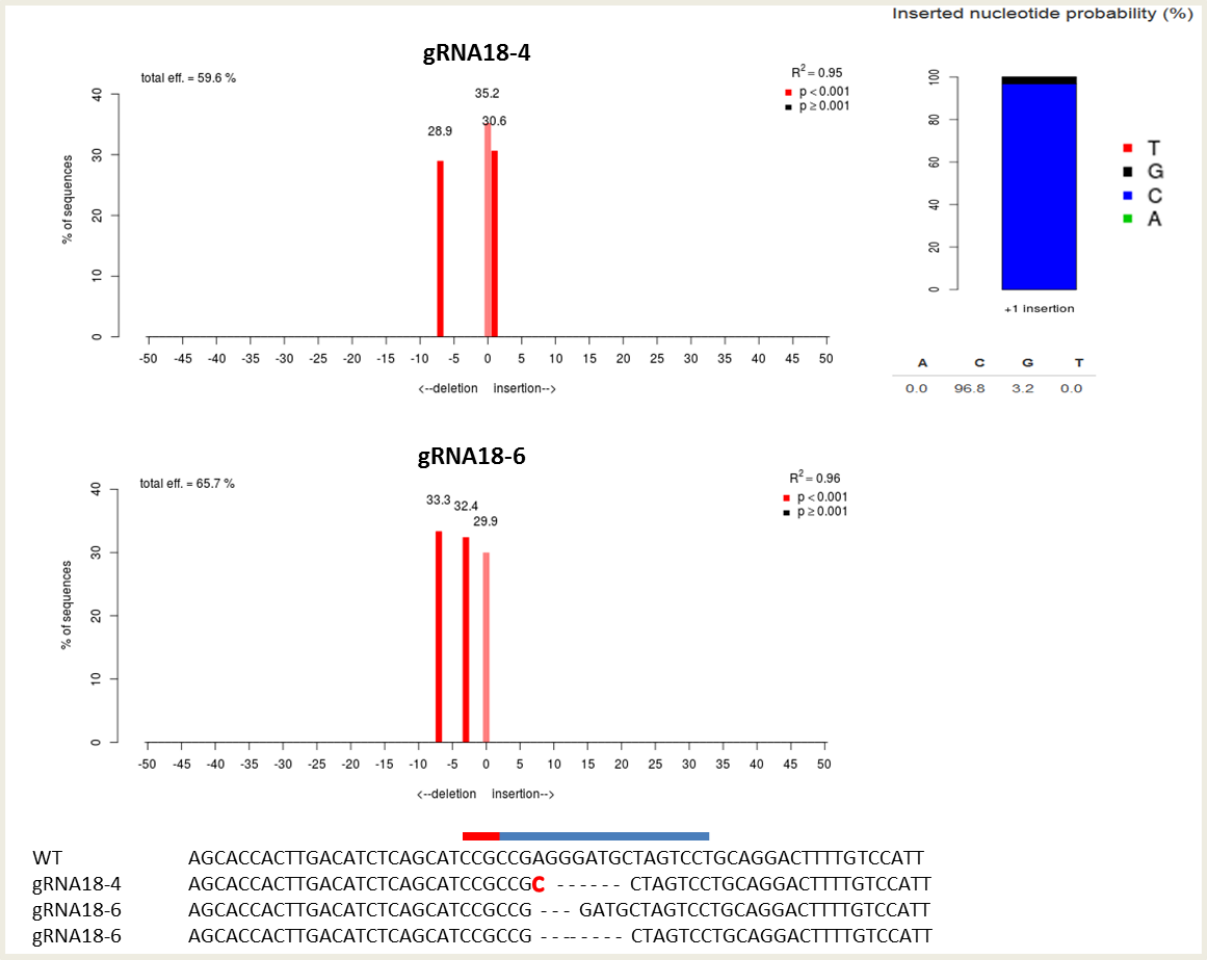


Figure 4.14. Decomposition window of TIDE software for identification and quantification of the predominant types of indels occurred in the DNA amplicons from gRNA18-4 and gRNA18-6 explants which turned positive to E7T1 assay. Dark red bars indicate the presence of indels in the sequence of either explants, light red bar positioned at zero on horizontal axis indicates the reference sequence. Statistical significance and total efficiency of base calling and quantification are given as R^2 and percentage value, respectively. Numbers on horizontal axis indicate the position of indels. Below, gRNA18 DNA sequence of a WT, gRNA18-4 and gRNA18-6 explants is indicated by a blue bar and PAM sequence is highlighted in red. An insertion of a cytosine (depicted in red) and one deletion of 7 nucleotides, indicated with dashes, are reported for gRNA18-4 and two deletions, of three and seven nucleotides, are given for gRNA18-6. On the top-right corner, a blue bar in another decomposition window indicates the type of one single insertion in gRNA18-4.

Amplification of the closest homologous sequence (*Solyc04g07040*) with the highset off-target score (0.7) and subsequent heteroduplex determination with E7T1 assay resulted in no putative off-target site detection as only the amplification product was observed by agorose gel electrophoresis (Fig. 4.13b).

Following these preliminary data, it is possible to affirm that the CRISPR-Cas9 technology was effective in causing indel modification in the target locus driven by the gRNA18 and that the editing was locus specific since off-target sites were not identified, at least in the locus with highest off-target activity.

Functional characterization of the candidate gene: in silico *SlHB15* promoter analysis

A motif or cis-element is the regulatory element (RE) found within the promoter region of any gene that controls the expression of that particular gene. The search of such motifs in the promoter region of *SlHB15* may help inferring the functional role and the regulation of the gene. In order to search for consensus patterns of regulatory sequences, 3000 bp upstream the 3'-UTR sequence of the gene, defined as the promoter of *SlHB15*, were subjected to the NSite program of the Softberry platform. The analysis was performed also with the promoter region of same length belonging to the other members of the *HD-Zip III* family. The *SlHB15* promoter sequence bears 18 REs, of which nine were shared with other members of the gene family and nine were specific of this gene (Table 4.3). In particular, four REs were found also in the promoter of *SlHB14* and five in that of *IFL1*. The functional role of the REs, was inferred from the biological process and possibly the tissue specificity of reference genes carrying these motifs or transcription factors exhibiting a binding affinity with these REs. In this context, two REs, GA-5 and GA-6 (Table 4.4) are reported to be directly involved in regulating the expression of *SEEDSTICK* (*STK*), a gene with pleiotropic roles in ovule identity, flower organ growth and development which is regulated by the TF BPC1 (Favaro *et al*., 2003; Rounsley *et al.*, 1995; Pinyopich *et al*., 2003; Brambilla *et al*., 2007, Table 4.4). Such a TF would regulate also *STM*, the Arabidopsis orthologue of *LeT6*/*TKn2*, conferring the meristem differentiation and development of floral organs (Simonini *et al.*, 2014). In Arabidopsis, a BPC family triple mutant deregulate**s** *STM* producing pleiotropic developmental defects regarding the flower, including short anthers (Simonini *et al.*, 2014), a phenotype peculiar to *pat*. Moreover, a role to BPCs in the fine regulation of

cytokinin content in the meristem was assigned (Simonini *et al*., 2014), as both *ISOPENTENYLTRANSFERASE 7* (*IPT7*) and *ARABIDOPSIS RESPONSE REGULATOR 7* (*ARR7*) genes were shown to be overexpressed in the *bpc1-2 bpc2 bpc3* triple mutant. An increase of the cytokinin biosynthetic pathway in the *pat* mutant was addressed at least for the hypocotyl (Chapter 3).

Table 4.3. Number of shared regolarory elements (RE) in the promoter of the six tomato HD-Zip III homeobox genes, numer of total motifs and percentage of private Res.

Gene	SlHB14	SlHB15	IFL1	SlHB8	SlHB15-LIKE	SlHB9	Total n° of RE	% of private RE
SlHB14	11	4	2	5	2	1	24	54
SlHB15	4	9	5	1	2	0	1	50
IFL1	2	5	9	1	1	0	17	47
SlHB8	6	1	1	7	1	1	13	46
SlHB15-LIKE	2	2	1	1	5	0	13	62
SlHB9	1	4	0	1	0	2	9	78

Another RE, found for instance in *ga2ox1*, is recognized by Knotted-like homeobox (KNOX) TFs (Table 4.4). It is well documented how *KNOX* genes regulate GA biosynthesis by both suppressing GA biosynthesis genes and inducing GA catabolic gene activity such as ga2ox1 (Hay *et al*., 2002). The KNOX-GA module has been postulated to operate also in tomato, indicating an involvement of the *KNOTTED-1 LIKE* orthologue, *LeT6/TKn2,* in the regulation of fruit set being repressed in the *pat* ovary at anthesis and releasing the activation of *SlGA20ox2* (Olimpieri *et al*., 2007; Ruiu *et al*., 2015). The same expression phenotype was observed for *LeT6* and its paralogue, *LeT12* after flower fertilization and auxin/GA_3 treatments coupled with a higher expression of *SlGA20ox2* (Tang *et al*., 2015). It is noteworthy how a number of REs, such as L3, VWRE and AC-II (Table 4.4), are present in genes sharing the vascular system as the dedicated tissue compartment where they are expressed. The L3 motif is the binding site of *DNA binding with one finger 11* (*Dof11*) in soyben (*Glycine max*) (Wang *et al*., 2007), a member of a group of plant-specific transcription factors acting on the formation and functioning of the vascular tissues. More than half of the members of this TF family are expressed in the vascular system (Le Hir, 2013). In Arabidopsis*, At5g60200*, the orthologus of *GmDof11*, is

specifically involved in leaf vasculature formation (Gardiner *et al*., 2010). In addition, *GmDof11*, was shown to enhance lipid content in the seeds of transgenic Arabidopsis plants (Wang *et al*., 2007).

The vascular system-specific and wound-responsive (VWRE) cis-element (Table 4.4) has been identified as a novel cis-element for wound-induced and vascular system-specific expression of the tobacco peroxidase gene, *tpoxN1* (Sasaki *et al*., 2006). The VWRE element is recognized by two AP2/ERF domain proteins (Sasaki *et al*., 2007).

Table 4.4. Cis-regulatory elements (REs) identified in 3 kbp of *SlHB15* promoter with indication of cis regulatory elements, position on the sequence, reference (source) gene which is regulated by the RE, biological/molecular process and main organ or tissue where the source gene is involved, bibliographic reference regarding the regulation of source gene by the RE.

N°	Cis regulatory element	Position in the 3000 bp DNA region upstream SlHB15	Source gene	Biological/molecular process	Main organ/tissue specificity	Reference
1	AT-RICH G2	-75	rbcS1	Photosyntesis	mesophyll, fruit locule	Meier et al., 1995
2	UN I2	-94	rbcS1	Photosyntesis	mesophyll, fruit locule	Meier et al., 1995
3	L3	-976	Synthetic oligonucleotides	Aux regulated expression of fatty acid synthesis in seeds	seed	Wang et al., 2007; Baumann et al., 1999
4	CG-box	-1033	ARF19 (AUXIN RESPONSIVE FACTOR 19)	Activation of lateral root initiation	root	Okushima et al., 2007
5	ABF3/ABF 4 BS2	-1510	ANAC055, NAC1	ABA and JA response	plant	Jiang et al., 2009
6	CA-box	-1597	SHY2/IAA3 (SUPPRESSOR OF HY2)	Regulation of aux homeostasis in root formation	lateral root	Goh et al., 2012
7	CG-box	-1608	NAC1/STF1	Aux signalling transduction to promote lateral root formation	root, cotyledon, leaf	Song et al., 2008
8	VWRE	-1901	tpoxN1 (peroxidase)	Vascular system specific expression	vascular tissue	Sasaki et al., 2006
9	tef-box	-1993	eEF1AA1	Component of the cell translation apparatus in root primordia	root	Tremousaygue et al., 1999
10	LRE (repressor)	-2075	psaDb (Ferredoxin-binding subunit of PSI)	Photosyntesis	mesophyll	Nakamura et al., 2009
11	AC-II	-2139	PAL2 (Phenilalanine ammonia-lyase)	Phenylpropanoid synthesis	xylem	Hatton et al., 1995
12	C-RICH GG2	-2155	rbcS3A	Photosyntesis	mesophyll	Meier et al., 1995
13	KNOX BS	-2193	BD ga2ox1	GA inactivation	plant, ovary	Bolduc et al., 2009
14	Box II - GBF	-2237	rbcS-3.6	Photosyntesis	fruit locule	Meier et al., 1995
15	CT-rich	-2271	CrWRKY1	ABA, JA, GA and ET dependent expression	root	Suttipanta et al., 2011
16	GAGA element	-2285	gsa1 (Glu1-semialdehyde amin transferase)	Chlorophyll synthesis	mesophyll	Sangwan et al., 2002
17	$(GA)_5$	-2286	STK (SEEDSTICK)	Carpel and ovule identity	ovary	Favaro et al., 2003
18	$(GA)_6$	-2431	STK (SEEDSTICK)	Carpel and ovule identity	ovary	Favaro et al., 2003

Other REs are reported in the promoter of genes involved directly in the auxin signalling transduction to promote root initiation. In particular, the CG-box is necessary for the expression of *ARF19* and *NAC1* (Table 4.4), the former taking part in the formation of an Aux/IAA-ARF auxin-signalling module, the SOLITARY-ROOT (SLR)/IAA14–ARF7–ARF19 module, which regulates the nuclear migration and asymmetric cell divisions of the lateral root founder cells for lateral root initiation (Goh *et al*., 2012). *NAC1*, a new member of the *NAC* family , is an auxin induced transcription activator and mediates auxin signalling to promote lateral root development. Transgenic plants expressing sense or antisense *NAC1* cDNA show an increase or reduction of lateral roots, respectively (Xie *et al.*, 2000).

The CA-box is a regulatory motif of IAA3/*SHY2* (Table 4.4), another member of the Aux/IAA-ARF model, the auxin/indole-3-acetic acid (Aux/IAA)–AUXIN RESPONSE FACTOR (ARF) module, which regulates lateral root formation (Goh *et al*., 2012). IAA3/SHY2 represses the transcription of *PIN1*, a member of the auxin efflux carrier PIN family involved in the auxin cell homeostasis (Vieten *et al*., 2005), resulting in a change in auxin distribution that promote cell differentiation, thus increasing the number of lateral root founder cells (Dello Ioio *et al.*, 2008; Goh *et al*., 2012).

The CA-box and CG-box motifs are high-affinity binding sites for STF1 and LONG HYPOCOTYL5 (HY5) proteins (Table 4.4). HY5 is a bZIP (basic leucine zipper) transcription factor that activates photomorphogenesis and root development in Arabidopsis (Song *et al*., 2008). STF1 is a homolog of HY5 with a role in light and hormone signalling. The *hy5* null mutant shows an altered root morphology (Oyama *et al*., 1997). The *hy5* mutation affects several aspects of root morphogenesis, resulting in an elevated number of lateral roots, less responsiveness to gravitropic stimulus and touching, and longer root hairs in *hy5* seedlings than in WT. The pleiotropic effects in *hy5* are partly the result of an altered balance in the signalling of auxin (Sibout *et al*., 2006). Microarray analyses have shown that many auxin responsive and auxin-signalling genes are misexpressed in *hy5* mutants, an indication that the genes encoding auxin-signalling components are one group of the HY5 downstream genes (Cluis *et al*., 2004; Sibout *et al.*, 2006). HY5 is also involved in cytokinin signalling (Vandenbussche *et al*., 2007). Cytokinin treatment results in a number of growth responses to blue light, such as the inhibition of hypocotyl growth elongation (Chory *et al.,* 1994). It has been proposed that cytokinin increases the level of HY5 by reducing the degradation mediated by

CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), an E3 ubiquitin-protein ligase (Vandenbussche *et al*., 2007). Previously, STF1, a homologous bZIP protein that acts as a potential regulatory factor for hypocotyl elongation, was reported for soybean (Cheong *et al*., 1998). The CG-box is important for activating auxin-dependent regulatory loop that affects behavior of cells in the root meristem. These and previous (Chapter 3) observations indicate that the IAA3/SHY2–ARF signalling module regulates not only lateral root development by affecting auxin homeostasis but also may interacts with the cytokinin signalling pathway.

The tef-box (Table 4.4) represents an activating sequence and cooperates with another cis element, the telo-box, required for the gene expression of the translation elongation factor *Elongation factor 1-alpha 1* (*eEF1A*), involved in the translational apparatus in the cycling cells of root primordia (Tremousaygue *et al*., 1999).

Photosynthesis-related genes are strongly stimulated during fruit set (Ruiu *et al*., 2015; Klap *et al*., 2015). Regulatory elements such as GAGA, Box II, LRE, AT-RICH G2, UN I2 and C-RICH GG2 (Table 4.4) are recognized motifs in the promoter of *rbcS* gene family members,supporting the assumption that photosynthesis metabolism plays an important role in onset of both pollination-dependent and parthenocarpic fruit development.

Chapter 5

General discussion

Recently, by a candidate gene approach, the identity of *SlHB15* with the *Pat* locus has been hypothesized, for the presence of a nucleotide point mutation generating a missense substitution, which leads, on a predictive basis, to loss of the SlHB15 protein functionality. Phenotypic characterization of Arabidopsis mutants, carrying lesions in the *SlHB15* orthologue provided analogies with a number of phenotypes observed in *pat* (Ruiu, 2013). A first experiment of gene complementation in the *pat* mutant resulted apparently elusive because the manifestation of the WT phenotype was missing. Conversely, *SlHB15* cosuppression in the WT line produced a series of phenotypes among T_0 and T_1 plants that were reminiscent of the *pat* mutant, such as polycot seedlings, short anthers and ectopic formation of ovules in their adaxial surface, impaired ovule development and fertility and onset of ovary growth at anthesis, recalling the pleiotropic traits found in the *pat* mutant (Mazzucato *et al.*, 1998; Olimpieri *et al.*, 2007). All these phenotypes have been attributed to an altered polar auxin transport and GA imbalance (Mapelli *et al.*, 1978; Mazzucato *et al*., 1988; Olimpieri *et al.*, 2007). Transcription analysis of *SlHB15* and other genes involved in hormone (auxin, GA and cytokinin) homeostasis revealed an unexpected upregulation of Sl*HB15* coupled with activation of *SlLOG6* in the hypocotyl. *SlLOG6* is a gene inducing cytokinin synthesis and, in Arabidopsis, one of the *LOG* members, *LOG4*, proved to be transcriptionally induced by *HD-Zip III* genes (Carlsbecker *et al*., 2015). Further, with regard to auxin response, *SlIAA9* was found upregulated in the *pat* hypocotyl. In Arabidopsis, cytokinin activity and auxin signalling are two highly coordinated pathways. Recently it has been identified a regulatory loop between *HD-Zip III* genes (i.e. *PHB*) and cytokinin biosynthesis that creates robust domains of cytokinin activity allowing root growth by balancing processes of cell division and elongation/differentiation (Dello Ioio *et al*., 2012). Interplay between cytokinin and auxin to control the growth response is guaranteed by a member of the AUX/IAA TF family (Dello Ioio *et al*., 2008). Such an interplay has not been demonstrated in other organs of the plant yet (Bishopp *et al*., 2012) and it is not known if it can suit other species. Upregulation of *SlLOG6* and *SlIAA9,* in the *pat* mutant, might lead to a deviation from a steady state of cytokinin activity and

auxin signalling affecting the hypocotyl growth. Though more investigations at the molecular level are needed, misregulation of *SlLOG6* and *SlIAA9* might be the consequence of *SlHB15* upregulation. If so, this possibility should bear the explanatory hypothesis on the functional role of the point mutation affecting the $SlHB15^{pat}$ allele. The *pat* mutation was attributed a recessive inheritance (Philouze, 1968). Though with low intensity, expression of some phenotypes resembling the *pat* lesion (namely aberrant anthers and tendency to fruit seedlessness) in plants heterozygous for *SlHB15* lent support to the hypothesis that *pat* might represent a hypofunctional dominant negative allele. The structure of the HB15 protein is complex and possesses different functional domains. A dominant negative (antimorphic) effect was proposed for some Arabidopsis *HB15* mutants, *cna-1* and *rev-3* (Green *et al*., 2005) and deduced for *hoc* (Duclercq *et al*., 2011), where the altered allele might adversely affect the normal WT gene product. This is the case when the DNA-binding domain of the protein is left intact by the mutation but the homo/heterodimerization domain is altered or removed (or viceversa); as a consequence a fraction of protein dimers would be missing (Veitia, 2007). Generally, dominant negative alleles have stronger phenotypes than null ones. This was observed for *hoc* (Duclercq *et al*., 2011), *rev-3* (Green *et al*., 2005) and *athb2* (Morelli *et al*., 2008), a *class-II HD-ZI*P antimorphic mutant, but many other cases are reported in the literature. This might give reason for a, though milder, expression of the *pat* phenotypes in *SlHB15* cosuppressed plants. It cannot be ruled out that the absence of the ATHB15 protein in the knockout lines could be balanced by combinatorial redundancy (or antagonistic action) of other class III HD-ZIP proteins, resulting in a mild phenotype, as put in evidence by Zhu *et al*. (2013) in *Populus trichocarpa AtHB8* orthologue, *PtrHB7*. The *hoc* mutation is located 18 amino acids upstream of the PAS-like sequence of the MEKHLA domain. Because of similarities with the bacterial PAS domain, the PAS-like region could act in perception of environmental signals (Taylor and Zhulin, 1999) and/or support dimerisation (Pongratz *et al*., 1998). The *hoc* mutation removes a potential phosphorylation site from the MEKHLA domain. The phosphorylation of a TF can modulate its activity or its association with others factors (Schultze *et al*., 2008). The mutation in the $SlHB15^{pat}$ allele resides between the SAD and the MEKHLA domains and a potential phosphorilation site is added according to predicted changes of the 3D protein folding (Ruiu, 2013). This could have a functional effect in terms of dimerisation activity. Also, the mutation could promote the protein activity by altering the MEKHLA domain, which is reported to act as a negative regulator of the TF protein (Magnani and

Barton, 2011). HD-ZIP proteins could dimerise with the AP2 domain of two members of the APETALA (AP)2/ETHYLENE RESPOSNE FACTOR (ERF) protein family, ESR1 (ENHANCER OF SHOOT REGENERATION 1) and ESR2, possibly forming a HD-ZIP/AP2/ERF transcriptional complex (Chandler *et al*. 2007). The Arabidopsis AP2/ERF family is a large class of 144 TFs having diverse functions in plant development (Riechmann *et al*., 2000). These and the class III HD-ZIP transcription factors intervene in the same developmental processes (Banno *et al.*,2001; Boutilier *et al*.,2002; Hirota *et al.,* 2007). The fact that TFs from these two families can form heterodimers suggests that they could together form transcriptional complexes, whose variability could modulate different organogenetic processes.

Conclusion and perspectives

Parthenocarpy is the production of seedless fruits in the absence of pollination and/or fertilization. This process has been extensively studied in tomato because it offers a method to overcome unfavourable environmental conditions that reduce pollen production, anther dehiscence and, as a consequence, fruit set. Among the different sources of genetic parthenocarpy described in tomato, the *pat* mutation is of particular interest because of its strong expressivity, high fruit set and enhanced fruit quality. In this study, we have given functional evidence that *SlHB15*^{pat} may exert pleiotropic effects linked to the parthenocarpic fruit phenotype in tomato. A CRISPR-Cas9 gRNA sequence targeting the *SlHB15* locus has produced the first regenerants containing potential permanent mutations in the target site and more lesions are expected to be generated. These new alleles will be able to guarantee gene knock-outs with a higher efficiency and stability than cosuppression. However, our findings reveal a realistic role of *SlHB15* in the manifestation of the *pat* syndrome.

In view of both the potential dominant negative (antimorphic) effect of the *pat* mutation and the complementation of the *pat* mutant with the new (*SlHB15Promoter::5UTR'::SlHB15*^{WT}*::3UTR'*) construct, it would be interesting to perform a parallel test by introducing an analogous construct with the altered *SlHB15* (*SlHB15*^{pat}) allele in the WT line to analyze its possible (dose-dependent) antimorphic activity on the WT allele. Moreover, it would be interesting to explore the effects of *HB15/CNA/ICU4* knockout in other species with fleshy fruits.

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Appendix

Table S1. Ninety-six species in which parthenocarpy has been reported and classified according to the presence of either 1-2 seeds per fruit (monospermic species) or more seeds (plurispermic species), the two categories are indicated in the "Seed cat." column with "1" and "2", respectively; subcategories distinguishing species occurring in the wild or cultivated as either ornamental or consumption are indicated in the "Use" column with 1, 2 and 3, respectively.

N°	*Species*	Family	Phylogenetic group	Seed cat.	Use	Reference
1	*Acer griseum (Franch.) Pax 1902*	*Aceraceae*	Rosidae	1	2	Gansu et al., 2008
2	*Acer negundo L.*	*Aceraceae*	Rosidae	1	2	cited by Gustafson, 1939
3	*Acer saccharum Marshall*	*Aceraceae*	Rosidae	1	3	cited by Gustafson, 1939
4	*Actinidia arguta (Siebold & Zucc.) Planch. ex Miq. cv. issai*	*Actinidiaceae*	Magnoliidae	2	3	Mizugami et al., 2007
5	*Aechmea lindenii (E. Morren) Baker*	*Bromeliaceae*	Monocot	2	2	Lenzi et al., 2006
6	*Aethionema grandiflorum Boiss. & Hohen.*	*Brassicaceae*	Basal Eudicot	2	2	cited by Gustafson, 1939
7	*Ananas comosus L.*	*Bromeliaceae*	Monocot	2	3	cited by Gustafson, 1939
8	*Aristolochia macrophylla Lam.*	*Aristolochiaceae*	Magnoliidae	2	2	cited by Gustafson, 1939
9	*Artocarpus altilis (Parkinson) Fosberg*	*Moraceae*	Rosidae	2	3	cited by Gustafson, 1939
10	*Berberis vulgaris L. var. asperma*	*Berberidaceae*	Basal Eudicot	2	3	Ebadi et al., 2010
11	*Beta vulgaris L. var. saccharum*	*Chenopodiaceae*	Magnoliidae	2	2	Yudanova et al., 2011
12	*Betula pendula Roth.*	*Betulaceae*	Rosidae	1	2	Brown et al., 1982
13	*Bursera morelensis Ramirez*	*Burseraceae*	Rosidae	1	1	Ramos-Ordonez et al., 2008
14	*Capsicum annuum L.*	*Solanaceae*	Asteridae	2	3	cited by Gustafson, 1939
15	*Carica papaya L.*	*Caricaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
16	*Casimiroa edulis La Llave & Lex.*	*Rutaceae*	Rosidae	2	3	cited by Gustafson, 1939
17	*Citrullus vulgaris Schrad. ex Eckl. & Zeyh.*	*Cucurbitaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
18	*Citrus aurantiifolia (Christm.) Swingle*	*Rutaceae*	Rosidae	2	3	cited by Gustafson, 1939
19	*Citrus grandis Osbeck*	*Rutaceae*	Rosidae	2	3	cited by Gustafson, 1939

20	*Citrus limon (L.) Burm.*	*Rutaceae*	Rosidae	2	3	cited by Gustafson, 1939
21	*Citrus nobilis Lour.*	*Rutaceae*	Rosidae	2	3	cited by Gustafson, 1939
22	*Citrus keraji Hort. ex Tanaka*	*Rutaceae*	Rosidae	2	3	Yamamoto, 2002
23	*Citrus sinensis (L.) Osbeck*	*Rutaceae*	Rosidae	2	3	cited by Gustafson, 1939
24	*Citrus x clementina Hort. ex Tan.*	*Rutaceae*	Rosidae	2	3	Mesejo et al., 2013
25	*Cordyline australis (G. Forst.) Endl.*	*Agavaceae*	Monocot	2	3	Beever et al., 1995
26	*Corylus avellana L.*	*Betulaceae*	Rosidae	1	3	Lagerstedt, 1977
27	*Crocus sativus L.*	*Iridaceae*	Monocot	2	3	Ahmad et al., 2014
28	*Cucumis melo L.*	*Cucurbitaceae*	Basal Eudicot	2	3	Nerson, 2002
29	*Cucumis sativus L.*	*Cucurbitaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
30	*Cucurbita pepo L.*	*Cucurbitaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
31	*Dioscorea alata L.*	*Dioscoreaceae*	Monocot	2	3	Abraham et al., 1990
32	*Diospyros kaki Thunb.*	*Ebenaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
33	*Diospyros lotus L.*	*Ebenaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
34	*Diospyros virginiana L.*	*Ebenaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
35	*Dodonaea viscosa Jacq.*	*Sapindaceae*	Rosidae	2	2	cited by Gustafson, 1939
36	*Elaeis oleifera (Kunth, Cortés)*	*Arecaceae*	Monocot	1	3	Barcelos et al., 2015
37	*Eugenia aquea Burm. F.*	*Myrtaceae*	Rosidae	1	1	cited by Gustafson, 1939
38	*Eugenia javanica Lam.*	*Myrtaceae*	Rosidae	1	3	cited by Gustafson, 1939
39	*Eugenia malaccensis L.*	*Myrtaceae*	Rosidae	1	3	cited by Gustafson, 1939
40	*Euphorbia dulcis L. var. purpurata*	*Euphorbiaceae*	Rosidae	2	2	cited by Gustafson, 1939
41	*Ficus carica L.*	*Moraceae*	Rosidae	2	3	cited by Gustafson, 1939
42	*Fuchsia spp.*	*Onagraceae*	Rosidae	2	2	cited by Gustafson, 1939
43	*Garcinia mangostana L.*	*Guttiferae*	Basal Eudicot	1	3	cited by Gustafson, 1939

44	*Hedyosmum bonplandianum Kunth*	*Chloranthaceae*	Austrobaielyales	1	1	cited by Gustafson, 1939
45	*Impatiens holstii Engl. & Warb.*	*Balsaminaceae*	Rosidae	2	2	cited by Gustafson, 1939
46	*Impatiens oliveri C. H. Wright ex W. Watson*	*Balsaminaceae*	Rosidae	2	2	cited by Gustafson, 1939
47	*Impatiens walleriana Hook. f.*	*Balsaminaceae*	Rosidae	2	2	cited by Gustafson, 1939
48	*Juniperus osteosperma (Torr.) Little*	*Cupressaceae*	Gimnospermeae	1	1	Fuentes et al., 1998
49	*Lansium domesticum Correa*	*Meliaceae*	Rosidae	2	3	Bernardo et al., 1961
50	*Lobularia maritima (L.) Desv.*	*Brassicaceae*	Basal Eudicot	1	1	cited by Gustafson, 1939
51	*Malus domestica Borkh.*	*Rosaceae*	Rosidae	2	3	cited by Gustafson, 1939
52	*Mammillaria wildii A. Dietr.*	*Cactaceae*	Basal Eucot	2	2	cited by Gustafson, 1939
53	*Mangifera indica L.*	*Anacardiaceae*	Rosidae	1	3	Jing-Hao et al., 2010
54	*Mespilus germanica L.*	*Rosaceae*	Rosidae	2	3	cited by Gustafson, 1939
55	*Morus alba L.*	*Moraceae*	Rosidae	2	3	cited by Gustafson, 1939
56	*Morus nigra L.*	*Moraceae*	Rosidae	2	3	Griggs etal., 1973
57	*Musa sapientum L.*	*Musaceae*	Monocot	2	3	cited by Gustafson, 1939
58	*Nicotiana tabacum L.*	*Solanaceae*	Asteridae	2	3	cited by Gustafson, 1939
59	*Olea europea L.*	*Oleaceae*	Asteridae	1	3	cited by Gustafson, 1939
60	*Opuntia ficus-indica (L.) Mill.*	*Cactaceae*	Basal Eudicot	2	3	Weiss et al., 1993
61	*Oryza sativa L.*	*Graminaceae*	Monocot	1	3	cited by Gustafson, 1939
62	*Papaver rhoeas L.*	*Papaveraceae*	Magnoliidae	2	1	cited by Gustafson, 1939
63	*Pastinaca sativa L.*	*Apiaceae*	Rosidae	1	3	Zangerl et al., 1991
64	*Persea gratissima C. F. Gaertn.*	*Lauraceae*	Magnoliidae	1	3	cited by Gustafson, 1939
65	*Phillyrea media L.*	*Oleaceae*	Asteridae	1	1	cited by Gustafson, 1939
66	*Phoenix dactylifera L.*	*Palmae*	Monocot	1	3	cited by Gustafson, 1939
67	*Physalis ixocarpa Brot.*	*Solanaceae*	Asteridae	2	3	Mulato-Brito et al., 2007

68	*Piper methysticum (L.) G.Forst.*	*Piperaceae*	Magnoliidae	1	2	Prakash et al., 1994
69	*Pistacia atlantica Desf.*	*Anarcadiaceae*	Rosidae	1	1	Grundwag, 1975
70	*Pistacia chinensis Bunge*	*Anarcadiaceae*	Rosidae	1	1	Grundwag, 1975
71	*Pistacia khinjuk Stocks*	*Anarcadiaceae*	Rosidae	1	1	Grundwag, 1975
72	*Pistacia lentiscus L.*	*Anarcadiaceae*	Rosidae	1	1	Verdù et al., 1998
73	*Pistacia terebinthus L.*	*Anarcadiaceae*	Rosidae	1	1	Traveset, 1993
74	*Pistacia vera L.*	*Anarcadiaceae*	Rosidae	1	3	Polito, 1999
75	*Pisum sativum L.*	*Fabaceae*	Rosidae	2	3	cited by Gustafson, 1939
76	*Prunus cerasifera Ehrh.*	*Rosaceae*	Rosidae	1	3	cited by Gustafson, 1939
77	*Prunus persica (L.) Batsch*	*Rosaceae*	Rosidae	1	3	cited by Gustafson, 1939
78	*Pyrus communis L.*	*Rosaceae*	Rosidae	2	3	cited by Gustafson, 1939
79	*Ribes spp.*	*Saxifragaceae*	Basal Eudicot	2	3	cited by Gustafson, 1939
80	*Rosa canina L.*	*Rosaceae*	Rosidae	2	2	cited by Gustafson, 1939
81	*Salix cinerea L.*	*Salicaceae*	Basal Eudicot	2	1	Heslop-Harrison, 1924
82	*Solanum carolinense L.*	*Solanaceae*	Asteridae	2	1	Solomon, 1980
83	*Solanum lycopersicum L.*	*Solanaceae*	Asteridae	2	3	cited by Gustafson, 1939
84	*Solanum melongena L.*	*Solanaceae*	Asteridae	2	3	cited by Gustafson, 1939
85	*Solanum muricatum Aiton*	*Solanaceae*	Asteridae	2	3	cited by Gustafson, 1939
86	*Solanum nigrum L.*	*Solanaceae*	Asteridae	2	3	cited by Gustafson, 1939
87	*Solanum palustre L.*	*Solanaceae*	Asteridae	2	1	Hermsen et al., 1979
88	*Spondias mombin L.*	*Anacardiaceae*	Rosidae	1	1	cited by Gustafson, 1939
89	*Stratiotes aloides L.*	*Hydrocharitaceae*	Monocot	2	1	cited by Gustafson, 1939
90	*Tamarix dioica Roxb. ex Roth*	*Tamaricaceae*	Basal Eudicot	2	2	cited by Gustafson, 1939
91	*Ulmus minor Mill.*	*Ulmaceae*	Rosidae	1	1	Lopez-Almansa et al., 2003

92	*Vaccinium corymbosum L.*	*Ericaceae*	Asteridae	2	3	Ehlenfeldt et al., 2007
93	*Vaccinium virgatum Aiton*	*Ericaceae*	Asteridae	2	3	Cano-Medrano et al., 1997
94	*Vitis vinifera L.*	*Vitaceae*	Rosidae	2	3	cited by Gustafson, 1939
95	*Zea mays L.*	*Graminaceae*	Monocot	1	3	cited by Gustafson, 1939
96	*Zizyphus jujuba Mill.*	*Rhamnaceae*	Rosidae	1	3	Tian et al., 1987

Table S2. List of primers used for PCR analyses. Primers amplifying fragments of *Solyc03g120900, Solyc03g120920, Solyc03g120940* and *Solyc03g120950* are reported in section a); primers for cloning of *SlHB15* according to the GoldenBraid domestication strategy are reported in section b); *GBHB15PRO-1/10* primer pairs were used for amplifying the promoter containing the 5'UTR region, *GBHB15CDS-1/2* and *_-3/4* pairs amplified the two domesticated fragments of the coding sequence and *GBHB15UTR3-1/2* pair was used for the domestication of the 3'UTR region. Other 16 primers were used in combination also with the former for sequence verification of the amplified fragments and are reported in section c). Primers in section d) were used for qRT-PCR expression analysis and *qCAC-1/2* pairs amplified the *CAC* housekeeping gene (*Solyc08g006960*), used for qRT-PCR data normalization, according to Expósito-Rodríguez *et al*. (2008). For other primers not reported here, see text.

Primer name	Sequence		Ta (°C)
Solyc03g120900-1	5'-GCATGCCTAGTTCTCGGAATTA-3'	**a**	58
Solyc03g120900-2	5'-AGCAGATCAGAGCCTACAAGAGAG-3'		58
Solyc03g120900-3	5'-GATCAGGCTCATCCAGTAGGTG-3'		58
Solyc03g120900-4	5'-TGTAACATTCTGGACTGAAGGGTT-3'		58
Solyc03g120920-1	5'-AAATGGCACCTCATGGAGAG-3'		55
Solyc03g120920-2	5'-TTGCAGAAGGTACAACCCAAG-3'		55
Solyc03g120940-1	5-'CACTAAACACTCACCTCGACTCAG-3'		58
Solyc03g120940-2	5'-TTTCACATGCTACACACGATCC-3'		58
Solyc03g120940-3	5'-GACAGACTAATTGAAATGGAGGGA-3'		58
Solyc03g120940-4	5'-AGACAAATAGAGGCCAGTGAGAAA-3'		58
Solyc03g120940-5	5'-TGTCAGTCTCAGGTCAATGTGC-3'		58
Solyc03g120940-6	5'-ATGGCTGTGATCTATGAGGAGAA-3'		58
Solyc03g120940-7	5'-TGTCTGACCAAGTAGCGGTTTC-3'		58
Solyc03g120940-8	5'-CTCCACCTATGACGTCCAGG-3'		58
Solyc03g120940-9	5'-TGGACTCACTTGTCACTCACACA-3'		58
Solyc03g120940-10	5'-CTACCAGTCTACCATTACATGCCA-3'		58
Solyc03g120940-11	5'-AATGGCATGTAATGGTAGACTGG-3'		58
Solyc03g120940-12	5'-TGAGATAGAGAGGTTGTTTCCGA-3'		58
Solyc03g120950-1	5'-TCGTCTTCCACAATTCACG-3'		57
Solyc03g120950-2	5'-GATTCGTTTGGTCGGGAA-3'		57
Solyc03g120950-3	5'-TTGGTTGAGGTTGTTTCCCT-3'		57
Solyc03g120950-4	5'-CTCGTCCGTCTTTGGCTT-3'		57
GBHB15PRO_1	5'-GCGCCGTCTCGCTCGGGAGTCTTGTCCATGTCAATCTTACTTC-3'	**b**	64
GBHB15PRO_10	5'-GCGCCGTCTCGCTCACATTTTTTTCTTAAATCTTACAAACTTGAATGC-3'		64
GBHB15PRO_11	5'-GCGCCGTCTCGCTCGGGAGATACTTCCCATGCCTCTCCT-3'		64
GBHB15CDS_1	5'GCGCCGTCTCGCTCGAATGGCTTCCTGCAAGGATGG-3'		60
GBHB15CDS_2	5'-GCGCCGTCTCGCAAGACCAACCAGGCCACAA-3'		60
GBHB15CDS_3	5'-GCGCCGTCTCGCTTGAGCCAACGAGGGTATCCGAAATC-3'		60
GBHB15CDS_4	5'-GCGCCGTCTCGCTCAAAGCTTAGACAAATGACCAGTTGACAAA-3'		60
GBHB153UTR_1	5'-GCGCCGTCTCGCTCGGCTTTTACTGTTCTTTAGATCTGTTTTAAAG-3'		58
GBHB153UTR_2	5'-GCGCCGTCTCGCTCAAGCGCTACTCATATGAGTGCTTGTAG-3'		58

HB15PRO_3	5'-ATACTTCCCATGCCTCTCCTT-3'	**c**	56
HB15PRO_4	5'-GGAGAGGCATGGGAAGTATG-3'		57
HB15PRO_5	5'-GGGCATAATTGTACGCTGAAATC-3'		57
HB15PRO_6	5'-TTTCAGCGTACAATTATGCCC-3'		55
HB15PRO_7	5'-AGTATTCTAAATCTGGTGCGACG-3'		57
HB15PRO_8	5'-TGCTGACCTCTGTTCGTCG-3'		56
HB15PRO_9	5'-CATGCCTACAAGATCTTCCTCTC-3'		58
HBCDS15_2	5'-GGCTACTGGAACTGCTGTTGA-		57
HBCDS15_3	5'-AGTAGTGACTAGTGGTCAGC-3'		57
HBCDS15_4	5'-GGTCCAAGTATGCCACCTGT-3'		57
HBCDS15_5	5'-AATGGTATAGCTGATGAGGG-3'		57
HBCDS15_6	5'-CCATCCTTGTGAACTCTTCT-3'		57
HBCDS15_9	5'-AAGTTGGAAGGTCATTCTCC-3'		56
HBCDS15_8	5'-GCGATAATGCCACGAGATAT-3'		57
HBCDS15_11	5'-CTCGACAGTATGTCCGAAGC-3'		57
HBCDS15_10	5'-GGAGGTCTTCGTTTGCCATT-3'		56
qHB15_1	5'-GGAATGGATGAAAATGCTGTTGGA-3'	**d**	58
qHB15_2	5'-GAGTGAAATAATGCGAAACCCAGA-3'		58
qGA20ox1_1	5'-CATTGTGGTTATGCTAGTAG-3'		58
qGA20ox1_2	5'-GTAGTAGTGTTGTGTTGATC-3'		58
qLeT6_1	5'-CCATCGTCTCTTGACTGCTTATCT-3'		58
qLeT6_2	5'-AGGATCTTCTCCAATGATTCCACC-3'		58
qLOG6_1	5'-GTTGCTTGAAGTGATAACCTGG-3'		58
qLOG6_2	5'-TTGACTAGCTCCTTTGACGATG-3'		58
qARF8_1	5'-CTGCTCAAACCCAAATGCTGTC-3'		58
qARF8_2	5'-GGTAAGTGTGTTGGTGAGCCTG-3'		58
qIAA9_1	5'-TAGATGCTTTACCTGATTACGACA-3'		58
qIAA9_2	5'-TGCAGACAAACTCCAATATCAAAC-3'		58
qLAX3_1	5'-AATGGACAGTTGGGGAGTG-3'		58
qLAX3_2	5'-ATTAGATGGGTGAGAAGTGC-3'		58
qCAC_1	5'-GATGTCCTTATCAACCGTCTCTAC-3'		58
qCAC_2	5'-ACAAGAAAGAACAGCCTCCAATCT-3'		58

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OTHER ACTIVITIES

Teaching experience

Teaching assistant at University of Tuscia (DAFNE) in:

"Biotecnologie per il miglioramento delle piante agrarie" and "Miglioramento genetico e biotecnologie del seme", a.y. 2013-14.

"Biotecnologie per il miglioramento delle piante agrarie" and "Miglioramento genetico e biotecnologie del seme", a.y. 2014/15.

"Biotecnologie per il miglioramento delle piante agrarie"(SAA-L25 and BIOTEC-L2), a.y. 2016/17.

"Miglioramento genetico delle produzioni animali e vegetali" and "Produzioni vegetali" module (BAAS-LM7 and SAA-LM69), a.y. 2016/17.

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Journal Publications:

Mazzucato A, Cellini F, Bouzayen M, Zouine M, Mila I, Minoia S, Petrozza A, Picarella ME, Ruiu F, Carriero F (2015) *A TILLING allele of the tomato* Aux/IAA9 *gene offers new insights into fruit set mechanisms and perspectives for breeding seedless tomatoes*. Molecular Breeding 35:22 DOI 10.1007/s11032-015-0222-8.

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Poster Abstracts:

Picarella ME, Troise AD, Baldina S, Pucci A, Ferracane R, Fogliano V, Mazzucato A (2014) *Genotypic and ripening stage variation of quality-related metabolites in tomato fruits*. 58th SIGA Annual Congress, 15th-18th September 2014, Alghero (Sassari, Italy).

Pucci A, Picarella ME, Mazzucato A (2014) *Advances in the characterization of tomato mutants putatively affected in class B MADS-BOX transcription factors*. 58th SIGA Annual Congress, 15th-18th September 2014, Alghero (Sassari, Italy).

Grandillo S, Palombieri S, Riccini A, Cammareri M, Sampalmieri M, Picarella ME., Ruggiero A, Batelli G, Grillo S, Riccardi R, Spigno P, Patanè C, Poma I, Sunseri F, Finkers R, Blanca JM, Canizares J, Pons C, Monforte AJ, Granell A, Sulli M, Diretto G, Giuliano G, Mazzucato A (2016) *A morphological and genetic characterization of a large collection of tomato landraces.* Proceedings of the LX SIGA Annual Congress, 13th-16th September, 2016 Catania (Italy).

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Picarella ME and Mazzucato A (2017*) Insight on parthenocarpy: a possible "adaptive" response of seedless fruits and hybridity as a force driving departures from normal sexual plant reproduction*. SolCuc Meeting, 3rd-6th September 2017, Valencia (Spain).

Congresses, workshops, seminars, courses attended

Seminar by Francesca QUATTROCCHIO (Institute for Life Sciences, University of Amsterdam (NL). Vacuolar hyperacidification in plants…mistery solved! - Università degli Studi della Tuscia, 24th October 2014.

Seminar by Ronald KOES (Institute for Life Sciences, University of Amsterdam (NL). Plant inflorescence architecture….what makes the difference? - Università degli Studi della Tuscia, 24th October 2014.

58th SIGA Annual Congress, 15th-18th September 2014, Alghero (Sassari, Italy).

Course on "Applied statistics in agricultural research" at Università degli Studi della Tuscia, Viterbo (Italy), 16th-20th June 2014 (35 hrs).

PBG Network 5th annual meeting on "New Breeding Techniques: Genome Editing, Cisgenesis and Genomic Selection". University of Piacenza, Piacenza (Italy), 8 June 2016.

SolCuc 2017, XIV *Solanaceae* and III *Cucurbitaceae* Joint Conference. Valencia (Spain), 3rd-6th September 2017.

Projects

ATENA project. Title: Approcci TEcnologici Nuovi per l'Aumento della shelf-life del contenuto di servizio nei prodotti qualificanti il modello alimentare mediterraneo. Industria 2015 Bando Nuove Tecnologie per il Made in Italy" (MISE). University of Tuscia, Viterbo (Italy).

TRADITOM project. Title: Identification and valorisation of European traditional tomato varieties and their cultural practices. Task: tomato landraces phenotyping. University of Tuscia, Viterbo (Italy).

FILAS – MIGLIORA project. Title: Innovazioni tecnologiche per migliorare i processi produttivi e le qualità nutraceutiche e salutistiche dei prodotti di specie vegetali del territorio laziale. University of Tuscia, Viterbo (Italy).

Fellowships

20/09-13/10/2016, by EU COST programme, Action FA1106 "An integrated systems approach to determine the developmental mechanisms controlling fleshy fruit quality in tomato and grapevine" for research activities at IBMCP, University of Valencia (Spain).

02-14/09/2017 by LLP- ERASMUS programme, University of Tuscia, Viterbo (Italy) for research activities at IBMCP, University of Valencia (Spain).

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