Parthenocarpy and functional sterility in tomato

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CONTENTS

ABBREVIATIONS

aa	amino acids
ABL	Advanced Breeding Line
ADPG	*Arabidopsis* Dehiscence PolyGalacturonase
AFLP	Amplified Fragment Length Polymorphism
aos	*allene oxide synthase*
ARF8	*AUXIN RESPONSE FACTOR8*
BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
BSA	Bulked Segregant Analysis
cM	centiMorgan
coi1	*coronatine insensitive1*
COS	Conserved Ortholog Set
CAPS	Cleaved Amplified Polymorphic Sequence
dad1	*defective in anther dehiscence1*
dap	days after pollination
dCAPS	derived CAPS
dde	*delayed dehiscence*
ERE	Ethylene Responsive Element
fad	*fatty acid desaturation*
fwf	*fruit without fertilization*
GA	Gibberellic Acid
IAA	Indol-3-Acetic Acid
IL	Introgression Line
JA	Jasmonic Acid
MAS	Molecular Assisted Selection
MQM	Multiple QTL Mapping
opr3	*12-oxophytodienoic acid reductase3*
ORF	Open Reading Frame
pat	*parthenocarpic fruit* locus/loci
pat-1	Used to designate the *pat* gene of *Soressi* is this thesis
PG	PolyGalacturonase
ps-2	*positional sterility-2*
QTL	Quantitative Trait Locus
SCAR	Sequence Characterized Amplified Region
se	*style exertion*
SH	*Solanum habrochaites*
SL	*Solanum lycopersicum*
SNP	Single Nucleotide Polymorphism
TDPG	Tomato Dehiscence PolyGalacturonase
TFPG	Tomato Fruit PolyGalacturonase
WT	Wild Type

PREFACE

Parthenocarpy and functional sterility in tomato

Parthenocarpy is the development of the ovary into a seedless fruit without the need of pollination and/or fertilization. Parthenocarpy is a very attractive trait because it overcomes the problem of poor fruit set when the pollination is hampered due to adverse conditions. In tomato, breeders have been mostly looking for facultative parthenocarpy. In this kind of parthenocarpy, the ovary, if fertilized, will develop into a normal seeded fruit. However, in absence of pollination/fertilization, the ovary develops into a seedless fruit. Two main problems have so far strongly limited the development of parthenocarpic tomato cultivars:

- **Stability and uniformity of parthenocarpic production are difficult to obtain in tomato cultivars**. Several sources of parthenocarpy are known in tomato. However they are often associated with unfavorable characteristics or are poorly expressed in indeterminately growing plants. In addition, when seeded and seedless fruits develop on the same tomato cluster, seeded fruits are generally larger. A logical approach to overcome this problem would be to combine several parthenocarpy genes to obtain a higher level of parthenocarpy, but pyramiding genes is impossible without molecular assisted selection.
- **Parthenocarpy hampers the production of commercial seeds**. The development of tomato lines with a high parthenocarpy level is problematic for commercial seed production. With a high level of parthenocarpy, the swelling of the ovary, characteristic of fruit setting, often starts before anthesis. Therefore, at anthesis the ovary cannot be pollinated anymore and the production of seeds becomes impossible.

A combination of parthenocarpy with pollen sterility has the advantage of preventing the presence of seeded fruits and therefore would lead to a more uniform production of seedless fruits. However the seed production would become impossible. A way to solve this problem is

by the use of functional sterility, which is characterized by a normal development of viable pollen, but natural pollination is strongly restricted due to some deviation from the normal morphology and function of the flower. One of the best functional male sterility genes in tomato is *positional sterility 2* (*ps-2*), which is characterized by non-dehiscent anther bags.

The combination of parthenocarpy with *positional sterility-2* would prevent the development of seeded fruits and the production of seeds would still be possible by manual anther opening (**Figure 1**). Breeding for parthenocarpy in a functionally sterile background would focus on the parthenocarpic potential of the plants and not on a selection against seed set as it has been done so far.

This thesis is divided into five chapters:

- **Chapter 1** gives a detailed overview of parthenocarpy in tomato and on the research performed on that topic. The main sources of parthenocarpy in tomato are presented.

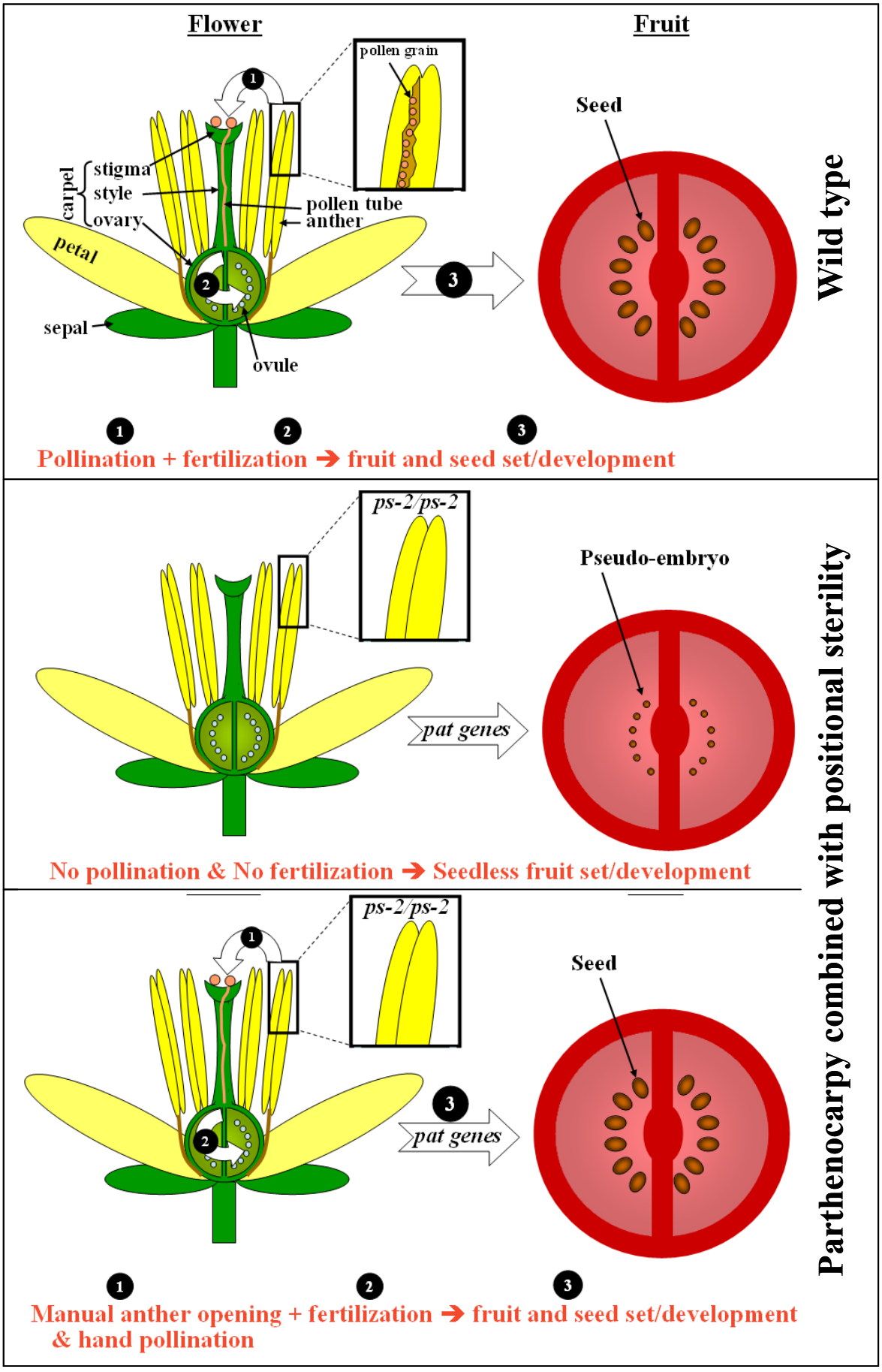
- **Chapter 2** describes the identification, characterization and mapping of new parthenocarpy genes originating from *S. habrochaites*, in tomato and leads to the development of molecular tools to assist in the introduction of those genes in modern lines.

- **Chapter 3** presents the fine mapping of *ps-2* and highlights the need for cloning in order to develop a universal applicable molecular marker to be used in breeding programs.

- **Chapter 4** describes the successful isolation and functional characterization of the *ps-2* gene, which is the result of a single mutation in a novel polygalacturonase gene.

- **Chapter 5** gives an overview discussion on the experimental chapters focusing on the use of the results for the breeding of parthenocarpic tomato cultivars.

Figure 1: General scheme on the combination of parthenocarpy and positional sterility in tomato.


Flower
Fruit
pollen grain
Seed
carpel
stigma
style
ovary
pollen tube
anther
petal
sepal
ovule
Wild type
1
2
3
Pollination + fertilization ➔ fruit and seed set/development
ps-2/ps-2
Pseudo-embryo
pat genes
No pollination & No fertilization ➔ Seedless fruit set/development
ps-2/ps-2
Seed
pat genes
Parthenocarpy combined with positional sterility
Manual anther opening + fertilization ➔ fruit and seed set/development
& hand pollination

CHAPTER 1

Parthenocarpic fruit development in tomato – A review

Benoit Gorguet, Adriaan W. van Heusden, Pim Lindhout

Abstract

Parthenocarpic fruit development is a very attractive trait for growers and consumers. In tomato, three main sources of facultative parthenocarpy, *pat-1, pat-2, pat-3/pat-4*, are known to have potential applications in agriculture. The parthenocarpic fruit development in these lines is triggered by a deregulation of the hormonal balance in some specific tissues. Auxins and gibberellins are considered as the key elements in parthenocarpic fruit development of these lines. An increased level of these hormones in the ovary can substitute for pollination and trigger fruit development. This has opened up genetic engineering approaches of parthenocarpy that have given promising results, both in quality and quantity of seedless fruit production.

Updated version of the published review in Plant Biology (2005) 7: 131-139

Introduction

The fruit is the result of the development of the ovary. A fruit provides a suitable environment for the development of seeds, and often, as Darwin observed, also supports efficient dispersal of mature seeds. In general, the development of fruits follows pollination and fertilization, and coincides with the maturation of the seeds.

In normal fruit development, the initiation of fruit set depends on the successful completion of pollination and fertilization. By definition, pollination is the transfer of a pollen grain from the anther to the stigma. Pollination occurs after formation of the pollen grain in the anther and its release. Fertilization occurs in the female gamete, the ovule, located within the carpel of the flower. Fertilization requires pollen germination, penetration and growth of the pollen tube in the stylar tissue towards the embryo sac in order to fuse with the egg cell (Dumas and Mogensen 1993; Mascharenhas 1993). Fertilization of the ovule generally triggers the development of the ovary into a fruit (Gillaspy et al. 1993).

Mutants can be used to study the processes involved in fruit set and development. Mutants known to alter hormone biosynthesis or hormone perception have been widely studied in the last decade. In some cases the hormonal deregulation occurring in those mutants resulted in a parthenocarpic fruit development, as in the *spindly* (*spy*) mutant in *Arabidopsis* (Jacobsen and Olszewski 1993).

The normal process of fruit development may occasionally be independent of pollination and fertilization. Two main phenomena, apomixis and parthenocarpy, can lead to the development of fruits without pollination and fertilization. Apomixis is defined as asexual reproduction through seeds (Nogler 1984), leading to the production of clonal progeny (Koltunow and Grossniklaus 2003). Parthenocarpy is the growth of the ovary into a seedless fruit in the absence of pollination and/or fertilization (Lukyanenko 1991). Parthenocarpy may occur naturally or can be induced artificially with the application of various hormones (Gustafson 1936, 1942; Nitsch 1952; Osborne and Went 1953). It is therefore suggested that the deregulation of the hormonal balance in the ovary of parthenocarpic plants substitutes for pollination and fertilization and so triggers fruit set and fruit development.

Unfavorable conditions, such as extreme temperatures, may prevent pollination and hence also fruit set. Parthenocarpy has been recognized for a long time as an interesting trait to avoid low fruit set in harsh conditions. In the case of facultative parthenocarpy, the development of parthenocarpic fruit occurs when conditions are adverse to pollination. In tomato, failure to set fruit is a common phenomenon under certain field and greenhouse

conditions (Howlett 1939; Rick 1978). Unfavorable environmental conditions for tomato fruit set include: low and high humidity, low light intensity, low or high temperatures, and high winds (George et al. 1984). Parthenocarpy is mainly used for crops where seedless fruits are desired by consumers, such as bananas, citrus, grapes, etc. (reviewed by Varoquaux et al. 2000). The shelf life of seedless fruits is longer than seeded fruits because seeds produce hormones that trigger senescence. In spite of the advantages, parthenocarpy is still of limited use in agriculture. Mutations causing parthenocarpic fruits often have pleiotropic effects and are often associated with unfavorable characteristics, such as male or female sterility or smaller and misshapen fruits (Varoquaux et al. 2000). Consequently, to exploit parthenocarpy, a lot more knowledge is required about the detailed mechanism and the associated pleiotropic effects. In tomato, the normal processes of reproduction are well studied and several genes are known that confer parthenocarpy (*pat* genes). In this review, we focus on modes of action of those *pat* genes to illustrate the possible mechanisms of parthenocarpic fruit development which may eventually lead to exploitation of parthenocarpy in tomato breeding.

Role of hormones in pollen formation, pollination, fertilization, and seed and fruit development

Pollen development takes place within the anther. Four anther wall layers enclose the fluid-filled locule, which contains the sporogenic cells that will undergo meiosis. Each microsporocyte undergoes two meiotic divisions over a period of approximately three days, producing a tetrad of four haploid cells. At that stage, those so-called microspores are still encased within a callose wall. When the callose wall is dissolved and the microspores are mature, the asymmetric division called microspore mitosis can occur. The bicellular product of that division is the pollen. In the following steps, further pollen maturation occurs. After final dehydration, the pollen grain is ready to be released (for review: Bedinger 1992).

Exogenous application of cytokinins and gibberellins to various wild type and some specific male-sterile plants has been used to study the role of these hormones in male reproductive development (for review: Sawhney and Shukla 1994). Sometimes, a delay or even elimination of anthesis can be caused by deficiencies in endogenous cytokinins and gibberellins (GAs). Lower endogenous cytokinin and GA levels have been found in the male-sterile mutant of tomato *sl-2* (*stamenless-2*; Sawhney 1974; Sawhney and Shukla 1994).

Moreover, tomato GA-deficient mutants (*gib-1* and *gib-2*) are male sterile and fertility can be restored by exogenous application of GAs (Nester and Zeevaart 1988; Jacobsen and Olszewski 1991). Recently, the role of plant hormones in reproductive development has been studied with the use of tissue specific hormone manipulation. The exogenous applications of plant growth hormones, like cytokinins and GAs, may influence many processes in plant growth and development (Huang et al. 2003). Therefore, to avoid abnormal growth phenotypes, the hormone concentration was only manipulated in specific tissues, such as anthers or pistils. For example, maize was transformed with *CKX1*, a gene that confers inactivation of cytokinin, under the control of anther- and pollen-specific promoters. Similarly, *Arabidopsis* and tobacco have been transformed by the introduction of *gai* gene (GA-insensitive), which negatively regulates GA responses, driven by anther- or pollen-specific promoters (Huang et al. 2003). Direct involvement of cytokinins and GAs in male reproductive development was demonstrated by abortion of anther and pollen in these hormone-insensitive transgenic plants (Huang et al. 2003).

In tomato there are more than 40 genes known to be involved in male sterility (*ms*). Only a few of them are considered useful for hybrid seed production (Georgiev 1991) as the male sterility is often not absolute and selfings can still occur.

After pollen formation, the final function of the anther is the dehiscence that results in release of the pollen (Goldberg et al. 1993, 1995). Studies have shown that a deficiency in endogenous jasmonic acid (JA) leads to a delay in anther dehiscence. Hence, the JA pathway plays a role in controlling the elongation and time of anther dehiscence within the flower (Xie et al. 1998; Feys et al. 1994; Sanders et al. 2000; Stintzi and Browse 2000; Ishiguro et al. 2001; von Malek et al. 2002; Park et al. 2002). Apparently, the JA produced in the stamens synchronizes pollen maturation, anther dehiscence and flower opening (Sanders et al. 2000; Ishiguro et al. 2001). Recently, ethylene has also been found to play a role in the timing of anther dehiscence. In an ethylene-insensitive mutant of tobacco, dehiscence of the anthers was delayed and no longer synchronous with flower opening (Rieu et al. 2003). In self-pollinating crops, delay in anther dehiscence prevents fertilization because the pollen is released after the pistil is receptive to pollen. However, the pollen of some JA mutants is still viable and can possibly fertilize neighboring flowers.

Several environmental factors influence pollination (for tomatoes, reviewed by George et al. 1984). For instance, high temperatures as well as low temperatures can affect the formation of the endothecium in tomato, thus preventing the release of pollen (Rudich et al.

1977; Charles and Harris 1972). Pollen tube growth and the development and functionality of gametes are also influenced by environmental factors (Iwahori 1966).

After pollen has been released, the pollen grains may land on the stigma where they adhere, hydrate, and germinate. Pollen tube growth can be stopped in case of self incompatibility, when genes in the pollen (or in the male parent) do not match the genes in the female parent (for review: Wheeler et al. 2001). The germinating and growing pollen tubes produce plant growth factors, most likely auxin and gibberellins (Nitsch 1970).

The gene *PsGA$_3$ox1* (*Pisum sativum GA$_3$ oxidase 1*) codes for an enzyme that converts GA_{20} to biologically active GA_1 (Olszewski et al. 2002). In pea, *PsGA$_3$ox1* is expressed in the pericarp and its expression may be regulated by the presence of seeds and auxin. Expression of *PsGA$_3$ox1* in the pericarp induces an increase in the level of active GA that is necessary for pericarp growth (Ozga et al. 2003). Developing seeds are therefore considered as a driving force for fruit growth (Nitsch 1950; Archbold and Dennis 1985) because they produce high levels of plant growth hormones (Eeuwens and Schwabe 1975; Sponsel 1983; Talon et al. 1990; García-Martínez et al. 1991a; García-Martínez et al. 1991b; Ben-Cheikh et al. 1997; Rodrigo et al. 1997). In *Arabidopsis*, fertilization induces an auxin-like signal in the pistil (O'Neill and Nadeau 1997) which is dominant over the action of GA and results in a higher cellular expansion and an alteration of the mesocarp structure (Vivian-Smith and Koltunow 1999; **Figure 1**).

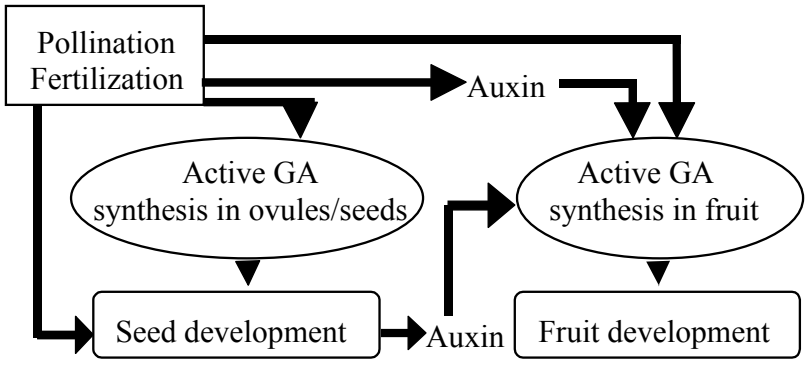


Figure 1: Model proposed for the regulation of fruit and seed development.

As pollination and seed formation via stimulation of the synthesis of active GA trigger fruit initiation and development, the artificial addition of GA should also initiate fruit set. This

has been shown in a wide range of agricultural plant species (Schwabe and Mills 1981). However, fruit development is not controlled by only a single growth hormone but is far more complex, as described in rape (Srinivasan and Morgan 1996) and pea (van Huizen et al. 1997). Similarly, in *Arabidopsis*, the addition of one single plant growth hormone, auxin, cytokinin or gibberellin, in the absence of pollination did not result in the exact same length, shape, and growth rate of the siliques as after pollination (Vivian-Smith and Koltunow 1999). The most similar silique development was obtained after application of GA_3 (Vivian-Smith and Koltunow 1999), confirming the crucial role of gibberellins.

Gibberellin is a key factor in fruit set and development. However, gibberellin comprises a large family of similar compounds and the specific function of each gibberellin varies over different plant species. In tomato, GA_3 induced mesocarp cell expansion with restricted cellular division (Bunger-Kibler and Bangerth 1982). Contrarily, in *Arabidopsis*, GA_3 primarily influenced mesocarp cell division, while mesocarp cell expansion was observed after auxin treatment (Vivian-Smith and Koltunow 1999).

The role of hormones in fruit set and development has mainly been studied using biosynthetic and hormone perception mutants of *Arabidopsis*. Already, more than ten years ago, the *Arabidopsis spindly* (*spy*) mutant which confers hypersensitivity to GA was very helpful in understanding fruit set and development. Compared to wild type, the *spy* mutant exhibits parthenocarpic fruit development and showed that the control of GA sensitivity can mimic the role of pollination and fertilization in fruit set and development. Recently, the *Spindly* homolog from tomato has been isolated (Greb et al. 2002), enabling further studies of fruit development in tomato.

The phenotype of mature fruits is mainly characterized by fruit size, fruit weight, number of locules, and number of seeds per fruit. The role of endogenous gibberellin in seed and fruit development has been studied in tomato using the GA-deficient *ga-1* mutant (Groot et al. 1987). The final fresh weight of the mutant fruits is positively correlated with the number of seeds per fruit. It was also observed that fruits containing GA-producing seeds reach a higher fresh weight than those containing GA-deficient seeds. The authors concluded that GA is involved in the later stages of fruit and seed development. In the auxin-resistant *diageotropica* (*dgt*) mutant of tomato, fruit set, size, and weight, number of locules and number of seeds are reduced (Balbi and Lomax 2003). Because the number of seeds affects the final fruit size of non-parthenocarpic tomato (Varga and Bruinsma 1986; Groot et al. 1987), it is suggested that the reduction in fruit weight can be partially explained by this

reduced number of seeds (Balbi and Lomax 2003). The smaller fruits had decreased cell division and expansion. However, only the early stages of fruit development were affected by the *dgt* mutation. The *dgt* allele also affects expression of certain members of gene families involved in regulation of ethylene biosynthesis and auxin response genes. This suggests that auxin- and ethylene-mediated gene expression play a role in the early stages of fruit development in tomato (Balbi and Lomax 2003).

The three main sources of parthenocarpy in tomato

Detailed reviews on the different types and sources of parthenocarpy in tomato were presented by George et al. (1984) and Lukyanenko (1991). Three sources have been widely studied because of their perspectives for practical application to produce seedless fruits: '*Soressi*' or '*Montfavet 191*', '*Severianin*' and '*RP75/59*'. Philouze (1985) considered these three sources as the only ones able to give parthenocarpic fruits after emasculation, with nearly the same properties as fruits obtained after pollination and fertilization.

The *pat-1* gene

Soressi and Salamini (1975) described the short anther (*sha*) mutant in tomato. This mutant has abnormal stamens and produces parthenocarpic fruits. This parthenocarpic phenotype was thought to be caused by two closely linked recessive genes, *sha* and *pat-1* (*parthenocarpic fruit-1*). However, Pecaut and Philouze (1978) independently obtained a mutant with the same phenotype, designated '*Montfavet 191*'. These independent mutations were allelic (Pecaut and Philouze 1978; Philouze and Pecaut 1986) and the characters 'short anthers' and 'parthenocarpic fruits' could never be separated. It was concluded that the described phenotype was caused by a recessive mutation in a single gene, designated *pat-1*, with pleiotropic effects.

Cytological studies have shown that the start of ovary growth in *pat-1* mutants occurs at the pre-anthesis floral stage compared to two days post anthesis in the wild type (Mazzucato et al. 1998). This results in a higher ovary weight and in higher numbers of pericarp cell layers in *pat-1* ovaries (Mapelli et al. 1978). This *pat-1* mutant also shows irregular meiosis which results in a lower number of viable female gametes (Mazzucato et al. 1998). Apparently, aberrations in the ovule production play a role in seed set of *pat-1* fruits. However, fertilization in *pat-1* ovaries is strongly impaired, even in the ovules that appear normal, probably due to a defective pollen tube-placenta interaction (Mazzucato et al. 2003).

Therefore, even in conditions favorable for seed production, *pat-1* genotypes give a very low seed set (Mazzucato et al 1998; Philouze and Pecaut 1986). The *pat-1* mutant produces tomato fruits which are about two-thirds the normal size and weight (Bianchi and Soressi 1969; Falavigna et al. 1978; Philouze and Pecaut 1986). A reduction in fruit size combined with difficulties to obtain seeds has made the *pat-1* gene less attractive for breeding (Philouze and Pecaut 1986). Recently, the *pat-1* gene has been mapped on the long arm of Chromosome 3 (Beraldi et al. 2004), opening the way towards cloning of the gene.

The *pat-2* gene

Another source of parthenocarpy has been found in the tomato cultivar '*Severianin'*. Philouze and Maisonneuve (1978) and Nuez et al. (1986) showed that a single recessive *pat-2* gene was responsible for parthenocarpy in '*Severianin'* while Vardy et al. (1989b) hypothesized a model based on two recessive genes, one having a major effect (*pat-2*) and one a minor effect (*mp*). Philouze et al. (1988) showed that the choice of the recurrent parent in which the *pat-2* gene is introduced is very important for plant vigor. In the genetic background of the tomato line *Apedice*, the plants are smaller and less vigorous, resulting in a reduction in yield, fruit set, and firmness of the fruits (Philouze et al. 1988). No differences in yield and vigor were found when *pat-2* was introduced into the genetic background of tomato lines '*Monalbo'* or '*Porphyre'*.

Parthenocarpy may interact with growth habit. Lin et al. (1984) found that in a population segregating for growth habit and *pat-2*, the majority of the parthenocarpic plants had determinate growth. However, no genetic linkage was found between parthenocarpy and the locus controlling the determinate growth habit (*sp*). Probably, determinate growth allows better expression of parthenocarpy.

The main advantage of exploiting parthenocarpy from *Severianin* is the potential that the deleterious pleiotropic effects of *pat-2* can be overcome by a suitable genetic background.

The *pat-3* and *pat-4* genes

The tomato line *RP75/59* was found in the progeny from a cross between 'Atom' x 'Bubjekosoko'. A high level of parthenocarpy was observed without obvious effects on fruit size and appearance (Philouze 1983b, 1985). However, in this new source of parthenocarpy an association was observed between seed set and size of fruits within the same truss or plant: When both seeded and seedless fruits are present on the same plant, the seeded fruits grow

bigger than the seedless fruits (Philouze 1989). Genetic studies on *RP75/59* showed that two to five genes control this trait. (Nuez et al. 1986; Philouze 1989; Vardy et al. 1989a). The differences in the number of genes involved in parthenocarpy are probably due to the very difficult assessment of the trait. *RP75/59* is facultative parthenocarpic, therefore, to obtain seedless fruits, the plants have to be grown in conditions adverse to pollination and/or fertilization, or the flowers have to be emasculated. Nowadays, a genetic model with the two genes *pat-3* and *pat-4* in *RP75/59* is commonly accepted. Philouze (1989) showed that *pat-2* and *pat-3/pat-4* are not allelic. The polygenic inheritance of the RP75/59 parthenocarpy source and the different fruit sizes of seeded and seedless fruits on the same truss make the use of *pat-3/pat-4* genes less attractive for breeding.

Mechanism of parthenocarpy

Fos and Nuez (1996) found a differential expression of a 30-kDa product in flowers in the pre-anthesis stage of *pat-2* plants in comparison to wild type flowers. A similar effect was found for *pat-3/pat-4* flowers. Apparently, even before pollination, specific events responsible for the development of parthenocarpic fruits occur. At anthesis, at least six *in vitro* translation products from flowers and ovaries were differentially expressed in a non-parthenocarpic line and a *pat-2* near isogenic line (Fos and Nuez 1997). Conceivably, those differentially expressed *in vitro* translation products play a role in the higher level of GA_{20} and the lower level of GA_{19} in the unpollinated ovaries of *pat-2* (Fos et al. 2000). *pat-2* may increase GA_{20}-oxidase activity in unpollinated ovaries, resulting in greater synthesis of GA_{20}, the precursor of an active GA (Fos et al. 2000). Several authors have reported that GA_3 induces the development of seedless fruits (Bunger-Kibler and Bangerth 1982; Sjut and Bangerth 1982; Alabadi et al. 1996; Fos et al. 2000). Also for the line *RP75/59*, it was suggested that *pat-3/pat-4* is responsible for an increase in the concentration of GA_1 and GA_3 in the ovaries before pollination (Fos et al. 2001). Recently, the expression analysis of genes encoding key enzymes involved in GA biosynthesis showed a high and constitutive expression of *GA_{20}-ox1* in the ovaries of the *pat-1* mutant, when in WT ovaries this gene was only up-regulated at pollination/fertilization (Olimpieri et al. 2007). In addition, the authors studied the effect of this differential gene expression on the accumulation of GAs: before anthesis, GA synthesis was interrupted between GA_{19} and GA_{20} in WT ovaries. In contrast, in *pat-1* ovaries, GA_{20} and the active GA_1 were found at high concentration (Olimpieri et al. 2007). The GA

biosynthesis pathway seems to be the key in initiation of development of parthenocarpic fruits in all three sources (*pat-1*, *pat-2* and *pat-3/pat-4*).

Polyamines are also involved in early seedless fruit development. Polyamines are necessary for parthenocarpic growth of *pat-2* ovaries. Fos et al. (2003) showed that adding polyamines induces partial parthenocarpic fruit set and growth of wild type ovaries in the tomato cultivar *Madrigal*. It is not certain whether gibberellins and polyamines act independently on fruit set in tomato, but the lower efficiency of polyamines compared to gibberellins suggests that polyamines do not act as the primary signal (Fos et al. 2003). Alabadi et al. (1996) showed that certain biosynthesis pathways of polyamines are more active after auxin or gibberellin treatment. Possibly, *pat-2* stimulates the polyamine biosynthesis pathway via higher GA levels in unpollinated *pat-2* ovaries (Fos et al. 2003).

In conclusion*, pat-1, pat-2* and *pat-3/pat-4* genes stimulate one or more steps in the GA biosynthesis pathway, which results in enhanced expression of active GAs, and these GAs induce parthenocarpic fruit development (**Figure 2**).

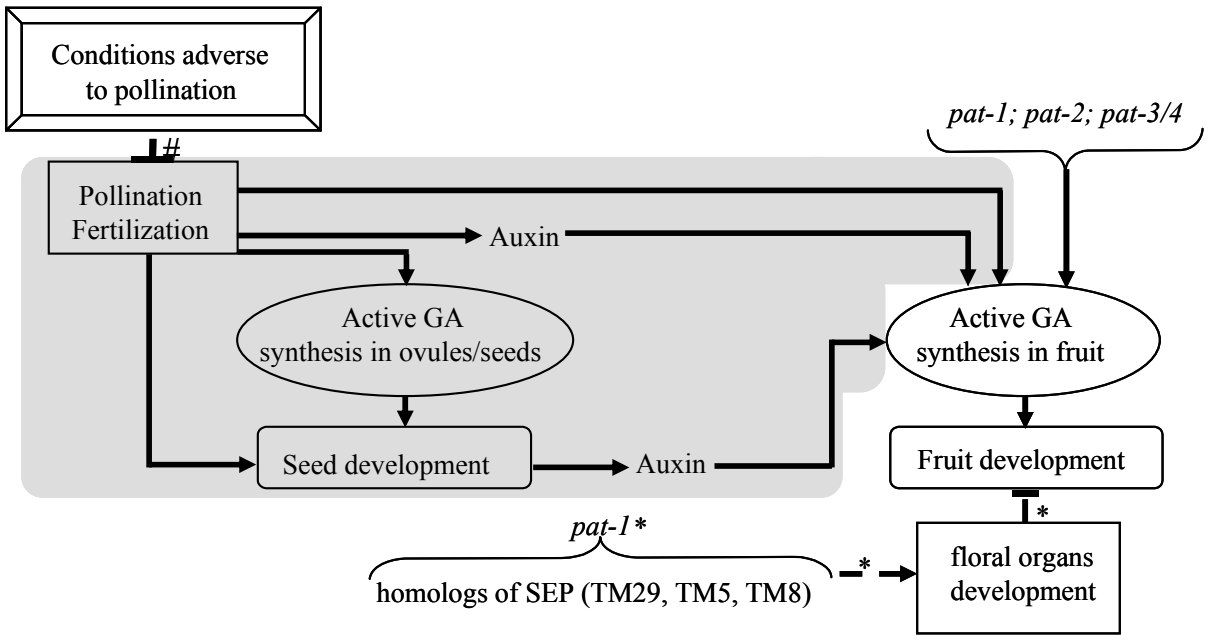


Figure 2: Model proposed for the parthenocarpic tomato fruit development. (#) Conditions adverse to pollination repress the processes of pollination and fertilization. The normal process of regulation of fruit and seed development does not occur (in gray). (*) *pat-1*, a possible allele of a putative gene with homeotic function is here compared to down regulated homologs of SEP. Abnormal floral organs development, which occurs in the case of those mutated genotypes, does not repress parthenocarpic fruit development.

Besides GAs, auxin also plays a role in the development of parthenocarpic fruits, i.e. parthenocarpy can be artificially induced by an application of auxin (Nitsh 1972). In addition,

during pea pod growth, GA_{20}-oxidase activity is regulated by both auxin and GA_3 (van Huizen et al. 1995; van Huizen et al. 1997; García-Matínez et al. 1997). Generally, auxin maintains the level of GA_3-oxidase and, consequently, GA_1 biosynthesis (Ross et al. 2000). However, the specific interaction between gibberellins and auxin may vary depending on the organ and the plant (Ross and O'Neill 2001). Vivian-Smith and Koltunow (1999) concluded that an auxin-like effect would cause the required level of active GA in *Arabidopsis* mesocarp to allow cellular expansion and alteration in mesocarp structure. Recently the role of auxin in parthenocarpic fruit development was highlighted with the implication of the *ARF8* gene in *Arabidopsis* and *IAA9* gene in tomato: in the *Arabidopsis* mutant *fruit without fertilization* (*fwf*), a mutation in *AUXIN RESPONSE FACTOR8* (*ARF8*) resulted in the uncoupling of fruit development from pollination and fertilization and gave rise to parthenocarpic fruit (Goetz et al. 2006). When functional, ARF8 acts as an inhibitor to stop further carpel development in the absence of fertilization. It is suggested that ARF8 binds to Aux/IAA proteins to form a protein complex that will bind to the promoters of a range of primary auxin-responsive genes that play an essential role in fruit initiation and development. This will repress the expression of those later genes until pollination. The pollination event, by triggering an auxin burst in floral organs (O'Neill 1997) could induce the degradation of the Aux/IAA protein (Gray et al. 1999), thus, abolishing the repression of the auxin-responsive fruit initiation genes. A good candidate for the Aux/IAA proteins that bind to ARF8 was found in tomato: down-regulation of the *IAA9* gene in tomato resulted in parthenocarpic fruit development. In addition, IAA9 was found to act as a transcriptional repressor of auxin signaling (Wang et al. 2005).

As reported above, Groot et al. (1987) showed that during normal fruit development, the final size of a fruit is positively correlated with the number of seeds in that fruit. Recently, a similar relationship was suggested for parthenocarpic fruit development. The seedless fruits of *Severianin* contain pseudoembryos, seed-like structures which are similar to those found in auxin-induced parthenocarpic fruits (Lin et al. 1983). These pseudoembryos are formed from the innermost layer of the integuments of ovules (Asahira et al. 1967). It is suggested that pseudoembryo development is closely related to fruit set and development (Kataoka et al. 2003). Thus, pseudoembryos may act as a substitute for seeds in the control of fruit growth. The development of pseudoembryos in *Severianin* keeps pace with fruit growth and is under control of GA_3 and/or of uniconazole, an inhibitor of gibberellin biosynthesis (Kataoka et al. 2003). The authors suggested that pseudoembryos influence early growth of fruits as a substitute for developing seeds.

The importance of endogenous gibberellins in parthenocarpic fruit development may not necessarily last for the entire period of fruit growth. The level of endogenous gibberellins during and after the rapid growth phase (3-4 weeks after anthesis) may not be important in later fruit growth. On the contrary, a low level of endogenous gibberellin from 1-4 weeks after anthesis might inhibit fruit growth (Kataoka et al. 2004).

At the cell expansion stage of parthenocarpic tomato fruit, it has been found that auxin is mainly synthesized within cells of partitions (Kojima et al. 2003). At the end of the expansion stage, the apoplast of the pericarp also participates in auxin synthesis. The authors found lower auxin concentrations in locules, including the seed-like structures, than in pericarps and partitions. In pollinated fruits, auxin is probably synthesized in the developing seeds because they had the highest auxin concentration (Kojima et al. 1994). The analogy between the seed and the pseudoembryo in relation to fruit development is therefore only partial.

Differential expression of three genes in the tomato ovary of the *pat-1* mutant was also observed and further characterized by Testa et al. (2002). One of these genes, named 'Clone 91', showed a higher expression in *pat-1* ovaries than in wild type ovaries and has homology to Glutamic Acid Decarboxylase 3 (GAD3). GAD3 was identified as transiently down-regulated after GA_3 application in the GA-deficient *Arabidopsis* mutant *gib-1* (Jacobsen et al. 1996). GAD3 is homologous to the maize TS2 gene, which has a pivotal role in controlling abortion of female primordia in the maize tassel (De Long et al. 1993). In *pat-1* mutants, a treatment with GA_3 did not affect the level of GAD3 in the ovary nor the typical phenotype of the *pat-1* mutant, namely short anthers, aberrant ovules, and parthenocarpy (Mazzucato et al. 1999). The already high endogenous level of GAs in parthenocarpic ovaries, reported by Mapelli et al. (1978) and Olimpieri et al. (2007), might be the cause for the lack of response.

Another mechanism may also trigger parthenocarpic fruit development in the *pat-1* mutant. Mazzucato et al. (1998) proposed that *pat-1* might be a mutation of a gene with homeotic functions, similar to the mutation in a TM8 MADS-box gene that results in deformation of the pistil and complete male and female sterility (Lifschitz et al. 1993). In a down-regulated TM8 transgenic tomato plant, parthenocarpic development of the fruits occurred before opening of the flower. Parthenocarpic fruits have also been observed in tomato flowers expressing antisense TM5 (Pnueli et al. 1994) and TM29 (Ampomah-Dwamena et al. 2002). Both TM8 and TM5 are tomato *Sepallata* (*Sep*) orthologs and have a

function in floral organ development. It is suggested that these genes inhibit parthenocarpic fruit development (**Figure 2**).

In the *Arabidopsis fwf* mutant, presented earlier, parthenocarpy is facultative and parthenocarpic siliques are 40% shorter than in the wild type. Similar to the *pat-1* mutant in tomato, the seed set under normal pollination conditions was reduced in the *fwf* mutant. Extended outer integuments were observed in the ovules of that mutant (Vivian-Smith et al. 2001).

The interaction between *pat-2* and *ls*

A mutant at the *Lateral Suppressor* (*ls*) locus, suppresses secondary meristem initiation in tomato (Schumacher et al. 1999). Plants of *ls* mutants also form flowers that do not initiate petal formation (Szymkowiak and Sussex 1993). Parthenocarpic fruit development in *pat-2* plants is inhibited by *ls* (Philouze 1983a). So, functional LS activity is required for seedless fruit development in tomato *pat-2* lines.

LS is a member of the *GRAS* gene family and the acronym GRAS is based on the locus designations of the three genes: *GAI*, *RGA* and *SCR*, referring to *Gibberellin Insensitive*, *Repressor of GA1* and *Scarecrow*, respectively.

The *GAI* gene regulates GA signaling (Sun 2000). As *LS* and *GAI* are members of the same family, this supports the hypothesis that the LS protein may play a role in the mechanism of localized regulation of GA responsiveness (Schumacher et al. 1999). This is also supported by the observation that, in the *ls* mutant, drastic changes in the levels of several plant hormones take place (higher levels of auxin and GA and lower levels of cytokinins; Tucker 1976).

A mutation at the *GAI* locus, *gai-1*, blocks responses to GA (Peng et al. 1997) and plants homozygous for *gai-1* do not develop GA_3-induced parthenocarpic siliques (Vivian-Smith and Koltunow 1999). The GA-deficient mutant requires exogenous GA to produce siliques following fertilization (Barendse et al. 1986). Apparently, a minimal level of endogenous biosynthesis of GAs is required for parthenocarpic and pollination-induced silique development (Vivian-Smith and Koltunow 1999).

In conclusion, changes in the regulation of GA, as observed in *ls* mutants, inhibit parthenocarpic fruit development in tomato lines carrying *pat-2*.

Genetic engineering of parthenocarpy

Spena and Rotino (2001) reviewed the potential applications of recombinant DNA technology for the development of parthenocarpic plants. They made a distinction between two approaches: the first is based on unbalanced embryo development and/or prevention of seed production, and the second consists of modulating the phytohormone content and/or activity in a specific organ in order to trigger parthenocarpic fruit development. The two examples discussed below are based on the second approach.

As stated earlier, parthenocarpic fruit development is mainly dependent on the level of auxin and gibberellin in the ovary during anthesis and fruit development. This has been demonstrated with parthenocarpic mutants and with parthenocarpic fruit development after exogenous application of auxin or gibberellin. It is therefore expected that parthenocarpy can be induced in transgenic plants in which a gene involved in the biosynthesis of either of these two plant growth hormones is expressed in the ovary (Varoquaux et al. 2000). Such transgenic plants should contain a gene which gives an increase in the level of a specific hormone and is under control of an ovary-specific promoter that is active during appropriate stages of flower development.

Genetic engineering aimed at obtaining parthenocarpic fruits was first successful in tobacco and eggplant, with the introduction of the *iaaM* gene. This gene is under control of the placenta and ovule-specific *DefH9* promoter from *Anthirrhinum majus* (Rotino et al. 1997). The *iaaM* gene codes for a tryptophan mono-oxigenase that converts the tryptophan into indolacetamide, which is then converted to auxin. The *DefH9* promoter is active during early stages of flower development. Seedless fruits were produced from emasculated flowers, and seeded fruits from pollinated flowers. In eggplant, both in the greenhouse and in open fields, a significant increase in yield was observed in transgenic plants compared to wild type (Donzella et al. 2000 ; Acciarri et al. 2002).

Genetic engineering offers the possibility of introducing the parthenocarpic trait into any plant species of agronomic interest (Rotino et al. 1997). For instance, in tomato, this has been achieved by introduction of the *DefH9-iaaM* construct. Transgenic parthenocarpic plants generally do not differ from the untransformed pollinated control with respect to fruit set and fruit quality (Ficcadenti et al. 1999; Rotino et al. 2005). However, the expression of the transgene can be too high in tomato cultivars which are more sensitive to high levels of auxins. As a consequence, malformations of the fruits can occur, comparable with the effects of an excess of exogenous auxin or by higher sensitivity to hormonal treatment (Pandolfini et

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

An alternative transgenic approach was based on the ovary-specific expression of the *rolB* gene. Transgenic plants expressing *rolB* show several symptoms characteristic for auxin treatment (Schmulling et al. 1988). No parthenocarpic fruit development was reported in transgenic tomatoes expressing *rolB* under control of its native promoter (van Altvorst et al. 1992). However, when the *rolB* gene was introduced into tomato under control of the ovary- and young-fruit-specific promoter of the Proline Rich Protein gene (*TPRP-F1*), a high level of expression of the *rolB* gene was detected during the early stages of fruit development and this resulted in completely parthenocarpic fruits (Carmi et al. 1997; Carmi et al. 2003; Barg and Salts 2000). Under both high and low temperatures, the performance of the transgenic lines, in respect with yield and fruit shape, was better than the control, untransformed lines (Shabtai et al. 2007). It is anticipated that *rolB* can also be used in other species where synthetic auxin is used to increase fruit set.

In conclusion, the process of fruit development is mainly dependent on the gibberellin and auxin biosynthesis pathways. Synthesis of active GA is under the influence of seed development. Parthenocarpic genes stimulate active GA synthesis and can therefore trigger development of fruits in the absence of pollination and fertilization.

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CHAPTER 2

Bigenic control of parthenocarpy in tomato

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Abstract

Parthenocarpy is the development of the fruit in absence of pollination and/or fertilization. In tomato, parthenocarpy is considered as an attractive trait to solve the problems of fruit setting under harsh conditions. We studied the genetic of parthenocarpy in two different lines, IL5-1 and IVT-line 1, both carrying *Solanum habrochaites* chromosome segments. Parthenocarpy in IL5-1 is under the control of two genes, one on Chromosome 4 and one on Chromosome 5. IVT-line 1 also contains two parthenocarpy genes, one on Chromosome 4 and one on Chromosome 9. In addition, we identified one stigma exsertion locus in IL5-1, located on the long arm of Chromosome 5, responsible for functional sterility, thus promoting parthenocarpic fruit development. It is likely that IL5-1 and IVT-line 1 share one parthenocarpy gene, near the centromere of Chromosome 4. By making use of the microsyntenic relationship between tomato and *Arabidopsis* in this genetic region, we identified *ARF8* as a potential candidate gene. ARF8 is known to act as an inhibitor for further carpel development in *Arabidopsis*, in absence of pollination and fertilization. A lesion in *ARF8* has been found to cause parthenocarpy in *Arabidopsis*. This candidate gene approach may lead to the first isolation of a parthenocarpy gene in tomato and will allow further use in several other crop species.

To be submitted

Introduction

In normal fruit development, the initiation of fruit set depends on the successful completion of pollination and fertilization. However, these processes depend on narrow environmental constrains (Picken 1984). Good pollen production is restricted to a specific temperature range, and air circulation is necessary to ensure pollen shedding. In tomato, failure to set fruit is therefore a common phenomenon under certain field conditions (high or low humidity combined with low or high temperatures) and in unheated greenhouses or tunnels during winter or early spring cultivation (George et al. 1984). Parthenocarpic fruit development, which is the growth of the ovary into a seedless fruit in absence of pollination and/or fertilization, offers an opportunity to overcome this problem of poor fruit set under harsh conditions. In tomato three sources of natural parthenocarpy have been widely studied because of their perspectives for practical application to produce seedless fruits (reviewed by Gorguet et al. 2005): *Soressi* or *Montfavet 191* (*pat-1*), *Severianin* (*pat-2*) and *RP75/59* (*pat-3/pat-4*). In addition, two other sources of parthenocarpy in tomato, IVT-line 1 and 2 (Zijlstra 1985), were found to give a higher and more stable level of parthenocarpy than *Soressi* and *Severianin*, though no detailed studies has ever been reported on them. Parthenocarpy in IVT-line 1 originated from *S. habrochaites* and was thought to be monogenic. Parthenocarpy in IVT-line 2 originated from *S. peruvianum* and was assumed to be polygenic. To date, the only mapped gene for parthenocarpy in tomato is *pat-1*, which is localized on the long arm of Chromosome 3 (Beraldi et al. 2004).

Recently Finkers et al. (2007b) have developed a set of introgression lines for *S. habrochaites* accession LYC4 in the *S. lycopersicum* cv. Moneymaker genetic background. Parthenocarpic fruit development and stigma exsertion was observed in one of the introgression lines (IL5-1), which carries the short arm of Chromosome 4 of *S. habrochaites* in homozygous state and the complete Chromosome 5 of *S. habrochaites* in homozygous or heterozygous state (R. Finkers, personal communication).

In this study we characterized and mapped four novel parthenocarpy genes responsible for the seedless fruit development in IL5-1 and IVT-line 1. In addition the position of the stigma exsertion locus was also identified. The syntenic relationship between the *pat* genes containing tomato region and the *ARF8 Arabidopsis* parthenocarpy gene was investigated. *ARF8* was determined as a likely candidate for two of the identified parthenocarpy genes.

Materials and Methods

Plant materials

For the mapping procedure in *Solanum habrochaites* LYC4, we originally used two BC_5S_1 populations that were part of the introgression line (IL) development program of Finkers et al. (2007b). These ILs contain chromosome fragment(s) of *Solanum habrochaites* LYC4, hereafter referred as *SH*, in a *Solanum lycopersicum cv*. Moneymaker genetic background, hereafter referred as *SL*. The two BC_5S_1 populations had previously been used for the selection of IL5-1 and IL5-2 (Finkers et al. 2007b) and will be hereafter denoted as populations 5-1 and 5-2. Population 5-1, segregating for the short arm of Chromosome 4 and the entire Chromosome 5, consisted of 174 plants. Population 5-2, segregating for the long arm of Chromosome 5, was composed of 183 plants. Plants were grown in a greenhouse in Wageningen, The Netherlands, under controlled conditions. Subsequently a set of relevant BC_5S_2 recombinant plants were selected and further studied.

For the mapping of parthenocarpy genes in IVT-line 1, we used an F_2 population coming from a single cross between the parthenocarpic IVT-line 1, and the non parthenocarpic *Solanum lycopersicum* cv. Moneymaker. The F_2 population of this cross was composed of 160 plants and grown under controlled conditions in a greenhouse in Wageningen, The Netherlands.

Flower morphology

Fresh flowers were collected and analyzed at pre-anthesis on the third flower truss of BC_5S_1 plants. Style length, ovary length and stamen length were measured to the nearest 0.1 mm. Stigma exsertion was determined by subtracting stamen length from the sum of the style length plus the ovary length, as presented by Chen and Tanksley (2004).

Characterization of parthenocarpy

To minimize pollination, in order to promote parthenocarpy, flowers were not vibrated. To classify the level of parthenocarpy, the first five fruit clusters of a plant were analyzed for fruit size, number of fruits per cluster, number of flowers per cluster and the presence of seeds. The size of the fruits was measured to the nearest 1 mm. Fruits were scored at mature stage. The trait parthenocarpy was calculated quantitatively, as the percentage of seedless fruits from the total number of flowers per cluster. Subsequently we calculated the average

percentage of seedless fruit set per plant, over the first five clusters. This percentage is hereafter referred as "parthenocarpy level".

DNA extraction

Two DNA isolation techniques were used. For most experiments total DNA was isolated from two young tomato leaves by using a CTAB DNA isolation method as described by Steward and Via (1993), adjusted for 96-well format using 1.2 ml COSTAR cluster tubes (Corning Incorporated). Leaf samples were crushed using a Retsch.

DNA isolation, for the selection of relevant BC_5S_2 progenies, was performed by a rapid alkaline (NaOH) based extraction method (Wang et al. 1993). This method was up-scaled to a 96-well format as described by Gorguet et al. (2006).

Molecular marker analysis

Genotypes were determined using PCR-based markers. Primers and enzymes of CAPS and SCAR markers TG441, CD64, CD31, TACL2, TG538, TG318 and TG358 have been described by Coaker and Francis (2004), and Brouwer and St. Clair (2004). Other CAPS and SCAR markers were generated based on RFLP and COS marker sequences previously mapped by Tanksley et al. (1992) or Fulton et al. (2002). The sequences of the RFLP and COS markers were available on the "SOL Genomics Network" (Mueller et al. 2005; http://sgn.cornell.edu). The conversion of RFLP and COS markers into CAPS and SCAR markers was performed as described in Gorguet et al. (2006). See **Table 1**. Each PCR reaction (25 µl) contained 10-20 ng of genomic DNA, 1x PCR-reaction buffer, 0.4 µM of each forward and reverse primer, 0.2 mM dNTPs and 0.5 unit Taq polymerase in demi water. PCR conditions were: hot start of 5' at 94 °C, followed by 39 cycles of 30" at 94 °C, 30" at annealing temperature (**Table 1**), 30" at 72 °C and a final extension of 7' at 72 °C. About 3 µl of PCR product was digested in a total volume of 15 µl for at least 3h with 1-2 units of restriction enzyme. After digestion, DNA fragments were separated on a 2-3% agarose gel. *Arabidopsis* orthologs of the COS markers used in this study were available on the "SOL Genomics Network" (Mueller et al. 2005; http://sgn.cornell.edu).

Reverse primers for microsatellite markers were labeled with IRD700 or IRD800. PCR reactions (10 µl) were prepared in the same proportion as described for CAPS markers, only with 0.1 µM forward and labeled reverse primer. PCR conditions were: hot start of 3' at 94 °C, followed by 30 cycles of 45" at 94 °C, 45" at 53 °C, 1' at 72 °C and a final extension of 3' at 72 °C. After the PCR, 10 µl LI-COR loading dye was added and the IRD700 labeled

fragments were analyzed on a LI-COR 4200 DNA sequencer, essentially following the method published by Myburg and Remington (2000).

AFLP markers were determined as described by Gorguet et al. (2006), using the same primer combinations as in Finkers et al. (2007a).

Table 1: Primer sequences and PCR reaction parameters for CAPS and SCAR markers

Marker type	Name	Use	Primer (5'-3') Forward, Reverse	Size[1] (bp)	TA[2] (°C)	Restriction enzyme	Chr[3]
CAPS	TG609	IVT-1	ATATGACTAGGAGGCAATGACTGA TTGCCTACTTATAACCCTGTGGA	400	52	*Alu*I	4
CAPS	CT258	IVT-1	CAATGAATCATCTGTGGTGATT TGCATTCCTCTGTGGATGCT	200	55	*Hinf*I	4
CAPS	At3g24010	IVT-1	ATGCAATCAGGATTGCTGATG CTGATCGAGCTGCTGAATATG	1000	55	*Taq*I	9
CAPS	T0156	IVT-1	GCGGTTGATTCACATCGTAA CCTGTAGCACCCAAAGGATG	1100	55	*HpyCH4*IV	9
CAPS	TG328	IVT-1	GAATGTCTAGTACCAGACTTAT AGTTCAATGTCCCTAGTTATAG	350	55	*Spe*I	9
CAPS	CT220	IVT-1	AAGCGAATTATCTGTCAAC GTTCCTGACCATTACAAAAGTAC	200	55	*Mse*I	9
CAPS	T1065	IVT-1	GACGGTGAAGGGTACCAAG CAGGAGTGCATGGGTAGGT	550	55	*Ssp*I	9
CAPS	At5g06360	IVT-1	GGCTATGCATGAAGAGTCATC GGCACCTCCCATTTTCCAGC	250	55	*Apo*I	9
CAPS	CT229	ILs	ATGGGCTGGGATCGTAGTAAA AAGCTTGCGATTCCCATAACAT	336	55	*Mwo*I	4
CAPS	T0208	ILs	AACGCCCCAGCCTGACTACA CTGGGGAGGTTTCGATTTCTG	514	55	*Hind*III	4
CAPS	TG483	ILs	CACTCCCATGGCAGATAAAA AGTGAAGTAAAACAAAGCCAAAAT	334	59	*Hph*I	4
CAPS	T0703	ILs	ATTTTTACGGGCAAGCGACTG CGTTGATCCCTCTATAATGGTG	456	55	*HpyCH4*IV	4
CAPS	T1068	ILs	CAAAGCAATGGGCAATGGT ACACAGCAGTTTCAGTAGGAC	500	55	*Hinc*II	4
CAPS	CT175	ILs	CAGCTAAGCGTTGACAGTTGAGAA ATGGCCGCGGTTTGAGC	750	55	*Mse*I	4
CAPS	TG182	ILs	GCTCGGGCAACAGTGAAC GCTAAGCAAATGAAAAACCAGA	335	55	*Taq*I	4
CAPS	TG370	ILs	ATGCTGCTGCCGGTTCCACT ATCGGGTCTCTAATTTCAGCAC	352	55	*HpyCH4*IV	4
CAPS	T0958	ILs	GTGTCGAACCCTTGGCAACAAT AGTTCTTTCAGCTTTTGGGTTAA	650	55	*Rsa*I	4
CAPS	T0891	ILs	GACCGCTACCTCAACTTCT CACTCTAATACTCCACTCAACATA	1200	55	*Dra*I	4
SCAR	TG339	ILs	GAAACCTTACCCCTCTA CGCTGTTTCTTGCCATTT	500, 436[4]	46		4
SCAR	T0529	ILs	TGGAGAGGAACAGGCTAAATC CACTCCGGCAACTGAAATGT	1600, 1650[4]	55		4
CAPS	T0635	ILs and IVT-1	CCAGAACCTCGACTCATCA TAGCCTCACAGTCTCAGTCAA	300	55	*Hinc*II	4
CAPS	TG60	ILs	TTGGCTGAAGTGAAGAAAAGTA AAGGGCATTGTAATATCTGTCC	1500	55	*HpyCH4*IV	5
CAPS	CT138	ILs	ACCAGCCCCGGAAGATTTTA GCGGTCAACTTCAGCAACTAT	900	55	*Rsa*I	5

[1] Size of undigested PCR product
[2] PCR annealing temperature
[3] Chromosome number
[4] PCR product size on *S. habrochaites* and *S. lycopersicum* respectively

Data analysis and mapping

To normalize the distribution of the recorded trait, the parthenocarpy level (percentage of seedless fruits) was transformed to a logit scale: logit (p) = log (p/(100-p)), (with p the percentage of seedless fruits on the first five clusters per plant).

Genetic linkage maps were constructed with JoinMap 3.0 (Van Ooijen and Voorrips 2001), applying the Kosambi mapping functions. QTL mapping was performed using the interval mapping and multiple-QTL mapping procedures of MapQTL 5 (Van Ooijen 2004). A logarithm of odds (LOD) threshold value of 3.0 was set (Van Ooijen 1999). A two-LOD support interval was taken as a confidence interval for a putative QTL.

Models for QTL analysis are presented hereafter.

In the BC_5S_2 population, the linear model used for the phenotype Y of an individual was:

$$Y_{i(j)} = \mu + X_{i(j)}\alpha_j + e_i \qquad \textbf{[model 1]}$$

where μ is the population mean, $X_{i(j)}$ is the number of *SH* alleles at the major locus for individual *i(j)* and α_j is the effect of one allele of the major gene. This effect differs according to j, the genotypic status of the minor gene. j = 1 (i=1…61) when the minor gene is homozygous *SL*; j = 2 (i = 1…21) when the minor gene is heterozygous and j = 3 (i = 1…24) when the minor gene is homozygous *SH*. e_i is the residual.

In the F_2 population used for the mapping of two parthenocarpy genes, the model used for the phenotype Y of an individual was the factorial combination of the two loci:

$$Y = \mu + X_1X_2 + e \qquad \textbf{[model 2]}$$

Where μ is the population mean, X_1X_2 is the effect of the combinations of the two parthenocarpy genes and e is the residual.

Results

Parthenocarpic fruit development was observed in the introgression line IL5-1 developed by Finkers et al. (2007b; **Figure 1**). IL5-1 carries an *SH* introgression on the short arm of Chromosome 4 and the complete Chromosome 5 from *SH*. In addition, flowers of IL5-1 presented an exserted stigma from pre-anthesis stage on, which altered self-pollination (**Figure 1**). We hypothesized that the observed parthenocarpy in IL5-1 was due to a combination of parthenocarpy gene(s) and functional sterility. Because ILs were initially vibrated to promote pollination, parthenocarpic fruit development was only obvious with the presence of a certain form of sterility. IL5-2, carrying only the long arm of Chromosome 5 of *SH* was not parthenocarpic but showed stigma exsertion. The conclusion was that the gene responsible for stigma exsertion is located on Chromosome 5.

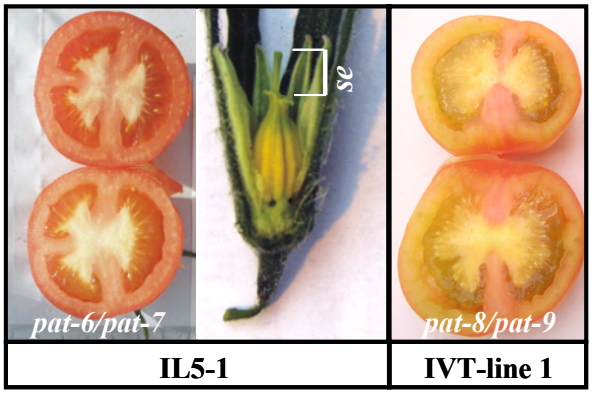

Figure 1: Seedless fruits observed on IL5-1 (left picture) and IVT-line 1 (right picture). Stigma exsertion observed on IL5-1 (middle picture).

To map and characterize the parthenocarpy and functional sterility genes observed in this material, we generated a genetic linkage map of the introgressed regions of the two ILs by making use of two BC_5S_1 populations: population 5-1 and population 5-2 segregating for the *SH* introgressions of IL5-1 and IL5-2 respectively.

Both populations were screened at juvenile stage with SCAR marker TG318, to select for plants with a homozygous or heterozygous *SH* introgression on Chromosome 5. The final population 5-1 and population 5-2 consisted of 74 and 66 plants, respectively. Hereafter, "population 5-1 and 5-2" refer to these selected plants. TG318 was chosen to screen the population due to its central position on Chromosome 5. Therefore by skipping the plants homozygous *SL* at TG318 locus on Chromosome 5, we enriched the population for plants with functional sterility to promote visible parthenocarpic fruit development. Subsequently,

parthenocarpy was evaluated in population 5-1 and functional sterility in populations 5-1 and 5-2.

Screening and segregation of parthenocarpy

Parthenocarpy was characterized quantitatively, as the percentage of seedless fruits in the first five clusters over the total number of flowers in those clusters. (**Figure 2**). The distribution of the parthenocarpy level ranged from 0 to 90.5% and the average size of the parthenocarpic fruits (4.73 cm) did not significantly differ (P>0.05) from the size of the seeded fruits (4.75 cm).

To improve the normality of the parthenocarpy level, this percentage was transformed into a *logit* scale and this last parameter was used for the QTL mapping procedure.

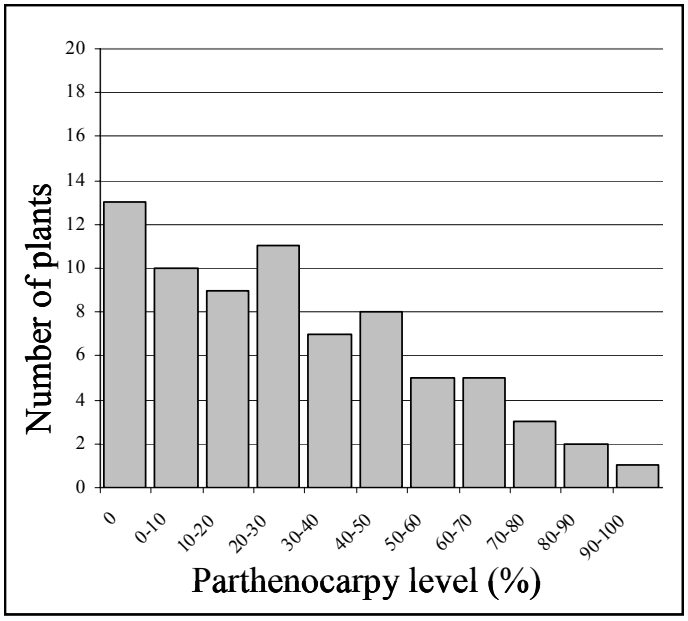

Figure 2: Frequency distribution of the parthenocarpy levels (percentages) over the first five clusters in the BC_5S_1 population 5-1 (*n* =74). i.e. "0" refers to plants without seedless fruit; "0-10" refer to plant with at least 1 seedless fruit and less than 10% of the flowers setting seedless fruits.

Genetic linkage map construction

The initial step in the development of linkage maps in the regions of the *SH* introgressions was to identify markers on the borders of the introgression. We developed a set of RFLP- and COS-derived PCR primer combinations in the expected regions of the introgressions and determined whether the loci were in or out. The border of the introgression on Chromosome 4 in IL5-1 was determined between markers T0635 and TG609, respectively at 55cM and 56cM on the EXPEN2000 linkage map. On Chromosome 5, the border of the introgression in IL5-2 was identified between markers CD64 and CD31, respectively at 27cM and 39cM on the

EXPEN1992 linkage map. *SH* introgression of IL5-1 is likely to cover the entire Chromosome 5 (Finkers et al. 2007b).

The genetic linkage map of the *SH* Chromosome 4 introgression of IL5-1 was generated using the population 5-1 (*n* =74). Thirteen RFLP or COS markers located on the short arm of Chromosome 4 in the EXPEN2000 map were converted into CAPS or SCAR markers and mapped in population 5-1. Two microsatellite markers, SSR43 and SSR72 (http://sgn.cornell.edu; Mueller et al. 2005), were added. The introgression on Chromosome 4 spanned 22.4cM which is almost the complete short arm of Chromosome 4, from the telomere to CAPS marker T0635. (**Figure 3a**).

The genetic linkage map of the *SH* Chromosome 5 was constructed using populations 5-1 and 5-2. A total of nine CAPS or SCAR markers were developed either based on available information (Coaker and Francis 2004; Brouwer and St. Clair 2004) or based on the RFLP sequence (Tanksley et al. 1992). The *SH* introgression of IL5-2 spanned 26.2 cM on the long arm of Chromosome 5, from the telomeric end to CAPS marker CD31. The limit of the *SH* introgression of IL5-1 on the short arm of Chromosome 5 (distal to TG441), was not determined, therefore the introgression spanned at least 57.4 cM. (**Figure 3a**).

The order of the markers on Chromosome 4 and 5 were in accordance with the Tomato-EXPEN2000 map and EXPEN1992 map of the "SOL Genomics Network" (http://sgn.cornell.edu). Overall the map distances in the Chromosome 4 and 5 introgressions were reduced by 58% compared to the EXPEN2000 reference map and 31% in the Chromosome 5 introgression (TG441-CT138) in comparison to the high-density RFLP tomato map (Tanksley et al. 1992).

Mapping of parthenocarpy

By applying Interval Mapping, one QTL for parthenocarpy (designated *pat-6*) was identified on Chromosome 4 (**Figure 3a**), close to the centromere, with the highest LOD value at CAPS markers T0958/T0891/T0635 (**Table 2**). This QTL explained 48.9% of the total variation. By using one of the three peak markers as cofactor, in an MQM mapping procedure, no extra QTL was detected in the introgressions.

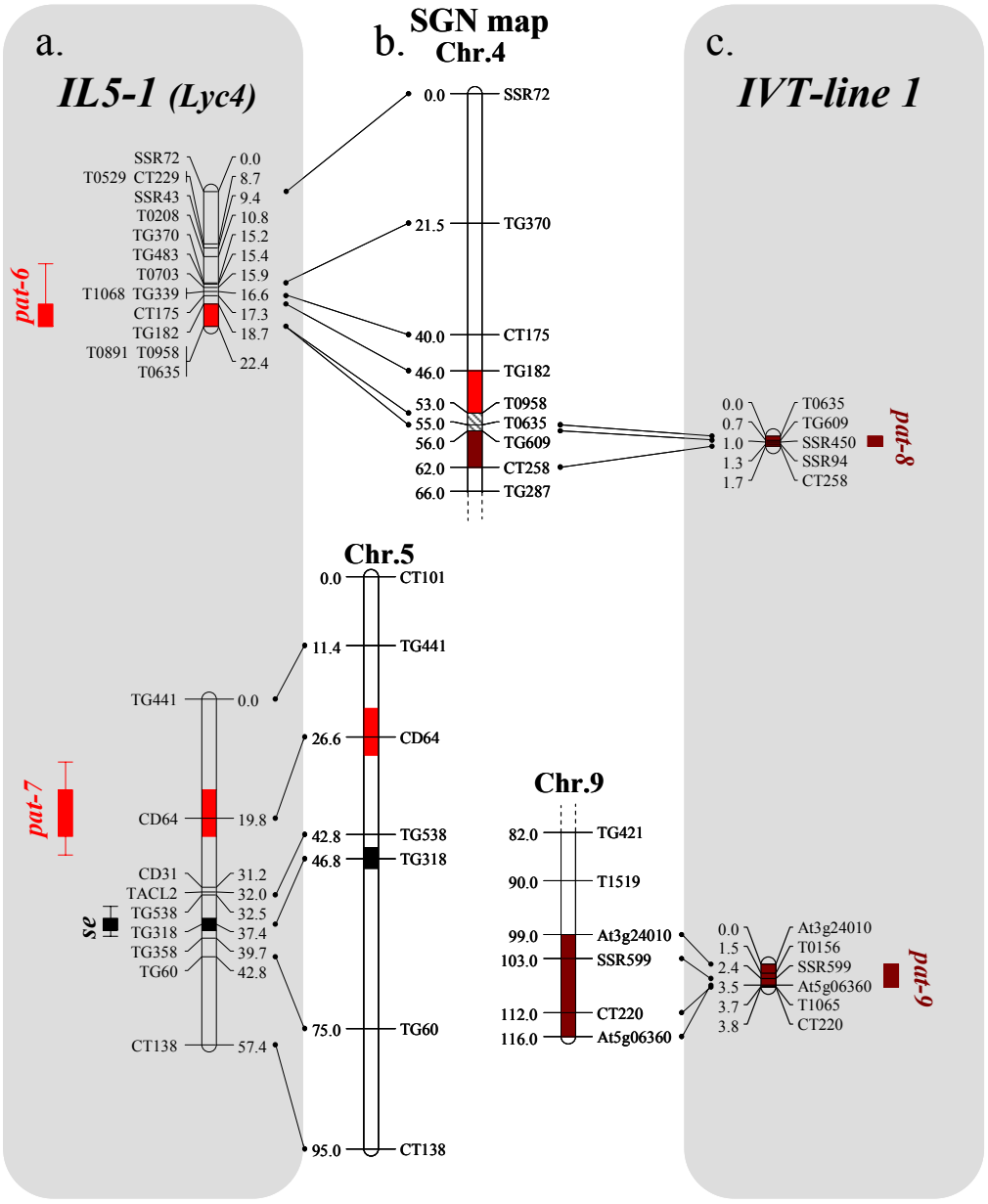

Figure 3: **a**: Genetic linkage groups of Chromosome 4 and Chromosome 5 *SH* introgressions of IL5-1 developed on population 5-1 (BC_5S_1). The position of the parthenocarpy QTLs (*pat-6* and *pat-7*) is indicated in light grey on the left of the linkage map. *pat-6* was mapped in the BC_5S_1 population and *pat-7* in the BC_5S_2 population. The location of the stigma exsertion locus (*se*) is indicated in black. The QTL bars indicate an interval in which the inner, thicker bar, shows a one LOD support confidence interval and the outer bars, thinner, shows a two LOD support confidence interval. **b**: SGN reference map for the short arm and centromeric region of Chromosome 4, the complete Chromosome 5 and the telomeric region of the long arm of Chromosome 9 (http://sgn.cornell.edu). The putative positions of the identified QTLs are represented by respective color codes. Because the one LOD confidence interval of *pat-6* and *pat-8* are overlapping, this overlap is indicated with dashed black lines. **c**: Genetic linkage groups of Chromosome 4 and Chromosome 9 *SH* introgressions of IVT-line 1 developed on the F_2 population. The position of the parthenocarpy QTLs (*pat-8* and *pat-9*) is indicated in dark grey. Map positions are given in cM. Maps and QTL alignments were performed with MapChart.

Table 2: Phenotypic means, peak LOD value, percentage explained variance and genetic action of the *logit* of 'parthenocarpy level' for *pat-6*, detected by Interval Mapping in population 5-1

	Genotype						LOD	Explained variation
Trait	SL/SL	n	SL/SH	n	SH/SH	n		
Logit (Parthenocarpy level %)	-1.73	(16)	-0.40	(45)	-0.18	(13)	10.8	48.9%
Parthenocarpy level[1]	1.8%		28.3%		39.8%			

[1] The logit numbers are transformed back into parthenocarpy level (percentages)

Confirmation of parthenocarpy genes in BC_5S_2

In order to confirm and narrow down the confidence interval of *pat-6* on Chromosome 4 and to study the potential interaction of *pat-6* with genes on Chromosome 5, we developed a set of recombinant progenies with small homozygous *SH* introgressions on the short arm of Chromosome 4. To develop this set of progenies, BC_5S_2 plants, recombinant for the Chromosome 4 introgression, were screened at seedling stage using molecular markers. Homozygous recombinant plants were selected and divided into 9 classes (**Figure 4**). Potential dominant parthenocarpy genes on Chromosome 5 could not be identified in the BC_5S_1 population because most plants were homozygous *SH* or heterozygous for Chromosome 5 due to the enrichment for plants with functional sterility. The evaluation of the recombinant progenies segregating randomly for Chromosome 5 allowed us to look for potential parthenocarpy genes on Chromosome 5. Selected plants were genotyped and evaluated for level of parthenocarpy from April to July 2006 (hereafter referred as Spring/Summer). After making cuttings the recombinant progenies were grown and re-evaluated in winter 2006/2007 (hereafter referred as Winter). The level of parthenocarpy in each recombinant progeny is presented in **Figure 4**. Only Chromosome 4 recombinant progenies carrying the *SH* chromosome segment TG182 – T0635 produced parthenocarpic fruits. This narrows down the position of the parthenocarpy gene *pat-6* to 3.7cM in population 5.1. The parthenocarpy level was significantly higher in spring/summer than in winter ($P<0.05$). More strikingly however, was the great variation of parthenocarpy level, from one progeny to another. This did not depend on the size of the *SH* fragment on Chromosome 4, eliminating the possibility of having a second parthenocarpy gene on that chromosome. Therefore we investigated the potential interaction of *pat-6* with Chromosome 5 in progenies 1 to 5. To study this interaction we applied the Multiple-QTL model (MQM) mapping function and used marker T0635 (one of the peak markers for *pat-6*) as co-factor. A QTL linked to marker CD64 on Chromosome 5 showed a significant effect on the expression of the parthenocarpy trait (**Figure 3a**). This QTL is hereafter referred as *pat-7* and was detected in

spring/summer and winter.

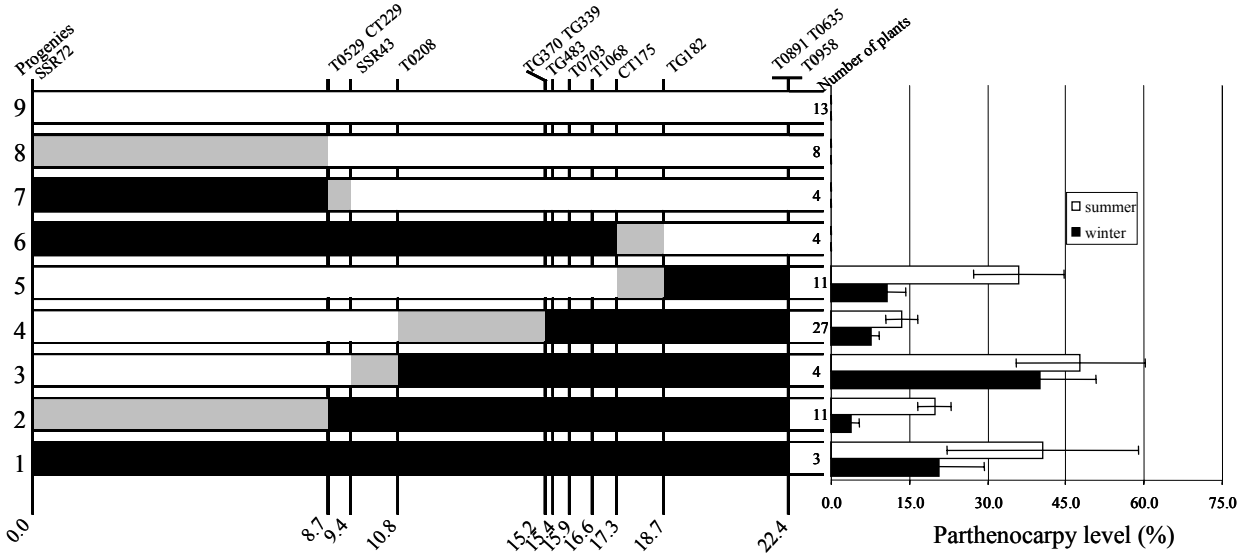

Figure 4: **Left**: Graphical genotypes of BC_5S_2 progenies for the short arm of Chromosome 4. Color code: Black stands for homozygous *SH*, white for homozygous *SL* and grey for unknown because the exact position of the recombination between the two closest flanking markers is unknown. Numbers under the bars indicate the position of the above mentioned markers, in cM. The reference number of each progeny is indicated at the far left of the graphical genotypes and the number of plants per progeny at the far right. **Right**: Average parthenocarpy level (%) over the first five clusters per specific progeny, in spring/summer (white) and winter (black), indicated with Standard Error bars.

In order to study the effects and interaction of *pat-6* and *pat-7* (**Table 3**), we searched for the best linear model to explain the observed variation. The *pat-6* gene is clearly the main gene in this interaction, but the size of its effect depends on the alleles of the *pat-7* locus. A higher level of parthenocarpy is observed when the two *SH* alleles of *pat-6* are present in combination with at least one *SH* allele of *pat-7*. In spring/summer, the parthenocarpy level observed on these plants is on average 41% with one *SH* allele of *pat-7* and 46% with two *SH* alleles (no significant difference). When *pat-6* is homozygous *SL*, the *pat-7* gene alone never shows parthenocarpy. Regarding these two observations *pat-6* can be considered as the major gene because it accounts for most of the effect on the parthenocarpy level, and *pat-7* can be considered as a minor gene that affects the parthenocarpy level of the *pat-6* alleles. **Model 1** was chosen among the simple linear models, because in this model the variable is explained by one major gene, which effect is depending on one minor gene. The coefficient of correlations for spring/summer and winter were respectively 64.8% and 57.9% which confirms that **model 1** was appropriate to this case. The details of this model are given in **Table 4**.

Table 3: Observed parthenocarpy level (percentages) for each combination of *pat-6* and *pat-7 allele's*, in the BC_5S_2, in spring/summer and winter

pat-6	*pat-7* (*SL*/*SL*)					*pat-7* (*SL*/*SH*)					*pat-7* (*SH*/*SH*)				
	spr/sum[1]		winter		n[3]	spr/sum		winter		n	spr/sum		winter		n
	Mean	SE[2]	Mean	SE		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
SL/*SL*	**0.0**	0.0	**0.0**	0.0	(13)	**0.0**	0.0	**0.0**	0.0	(6)	**0.0**	0.0	**0.0**	0.0	(14)
SL/*SH*	**4.1**	2.3	**2.4**	1.2	(7)	**4.8**	4.8	**16.1**	13	(2)	**45.5**	0.0	**37.5**	0.0	(1)
SH/*SH*	**11.6**	1.9	**4.6**	0.9	(41)	**41.0**	6.4	**14.8**	3.0	(13)	**46.0**	6.7	**27.2**	6.6	(9)

[1] spr/sum: Spring/summer
[2] SE: Standard Error
[3] n: Number of individuals per genotype

Table 4: Significance of model 1 and estimates of the parameters

	Spring/Summer	Winter
Probability of F	<0.001	<0.001
Coefficient Correlation	64.8%	57.9%
Constant (μ)	-6.801	-6.658
Effect of *SH* allele of *pat-6* (α_j):		
- When *pat-7* is *SL*/*SL* (α_1)	1.782	1.235
- When *pat-7* is *SL*/*SH* (α_2)	3.101	2.439
- When *pat-7* is *SH*/*SH* (α_3)	3.408	2.787

Characterization and mapping of functional sterility

Functional sterility, procured by exserted stigma, was evaluated in populations 5-1 and 5-2 by measuring the length of the stamen, style and ovary at pre-anthesis on the third cluster. The size of the exserted stigma was calculated by subtracting the stamen length from the length of the style and ovary. *SH* flowers have exserted stigmas, whereas the stigma of *SL* flowers is inside the anther cone at pre-anthesis. Interval Mapping showed one major QTL for stigma exsertion (*se*), on Chromosome 5 (**Figure 3a**) linked to marker TG318. Plants homozygous for the *SH* introgression at marker TG318 produced flowers with stigmas significantly more exserted than heterozygous or homozygous *SL* plants at that marker (LOD 19.2; **Table 5**).

Table 5: Phenotypic means, peak LOD value, percentage explained variance and genetic action of 'stigma exsertion', detected by Interval Mapping in BC_5S_1 population 5-1 and 5-2

Trait	Genotype						LOD	Explained variation
	SL	n[1]	H	n	SH	n		
Stigma exsertion (mm)	-0.53	(3)	0.45	(73)	2.04	(64)	19.23	47.9%

[1] n: Number of individuals per genotype category

Sterility was also characterized qualitatively in population 5-2 by determining the presence or absence of seeds. Plants without seeded fruits over the five characterized clusters were differentiated from plants producing at least one fruit with seeds. Population 5-1 was not

used for this purpose, because this population was also segregating for parthenocarpy which influences the setting of fruits with seeds. This trait co-segregated with CAPS marker TG318 on Chromosome 5 and thus with the QTL for stigma exsertion.

IVT-line 1

IVT-line 1 (**Figure 1**) has been developed from an interspecific cross between *Solanum habrochaites* (accession unknown) and *SL*, followed by several generations of back crosses followed by at least one selfing, in the early 1980's (Zijlstra 1985). In a first step towards the mapping of the parthenocarpy gene(s) present in IVT-line 1, we wanted to identify the positions of the *SH* introgressions. We screened a large number of known *S. habrochaites* AFLP markers on IVT-line 1. Seven *SH* introgressions could clearly be identified, on Chromosomes 4, 5, 6, 9 and 11. Only introgressions where known *S. habrochaites* AFLP markers were present can potentially be retrieved, therefore we cannot exclude the possible presence of other, small *SH* introgressions.

An F_2 population composed of 160 plants, coming from the cross between IVT-line 1 and *SL,* was grown, and the parthenocarpy level of the plants was evaluated in spring 2006 in Wageningen, The Netherlands, following the same procedure as previously described. The parthenocarpy level in the F_2 population ranged from 0 to 97%. About 44% of the plants did not produce any parthenocarpic fruit (**Figure 5**). Fruits with and without seeds, within a same cluster were significantly different in size ($P<0.05$), with averages of 4.36 cm and 4.11 cm, respectively.

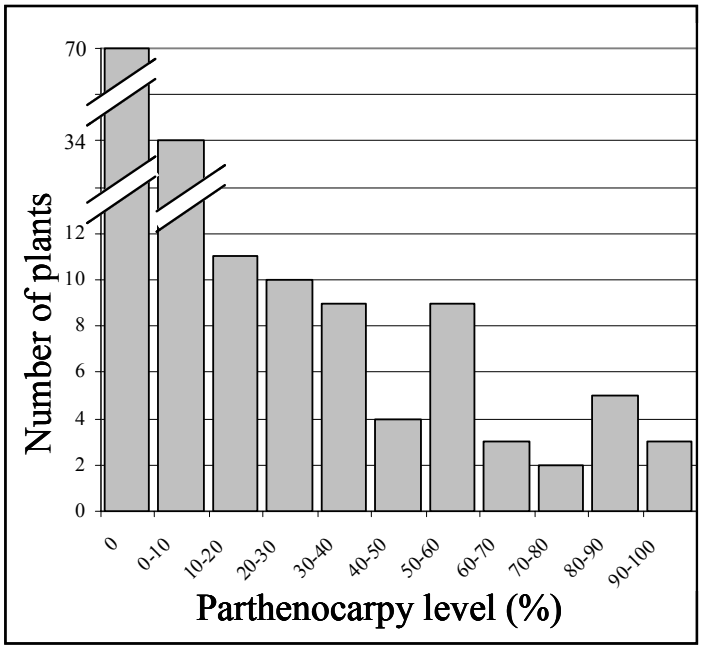

Figure 5: Frequency distribution of the parthenocarpy levels (percentages) over the first five clusters in the F_2 population of IVT-line 1 (*n* =160).

Because of the presence of an *SH* introgression around the centromere of Chromosome 4, where *pat-6* was previously mapped, we hypothesized that parthenocarpy in IVT-line 1 might also be under the control of *pat-6* or an allelic variant of it. After confirming that marker T0635 was in the *SH* introgression of IVT-line 1, we tested the association between marker T0635 (peak marker for *pat-6*) and the segregation of parthenocarpy observed in the F_2 population. This association was highly significant.

Although the association was significant it was clear that not all plants homozygous *SH* for T0635 produced parthenocarpic fruits, which may be due to the mode of action of another locus. To localize this other locus, we screened AFLP primer combinations on the set of F_2 plants homozygous *SH* at the T0635 locus. One AFLP marker, P18M51-219 was clearly associated with the parthenocarpy level of the selected plants and was known to be located in a Chromosome 9 introgression. Parthenocarpy in IVT-line 1 is therefore under the control of at least two genes, one located near the centromere of Chromosome 4 and one near the telomere of the long arm of Chromosome 9.

To map these two parthenocarpy loci more accurately, we developed a linkage map for the introgressions on Chromosomes 4 and 9 (**Figure 3c**). The borders of the introgression on Chromosome 4 were between markers T0635 and T0958 and between CT258 and TG287. On Chromosome 9, the upper limit of the introgression was located between markers T1519 and At3g24010 and likely spanned the rest of the telomeric region of the long arm of Chromosome 9. The two linkage groups were generated using eight CAPS markers, converted from RFLP or COS sequences and three SSR markers (SGN database; Mueller et al. 2005). The order of the markers on Chromosome 4 was identical as in the SGN reference map. In the introgression of Chromosome 9, few inversions of marker orders were observed. The introgression of Chromosome 4 spanned 1.7cM (7cM on the SGN reference map) and the introgression on Chromosome 9 was 3.8cM (17 cM in the reference map). This means a suppression of recombination of about 75%.

Parthenocarpy genes were mapped using MapQTL. We used a *logit* scale of the parthenocarpy level to improve the normality of the distribution. Because of the small genetic sizes of the two introgressions on Chromosomes 4 and 9, both complete introgressions were highly significant and it was not possible to narrow down the position of the two parthenocarpy genes. These two parthenocarpy genes are hereafter denoted as *pat-8* and *pat-9*, respectively for the gene located on Chromosome 4 and Chromosome 9. Because it was not possible to narrow down the genetic regions in which *pat-8* and *pat-9* were located, we

excluded the plants recombinant for one or both introgressions in the following analysis, and plants with an ambiguous scoring, in order to improve the accuracy of the calculations. In total, out of 160 F_2 plants, 137 plants were selected. An ANOVA showed that both loci had a highly significant effect on parthenocarpy and also the interaction between both loci was highly significant. Therefore among simple linear models we selected one in which the observed variation is explained by the interaction between two genes (**model 2**). The coefficient of correlation (R^2) was 73.7%, which confirms that the model chosen fitted well the observed variation. The observed and predicted effect, based on **model 2**, of the different allele's combinations between *pat-8* and *pat-9* is presented in **Figure 6** and **Table 6**. The absence of *SH* allele in either of the *pat-8* or *pat-9* loci results in no parthenocarpic fruits. The highest level of parthenocarpy is obtained when both genes are homozygous *SH* (76% of parthenocarpic level).

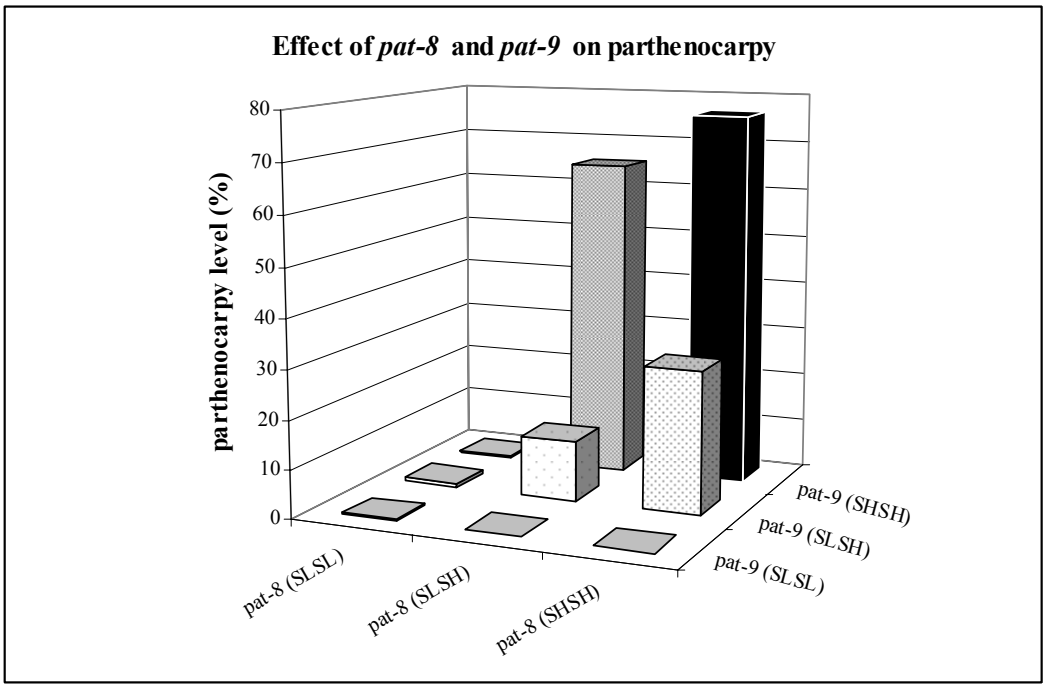

Figure 6: Interaction between *pat-8* and *pat-9* in the F_2 population (n=137). Each column stands for the parthenocarpy level (%) per allele combination recorded in spring (2006).

Table 6: Observed and predicted parthenocarpy levels (percentage) for each combination of *pat-8* and *pat-9* allele's, in the F_2 population (n=137)

pat-8	*pat-9* (*SL*/*SL*)				*pat-9* (*SL*/*SH*)				*pat-9* (*SH*/*SH*)			
	Pred[1]	Obs[2]	SE	n	Pred	Obs	SE	n	Pred	Obs	SE	n
SL/*SL*	***0.0***	**0.3**	0.2	(14)	***0.0***	**0.8**	0.4	(24)	***0.0***	**0.4**	0.4	(4)
SL/*SH*	***0.0***	**0.2**	0.2	(14)	***0.1***	**12.3**	2.7	(40)	***87.5***	**65.2**	6.0	(12)
SH/*SH*	***0.0***	**0.0**	0.0	(8)	***7.1***	**29.2**	4.4	(17)	***97.1***	**76.6**	12.8	(4)

[1] Predicted effect from model 2, initially calculated in logit scale, transformed back into percentages
[2] Observed mean for each combination of alleles (percentages)

Candidate gene analysis

One main parthenocarpy gene, known as *fwf* or *ARF8*, was recently isolated in *Arabidopsis* (Goetz et al. 2006). The use of COS markers in the present mapping work and the increasing availability of BAC sequence information linked to tomato markers, allowed us to study the potential microsynteny between the *ARF8 Arabidopsis* region and the *pat*-genes tomato regions. *Arabidopsis* orthologs of markers T0953 and C2_AT5G37360, both located in the *pat-6*/*pat-8* region, were found to be closely linked to *ARF8* (**Table 7**). In addition we found one extra *Arabidopsis* ortholog in the sequence of BAC clone HBa311A10, linked to marker C2_AT3G54770 in the *pat-6*/*pat-8* region, also located near *ARF8*. Eventually we could build a microsyntenic map that strengthens the hypothesis that *ARF8* may well be a homolog to *pat-6* or *pat-8* (**Figure 7**).

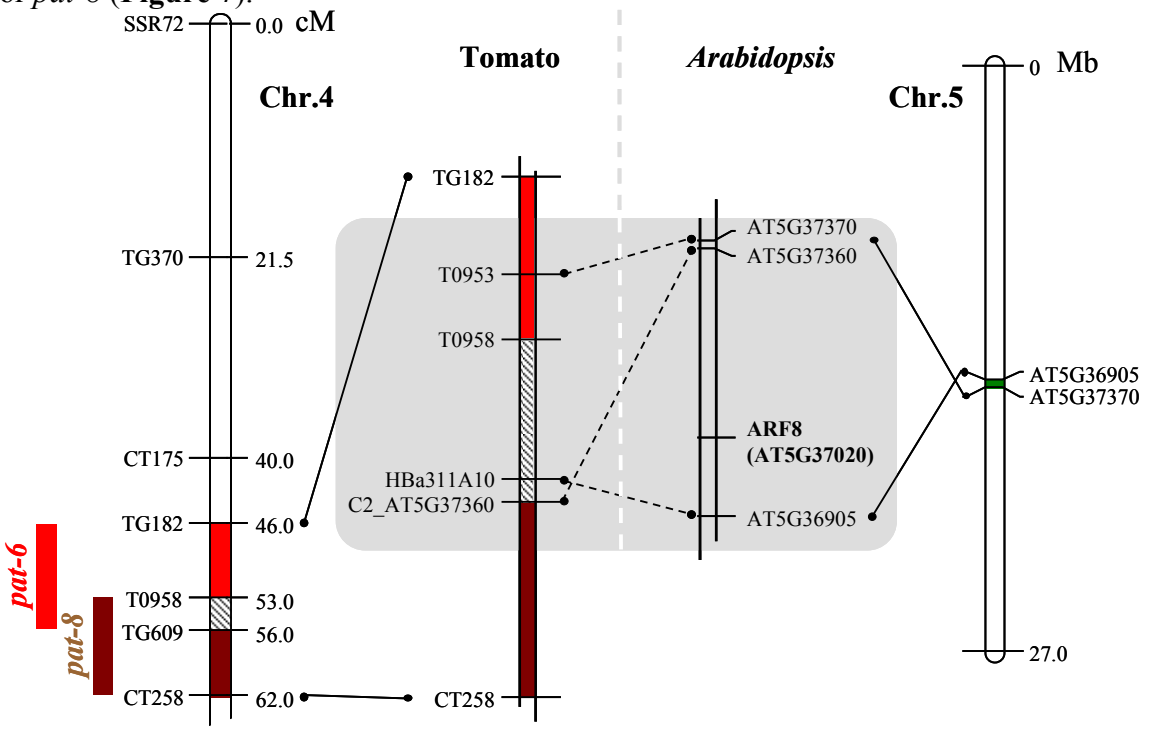

Figure 7: Microsynteny between the *pat6*/*pat-8* tomato region and *ARF8 Arabidopsis* region. A zoom in the two regions of interest, with the microsyntenic relations, is highlighted in gray.

Table 7: *Arabidopsis* orthologs of tomato markers in the *pat-6*/*pat-8* region compared to *ARF8*

Tomato		*Arabidopsis* genes			
Marker Chr.4	Position (cM)	Accession No.[2]	Gene name	Chr.	AGI coordinates (bases)
T0953	49.0	AT5G37370		5	14748652 - 14846779
HBa311A10[1]	55.3	AT5G36905		5	14575028 - 14577414
C2_AT5G37360	56.0	AT5G37360		5	14822459 - 14825466
		AT5G37020	***ARF8***	**5**	**14647258 - 14651617**

[1] BAC clone HBa311A10 is linked to marker C2_AT3G54770 which has been mapped at 55.3cM on Chr. 4 on the EXPEN2000 reference map.
[2] *Arabidopsis* orthologs were found in the SOL genomics network (www.sgn.cornell.edu)

Discussion

In this study we identified parthenocarpy in an introgression line of *Solanum habrochaites* in a *Solanum lycopersicum* genetic background. The recognition and scoring of parthenocarpy was simplified due to the functional sterility of this line. At least two genes were responsible for parthenocarpy in this line: one major gene, referred as *pat-6*, located close to the centromere of Chromosome 4, and one minor gene, referred as *pat-7*, on the short arm of Chromosome 5. The *pat-6* gene was initially mapped in a BC_5S_1 population, enriched for plants with functional sterility. This enrichment was done by selection for a marker on Chromosome 5 and thus also affecting the *pat-7* locus because the only plants that remained were heterozygous or homozygous *SH* at the *pat-7* locus. The *pat-7* locus was later on mapped in another set of specific progenies. The model used to explain the phenotypic variation in the selected progenies improves the understanding of the genetic control of parthenocarpy in this introgression line. In this model the variation is explained by a major locus, *pat-6* and a minor locus, *pat-7*. The effect of *pat-6* depends on the number of *SH* alleles at the *pat-7* locus. When at least one *SH* allele is present at the *pat-7* locus, the effect of *pat-6* is higher. There is hardly difference in the effect of *pat-6* between plants carrying one or two *SH* alleles at the *pat-7* locus. (**Table 4**). This explains why the *pat-7* locus was not detected in the BC_5S_1 population because the enrichment for functional sterility also resulted in an enrichment of *SH* alleles at the *pat-7* locus.

We mapped the stigma exsertion locus close to marker TG318, on Chromosome 5. When sterility was considered as a qualitative trait the sterility locus also co-segregated with marker TG318. This supports the hypothesis that the exserted stigma completely prevents self-pollination. Bernacchi and Tanksley (1997) have also characterized stigma exsertion in an introgression line of *S. habrochaites* acc. LA1777 in a *S. lycopersicum* genetic background. They have mapped the main stigma exsertion locus on Chromosome 2 and did not report a functional sterility locus on Chromosome 5. However, the introgression lines set of Montforte and Tanksley (2000) do not cover the complete genome and amongst others the central part of Chromosome 5 is missing. It is therefore impossible at this stage to speculate whether the stigma exsertion locus identified in our study is specific to accession LYC4, or inherent to the *Solanum habrochaites* species.

The position of *pat-6* led to the identification of *pat-8*, one of the two parthenocarpy genes identified in IVT-line 1. However the genetic control of parthenocarpy in this material

appears to be different from IL5-1. We identified two major genes, one close to the centromere of Chromosome 4, referred as *pat-8*, and one close to the telomere of the long arm of Chromosome 9, referred as *pat-9*. Both genes are of equal importance in the parthenocarpic expression. We could not determine whether *pat-8* is the same as *pat-6*. There are reasons to believe that there are at least allelic differences. The presence of *pat-6* alone is enough to obtain a certain level of parthenocarpy, but *pat-8* alone does not give any parthenocarpic fruit development. Whether this is due to different genetic background or different alleles or even different genes is not clear. In addition we observed that the size of seedless fruits produced in *pat-6*/*pat-7* plants was similar to the size of the fruits with seeds on the same material, but a significant smaller size was observed in the *pat-8*/*pat-9* material. To give a definitive answer to the question whether *pat-6* and *pat-8* are alleles from the same gene we need to fine-map them. However fine mapping will be hampered by the strong suppression of recombination that was observed in the populations used in this study. Reduction in recombination has been observed previously in introgressed intervals from wild tomato species (Rick 1969; Chetelat and Meglic 2000; Monforte and Tanksley 2000). Sequence divergence between wild and cultivated *Solanum* species has been suggested as cause for this phenomenon (Paterson et al. 1990).

The increasing availability of sequence information provided by the sequencing of BAC clones anchored to molecular markers, and the recent development and mapping of COS markers, helped us to study the syntenic relationship between tomato and *Arabidopsis* at *pat* genes regions. We aimed at studying the potential synteny of *pat* genes region with the *Arabidopsis* parthenocarpy locus, *ARF8*, region. Two COS markers and one putative gene of the *pat-6*/*pat-8* region were orthologs to *Arabidopsis* genes closely linked to *ARF8* (**Figure 7**). This finding provides evidence that *pat-6* and/or *pat-8* may be homologs of *ARF8*. *ARF8* has been found to act as an inhibitor for further carpel development, in *Arabidopsis*, in the absence of fertilization (Goetz et al. 2006). A lesion in *ARF8*, found in the *fruit without fertilization* mutant (*fwf*) has resulted in the uncoupling of fruit development from pollination and fertilization and therefore has given rise to seedless fruit. Further fine mapping and isolation of the parthenocarpy genes are necessary to confirm the potential homology of the present genes with *ARF8*. Alternatively the isolation of the tomato *ARF8* gene and further sequencing on IL5-1 and IVT-line 1 may already give information on the functionality of this candidate gene in both parthenocarpic lines. In addition, the mapping of the tomato *ARF8* homolog would also be sufficient to discard the possibility of homology between the *pat-6*/*pat-8*

gene(s) and *ARF8*, in case the tomato *ARF8* homolog would not co-segregate with the parthenocarpy locus.

In the present experiment, the parthenocarpy level was higher with *pat-8*/*pat-9* than with *pat-6/pat-7*. With *pat-8*/*pat-9*, we observed an average of 76.6% parthenocarpic fruit set in spring compared to 46% for *pat-6/pat-7*, in summer. In both cases, the higher the number of *pat* alleles is, the higher the level of parthenocarpy will be. We can only speculate about the level of parthenocarpy in one single line with all four *pat* genes. However, a too strong parthenocarpic expression often results in a fruit set that precedes anthesis. Therefore in such material the production of seeds, when needed, is hampered even when pollination is promoted. This limits greatly the interest for breeders who ultimately want to commercialize seeds. It would be valuable to study the level of parthenocarpy in a hybrid that combines several parthenocarpy genes, most of them or even all in heterozygous state. If such a hybrid displays a high level of seedless fruit set, it becomes possible to commercialize it by developing two parental lines that carry different parthenocarpy genes each, or one parental line that combines most of the parthenocarpy genes in a homozygous condition and the other parental line, used as mother line, carrying only few parthenocarpy genes with still a high level of seed production. Of course a reasonable level of seed production on the father is also needed. A detailed study of the parthenocarpy level of these new sources of parthenocarpy in different environments, different genetic background, as well as combining the four parthenocarpy genes or making new combinations will allow comparisons with the reference parthenocarpy lines such as *Soressi* (*pat-1*), *Severianin* (*pat-2*) and *RP75/59* (*pat-3*/*pat-4*). This will show the potential of these newly identified genes for practical breeding.

So far, only one parthenocarpy gene, *pat-1*, was mapped in tomato (Beraldi et al. 2004). In this study we mapped four extra parthenocarpy genes in tomato, which brings valuable input for the development of parthenocarpic tomato varieties and offers possibilities, because of the syntenic relationship within other Solanaceae crops.

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CHAPTER 3

High resolution fine mapping of *ps-2*, a mutated gene conferring functional male sterility in tomato due to non-dehiscent anthers

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Abstract

Functional male sterility is an important trait for the production of hybrid seeds. Among the genes coding for functional male sterility in tomato is the positional sterility gene *ps-2*. *Ps-2* is monogenic recessive, confers non-dehiscent anthers and is the most suitable for practical uses. In order to have tools for molecular assisted selection (MAS) we fine mapped the *ps-2* locus. This was done in an F_2 segregating population derived from the interspecific cross between a functionally male sterile line (*ps-2/ps-2; S. lycopersicum*) and a functionally male fertile line (*S. pimpinellifolium*). Here we report the procedure that has lead to the high resolution mapping of the *ps-2* locus in a 1.65cM interval delimited by markers T0958 and T0635 on the short arm of Chromosome 4. The presence of many COS markers in the local high resolution map allowed us to study the synteny between Tomato and *Arabidopsis* at the *ps-2* locus region. No obvious candidate gene for *ps-2* was identified among the known functional male sterility genes in *Arabidopsis*.

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Introduction

In plants, pollen maturation is normally followed by its release by dehiscence of the anthers (reviewed by Goldberg et al. 1993). This normal process of pollen formation and release may be hampered, resulting in male sterility. This phenomenon has been, for a long time, recognized as an important trait for hybrid seed production as self-pollination of male sterile plants is prevented. Male sterility can be divided into two groups: pollen sterility and functional male sterility. In tomato (*Solanum lycopersicum*, formerly *Lycopersicum esculentum*) more than 40 genes coding for pollen sterility have been identified (Georgiev 1991); however, the use of this trait in tomato breeding programs is restricted due to the difficulties of maintaining the pollen sterile lines as restorer genes have not been identified yet. Functional male sterility is characterized by a normal development of viable pollen that is not released for natural pollination due to abnormal morphology and functioning of the anthers (Georgiev 1991). One of the best characterised functional male sterility genes in tomato is *positional sterility 2* (*ps-2*), originating from a spontaneous mutation in the Czech tomato cultivar "Vrbicanske nizke", which is characterized by non-dehiscent anther bags (Atanassova 1999). Its low level of self pollination and the possible use of the viable pollen by manual anther opening, renders the *ps-2* gene the most suitable male sterility gene for tomato hybrid seed production. At least 19 tomato hybrid cultivars have been developed with the use of male sterility, mainly in Eastern Europe. Seventeen of them were based on the *ps-2* gene (Atanassova 1999).

The molecular mechanism of *ps-2* in tomato is unknown, but similar mutant phenotypes have been observed and studied mainly in *Arabidopsis,* such as the mutant *myb26* (Steiner-Lange et al. 2003), the mutants *coi1* (Feys et al. 1994; Xie et al. 1998) and *dad1* (Ishiguro et al. 2001), the double mutants *opr3*/*dde1* (Sanders et al. 2000; Stintzi and Browse 2000) and *aos*/*dde2-2* (von Malek et al. 2002; Park et al. 2002) and the triple mutant *fad3*/*fad7*/*fad8* (McConn and Browse 1996). They are characterized by defects in filament elongation, timing of anther dehiscence and often show reduced pollen viability. All, except the mutant *myb26,* are in one way or another involved in the jasmonic acid pathway. The mutant *coi1* (Xie et al. 1998) is insensitive to Jasmonic Acid, while the others are defective in Jasmonic Acid synthesis (Feys et al. 1994; Ishiguro et al. 2001; McConn and Browse 1996; Park et al. 2002; Sanders et al. 2000; Stintzi and Browse 2000; von Malek et al. 2002).

Atanassova (1999) observed up to 26% of self pollination in the most extreme cases, on cultivar "Vribicanske nizke" (*ps-2*/*ps-2*) under field conditions. In contrast, no self

pollination was found in a *ps-2* Advanced Breeding Line (*ps-2*ABL) bred for this trait (data not shown), which means that the problem of self pollination in *ps-2*/*ps-2* lines can be overcome by breeding selection. Because the expression of the *ps-2* gene depends on the genetic background, the availability of molecular markers closely linked to this gene is essential for breeding purposes.

Previously, Atanassova (1991) demonstrated a close linkage between the recessive gene *ps-2* and the *ful* gene on the short arm of tomato Chromosome 4. However this *ful* gene cannot be used as a marker in practical breeding programs. The availability of molecular markers closely linked to the *ps-2* gene is relevant for functional studies as well as for breeding purposes. The marker assisted introduction of the *ps-2* allele in parent lines would greatly enhance the use of this trait for the production of modern hybrid seeds.

In order to accurately map the *ps-2* gene on the tomato genome, we generated an interspecific F_2 population originating from a cross between a *ps-2* Advanced Breeding Line (*ps-2*ABL) (*S. lycopersicum*) and *S. pimpinellifolium* as male fertile parent. Here we report the high resolution fine mapping of the *ps-2* gene in a window of 1.65 cM on tomato Chromosome 4. The syntenic relationship between tomato and *Arabidopsis*, at the *ps-2* locus region, was determined and the knowledge of functional male sterility genes in *Arabidopsis* was used to search for candidate gene for *ps-2*. The possibility of map-based cloning of the *ps-2* gene is discussed.

Materials and Methods

Plant Material

We developed an F_2 mapping population from a single cross between the tomato (*S. lycopersicum*) true breeding line *ps-2*ABL and *S. pimpinellifolium* accession GI.1554. The *ps-2*ABL is homozygous for the *ps-2* mutation. *S. pimpinellifolium* accession GI.1554 is a close wild relative of *S. lycopersicum* and homozygous for *Ps-2/Ps-2* and hence functionally male fertile. The obtained F_2 population, composed of about 3070 plants, was used for the high resolution fine mapping of the *ps-2* locus. Plants were grown on rock wool, under controlled environment, in greenhouses in The Netherlands.

Another 176 advanced breeding lines, among which eight were *ps-2*/*ps-2*, was used to test the association between microsatellite markers and the *ps-2* locus.

A population of 98 Recombinant Inbred Lines (RILs) generated by four generations of single seed descent (F_6), originating from the cross between *S. lycopersicum cv*. Moneymaker

and *S. pimpinellifolium* accession GI.1554 was used to map AFLP markers on the tomato genome. This population has previously been generated and used as mapping population. A genetic linkage map composed of 323 AFLP markers and 69 RFLP markers was already available with this population (Finkers et al. 2002).

Genomic DNA extraction and molecular markers development

Total DNA was isolated by two different methods, following the NaOH extraction protocol (Wang et al. 1993) or the CTAB extraction protocol (Steward and Via 1993):

The NaOH DNA extraction was performed essentially as described by Wang et al. (1993). The protocol was slightly modified and up-scaled to 96 wells COSTAR plate format: Only one plastic ball was placed in each COSTAR tube (1mL) before collecting fresh leaf samples of about 0.5 cm^2. 20µl of NaOH (0.5 N) was added to the leaf material and the samples were crushed using a Retsch. 80µl of Tris buffer (100 mM) was added after crushing. Five µl of that mix was transferred to 100 µl of Tris (100 mM). One µl of this final mix was used for PCR amplification.

The CTAB DNA extraction protocol was performed mainly as described by Steward and Via (1993) up-scaled to a 96 wells COSTAR plate format. Leaf samples were crushed using a Retsch.

AFLP products were prepared essentially as described by Vos et al. (1995). *Eco*RI and *Pst*I primers were labeled with IRD700 or IRD800. AFLP fragments were separated on LI-COR 4200 DNA sequencer essentially as described by Myburg and Remington (2000). AFLP markers were named following the nomenclature presented by Haanstra et al. (1999). Positive AFLP primer combinations detected by BSA are described in **Table 1**.

To perform a bulked segregant analysis (BSA, Michelmore et al. 1991), two pools of ten functionally male sterile plants and two pools of ten functionally male fertile plants were made for AFLP screening by mixing equal amounts of AFLP pre-amplification products of the individual plants. After identification, informative AFLP markers were tested on 50 functionally male sterile F_2 (*ps-2/ps-2*) plants in order to determine the genetic distance between the identified AFLP markers and the *ps-2* locus.

To develop locus specific PCR markers, primers were designed based on the RFLP sequences publicly available in the SGN database (Mueller et al. 2005) and amplification products were generated from genomic DNA of the two parent lines. None of the PCR amplification products showed length polymorphism between *ps-2*ABL and *S.*

pimpinellifolium GI.1554. Therefore, the PCR fragments of both parents were sequenced (BaseClear BV, The Netherlands). When polymorphisms were found between the sequences of PCR products of the two parental lines, appropriate restriction enzymes were selected to develop CAPS markers (**Table 2**). When no appropriate restriction enzyme was found, dCAPS markers were developed if possible (Neff et al. 2002). The sequences of the PCR products of *ps-2*ABL were compared to the marker sequences of the SGN database in order to valid the primer specificity, using the SGN web BLAST interface (Mueller et al. 2005) (http://sgn.cornell.edu/tools/blast/simple.pl).

Table 1: Details on AFLP adaptors and primers giving linked AFLP markers to the *ps-2* locus

Primer and adaptor name	Use	Primer or adaptor sequence
*Mse*I adaptor	Adaptor	GAC GAT GAG TCC TGA G TA CTC AGG ACT CAT
M00	Universal primer	GAT GAG TCC TGA GTA A
M02	1 selective base	M00 + C
M47	3 selective bases	M00 + CAA
M51	3 selective bases	M00 + CCA
M52	3 selective bases	M00 + CCC
M54	3 selective bases	M00 + CCT
M60	3 selective bases	M00 + CTC
*Eco*RI adaptor	Adaptor	CTC GTA GAC TGC GTA CC CTG ACG CAT GGT TAA
E00	Universal primer	GAC TGC GTA CCA ATT C
E01	1 selective base	E00 + A
E32	3 selective bases	E00 + AAC
E33	3 selective bases	E00 + AAG
E36	3 selective bases	E00 + ACC
Pst I adaptor	Adaptor	CTC GTA GAC TGC GTA CAT GCA CAT CTG ACG CAT GT
P00	Universal primer	GAC TGC GTA CAT GCA G
P13	2 selective bases	P00 + AG
P15	2 selective bases	P00 + CA
P19	2 selective bases	P00 + GA

In order to increase the rate of polymorphisms between the two parental lines, primers of converted COS markers were specifically designed to amplify putative intron regions. The putative intron regions were detected with the "intron finder tool" (http://sgn.cornell.edu/tools/intron_detection/find_introns.pl ; Mueller et al. 2005).

PCR mix for CAPS and dCAPS markers was prepared as follows: Per reaction about 100 ng of DNA was mixed, in a total volume of 15 µl, with 10 ng of each primer, 1 µl of dNTPs (5 mM), 0.5 unit of *Taq* polymerase (SuperTaq, Enzyme Technologies Ltd, UK) and 1 x superTaq PCR reaction buffer. The PCR reaction started with a hot step at 94°C for 5 min., followed by 39 cycles of 30 sec. at 94°C, 30 sec. at appropriate annealing temperature (**Table**

2) and 30 sec. or 1 min. (depending on the expected PCR product size) at 72°C. The PCR reaction ended with a final extension of 7 min. at 72°C. About 3 µl of the amplified product was digested for 3 hours with one to two units of the appropriate restriction enzyme (**Table 2**) and appropriate restriction buffer, in a total volume of 15 µl. DNA fragments of CAPS and dCAPS markers were separated on a 1.5 % and 2 % agarose gel respectively, stained with ethidium bromide and visualized by UV light.

Table 2: PCR markers used in the genetic linkage maps

Marker name	Use	Primer sequence	Annealing T°C	Size (bp)	Restriction enzyme
Primers and enzymes used on *ps-2* mapping subpopulation					
TG483	CAPS	Fw: CACTCCCATGGCAGATAAAA Rv: AGTGAAGTAAAACAAAGCCAAAAT	59	334	*HPY*CH4IV
TG339	CAPS	Fw: AACATAGTAGCGTAATCCCACAGT Rv: ATTTATTTTCACGAAGCAAGTAGT	50	360	*Hinf* I
CT175	CAPS	Fw: CAGCTAAGCGTTGACAGTTGAGAA Rv: ATGGCCGCGGTTTGAGC	54	339	*Alu* I
CT192	CAPS	Fw: AGGGTCCTGTTGTCACTGTC Rv: CAATTGCCATCTCACCTAAA	55	200	*Hpy*188I
TG609	dCAPS	Fw: GATATGACTAGGAGGCAATGACTGA Rv: TGATAGTCAAAGATCACAGACATTTAGATT	54	200	*Hinf* I
TMS26	SSR	Fw: TTCGGTTTATTCTGCCAACC Rv: GCCTGTAGGATTTTCGCCTA	55	270	
TOM316	SSR	Fw: GAGTTGTTCTTTGGTTGTTT Rv: TAGATTTTTCGTGTAGATGT	46	215	
SSR94	SSR	Fw: AATCAGATCCTTGCCCTTGA Rv: AGCTGAGAAAGAGCAGCCAT	55	187	
TMS22	SSR	Fw: TGTTGGTTGGAG AAACTCCC Rv: AGGCATTTAAACCAATAGGTAGC	55	160	
EST259379	SSR	Fw: TTGGTCTCCCTCTTTATGCC Rv: GGCTTCATTGATGAACCCAT	55	150	
SSR450	SSR	Fw: AATGAAGAACCATTCCGCAC Rv: ACATGAGCCCAATGAACCTC	58	270	
TOM160	SSR	Fw: TGCTGAAGAATACAATGTTACC Rv: ATTGTTGGATGCTCAGTTTG	48	210	
TOM268	SSR	Fw: AGGGTATGAGATGAGACAAT Rv: TTTTACCTTCTTTACTTGGA	48	195	
Primers and enzymes used on recombinant subpopulation					
TG182	dCAPS	Fw: CGAACTGATCCTAATGCCCTGGTA Rv: CAGTTAAGAGAAGAACTGTCACTCA	55	200	*Dde*I
T1070	dCAPS	Fw: AATGGAGTTTCCAGTTGTAGA Rv: TGAACACAAAGACGATACCA	55	160	*Acc*I
T0958	dCAPS	Fw: GTGTCGAACCCTTGGCAACAAT Rv: AGTAAACTGTAGCTGACATTGGG	54	200	*Hph* I
T0953	CAPS	Fw: AAGTTCTTCACAATGAAACTTAC Rv: ACTTTCATTAATGGTCCTTAGGTC	55	350	*Sec* I
T0891	CAPS	Fw: GACCGCTACCTCAACTTCT Rv: CACTCTAATACTCCACTCAACATA	55	1200	*Ssi* I
cLED-7-G23	CAPS	Fw: GGATGAAGTTAGGGATCGTGTTA Rv: GCACACTGTATAAATCCATAGGT	55	250	*Mse* I
T0635	dCAPS	Fw: AATCCGAAAGTTTTGCCTACAG Rv: CAAGTGTGTCTGCAACTGGCTT	55	200	*Mse* I

The PCR mix for microsatellite markers was prepared in a total volume of 10 µl per reaction, by mixing 50 ng of DNA with 1 ng of each primer, 1 µl of dNTPs (5 mM), 0.5 unit of *Taq* polymerase (SuperTaq, Enzyme Technologies Ltd, UK) and 1x superTaq PCR reaction buffer. PCR profiles were as described by Areshchenkova and Ganal (1999; 2002), Suliman-Pollatshek et al. (2002) and the SGN database (Mueller et al. 2005). Reverse primers were labeled with IRD700 or IRD800. PCR products were run on a LI-COR 4200 DNA sequencer.

Evaluation of functional male sterility and map construction

Two types of phenotypic observation were performed to evaluate functional male sterility in the F_2 segregating population: (1) the ability of the anthers to release pollen; (2) the presence of seeded fruits:

To evaluate the ability of the anthers to release pollen, several flowers were collected per plant. Flowers were collected at anthesis and pollen release was tested by gently shaking the anther cones manually. Anthers classified as functionally male fertile could easily release pollen while the functionally male sterile anthers remained closed.

When the presence or absence of seeded fruits was used as phenotypic observation, plants with less than 10% of flowers setting seeded fruits were considered as functionally male sterile while plants with more than 50% of flowers setting seeded fruits were scored as functionally male fertile. Plants with an intermediate phenotype, between 10 and 50% of flowers setting seeded fruits, were considered as unknown.

We used JoinMap 3.0 (van Ooijen and Voorrips 2001) to generate the genetic linkage maps presented in this study, applying the Kosambi mapping function.

Microsynteny between tomato and *Arabidopsis* and candidate gene analysis

All COS marker sequences mapped in this study were scanned against the *Arabidopsis* genome sequence and *Arabidopsis* transcripts sequences from TAIR (http://www.Arabidopsis.org; Huala et al. 2001) using TBLASTX interface, in order to identify orthologs in *Arabidopsis*. The significance thresholds were identical to the ones set by Fulton et al. (2002). Positions and order of these orthologous genes in *Arabidopsis* were subsequently compared to the positions of the functional sterility genes identified in *Arabidopsis*.

Results

To perform the fine mapping of the *ps-2* locus, we developed an F_2 segregating population from the cross between *ps2*ABL (*S. lycopersicum*) and accession GI 1554 (*S. pimpinellifolium*). *S. pimpinellifolium* was used as wild type donor because the level of DNA polymorphism may be substantially higher in this interspecific mapping population, which is essential for the development of molecular markers. In addition, we preferred to use *S. pimpinellifolium* as a close relative of the cultivated tomato as this enabled us to work with a segregating population with vigorous and fertile progeny plants in order to maximize the accuracy of the phenotyping in the segregating population. The experimental approach followed in this study is presented in **Figure 1**.

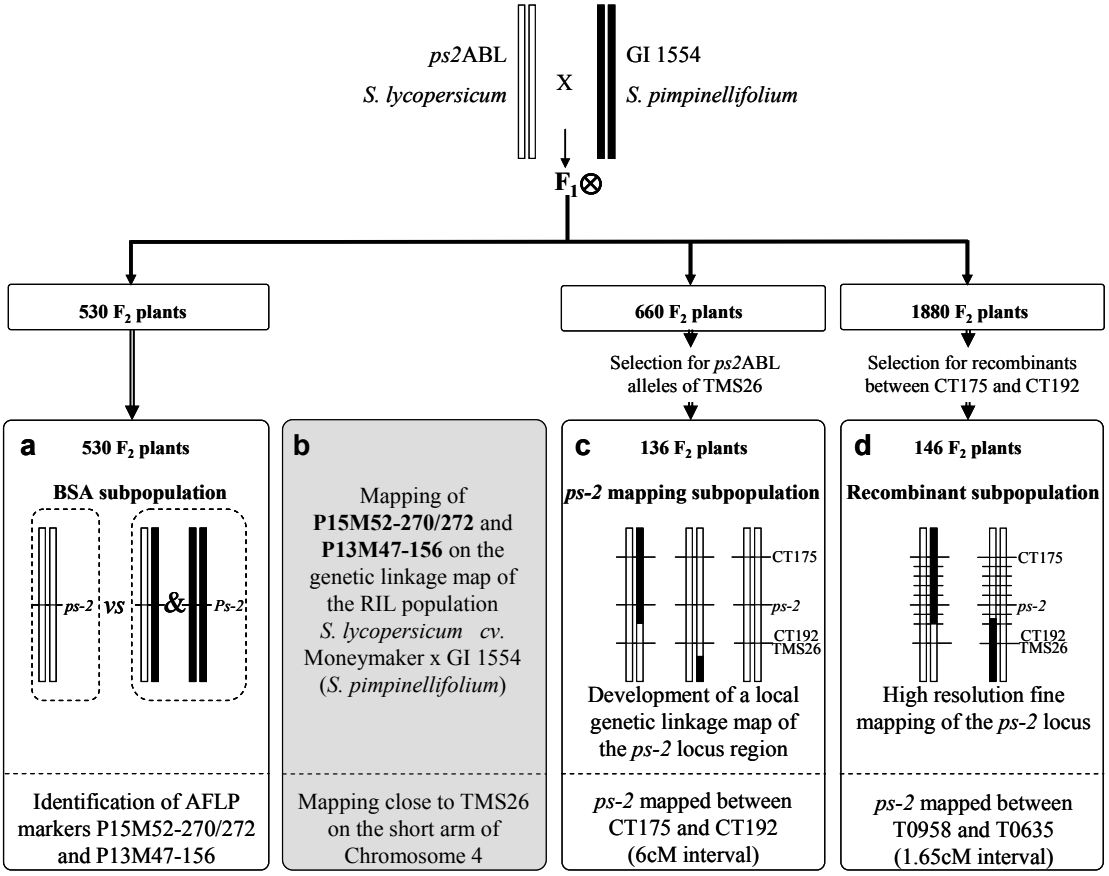

Figure 1: Representation of the populations and subpopulations used to fine map the *ps-2* gene (see also text). F_2 plants originate from the cross between *ps2*ABL, functionally male sterile (*S. lycopersicum*), and accession GI 1554 (*S. pimpinellifolium*). F_2 plants are divided into three subpopulations: the BSA subpopulation, the *ps-2* mapping subpopulation and the recombinant subpopulation. The fine mapping procedure was as follows:
a: the BSA subpopulation was used for the identification of AFLP markers closely linked to the *ps-2* locus.
b: these AFLP markers were mapped close to TMS26 on the short arm of Chromosome 4 in the RIL population *S. lycopersicon cv*. Moneymaker x *S. pimpinellifolium* (accession GI 1554)
c: the *ps-2* mapping subpopulation, obtained by screening F_2 plants for the presence of the *S. lycopersicum* alleles of the TMS26 locus, was used for the construction of a linkage map.
d: the recombinant subpopulation, composed of F_2 plants recombinant between CT175 and CT192, was used for high resolution mapping.

Identification of AFLP markers by BSA

The BSA subpopulation was used to identify AFLP markers closely linked to the *ps-2* locus. This subpopulation was composed of 530 F_2 plants. DNA of these F_2 plants was extracted according to the CTAB protocol. Functional male sterility was evaluated on these plants by testing the ability of the anthers to release pollen. Out of the 530 F_2 plants, 85 were clearly characterized as functionally male sterile, representing 16% of the total population, which is significantly lower than the expected 25% of the Mendelian ratio (X^2 = 22.7). This low percentage is likely to be due to a skewed segregation in the direction of *S. pimpinellifolium* alleles.

Two bulks of ten functionally male sterile plants and two bulks of ten functionally male fertile plants were formed and 220 AFLP primer combinations were tested in a BSA approach. The use of two times two bulks reduced the chance to find false positive markers. The AFLP primer combinations used in the BSA were *Pst*I/*Mse*I and *Eco*RI/*Mse*I. Because the functional male sterility is a recessive trait, a BSA approach only allowed us to find markers in repulsion of the *ps*-2 allele. Therefore the identified AFLP bands linked to the *ps-2* locus were specific to *S. pimpinellifolium* accession GI 1554. On average we observed eight AFLP bands specific to *S. pimpinellifolium* per *Eco*RI/*Mse*I primer combination and four bands per *Pst*I/*Mse*I primer combination.

Ten positive AFLP markers present in the functionally male fertile bulks and absent in the functionally male sterile bulks were identified and tested on 50 functionally male sterile plants of the BSA subpopulation. Three AFLP markers showed more than three recombinations on the 50 tested plants and were therefore not used in further analysis. The seven remaining AFLP markers, considered closely linked to the *ps-2* locus, were: P19M54-90, P19M51-185, E32M60-180, E36M47-240, E33M51-240, P15M52-270/272, and P13M47-156. The final number indicated after each primer combination represents the size of the AFLP fragment. AFLP marker P15M52-270/272 was a co-dominant marker in which the fragments of *S. lycopersicum* and *S. pimpinellifolium* were respectively 270bp and 272bp long.

Mapping of P15M52-270/272 and P13M47-156 in the RIL population

In order to determine the genetic position of the *ps-2* locus in the tomato genome, two of the seven AFLP markers detected by BSA were mapped in the RIL population. A genetic linkage map mainly based on AFLP markers has been developed on this later population and the

chromosome numbers and orientation of the chromosomes have been assigned. Both markers, P15M52-270/272 and P13M47-156 co-segregated and mapped on the short arm of Chromosome 4 between TG483 and the putative centromere (**Figure 2a**). TG483 was distant of 20 cM from the putative centromere in the RIL linkage map. The position of the putative centromere was suggested by the clustering of the *Eco*RI/*Mse*I AFLP markers (Haanstra et al. 1999) on the RIL genetic linkage map (**Figure 2a**).

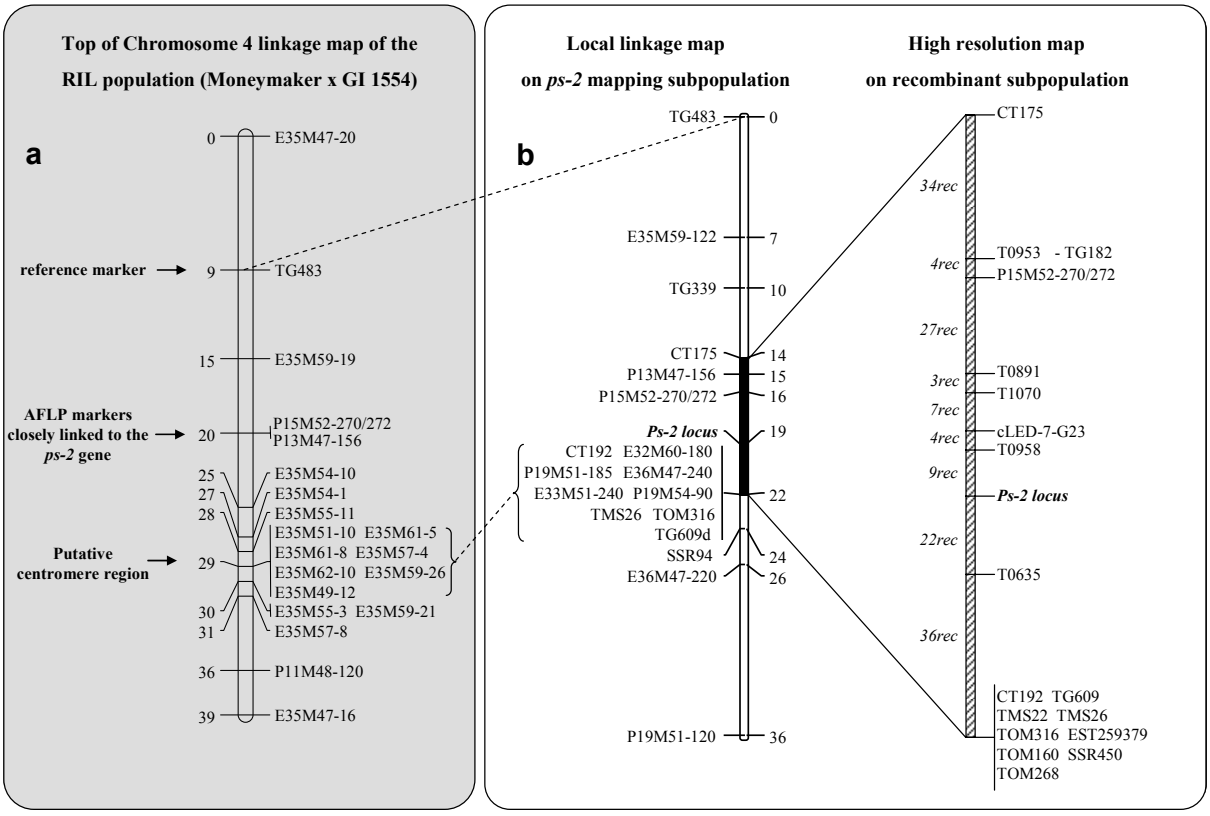

Figure 2: **a**: Genetic linkage map of the top of Chromosome 4 in the RIL population (Moneymaker [*S. lycopersicum*] x GI 1554 [*S. pimpinellifolium*]). AFLP markers P15M52-270/272 and P13M47-156 were mapped on this linkage map (LOD>8). The other AFLP markers of this linkage map do not follow the same nomenclature as presented in Materials and Methods section. Numbers along the linkage map indicate the position of the markers, in cM, relative to E35M47-20.
b: On the left, the local linkage map of the *ps-2* region developed on the *ps-2* mapping subpopulation (LOD>6). The clustering of AFLP and microsatellite markers corresponds to the putative centromere of Chromosome 4. Numbers along the local linkage map indicate the positions of the markers, in cM, relative to TG483. On the right, the high resolution map of the *ps-2* region developed on the recombinant subpopulation, delimited by CT175 and CT192. Numbers along the map indicate the number of recombinant plants identified in the respective intervals.

Linkage map of the *ps-2* locus region

In order to accurately generate a local genetic linkage map of the *ps-2* locus region, we developed the *ps-2* mapping subpopulation, enriched for functionally male sterile plants. For this purpose, about 660 F_2 plants were screened at an early plant stage with the microsatellite marker TMS26, located in the centromeric region of Chromosome 4 (Areshchenkova and Ganal 1999), which is at a distance of 9 cM to P15M52-270/272 and P13M47-156 on the RIL genetic linkage map (**Figure 2a**). DNA of these F_2 plants was extracted following the CTAB protocol. We used the microsatellite marker TMS26 to perform the selection for the *ps-2* mapping subpopulation, rather than P15M52-270/272 or P13M47-156, because simple co-dominant PCR markers are more accurate and easier to use than AFLP markers.

One hundred and thirty six plants were homozygous for the *ps2*ABL allele of TMS26. These plants were maintained in the greenhouse and allowed to grow further. Ten F_2 plants carrying at least one allele of *S. pimpinellifolium*, ten plants of both parents and of the F_1 were included as controls. The selected plants were scored for functional male sterility. These selected plants are hereafter denoted as the *ps-2* mapping subpopulation. By selecting for the *ps2*ABL allele of TMS26, we enriched the F_2 population with functionally male sterile plants and discarded most of the homozygous *Ps-2/Ps-2* F_2 plants. We could therefore perform a more efficient phenotyping because the functionally male fertile plants, among the selected F_2 plants, were in most of the cases heterozygous at the *ps-2* locus.

Out of the 136 plants homozygous for the *ps2*ABL allele of TMS26, 117 could be clearly phenotyped: The phenotype was based on the presence/absence of seeded fruits on the plants: 103 F_2 plants were scored as functionally male sterile and 14 as functionally male fertile. Nineteen plants had an intermediate phenotype and were therefore discarded for further analysis. To accurately map the *ps-2* locus, we developed a local genetic linkage map in the *ps-2* mapping subpopulation, with AFLP, RFLP derived CAPS markers and microsatellite markers according to the following approach: the seven linked AFLP markers detected by BSA were mapped on the *ps-2* mapping subpopulation. Six of the seven AFLP markers were dominant and one marker, P15M52-270/272, was co-dominant. The six dominant AFLP markers were specific to *S. pimpinellifolium* accession GI 1554. However, with the TMS26 selection performed to develop the *ps-2* mapping population, most plants were homozygous for the *S. lycopersicum* alleles in this region and, consequently, only few plants showed the presence of *S. pimpinellifolium* alleles near TMS26. Therefore, any *S. pimpinellifolium* allele in the *ps-2* plants reflected a recombination between this marker and

TMS26. This greatly enhanced the resolution of the mapping of the AFLP markers as well as the *ps-2* locus.

Microsatellite markers known to be located in or distal to the centromere of Chromosome 4 were tested for polymorphisms between the *ps2*ABL and the wild type *S. pimpinellifolium* parent (GI 1554). In total 15 microsatellite markers from Arashenkova and Ganal (1999; 2002), Suliman-Pollatshek et al. (2002) and the SGN database, were tested. Thirteen of them resulted in an amplification product on both parents. Four were not polymorphic between *ps2*ABL and accession GI 1554: SSR603, Tom89, Tom95 and Tom332. One marker, SSR86, did not map in the *ps-2* locus region. In total eight microsatellite markers were mapped in the *ps-2* mapping subpopulation: EST259379, TMS22, TMS26, SSR94, Tom316, SSR450, Tom160 and Tom268. Seven of them co-segregated in the putative centromere region and one, SSR94, mapped two cM distal to the putative centromere on the long arm of Chromosome 4 (**Figure 2b**).

In addition, some RFLP markers located on the RFLP map of Tanksley et al. (1992) between TG483 and the centromere of Chromosome 4 were converted into PCR markers. The RFLP markers TG483, TG339, CT175, CT192 and TG609 were successfully converted into CAPS or dCAPS markers and mapped on the *ps-2* mapping subpopulation. The position of these markers near the *ps-2* locus was relatively well conserved in comparison to the RFLP map of Tanksley et al. (1992) except for CT192, which mapped in the centromeric region of Chromosome 4 in the *ps-2* mapping subpopulation, though it has been mapped at five cM above the centromere in the RFLP map of Tanksley et al. (1992).

Eventually a local genetic linkage map was generated, with the *ps-2* gene mapped between the AFLP marker P15M52-270/272 and the cluster of markers (one genetic locus) corresponding to the centromere, in a window of 6 cM (**Figure 2b**).

To develop more markers closer to the *ps-2* locus we performed another BSA with *Pst*I/*Mse*I AFLP primer combinations. Contrasting bulks from the *ps-2* mapping subpopulation were constructed for the region between P15M52-270/272 and CT192 in such a way that only AFLP markers located between P15M52-270/272 and CT192 could be identified (data not shown). More than 180 AFLP primer combinations were tested, but no positive marker was found.

High resolution mapping of *ps-2* in a recombinant subpopulation

As we wanted to increase the genetic resolution of the *ps-2* locus, more than 1880 F_2 plants were screened at early plant stage for a recombination event between CT175 and CT192. To save time, DNA of these 1880 F_2 plants was extracted following the NaOH protocol. CT175 and CT192 were used for the recombinant screening because they were the closest flanking PCR markers and could easily be scored on agarose gels. None of the microsatellite markers in the centromere of Chromosome 4 could be scored on agarose gels. In total, 146 F_2 plants were selected. DNA of those 146 F_2 plants was extracted following the CTAB protocol in order to obtain stable high quality DNA. The selected recombinant plants were homozygous *S. lycopersicum* for either CT175 or for CT192 while heterozygous for the other marker. In this way all recombinants were informative for the *ps-2* locus.

The recombinant subpopulation was evaluated for functional male sterility based on the ability of the anthers to release pollen and based on the presence/absence of seeded fruits. In a first scoring, 54 plants were scored as functionally male sterile, 79 as functionally male fertile and 13 as ambiguous. These 13 last plants were maintained by cuttings and evaluated a second time. Nine were functionally male sterile, three were functionally male fertile and one remained ambiguous. The combination of both evaluations, ability of the anthers to release pollen and presence of seeded fruits improved the accuracy of the scoring of these plants.

To develop a high resolution linkage map, CT175, CT192, TG609, P15M52-270/272 and the microsatellite markers used for the *ps-2* mapping subpopulation, EST259379, TMS22, TMS26, SSR94, Tom316, SSR450, Tom160 and Tom268, were screened on the recombinant subpopulation. In addition, we intended to convert every COS markers (Fulton et al. 2002) located between TG182 and T0635 on the Tomato-Expen 2000 map (SGN database) into CAPS or dCAPS markers. We successfully converted six out of eight COS markers: T0953, T0891, T1070, cLED-7-G23, T0958 and T0635. No polymorphism was found for T1571 and T0964. The RFLP marker TG182 was also converted into a dCAPS marker. Polymorphisms between the parents were generally found in the intron regions, and it was often necessary to sequence several introns to find a polymorphism. The newly developed CAPS and dCAPS markers were mapped on the recombinant subpopulation. Eventually, we developed a high resolution map of the *ps-2* locus region, in which distances between loci are indicated in terms of number of recombinant plants (**Figure 2b**).

The order of the COS derived CAPS markers of the recombinant subpopulation was not entirely similar to the order in the Tomato-Expen 2000 map. Eventually the *ps-2* locus

mapped in a 1.65cM window delimited by T0958 and T0635 at about 0.48cM (9 recombinants) and 1.17cM (22 recombinants) respectively.

Microsynteny between tomato and *Arabidopsis* at the *ps-2* locus region

The presence of six COS markers in the high resolution map developed in this study allowed us to analyse the syntenic relationship between tomato and *Arabidopsis* at the *ps-2* locus region. Orthologs of the tomato COS markers, in *Arabidopsis*, and their corresponding Bacterial Artificial Chromosome (BAC) clones, were identified using a TBLASTX interface. Five of the six COS markers had only one significant *Arabidopsis* BAC clone match. COS marker T0958 matched to two unrelated *Arabidopsis* BAC clones (**Table 3**). When compared to the *Arabidopsis* gene database, only one corresponding ortholog was identified in *Arabidopsis*, for all six markers (**Table 3**). Four of the six identified orthologs were located on Chromosome 5 of *Arabidopsis*. Among them, the orthologs of the two closest markers flanking the *ps-2* locus, T0958 and T0635 were separated by 1.6megabases (mb).

The positions of the functional male sterility genes in *Arabidopsis* were determined (**Table 4**) and compared to the position of the six *Arabidopsis* orthologs previously identified. Two of these candidate genes are located on Chromosome 5 of *Arabidopsis* (**Figure 3**). Among them, *fad8* is distant of 1.2mb from the ortholog of T0958.

Table 3: Sequence similarities between tomato COS markers and *Arabidopsis* BAC clones and genes

Tomato	*Arabidopsis* BAC clones					*Arabidopsis* genes			
				TBLASTX				TBLASTX	
Marker	Accession No.	Chr	AGI coordinates (bases)	E - value	Score	Accession No.	AGI coordinates (bases)	E - value	Score
T0953	AB017069.1	5	14748652-14846779	3E-78	144	BT008856	14829915-14833750	3E-96	357
T0891	AB016886.1	5	19339994-19432496	8E-83	196	AY091394	19365145-19367895	2E-88	322
T1070	AC013483.5	3	2448977-2570030	7E-30	104	BT015904	2552483-2554927	7E-35	144
cLED	AC004512	1	24237602-24324793	5E-41	122	BT012855	24252515-24254065	3E-50	188
T0958	AB010077.1	5	15964645-16052024	3E-38	158				
	AB006697	5	2732479-2813448	2E-34	69	AY128719	2806324-2813227	1E-56	217
T0635	AB006704.1	5	4356798-4446056	1E-50	198	AY078971	4387337-4392230	3E-51	198

Table 4: Position of the functional male sterility genes in *Arabidopsis*

Gene name	Accession No.	Reference	Chr.	AGI coordinates (bases)
MYB26	Z95749	Steiner-Lange et al. (2003)	3	4576751 - 4578034
COI-1	AF036340	Xie et al. (1998)	2	16679040 - 16682826
DAD-1	AB060156	Ishiguro et al. (2001)	2	18486128 - 18487268
OPR3	AF132212	Sanders et al. (2000); Stintzi and Browse (2000)	2	2359111 - 2362176
AOS	AB007647	von Malek et al. (2002); Park et al. (2002)	5	17114823 - 17116623
FAD3	L22931	McConn and Browse (1996)	2	12788668 - 12792129
FAD7	AC073395	McConn and Browse (1996)	3	3499807 - 3502458
FAD8	AF056565	McConn and Browse (1996)	5	1664148 - 1666891

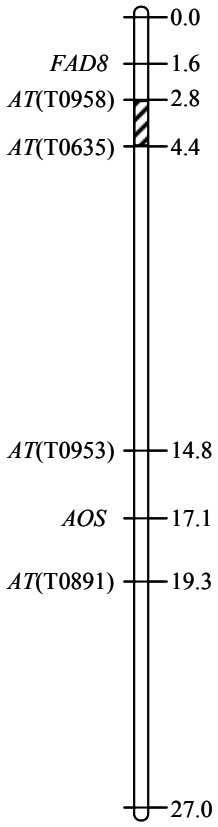

Figure 3: *Arabidopsis* Chromosome 5 represented with the orthologs of the tomato markers T0958, T0635, T0953 and T0891, and the functional male sterility genes of *Arabidopsis* identified on that chromosome. Numbers along the map indicate the position of the genes in megabases (mb) relative to the top of the chromosome. Orthologs of the tomato markers are indicated by "*At*" followed by the name of the marker between parentheses.

Association of the microsatellite markers with the *ps-2* allele in a population of advanced breeding lines

Microsatellite markers have a relatively high level of polymorphism among cultivated tomato lines (Vosman et al. 1992). Therefore in order to test whether the eight microsatellite markers, located near the *ps-2* locus (**Figure 2b**), could be used for molecular assisted selection of the *ps-2* allele, we studied the association of alleles of those microsatellite markers with the *ps-2* allele on a collection of advanced breeding lines. Among those advanced breeding lines, the functionally male sterile lines (including the *ps-2*ABL used to develop the mapping population in this study) were all developed from the same donor line with the *ps-2* trait. Therefore all the lines were most likely in Hardy Weinberg disequilibrium and the chance to identify association between the microsatellite markers and the *ps-2* allele was rather high.

The eight microsatellite markers present in the *ps-2* local map were screened on a subset of eight randomly selected advanced breeding lines (*S. lycopersicum*): four functionally male sterile lines and four male fertile lines. Two out of eight microsatellite markers, TOM316 and TOM268, showed polymorphism among this subset of advanced breeding lines. These two microsatellite markers were then screened on a collection of 176 advanced breeding lines, in which eight were functionally male sterile. Eight different alleles

were identified with TOM268 and four alleles for TOM316 over the set of advanced breeding lines. We did not identify one common allele that discriminated the eight functionally male sterile lines from the non male sterile plants. In conclusion, no association was found between the microsatellite markers alleles of the centromere of Chromosome 4 and the *ps-2* allele.

Discussion

In this study we developed a high resolution linkage map of the *ps-2* locus region and localized the *ps-2* gene in a window of 1.65 cM on the short arm of Chromosome 4, between T0958 and T0635. Only 1.65cM separate the two flanking markers T0958 and T0635 in the present study, though these two markers are distant of 4cM in the Tomato-Expen 2000 map (Fulton et al. 2002). The order of the COS markers was rather in accordance with the Tomato-Expen 2000 map, except for T0958. The present map was built with a resolution of 1880 F_2 plants. By contrast, the Tomato-Expen 2000 map has been generated using only 80 F_2 plants, which may explain the differences in positions of some markers within a small genetic interval. In addition, the use of a different wild species, in the present study, can explain the differences of genetic distances.

The accuracy of the phenotyping was the most important obstacle for the high resolution fine mapping of the *ps-2* locus. Though the expression of the *ps-2* gene in an *S. lycopersicum* genetic background can prohibit completely the dehiscence of the anthers and therefore the development of seeded fruits (data not shown), it was rather common, in this experiment, to observe seeded fruits on homozygous *ps-2/ps-2* F_2 plants. The *S. pimpinellifolium* genetic background may be the reason for the incomplete positional sterility observed in those cases. Influence of the genetic background for the expression of *positional sterility 2* has already been observed by Atanassova (1999) within *S. lycopersicum*, where up to 26% of self pollination was observed in the most extreme cases, on cultivar “Vrbicanske nizke” (*ps-2/ps-2*) under field conditions during a hot summer. The most accurate phenotyping was performed on the recombinant subpopulation, by combining the observation of the anther appearance and its ability to release pollen, and the presence/absence of seeded fruits.

We chose *S. pimpinellifolium* as a wild type donor in order to still have a good phenotypic expression of the trait in the segregating population. However this has cost us significant extra work in comparison to other wild relatives such as *S. pennellii*, for the development of markers polymorphic in the segregating F_2 population due to the rather low

level of DNA polymorphism between *S. lycopersicum* and *S. pimpinellifolium*, as found by Chen and Foolad (1999). Starting by the AFLP-BSA, where the amount of absence/presence polymorphism of AFLP bands between the two parents was rather low: 14 polymorphic bands per primer combination of *Eco*RI/*Mse*I, including the bands specific to *ps2*ABL and the bands specific to *S. pimpinellifolium,* and eight polymorphic bands per *Pst*I/*Mse*I primer combination. In comparison, an average of 42 polymorphic bands was identified per *Eco*RI/*Mse*I primer combination and 27 per *Pst*I/*Mse*I primer combination, between *S. lycopersicum* cv. Allround and *S. pennellii* LA716 (Haanstra et al. 1999). Also for the conversion of COS markers into CAPS or dCAPS markers, many introns sequences had to be compared between the two parents before identifying polymorphism. This low level of DNA polymorphism may explain why we could not identify closer AFLP markers to the *ps-2* gene during the second BSA procedure. In addition, this level of polymorphism will probably be one of the major obstacles during the map based cloning of the *ps-2* gene which will require frequent CAPS development.

The selection for *ps2*ABL (*S. lycopersicum*) alleles of TMS26, to develop the *ps-2* mapping subpopulation, helped us considerably to develop an accurate linkage map composed mainly of dominant AFLP markers specific to *S. pimpinellifolium*. With such a selection, the limitation in the mapping accuracy conferred by dominant markers was overcome.

The syntenic relationship between tomato and *Arabidopsis* at the *ps-2* locus region was investigated thanks to the presence of COS markers in the high resolution map. No global synteny was found between tomato and *Arabidopsis* over the entire *ps-2* locus region. However, the orthologs of the two closest markers flanking the *ps-2* gene were only separated by 1.6mb on Chromosome 5 of *Arabidopsis*. The foreseen map-based cloning of the *ps-2* gene will provide us extra sequence information to study this syntenic relationship more in depth. Microsynteny between tomato and *Arabidopsis* has already been proven to be of great help for the development of molecular markers in tomato (Ku et al. 2001).

No association was found between two microsatellite markers, TOM316 and TOM268, and *ps-2*, in the 176 advanced breeding lines. This finding confirms the need for extremely tight linkage to the locus of interest to get significant association between a marker and the *ps-2* gene, for practical use in applied breeding, as described in maize by Remington et al. (2001). In addition the development of markers for molecular assisted selection will be also limited by the extremely low level of DNA polymorphism within *S. lycopersicum*. The cloning of the *ps-2* gene will provide us the sequence information necessary for the

development of a universal marker to assist to the introduction of this gene into breeding lines. In addition the sequence of the *ps-2* gene will give us more insights into the physiology of anther dehiscence in tomato. Similar phenotypes have been observed in some *Arabidopsis* mutants where in most of the cases the Jasmonic Acid pathway was involved (Feys et al. 1994; Ishiguro et al. 2001; von Malek et al. 2002; McConn and Browse 1996; Park et al. 2002; Sanders et al. 2000; Stintzi and Browse 2000 and Xie et al. 1998). Once the *ps-2* gene is cloned, it will be interesting to know whether this gene is ortholog to one of those genes already identified in *Arabidopsis*. None of the known candidate genes in *Arabidopsis* is located between the orthologs of T0635 and T0958. However the gene *fad8* and the ortholog of T0958 were only separated by 1.2mb, which makes it a possible candidate gene for *ps-2*.

Better insights into the physiological process responsible for the non-anther dehiscence of *ps-2* could also be obtained by testing whether application of Jasmonic Acid on the mutated flowers would restore the wild phenotype and trigger anther dehiscence, as observed in some *Arabidopsis* mutants.

With the availability of the high resolution map of the *ps-2* locus, developed on a recombinant subpopulation (this study), a map based cloning procedure can be initiated, which will lead us to the cloning of the *ps-2* gene. Assuming an even distribution of the recombination events between T0958 and T0635 in the recombinant population and considering an average physical distance of 750kb/cM (Tanksley et al. 1992), we may have a physical interval of 35kb between each recombination event. This physical distance may even be smaller as it is usually observed in the genes rich regions (Peters et al. 2003). Sequences of the COS markers closely linked to the *ps-2* locus in this study have been used as probes for the hybridization of BACs, in the frame of the tomato sequencing project (Mueller et al. 2005). Markers T0891, T1070 and T0635 have successfully matched BAC clones: BAC clones linked to T0635 and T1070 were not part of the same contig indicating that the physical distance separating T1070 and T0635 is larger than an average BAC clone size. Markers T0891 and T1070 are linked to BAC clones of the same contig though these markers are separated by three recombination events in the high resolution linkage map. This may indicate that the recombination frequency in the region of the *ps-2* locus is high enough to intend to clone the *ps-2* gene by map-based mean

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CHAPTER 4

Molecular characterization of the gene responsible for functional sterility, due to non-dehiscent anthers, in tomato

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Abstract

The recessive mutation *ps-2*, which appeared spontaneously in tomato, confers functional male sterility due to non-dehiscent anthers. In this study we isolated and characterized the *PS-2* gene. A single nucleotide mutation in a novel tomato polygalacturonase gene is responsible for the *ps-2* phenotype. We named this new gene *TDPG* (*Tomato Dehiscence Polygalacturonase*). The mutation in *TDPG* is responsible for an alternative splicing during maturation of the pre-mRNA, which leads to an aberrant mRNA. The presence of ethylene and jasmonate regulatory elements in the promoter region of *TDPG* suggests that this gene is under control of both hormones. Differentiation between *ps-2* and wild type anthers only appears in the final developmental stage in which the stomium remains closed in the mutant. Because *TDPG* transcripts were also detected in maturing fruits we suggest a similar role in fruit cell wall degradation. To our knowledge, this is the first functional sterility gene isolated in the *Solanaceae* family. The specific expression of the *Arabidopsis* homolog of *TDPG* in the anther dehiscence zone suggests a conserved mode of action over the plant kingdom, which means that the repression of *TDPG* homologs may be a potential way to introduce functional sterility in other species.

To be submitted

Introduction

In higher plants mature pollen are released from the anther by dehiscence, which consists of a succession of cell destructions occurring successively in the tapetum, the septum and ultimately in the stomium. In tomato, after degeneration of the tapetum, exothecium cells are reinforce, creating a breaking force in the stomium that eventually leads to pollen release (Keijzer, 1987).

Recent progress was made in understanding the molecular control of anther dehiscence, which involves mainly the discovery of the implication of Jasmonic Acid (JA) and ethylene. Several mutants affecting diverse steps in the synthesis of JA in the anthers were identified in *Arabidopsis*. The mutants displayed a delay of anther dehiscence (reviewed by Scott et al. 2004). The role of ethylene signaling in this phenomenon was highlighted by Rieu et al. (2003) who observed a delay in the dehiscence of anthers of ethylene insensitive tobacco plants.

Polygalacturonases (PGs) belong to one of the largest hydrolase families (Torki et al. 2000; Markovic and Janecek 2001). PG activities have been shown to be associated with a wide range of plant developmental programs (reviewed by Hadfield and Bennett 1998), among them, anther dehiscence. Activity of PGs has been observed in the dehiscence zone of anthers of maize, tobacco, oilseed rape and *Arabidopsis* (Dubald et al. 1993; Sander et al. 2001). However their role in the dehiscence process has never been studied in detail.

We have recently fine mapped the *positional sterility-2* gene, conferring non-dehiscent anthers in tomato (Gorguet et al. 2006). Here we report the isolation of the *ps-2* gene by positional cloning. A single mutation in the coding sequence of a novel polygalacturonase gene is responsible for this phenotype. We found that this mutation affects one of the intron splicing recognition sites of the gene giving rise to an aberrant mRNA, lacking one of the exons. Microscopic observation of the *ps-2* phenotype revealed similarities with what was already found in *Arabidopsis* and tobacco mutants (reviewed by Scott et al. 2004; Rieu et al. 2003). This new PG gene, hereafter designated as *Tomato Dehiscence PG* (*TDPG*) is also expressed in maturing fruits. The expression control of *TDPG* by JA and ethylene as well as the involvement of *TDPG* in the process of fruit ripening are discussed.

Materials and Methods

Plant materials

The F_2 recombinant sub-population developed from the cross between *ps-2*ABL (*S. lycopersicum*, homozygous for the *ps-2* mutation) and *S. pimpinellifolium*, was used for genetic mapping. This population, segregating for *ps-2*, is composed of 146 F_2 recombinant plants in the *ps-2* locus region (Gorguet et al. 2006).

Another 176 Advanced Breeding Lines (ABLs), eight of them being *ps-2*/*ps-2*, were used to test the association between the identified SNP and the *ps-2* locus.

Anthers of *ps-2*ABL and cv. Moneymaker were used for microscopic observations.

Microscopy

Plant material was fixed for 24 h at 4°C in 0.1 M phosphate buffer, pH 7.0, containing 4% paraformaldehyde. Samples for scanning electron microscopy were processed as described in Dornelas et al. (2000), and digital images were obtained using an Orion Framegrabber (Matrox Electronic Systems, Unterhaching, Germany). Samples for light microscopy were embedded in Technovit 7100 (Hereaus Kulzer, Wehrheim, Germany), sectioned, stained with toluidine blue, and mounted in Euparal (Chroma-Gesellschaft, Kongen, Germany).

BAC library screening and contig construction

We used the tomato *Hind*III BAC library constructed from genomic DNA of the cultivar accession Heinz 1706. The Heinz library is a 15 genome equivalent with an average insert size of 114.5kb (Budiman et al. 2000). Screening of the BAC library was performed by PCR amplification, first on plate pools and then on individuals. Plasmid DNA of the positive BAC clones was then isolated and used for further analysis.

BAC ends sequences of the positive BAC clones were obtained from the SGN database (Mueller et al. 2005). Conversion of the BAC ends sequences into CAPS or dCAPS markers was performed as described by Gorguet et al. (2006). Details of the PCR markers derived from BAC ends sequences are presented in **Table 1**.

Fingerprinting patterns of individual BAC clones were generated essentially as described by Brugmans et al. (2006), using the *Hind* III/*Taq*I enzyme combination.

BAC DNA sequencing and analysis

The size of BAC clone 143M15 was estimated by pulse field gel electrophoresis. BAC clone 143M15 was sequenced via the shotgun-sequencing method by Greenomics (The Netherlands). PCR markers derived from the candidate genes identified on the BAC sequence were developed as described by Gorguet et al. (2006). To identify putative genes, the final BAC DNA sequence was scanned against the tomato Unigene database from SGN (Mueller et al. 2005) and the *Arabidopsis* gene models database from TAIR (http://www.Arabidopsis.org; Huala et al. 2001), using the TBLASTX interface of SGN (Mueller et al. 2005), with a significance threshold of $1E^{-10}$. PCR markers based on putative ORF sequences were developed and screened on the recombinant population as described by Gorguet et al. (2006). Details of these PCR markers are given in **Table 1**.

Table 1: PCR markers used in the genetic linkage maps

Marker name	Use	Primer sequence	Size (bp)	Restriction enzyme
Markers developed from BAC ends sequences				
67F23-T	CAPS	Fw: CTACTCTTCCGCCATAACTG Rv: GATCCAAACGAACAAAAGTCA	599	*Hinc*II
67F23-S	CAPS	Fw: TCATTCCGTTGCTGAATGAGA Rv: ATAACTTATATCACTCCCAATCA	413	*Dra*I
69C22-T	dCAPS	Fw: TCTTTCGATATTTTTCAGAACTAA Rv: TGAGATGTTTGCAATAACATTCT	200	*Dde*I
143M15-S	CAPS	Fw: CATCGAAGTAACAGAGATATTA Rv: CCATAGGGATTATGATGTGTA	369	*Mwo*I
114C15-T	CAPS	Fw: GCACTGAAGAATGGATAGACTC Rv: GGAATTGACCAAAAAGGATAGC	457	*Mnl*I
118A17-T	CAPS	Fw: GGCATGGTGAAGTCCACATT Rv: GTGTCACAGGTTTGGTTCAT	739	*Hae*III
15N23-T	CAPS	Fw: GGCAGATATCTGCAATACGT Rv: ATCATGAACAGCAAAACAACCA	576	*Taq*I
Markers developed from BAC 143M15 sequence				
ORF1	CAPS	Fw: CTGTATCTATGACGAGGAGA Rv: GATCCTGAAGCTGAAGCTT	625	*Hae*III
ORF2	CAPS	Fw: AATATTTTCAACTTTCAAATCTCTT Rv: ACGAAGGCATGATTGTCGTTA	206	*Mnl*I
ORF3	CAPS	Fw: GTTGAACTTATACCACTAGGA Rv: GTGCGGTCTCATCAACTCAA	937	*Nde*I
ORF4	dCAPS	Fw: GAACACTTAGGTTAAAATATAGC Rv: CCTACTATCCTTCTTGTAATCT	217	*Alu*I
ORF5	CAPS	Fw: CTTAAAGGCACACTTAGATTCA Rv: CTGAGAATTCTCTTGACTGCA	962	*HpyCH*4IV
ORF4(1)	SCAR	Fw: GCTTTATTCATAGTAAATTCTGT Rv: TCAGACAAATCATCGTATATTGA	805/885	
ORF4(2)	SCAR	Fw: TCCATTTGTAGTTTCATAAAGC Rv: CCAAGCGGATAATTAATGTCA	465 / 515	
Marker developed for *ps-2*ABL allele identification within *S.lycopersicum*				
ps-2 marker	CAPS	Fw: CAAATTGGATGAGAGTTTTGAA Rv: CATTTTACAAGTGTAACAACTTG	695	*HpyCH*4IV

Candidate gene analysis and phylogenetic analysis

The complete genomic DNA sequence of ORF4, as well as up-stream and down-stream sequences containing respectively promoter and terminator was amplified and sequenced from Moneymaker and *ps-2*ABL using several successive overlapping primer pairs giving products of around 900bp. The resulting DNA sequences of Moneymaker and *ps-2*ABL were assembled with DNAStar. The FGENESH software was used to identify the putative exons and introns of the candidate gene (Salamov and Solovyev 2000).

Tomato polygalacturonase protein sequences with known tissue expression, as well as the best hits of a protein BLAST search with the candidate protein, were selected to conduct a phylogenetic analysis. Only the Pfam Glycosyl Hydrolase 28 domains, of the selected protein sequences, were used for the analysis. The Pfam GH28 domain of each protein sequence was identified with the protein Blast interface of NCBI. Selected amino acid sequences of Pfam GH28 domains were aligned using ClustalW multiple sequence alignment software (Higgins et al. 1994).

PAUP software package version 4 (Swofford 2002) was used to construct a 50% majority-rule consensus phylogenic tree using maximum parsimony (1000 bootstrap replicates and 250 addition sequences replicates). Cedar PG protein sequence was used and defined as outgroup.

The amino acid sequences and their protein identification numbers were: Kiwi fruit (AAC14453; Atkinson and Gardner 1993), grape berry fruit (AAK81876; Nunan et al. 2001), soybean pods (AAL30418; Christiansen et al. 2002), peach fruit (CAA54448; Lester et al. 1994), apple fruit (AAA74452; Atkinson 1994), pear fruit (BAC22688; Hiwasa et al. 2003), Arabidopsis dehiscence zone ADPG1 (CAA05525; Sander et al. 2001), oilseed rape dehiscence zones RDPG1 (CAA65072; Petersen et al. 1996), oilseed rape pod (CAA90272; Jenkins et al. unpublished), turnip silique valve desiccation (CAD21651; Rodriguez-Gacio et al. 2004), Bell pepper fruit (BAE47457; Ogasawara S and Nakajima T, unpublished), tomato fruit TFPG (CAA32235; Bird et al. 1988), tomato pistil (AAC70951; Hong and Tucker 2000), tomato abscission zones TAPG1, 2, 4, 5 (AAC28903, AAB09575, AAB09576, AAC28906; Hong and Tucker 1998), tomato wound leaf (AAD17250; Bergey et al. 1999) and tomato seed (AAF61444; Sitrit et al. 1999)

Total RNA isolation, cDNA synthesis and quantitative PCR analysis

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Between 50 and 100 mg of each tissue (anthers, fruit, flower abscission zone and leaf abscission zone) was used per RNA isolation reaction. Only 1μg of total RNA, after DNase I treatment (Boehringer Manheim), was used per sample for the synthesis of cDNA. First strand cDNA template was synthesized using random hexamers as primers and Multiscribe^{TM} Reverse Transcriptase (Applied Biosystems). The nearly complete coding sequence of ORF4 was amplified to study the intron splicing using the forward primer: TAGCTCCAAAGCTATCCACAT, located on the first exon, starting 47 nucleotides down-stream the start codon and the reverse primer: TGGAGAATGTGAAATTGTTAGG, located on the last exon, stopping 100 nucleotides up-stream the stop codon (**Supplementary Figure 1**). Nearly-complete CDS of ORF4 was amplified with standard PCR reaction (55°C annealing temperature and 35 cycles)

Real-time experiments were conducted in an iCycler MyiQ detection system (Bio-Rad), using the SYBR green PCR master mix kit (Applied Biosystems). Primer sequences were: forward primer 5'-TTTTGCCATTGCCATTGATA-3', reverse primer 5'-TGTGGTGTCCCAGAACAAGA-3' (ORF4); and forward primer 5'-ACCACTTTCCGATCTCCTCTC-3', reverse primer 5'-ACCAGCAAATCCAGCCTTCAC-3' (β-actin). Relative quantification of the ORF4 transcript level was calculated with the internal β-actin control by applying the $2^{-\Delta CT}$ formula. Purity of the PCR products was verified with their melting curves. The experiment was performed in duplo. PCR controls were performed in absence of added reverse transcriptase to ensure RNA samples to be free of DNA contamination. In addition the ORF4 primers were designed on either sides of an intron so that the PCR product obtained on genomic DNA was significantly longer than on cDNA and that the difference could easily be observed on gel.

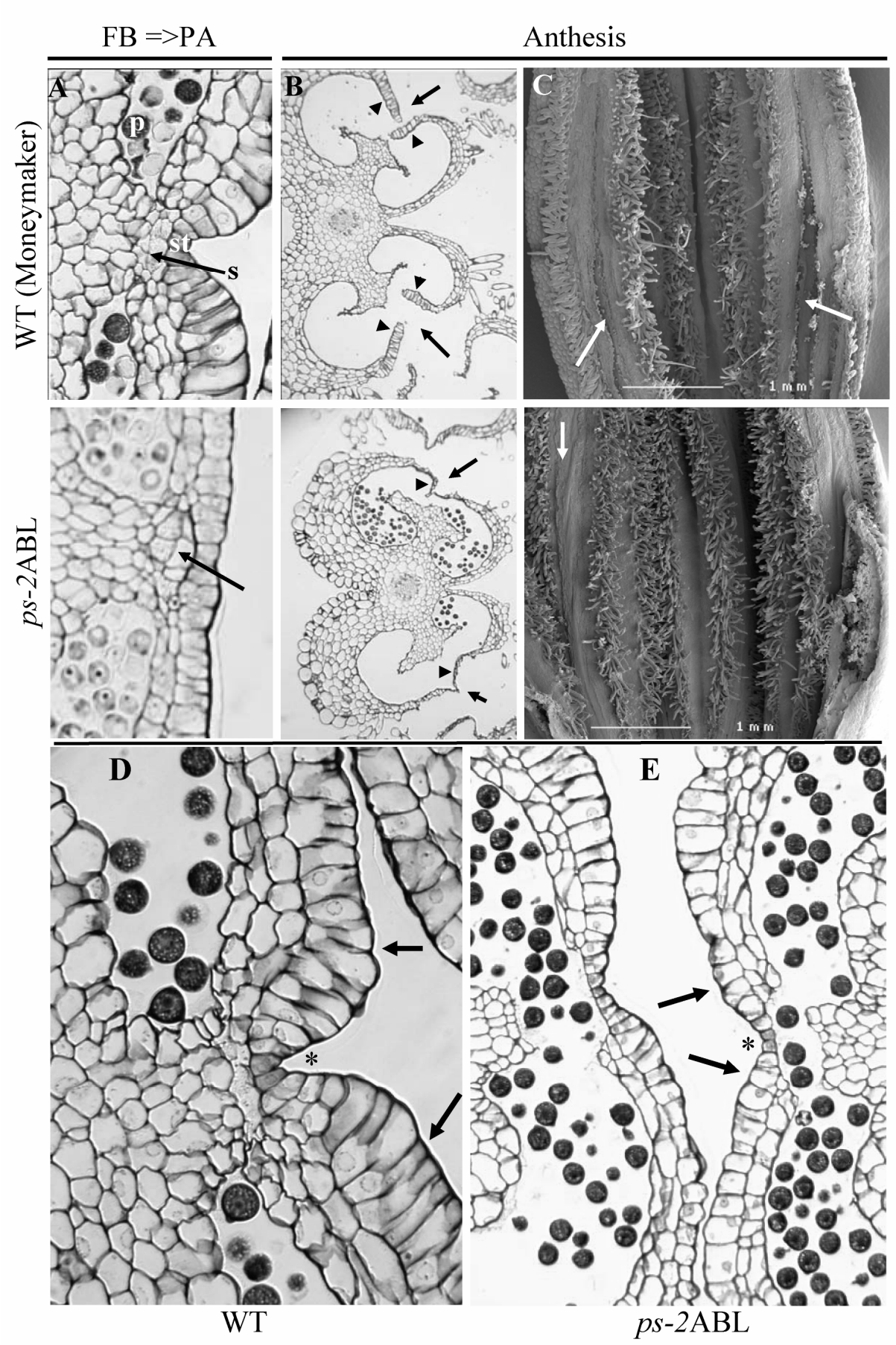
Results

Microscopic observation of the *ps-2* phenotype

Transverse sections of anthers of wild-type (Moneymaker) and mutant (*ps-2*ABL) were prepared and stained with toluidine blue to identify the stage at which anther development/dehiscence in the mutant is blocked. Anthers at anthesis were prepared for scanning electron microscopy.

Early anther development and the fusion of the locules was similar in mutant and wildtype: Breakage of the septum by which the locules, containing the microspores, fused, did occur in the mutant at a similar stage as in the wild type at pre-anthesis. Crystals appeared in the septum cells of mutant and wild-type, and pollen development appeared normal (**Figure 1a**). However, development of the locule walls altered. Exothecium cells of Moneymaker formed anticlinal cell wall thickenings which reinforce the exothecium cells (**Figure 1d**). As a consequence, the locule wall becomes rigid and trigger the stomium rupture, thus anther opening, at anthesis. Such anticlinal cell wall thickenings did not appear in *ps-2*ABL (**Figure 1e**). As a result the mutant stomium did not open at anthesis and the pollen remained in the anthers (**Figures 1-b, -c**). After anther opening, the exothecium in the wildtype was still discernable as a rigid structure because of the cell wall thickenings whereas the exothecium of the mutant was flattened and bent; The exothecium cells lacked rigidity, at the anthesis stage, needed to create a breaking force on the stomium.

Figure 1: Microscopic observation of anthers of *ps-2*ABL and wild type (WT; Moneymaker).
a: Cross section of anthers at late flower bud (FB), early pre-anthesis (PA) stained in toluidine blue. Arrows refer to the crystals that can be seen in the septum cells of WT and *ps-2*ABL. s: septum; st: stomium; p: pollen.
b: Cross section of anthers at anthesis stained in toluidine blue. Arrows indicate the opening of the anther in WT and the stomium that remains closed in *ps-2*ABL because stomium cells are not torn apart. Note the rigid locule walls in wildtype and the flattened ones in the mutant (arrowheads).
c: Longitudinal view of anther cones at anthesis observed by SEM. Arrows indicate the longitudinal opening of the anthers in WT with pollen presented along the opening edges, and the closed anthers in *ps-2*ABL. Some pollen are visible in the mutant anther at the right hand side, due to cutting during preparation.
d: Developing wild type anther showing the stomium region. Note that this stage is just before locule fusion. Cell wall thickenings in the exothecium are present (arrows). The stomium is indicated by *
e: Developing Mutant anther showing the stomium region. Note that there is fusion of the locules but development of cell wall thickenings in the exothecium cells is reduced/absent (arrows) The stomium is indicated by *.


FB =>PA
Anthesis
WT (Moneymaker)
ps-2ABL
A
p
st
s
B
C
1 mm
1 mm
D
E
*
*
WT
ps-2ABL

Physical mapping and candidate gene identification

We have previously mapped the *ps-2* locus to an interval of 1.65cM defined by the COS derived CAPS markers T0958 and T0635 on the short arm of Chromosome 4 (Gorguet et al. 2006). A physical map for the *ps-2* locus region was built using the Heinz BAC library. The library was screened by PCR amplification with the closest markers relative to the *ps-2* locus and by computational means using the sequences of those markers. Positive BACs were then anchored to the genetic map by converting BAC ends into PCR markers (**Figure 2**) and by screening these markers in the recombinant population. BAC fingerprints were also compared to evaluate the overlapping of BACs from the same contig and to verify whether BACs were part of the same contig. The closest BAC end relative to the *ps-2* locus was then used for a second round of screening. Eventually, the entire contig spanned 1.70 cM (32 recombinants) from the COS derived CAPS marker T1070 to BAC end 15N23-T (**Figure 2**). BAC 143M15 did obviously span the *ps-2* locus and was therefore sequenced.

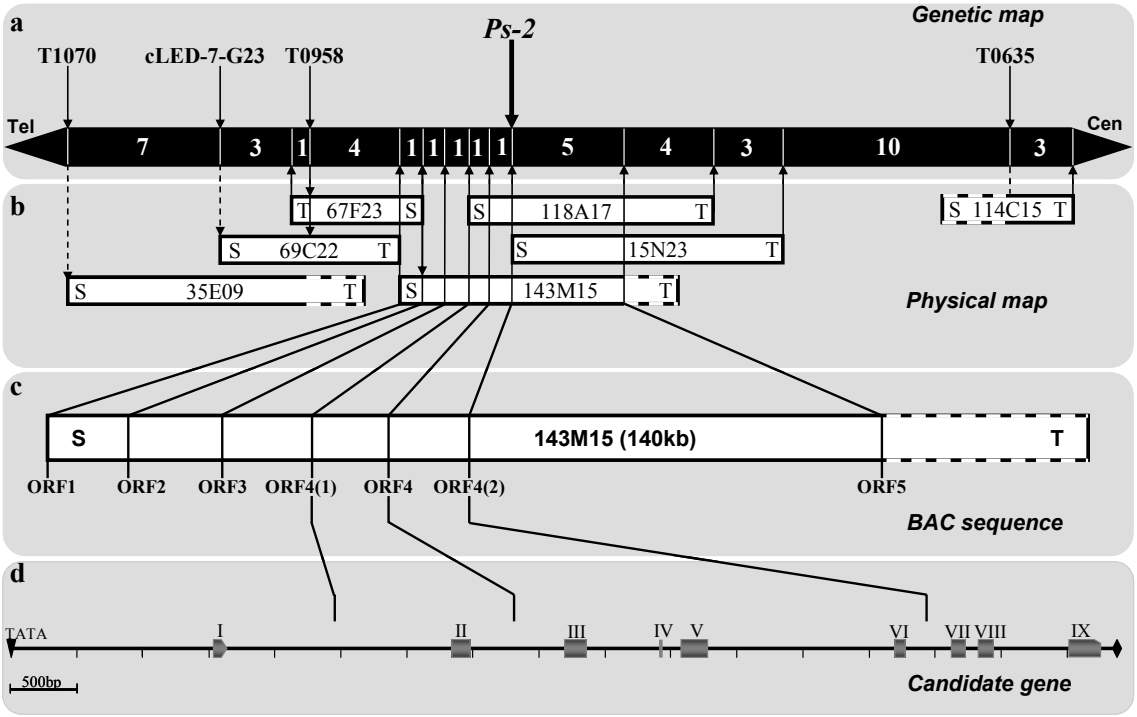


Figure 2: Cloning of the *ps-2* gene, from genetic map to gene structure.

a: Genetic map developed in a recombinant F2 population (*ps-2*ABL x *S. pimpinellifolium*; Gorguet et al. 2006). In white are the numbers of recombinant plants between each marker
b: Physical map: Arrows in dashed represent the computational anchoring of BACs. Arrows in full line represent the anchoring of BACs by molecular markers. (S: SP6; T: T7).
c: Genetic positions of the ORFs on BAC clone 143M15
d: Structure of candidate gene ORF4. Cylinders with roman numbers represent exons. Positions of the putative TATA box and PolyA signal sequence are indicated by an inverted triangle at the beginning of the sequence and a rhombus at the end of the sequence, respectively.

In order to identify the genes present in BAC clone 143M15, the BAC DNA sequence was scanned against the Tomato SGN Unigene database, using a BLASTN interface, and against the *Arabidopsis* gene models database, using a TBLASTX interface. Two tomato coding sequences and five *Arabidopsis* genes matched the BAC clone sequence (**Table 2**). The five candidate genes were named ORF1 to ORF5. In addition, six sequences of a retrotransposon family were also identified but were not taken into account in the further study. The positions of the five corresponding *Arabidopsis* genes were not contiguous in the *Arabidopsis* genome which showed the absence of synteny between the two species in this specific region. Moreover, none of them was homologous to one of the functional male sterility genes identified in *Arabidopsis* (listed in Gorguet et al. 2006).

Table 2: Identification of candidate genes present on BAC 143M15 based on *Arabidopsis* gene models

Candidate gene	Tomato	*Arabidopsis*					
	Unigenes Acc. no.	Accession no.	Gene function	Chr.	AGI coordinates (bases)	TBLASTX *E* value	Score
ORF1	U323899	AF014399	magnesium-chelatase	1	2696415-2700961	0	214
ORF2		NM112785	transcriptional factor B3 family	3	6548875-6551847	3E-21	105
ORF3	U317249	AF326883	remorin family protein	2	17477944-17480014	9E-37	110
ORF4		NM111676	polygalacturonase	3	21294315-21296918	1E-79	108
ORF5		AB017502	glycosyl hydrolase family 3	5	7107378-7111311	0	276

In order to localize the candidate genes in the high resolution map, we converted the putative gene sequences (or the sequences nearby) into PCR markers and mapped them in the recombinant population. Every candidate genes mapped at different positions in the high resolution linkage map and therefore we could easily identify the likeliest candidate for *ps-2* based on their positions in the genetic map. ORF4, a putative polygalacturonase gene, mapped the closest to the *ps-2* locus (**Figure 2**). Subsequently, the putative introns and exons were identified using the FGENSH software of Softberry. The candidate gene ORF4 is composed of nine exons and eight introns, covering a genomic distance of 6716 nucleotides from putative start to stop codon, for a coding sequence of 1179 nucleotides. The SNP used to develop the PCR marker ORF4 was located in the second putative intron. We developed and mapped two extra PCR markers, one based on a deletion of 76bp in the first intron in the *S. pimpinellifolium* allele [ORF4(1)] and one based on an insertion of 38bp in the sixth intron in the *S. pimpinellifolium* allele [ORF4(2)]. The three PCR markers, ORF4(1), ORF4 and ORF4(2) mapped at an interval of one recombinant between each other, indicating that at

least two recombinations had occurred within the candidate gene ORF4 in the recombinant population (**Figure 2**). ORF4(2) co-segregated with the *ps-2* locus on the high resolution map.

Mutation in ORF4 and molecular marker development

To consolidate the hypothesis that ORF4 corresponds to the *ps-2* gene, we searched for sequence alterations in the *ps-2*ABL allele. The entire 9kb, from the putative promoter to the putative terminator of ORF4 was sequenced from *cv.* Moneymaker and *ps-2*ABL. One single mutation was identified in the last nucleotide of the fifth putative exon of ORF4, in which the nucleotide Guanine was replaced by Cytosine. To test the association between the identified SNP in ORF4 and the *ps-2* trait, we developed a molecular marker based on that SNP, in such a way that the *ps-2*ABL allele and the wild type allele in *S. lycopersicum* could easily be differentiated on gel. This marker was tested on a set of 176 ABLs among which eight were *ps-2*/*ps-2*. These eight male sterile plants showed the same marker pattern, distinct from the other ABLs, which confirms that the SNP is present in the *ps-2* lines tested. This marker can now easily be used for molecular assisted introduction of the *ps-2* trait into modern tomato lines.

Alternative intron splicing

Because the sequence mutation in the *ps-2*ABL allele of ORF4 is located in one of the intron recognition splice sites, we hypothesized that this mutation could affect the pre-mRNA splicing of the gene. In order to verify this hypothesis we designed primers to amplify the nearly full-length cDNA clone of ORF4 (1032nt out of 1179nt): The forward primer was designed on the first exon of ORF4 and the reverse primer on the last exon (**Supplementary Figure 1**). RT-PCR was performed on cDNA made from RNA of anthers at post-anthesis from *ps-2*ABL and *cv*. Moneymaker. The obtained amplified product for *cv*. Moneymaker was of the expected size (1032bp), which was confirmed by sequencing. The amplified product of *ps-2*ABL was significantly smaller than the Moneymaker product, which suggested an alteration in intron splicing (**Figure 3**). The sequencing of the fragment amplified on cDNA of *ps-2*ABL showed that the fifth exon, on which the SNP is present, was absent in the cDNA sequence. This exon was removed together with the two flanking introns during the pre-mRNA maturation process (**Figure 4**). The wild type 5' sequence of this exon-intron splice junction is CAG/GTATCG, which is identical to one of the splice junction sequence identified in *Solanum tuberosum* (Brown 1986). The mutation found in the *ps-*

*2*ABL allele induces the following sequence: CA**C**/GTACG, which is not present in the list of intron splicing recognition sites. The absence of the fifth exon in the mature mRNA represents a deletion of 208 nucletides, which induces a frame-shift in the remaining coding sequence down-stream. This frame-shift causes a premature termination of translation after 14 aa due to a newly framed stop codon. The complete putative mutated protein is therefore 154 aa long in comparison to 392 aa for the wild type protein, and is likely to be non-functional.

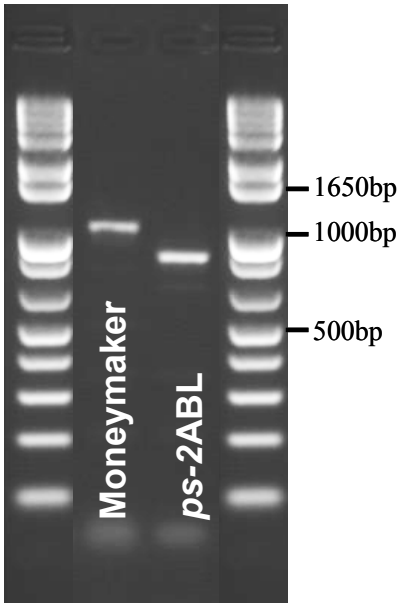


Figure 3: Size of the nearly complete coding sequence of ORF4 in Moneymaker and *ps-2*ABL. The positions of the primers on the gene sequence are indicated on the **supplementary Figure1**.

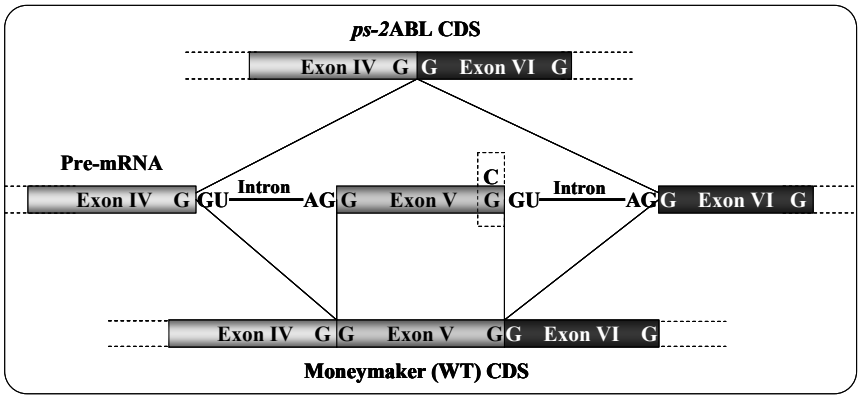


Figure 4: Intron splicing between exon IV and VI of ORF4, in Moneymaker (WT) and in *ps-2*ABL. The mutated nucleotide (G → C) is circled by a dashed rectangle.

ORF4 sequence analysis

A BLAST search with the putative candidate protein sequence of ORF4, in the protein database of NCBI, resulted in a list of PG proteins from several plants. The identified proteins have functions in fruit ripening and siliques/pods dehiscence. Among them, the ADPG1

protein had been found to be expressed in the dehiscence zone of siliques of *Arabidopsis* as well as the dehiscence zone of anthers (Sander et al. 2001). Amino acids of the PG protein domain (Pfam GH28), of the best BLAST hits were aligned together with the sequences of the candidate protein and the already known tomato PG proteins with identified functions, and one gymnosperm PG (cedar). A phylogenetic analysis was performed on the final alignment in order to place the candidate protein in one of the referenced PG clades. ORF4 was identified as a PG of clade B (**Figure 5**). Clade B is composed of cloned genes that encode PG expressed in fruit and dehiscence zone, as previously characterized by Hadfields and Bennett (1998). This was also observed in the phylogenetic tree. TFPG, the only tomato PG known to be expressed in fruits, was also part of the same clade. Alignment of Pfam GH28 domains of ORF4 and TFPG is presented in **Figure 6**. ORF4 and TFPG have a similarity of 59% in the entire protein sequence.

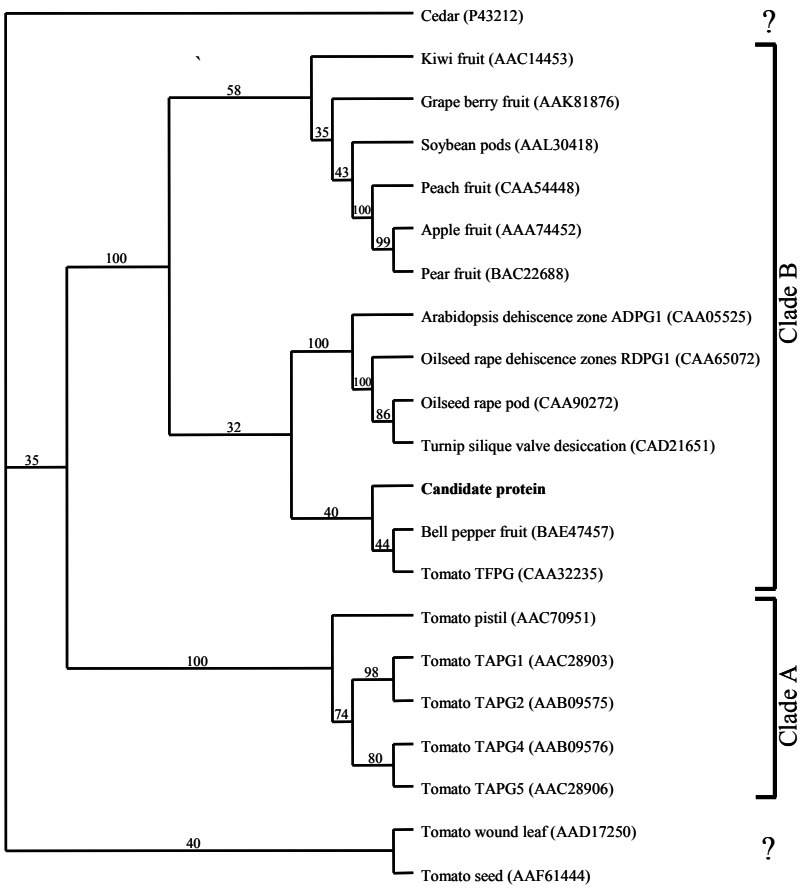


Figure 5: Phylogenetic tree for 20 angiosperm and one gymnosperm PG aa sequences. The phylogram is the result of a 50% Majority-rule consensus of 3296 trees (using tree weights). The PG sequences segregate into two major clades identified as the well characterized clades A and B. The candidate protein (TDPG) is part of clade B.

The putative derived protein of ORF4 contains the four conserved domains characteristic of PG proteins, as presented by Rao et al. (1996; **Figure 6**). The first conserved domain is located on the fifth exon, which is absent in the mutant protein and the three others

domains are located more C-terminally and therefore not in frame in the mutant sequence. Thus the mutant protein does not contain any of the four conserved domains that play a major role in the function of the protein. Analysis of the 2000 nucleotides up-stream the start codon of ORF4 revealed the presence of three Ethylene Responsive Elements (ERE; AWTTCAAA) at positions -667, -700 and -1955 relative to the start codon, one bZIP protein binding motif (TGACG) at -1632 and one G-box (CACGTG) at -1329. The presence of ERE motifs, bZIP protein binding motifs and G-box in the promoter sequence of ORF4 suggests that the transcription of ORF4 is stimulated by ethylene and jasmonate.

```
TFPG: CSSRTPVQFVVPKNKNYLLKQITFSGPCRSSISVKIFGSLEASSKISDYK-DRRLWIAFD 59
ORF4: CSSSHVVNFVVSQNKKYLLKPIKFYGPCKSSITMQIYGTLLASDDTSDYKKDSRHWLIFD 60
ps-2: CSSSHVVNFVVSQNKKYLLKPIKFYGPCKSSITMQIYGTLLASDDTSDYKKDSRHWLIFD 60

TFPG: SVQNLVVGGGGTINGNGQVWWPSSCKINKSLPCRDAPTALTFWNCKNLKVNNLKSKNAQQ 119
ORF4: SVQKLVVGGAGVINGNGKIWWQHSCKINKKLPCKVAPTALTFYKCNNLKVKDLKIENAQQ 120
ps-2: SVQKLVVGGAGVINGNGKIWWQHSCKINKKLPCKVAPTVMIASQLWMDLRRS stop    112

TFPG: IHIKFESCTNVVASNLMINASAKSPNTDGVHVSNTQYIQISDTIIGTGDDCISIVSGSQN 179
ORF4: IHLLIEKCVGVEVTKLVVTSPENSPNTDGIHITSTQNIQISDSTIATGDDCISIVDGSQK 180
                              ■■■■■■■■■                 ■■■■■
                                      I                      II
TFPG: VQATNITCGPGHGISIGSLGSGNSEAYVSNVTVNEAKIIGAENGVRIKTWQGGSGQASNI 239
ORF4: VLATGITCGPGHGISIGSLGGGNSEAHVSDIHVNGAKLYETTNGLRIKTWPGGFGSASNI 240
          ■■■■■■■■■■■■■■                          ■■■
                    III                           IV
TFPG: KFLNVEMQDVKYPIIIDQNYCDRVE-PCIQQFS-AVQVKNVVYENIKGTSATKVAIKFDC 297
ORF4: KYQNVVMNNVKNPIIIDQNYCDQADGPCKAETDSAVEVKNVIYQNIKGTSATNDAISIKC 300

TFPG: STNFPCEGIIMENINLVGESGKPSEATCKNVH 329
ORF4: SKKIPCEGILMENVKLLGG------------- 319
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Figure 6: Alignment of Pfam GH28 domain of TFPG, ORF4 and *ps-2* (mutant of ORF4). Colour code is only effective for the comparison between TFPG and ORF4: In reverse colour are the identical amino acids. In grey are the conserved substitutions. In bold underlined are the four conserved domains of polygalacturonase as defined by Rao et al. (1996), identified by roman numbers.

Expression of ORF4 in anther, fruit and other tissues

We tested the presence of ORF4 transcript in several tissues including abscission zones of leaf and flowers, mature fruits and anthers at anthesis. No ORF4 transcript was detected in the abscission zones (**Figure 7**). The presence of the ORF4 transcript was confirmed in anthers as well as in mature fruit. In order to study the transcription level of ORF4 over different stages of anther and fruit development, we performed a quantitative expression analysis of ORF4 at four developmental stages of anthers: flower bud; pre-anthesis; anthesis; post-anthesis, and eight developmental stages of the fruit, from five dap (days after pollination) to 57 dap (mature fruit); 47 dap corresponded to breaker stage. In anthers, the transcription level of

ORF4 was tested on Moneymaker and *ps-2*ABL, and in the fruits only on Moneymaker. Results are presented in **Figure 8**. In anthers of cv. Moneymaker, ORF4 transcripts were already detected at flower bud stage. At pre-anthesis the level of ORF4 transcript was similar to the level in flower buds and the ORF4 transcript accumulation increased at anthesis and reached a maximum at post-anthesis. In anthers of *ps-2*ABL, ORF4 transcripts were also detected, except at flower bud stage. The transcript level in *ps-2*ABL anthers was lower than in the wildtype anthers at anthesis and post-anthesis (**Figure 8**). In fruit, no ORF4 transcripts were detected before 37 dap. From 37 dap, ORF4 transcripts were detected and increased significantly over time to reach a maximum at mature stage (57dap).

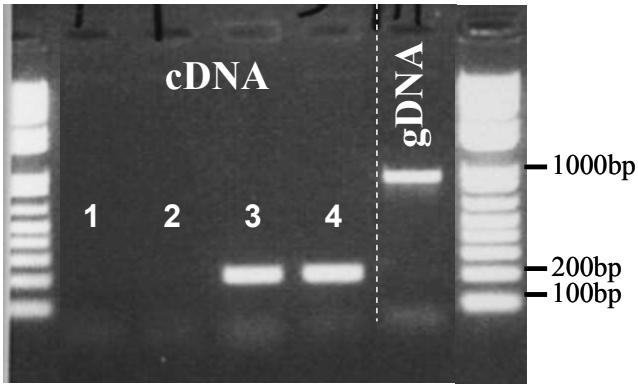

Figure 7: Tissue specific expression of ORF4. cDNA of Moneymaker from diverse tissues were subjected to PCR amplification using primers dedicated to quantitative PCR analysis.
1: Leaf abscission zone
2: Flower abscission zone
3: Anthers at anthesis
4 : Fruit at mature stage
gDNA: Genomic DNA control

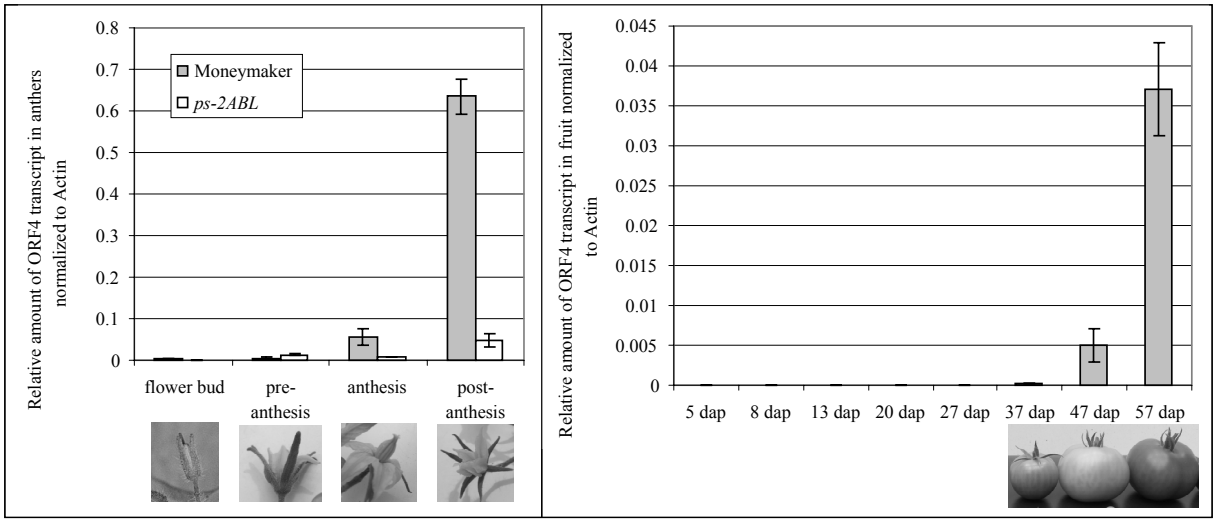

Figure 8: Real-time PCR expression data of ORF4 at different stages of anthers development on Moneymaker and *ps-2*ABL (left) and during fruit development and ripening of Moneymaker (right). dap: days after pollination.

Discussion

The *ps-2* Advanced Breeding Line produces anthers that do not undergo dehiscence. In this study we showed that anther dehiscence of *ps-2*ABL is blocked in the ultimate phase. The exothecium does not form cell wall thickenings which induce locule opening upon dehydration at anthesis and the stomium cells are not torn apart. In the absence of these two structural and physiological changes, the anther remains closed, because the epidermal cells lack the rigidity that could eventually break the stomium and liberate the pollen.

This phenotypic mutation is recessive and under the control of one single locus. In a previous study we fine mapped the *ps-2* gene on the short arm of Chromosome 4 (Gorguet et al. 2006). Here we report the isolation and functional characterization of the *ps-2* gene. This is the first functional sterility gene isolated in the *Solanaceae* family. The high resolution of the linkage map developed on the recombinant population allowed us to narrow down the position of the *ps-2* gene to one single candidate gene on the BAC sequence. The presence of at least two recombinations within ORF4 allowed us to restrict the location of the *ps-2* mutation to the end of the gene sequence. Still, we analyzed the complete gene and putative promoter sequence.

We found that the *ps-2* phenotype is the result of a single nucleotide mutation in a polygalacturonase gene unknown to date, composed of nine exons. This single nucleotide mutation is located on the last nucleotide of the 3' end of the fifth exon, affecting the intron splicing recognition site, which is changed from CA**G**/GTATCG to CA**C**/GTATCG (exon 3'/intron 5'). Though the Cytosine base is present in 11% of the intron splice sites at this specific position in plants, the combination "CAC" at the exon 3'end has never been detected in any splice site in plants (Brown 1986). The 5th exon is spliced out together with the two flanking introns. Analysis of *Arabidopsis* mutants with mutations around splice sites has revealed several examples of exon skipping in plant splicing (reviewed by Brown and Simpson 1998). Most of these mutations are located in the intron part of the recognition splice sites. To our knowledge, in plants, the only mutant showing exon skipping due to a mutation in the exon part of a recognition splice site, to date, was the *spy-1* mutant in *Arabidopsis* (Jacobsen et al. 1996) in which the CA**G**/GTTTGA (exon 3'/intron 5') recognition splice site at the end of the eighth exon was mutated into CA**A**/GTTTGA. The exon skipping observed in the mutated allele of ORF4 induces a frame-shift in the rest of the sequence, which has as consequence to create an early stop codon 14 aa further. The complete mutant protein is

therefore 154 aa long in comparison to 392 aa long for the wild type, and do not contain any of the four domains characteristic of PGs.

We did not clone the genomic sequence of ORF4 with its respective promoter, in order to perform a complementation experiment with the *ps-2*ABL. However, based on the accuracy of the high resolution map, the specific expression pattern of ORF4 in anthers and the non-functionality of ORF4 in *ps-2* ABL we believe to have proved that the mutation found in ORF4 is responsible for the functional sterility observed in *ps-2*ABL.

_ps-2_ is a PG of clade B

The isolated gene responsible for the *ps-2* phenotype is a PG unknown to date. We propose the acronym TDPG, for Tomato Dehiscence PolyGalacturonase. Phylogenetic analysis of TDPG revealed a close similarity with PGs of clade B as defined by Hadfield and Bennett (1998). Clade B is here composed of fruit PGs, among them the Tomato Fruit PG, as well as silique or pod dehiscence PGs. *ADPG*, the *Arabidopsis* homolog of *TDPG* is also expressed in the anther dehiscence zone (Sander et al. 2001). This suggests a conserved role of *TDPG* homologs over the plant kingdom. We suggest that the repression of *TDPG* homologs in other plant species may procure functional sterility in species where such a trait does not exist.

The other tomato PGs cluster in different clades. In accordance to the assertion that the divergence of PG family members occurred prior to the separation of the angiosperm species (Hadfield and Bennett 1998), TDPG is here more closely related to proteins of the same clade, from other species, than to tomato PGs from other clades. Most of the other tomato PGs are related to abscission (TAPG). In our study, expression of TDPG was not detected in flower and leaf abscission zones. However, in addition to anther tissues, we detected mRNA transcript of TDPG in fruits.

TDPG transcript accumulation increases along with the development of the anthers

We measured the relative level of TDPG transcript at different stages in anthers. TDPG transcript is already detected in anthers of Moneymaker at flower bud stage. The transcript level increases over stages to reach a maximum at post-anthesis, when anthers are dehisced. This increase of TDPG transcript accumulation is parallel to the septum and stomium degeneration as well as the thickening of the exothecium cell wall, observed in the anthers. TDPG transcript level in *ps-2*ABL anthers was detected from pre-anthesis on, but the transcript level remained very low in comparison to Moneymaker, at anthesis and post-

anthesis. Very likely the mutant mRNA is recognized as non-sense and degraded by nonsense-mediated mRNA decay (NMD). NMD functions as a quality control mechanism to eliminate abnormal transcripts (Lejeune and Maquat 2005).

TDPG is likely under the control of Ethylene and Jasmonate

Although it is speculative, the presence of ethylene and jasmonate responsive elements in the promoter region of *TDPG* might suggest that the transcription of *TDPG* could be influenced by both hormones. It has already been shown that ethylene was involved in the timing of anther dehiscence in tobacco (Rieu et al. 2003). More recently, in petunia, it has been shown that ethylene regulates the synchronization of anther dehiscence with flower opening (Wang and Kumar 2006). In addition, many studies already identified jasmonate as a key compound in the process and timing of anther dehiscence. Several mutants in JA biosynthetic enzymes have been identified for the study of this phenomenon. Scott et al. (2004) suggested that ethylene and JA may act redundantly in the control of anther dehiscence, which would explain why *Arabidopsis* mutants such as *dde-1*, which cannot synthesize JA within the stamens, or the tobacco ethylene insensitive mutant *Tetr*, eventually undergo anther dehiscence (Sander et al. 2004; Rieu et al. 2003).

In the present study we showed that anthers of *ps-2*ABL remain indehiscent, in contrast to delayed dehiscence in the other mutants, which strengthen the hypothesis that TDPG acts down-stream of Ethylene and JA in the control of anther dehiscence and that TDPG is the main actor of this process.

TDPG may also play a role in tomato fruit ripening

TFPG, the only tomato fruit polygalacturonase identified to date, has been characterized as one of the main actors in the process of fruit softening. Anti-sense repression of TFPG has lead to the production of tomato fruit with longer shelf life but the fruits did undergo ripening indicating that other actors also play a relevant role in the process of fruit softening (Smith et al. 1988). TDPG may well be one of these actors by contributing to the fruit cell wall degradation. TDPG transcript was detected in the late stages of fruit development in Moneymaker (**Figure 8**). Maximum transcripts were found at mature stage (57dap). Similarly TFPG has been detected only at ripening stages, starting at mature green or at breaker stage (Thompson et al. 1999; Eriksson et al. 2004). The level of TFPG was also found to increase over time from breaker stage to mature fruit, in *cv*. AC and Liberto (Thompson et al. 1999).

In tomato, although the regulation of PG mRNA accumulation by ethylene remained for a long time ambiguous, it has been demonstrated that TFPG accumulation was ethylene dependent in the process of fruit ripening (Sitrit and Bennett 1998). In accordance ERE motifs have been found in the promoter of TFPG (Montgomery et al. 1993). Ethylene is presented as the major plant hormone in the control of fruit ripening (reviewed by Giovannoni 2004). Expression of anti-sense RNA to the rate limiting enzyme in the biosynthetic pathway of ethylene inhibits fruit ripening in tomato and as consequence down regulates the production of TFPG (Oeller et al. 1991; Sitrit and Bennett 1998).

Earlier in this study we suggested that TDPG was under the control of ethylene due to the presence of ERE motifs in the promoter sequence. Repression of ethylene in the fruit is likely to inhibit both TFPG and TDPG and therefore prevents completely the process of fruit ripening. A simplified model for the hormonal control of TDPG in anther dehiscence and fruit maturation is presented in **Figure 9**.

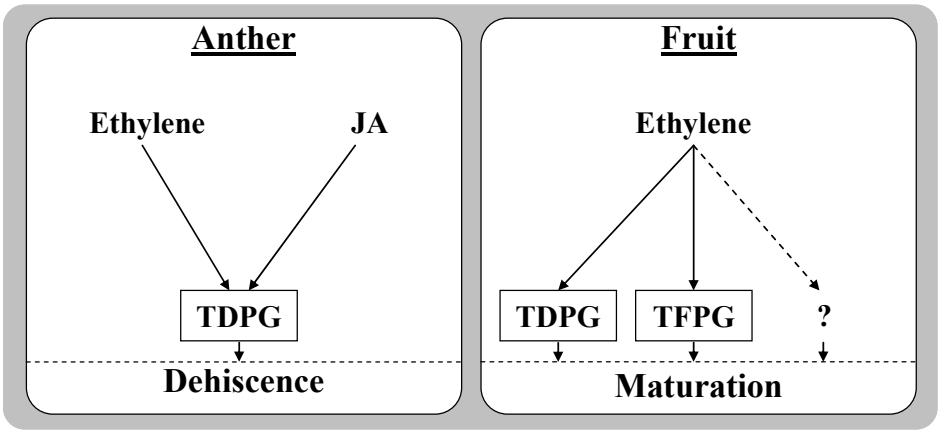

Figure 9: Simplified model for the hormonal control of anther dehiscence and fruit maturation in tomato in which TDPG is taking part.

It is not clear whether the role of TDPG in fruit maturation and shelf life is of similar importance than TFPG. The comparison between *ps-2*ABL after manual opening of the anthers and Moneymaker or any other normal tomato line is unreliable due to the difference of genetic background. Knock out of TDPG by anti-sense RNA or RNAi in tomato, or double mutants in which both TDPG and TFPG are impaired, could provide new insights to answer to that question. Time between hand pollination to mature fruit stage and fruit shelf life could be measured and compared to the untransformed control in order to evaluate the effect of TDPG in the fruit. Knock outs of homologs of TDPG in other plants species is also of

valuable interest to verify whether the control of anther dehiscence in its ultimate phase is conserved among species.

Acknowledgments:

This project was sponsored by Western Seed BV, The Netherlands. We thank Dr. Andre van Lammeren and Henk Kieft for microscopic analysis of the anthers, Antoine Deniau for generating the phylogenetic tree, Dr. Anne Kortstee and Marian Oortwijn for providing cDNA of Moneymaker fruits and Prof. Dr. Gerco Angenent for critical reading of the manuscript and useful suggestions.

Supplementary Figure 1: Alignment of the wild type sequence (WT) and the Mutant sequence of ORF4 at the nucleotide and protein levels

```
WT:     ATGGAGAAATTCAATGAAGAAGAAGATCAAGCTAAGGTTACAACAATTAATGTGGATAGC 60
Mutant: ATGGAGAAATTCAATGAAGAAGAAGATCAAGCTAAGGTTACAACAATTAATGTGGATAGC 60
Pt WT:  M  E  K  F  N  E  E  E  D  Q  A  K  V  T  T  I  N  V  D  S
Pt Mt:  M  E  K  F  N  E  E  E  D  Q  A  K  V  T  T  I  N  V  D  S
        Fw primer                                    ▼
WT:     TTTGGAGCTAAAGGTGATGGAAGTATAGATGATACAAATGCATTTCAAAAAGCATGGAAA 120
Mutant: TTTGGAGCTAAAGGTGATGGAAGTATAGATGATACAAATGCATTTCAAAAAGCATGGAAA 120
Pt WT:   F  G  A  K  G  D  G  S  I  D  D  T  N  A  F  Q  K  A  W  K
Pt Mt:   F  G  A  K  G  D  G  S  I  D  D  T  N  A  F  Q  K  A  W  K

WT:     GAAGCTTGTTCATCTTCACATGTTGTGAATTTTGTGGTGTCCCAGAACAAGAAATATCTT 180
Mutant: GAAGCTTGTTCATCTTCACATGTTGTGAATTTTGTGGTGTCCCAGAACAAGAAATATCTT 180
Pt WT:  E  A  C  S  S  S  H  V  V  N  F  V  V  S  Q  N  K  K  Y  L
Pt Mt:  E  A  C  S  S  S  H  V  V  N  F  V  V  S  Q  N  K  K  Y  L
                                                      ▼
WT:     CTCAAACCAATCAAATTTTATGGGCCATGCAAATCTTCCATTACAATGCAGATTTATGGA 240
Mutant: CTCAAACCAATCAAATTTTATGGGCCATGCAAATCTTCCATTACAATGCAGATTTATGGA 240
Pt WT:  L  K  P  I  K  F  Y  G  P  C  K  S  S  I  T  M  Q  I  Y  G
Pt Mt:  L  K  P  I  K  F  Y  G  P  C  K  S  S  I  T  M  Q  I  Y  G

WT:     ACCCTATTAGCATCTGATGATACTTCAGATTACAAGAAGGATAGTAGGCACTGGCTTATT 300
Mutant: ACCCTATTAGCATCTGATGATACTTCAGATTACAAGAAGGATAGTAGGCACTGGCTTATT 300
Pt WT:  T  L  L  A  S  D  D  T  S  D  Y  K  K  D  S  R  H  W  L  I
Pt Mt:  T  L  L  A  S  D  D  T  S  D  Y  K  K  D  S  R  H  W  L  I

WT:     TTTGATAGTGTTCAAAAATTGGTTGTTGGAGGAGCTGGAGTTATCAATGGCAATGGCAAA 360
Mutant: TTTGATAGTGTTCAAAAATTGGTTGTTGGAGGAGCTGGAGTTATCAATGGCAATGGCAAA 360
Pt WT:  F  D  S  V  Q  K  L  V  V  G  G  A  G  V  I  N  G  N  G  K
Pt Mt:  F  D  S  V  Q  K  L  V  V  G  G  A  G  V  I  N  G  N  G  K
                                                ▼                 ▼
WT:     ATTTGGTGGCAACATTCTTGCAAAATTAATAAAAAATTGCCATGCAAGGTAGCACCCACG 420
Mutant: ATTTGGTGGCAACATTCTTGCAAAATTAATAAAAAATTGCCATGCAAGGTAGCACCCACG 420
Pt WT:  I  W  W  Q  H  S  C  K  I  N  K  K  L  P  C  K  V  A  P  T
Pt Mt:  I  W  W  Q  H  S  C  K  I  N  K  K  L  P  C  K  V  A  P  T

WT:     GCCCTGACATTTTACAAGTGTAACAACTTGAAAGTGAAGGACCTTAAAATAGAAAATGCA 480
Mutant: ------------------------------------------------------------
Pt WT:  A  L  T  F  Y  K  C  N  N  L  K  V  K  D  L  K  I  E  N  A
Pt Mt:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

WT:     CAACAAATACATTTGCTAATTGAGAAGTGTGTTGGTGTTGAAGTTACAAAATTGGTAGTG 540
Mutant: ------------------------------------------------------------
Pt WT:  Q  Q  I  H  L  L  I  E  K  C  V  G  V  E  V  T  K  L  V  V
Pt Mt:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

WT:     ACTTCTCCAGAAAATAGCCCTAATACTGATGGAATCCATATAACTAGCACTCAAAATATT 600
Mutant: ------------------------------------------------------------
Pt WT:  T  S  P  E  N  S  P  N  T  D  G  I  H  I  T  S  T  Q  N  I
Pt Mt:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                          Domain I
                                ▼
WT:     CAAATTTCTGATTCCACCATTGCCACAGGTGATGATTGCATCTCAATTGTGGATGGATCT 660
Mutant: ----------------------------GTGATGATTGCATCTCAATTGTGGATGGATCT 452
Pt WT:  Q  I  S  D  S  T  I  A  T  G  D  D  C  I  S  I  V  D  G  S
Pt Mt:  -  -  -  -  -  -  -  -  -  -V  M  I  A  S  Q  L  W  M  D  L
                                   Domain II
```

```
                                                                    ▼
WT:     CAGAAGGTCTTAGCCACTGGCATTACTTGTGGACCAGGTCATGGAATTAGTATTGGAAGT 720
Mutant: CAGAAGGTCTTAGCCACTGGCATTACTTGTGGACCAGGTCATGGAATTAGTATTGGAAGT 512
Pt WT:  Q  K  V  L  A  T  G  I  T  C  G  P  G  H  G  I  S  I  G  S
Pt Mt:   R  R  S  stop                        Domain III

WT:     TTGGGAGGTGGAAATTCAGAAGCTCATGTGTCTGATATTCATGTAAATGGAGCTAAGCTT 780
Mutant: TTGGGAGGTGGAAATTCAGAAGCTCATGTGTCTGATATTCATGTAAATGGAGCTAAGCTT 572
Pt WT:  L  G  G  G  N  S  E  A  H  V  S  D  I  H  V  N  G  A  K  L
                                                    ▼
WT:     TATGAAACTACAAATGGACTTAGGATTAAGACTTGGCCGGGAGGATTTGGAAGTGCAAGC 840
Mutant: TATGAAACTACAAATGGACTTAGGATTAAGACTTGGCCGGGAGGATTTGGAAGTGCAAGC 632
Pt WT:  Y  E  T  T  N  G  L  R  I  K  T  W  P  G  G  F  G  S  A  S
                           Domain IV

WT:     AATATTAAGTATCAAAATGTGGTTATGAATAATGTCAAAAATCCAATAATTATAGACCAA 900
Mutant: AATATTAAGTATCAAAATGTGGTTATGAATAATGTCAAAAATCCAATAATTATAGACCAA 692
Pt WT:  N  I  K  Y  Q  N  V  V  M  N  N  V  K  N  P  I  I  I  D  Q
                                                    ▼
WT:     AATTATTGTGATCAAGCTGATGGTCCATGCAAAGCTGAGACTGACTCGGCAGTTGAAGTG 960
Mutant: AATTATTGTGATCAAGCTGATGGTCCATGCAAAGCTGAGACTGACTCGGCAGTTGAAGTG 752
Pt WT:  N  Y  C  D  Q  A  D  G  P  C  K  A  E  T  D  S  A  V  E  V

WT:     AAAAATGTGATTTATCAAAATATCAAAGGCACAAGTGCAACAAATGATGCAATAAGTATC 1020
Mutant: AAAAATGTGATTTATCAAAATATCAAAGGCACAAGTGCAACAAATGATGCAATAAGTATC 812
Pt WT:  K  N  V  I  Y  Q  N  I  K  G  T  S  A  T  N  D  A  I  S  I
                                                  Rv primer
WT:     AAGTGCAGCAAAAAAATTCCATGTGAAGGAATTTTGATGGAGAATGTGAAATTGTTAGGA 1080
Mutant: AAGTGCAGCAAAAAAATTCCATGTGAAGGAATTTTGATGGAGAATGTGAAATTGTTAGGA 872
Pt WT:  K  C  S  K  K  I  P  C  E  G  I  L  M  E  N  V  K  L  L  G

WT:     GGAAATGGTGAAACTCCAAATGGTATTTGGGGAAATATCAATAATCTTACGTGCAAAAAT 1140
Mutant: GGAAATGGTGAAACTCCAAATGGTATTTGGGGAAATATCAATAATCTTACGTGCAAAAAT 932
Pt WT:  G  N  G  E  T  P  N  G  I  W  G  N  I  N  N  L  T  C  K  N

WT:     GTTTTACCAGAATGTCAAAAAAACTCAAAAATTGTATAA 1179
Mutant: GTTTTACCAGAATGTCAAAAAAACTCAAAAATTGTATAA 971
Pt WT:  V  L  P  E  C  Q  K  N  S  K  I  V. stop
```

Pt WT: Protein sequence of the Wild Type (Moneymaker)
Pt Mt: Protein sequence of the Mutant (*ps-2*ABL)

- The nucleotide "G" in **bold** indicates the location of the mutation
- Conserved protein domains are indicated in **bold underlined**
- Positions of the introns are indicated with a black arrow above the sequence ▼
- Positions of the primers sequences used to amplify the nearly-complete coding sequence are indicated in gray

CHAPTER 5

General Discussion

In 1983, George and colleagues wrote a review entitled "Parthenocarpy in Tomato" in which they nicely presented the different types and sources of parthenocarpy available at that time. They introduced their work by highlighting the current interest in using parthenocarpy as an additional system to improve tomato fruit setting and possibly fruit quality, particularly for tomato production under stressful conditions. They presented *Montfavet* 191 or *Soressi* (*pat-1*), *Severianin* (*pat-2*) and *RP75/59* (*pat-3/4*) as the most interesting sources for practical use. Twenty years later, at the onset of this project, no important parthenocarpic tomato cultivars had been launched on the market and no tomato parthenocarpy gene had been mapped. In 2004 the position of *pat-1* on the tomato linkage map was published (Beraldi et al. 2004), giving new perspectives for the molecular understanding of this phenomenon in tomato. However the problems in the development of parthenocarpic tomato cultivars remained unchanged: **How to achieve a high, stable and uniform seedless tomato production and at the same time maintain a high level of commercial seed production**. This project aimed at developing new tools and knowledge to decipher this problem.

Perspectives for breeders

The identification and mapping of parthenocarpy genes, and ultimately the development of seedless tomato cultivars, is mainly hampered by the difficulty to evaluate the plants for such a trait. This difficulty resides in the fact that too many aspects are often considered under the term "parthenocarpy". The strict definition of parthenocarpy is the growth of the ovary into a seedless fruit in the absence of pollination and/or fertilization. Others aspects are often wrongly associated with parthenocarpy:

- The presence of parthenocarpy gene(s) in a plant does not necessarily mean that this plant will only produce seedless fruits. This makes it difficult to evaluate plants for this trait. Previous studies reported the need to emasculate the flowers in order to avoid self-pollination and giving only the possibility of seedless fruit development. In the present study, when we emasculated flowers it always resulted in very low seedless fruit set of poor quality (misshaped and small). Therefore, we preferred to limit self-pollination by avoiding any plant movement until evaluation. This did not completely prevent self-pollination resulting in several seeded fruits. Eventually we often observed on the same plant, seedless fruits, seeded fruits, knots and aborted flowers. Knots are seedless fruit-like organs characterized by sharp edges and a pale color. They usually take more time to turn red and are often much smaller than conventional fruits. However sometimes intermediate fruits, with a shape in between a

true seedless fruit and a knot were found which made those tomatoes difficult to classify. An additional problem is that it is not known whether the seeded fruits would have grown into a seedless tomato if not pollinated. The presence of seeded and seedless fruits on the same plants has often pushed the breeders to combine more parthenocarpy genes into the same tomato line in order to increase the parthenocarpy level. This has resulted in seedless tomato lines that can hardly produce any seed even after hand pollination. It is quit well possible that not only parthenocarpy genes were combined in those lines but also gene(s) preventing self-pollination. In such parthenocarpic lines it is often observed that fruits develop before anthesis stage, which completely prevents self-pollination.

The combination of parthenocarpy genes with positional sterility genes can overcome the problem of getting uniform parthenocarpic tomatoes without problems in seed production. In this study we characterized, mapped and isolated the *positional sterility-2* gene, which confers non-dehiscent anthers in tomato. The *ps-2* gene is likely to be the most suitable sterility gene to use in combination with parthenocarpy. The biology behind the *ps-2* gene is hereafter further discussed.

- Another aspect often wrongly associated with parthenocarpy, is the ability of parthenocarpic plants to produce good fruits in adverse weather conditions. It is true that parthenocarpy overcomes the problems of male and female sterility that occur under stress. However if the said parthenocarpic plant is not adapted to adverse conditions, this will affect its vigor and ultimately its fruit setting. The use of parthenocarpy genes to solve the problems of poor fruit set under harsh conditions has to be performed in a genetic background selected for those conditions.

Combination of parthenocarpy with functional male sterility

As proposed above, the combination of parthenocarpy with functional male sterility, more specifically with *ps-2*, would restrict the setting to only seedless fruits and allows the production of commercial seeds after manual opening of the anthers and hand pollination. The position of the *ps-2* gene relative to the position of some of the parthenocarpy genes, identified in the present study, gives room for some speculation: The *ps-2* gene was mapped on Chromosome 4, between markers T0958 and TG609 (**Chapter 3**). Remarkably the *pat-6* locus and *pat-8* locus confidence intervals span the *ps-2* locus (**Figure 1**). In the present study we could not determine whether *pat-6* and *pat-8* were allele variants of the same locus, or different loci. The combination of *pat-6*/*pat-8* with *ps-2* requires the fine mapping of the two

parthenocarpy genes in order to narrow down their confidence intervals in such a way that these confidence intervals do not span the *ps-2* locus. Fine mapping will probably give also the answer whether *pat-6* is allelic to *pat-8*. Screening an F_2 population coming from the cross between *ps-2*ABL and IL5-1, with the appropriated markers allows searching for a combination of both genes (*ps-2* and *pat-6*) in the same plant. A similar procedure can be followed with IVT-line 1 in case the *pat-8* locus is different from *pat-6*. Because the genetic distance between *ps-2* and *pat-6*/*pat-8* is very small, combining positional sterility with these two sources of parthenocarpy requires the screening of a high number of F_2 plants. The suppression of recombination observed in all the mapping populations used in this study (**Chapter 2 & 3**) will even complicate this procedure. Eventually, if the combining of *ps-2* with *pat-6*/*pat-8* succeeds, this would greatly ease further use of those genes in breeding programs, by simply selecting for the two flanking markers of this gene cluster. The *pat-7* and *pat-9* genes could also further be introduced into the same breeding material.

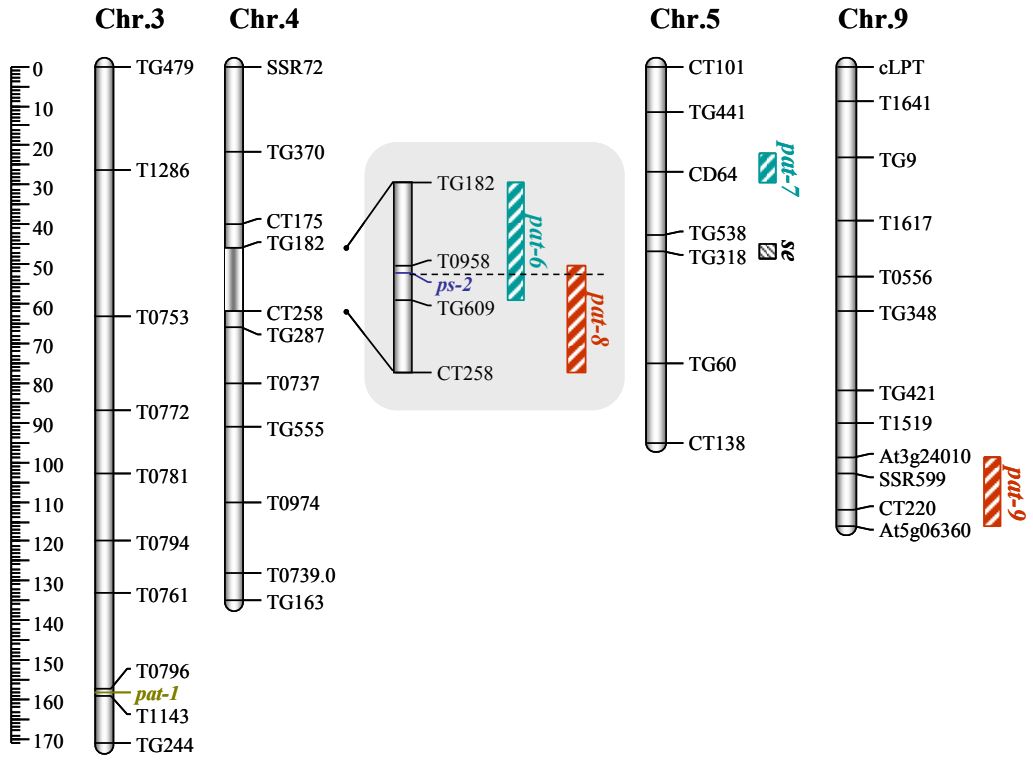

Figure 1: Map positions of the parthenocarpy loci in tomato and the *positional sterility 2* locus. In grey background is a zoom of the TG182-CT258 genetic interval on Chromosome 4. The ruler on the left is a centiMorgan scale.

Parthenocarpy genes in tomato

In this study we mapped four parthenocarpy genes in tomato (*pat-6, pat-7, pat-8* and *pat-9*), coming from two *S. habrochaites* accessions. Interestingly the LYC4 accession, of which genome fragments were introgressed in *S. lycopersicum var*. Moneymaker resulting in parthenocarpy, is not parthenocarpic itself and Moneymaker neither. Therefore the parthenocarpy observed on IL5-1 is not strictly due to the introgression of parthenocarpy genes from LYC4, but due to the combination of those two genes, *pat-6* and *pat-7*, with genes present in *S. lycopersicum var.* Moneymaker (no information was available on the parthenocarpic status of the *S. habrochaites* accession used for the development of IVT-line 1). We hypothesize that the genes coming from one of the parents are responsible for a high level of basal Auxin or GA biosynthesis, and the genes coming from the other parent set a high sensitivity of the plant to those hormones, in such a way that the hormone threshold to trigger fruit set is lower than the basal level of hormone production.

As mentioned earlier, any gene involved in the biosynthesis or the sensitivity to Auxin or GA is a putative candidate gene for parthenocarpy. In **Chapter 1**, the way these genes may interfere in the development of parthenocarpic fruit is presented. **Chapter 1** focused mainly on the involvement of the GA biosynthesis pathway because much attention has recently been paid to it in the study of *pat-2* and *pat3/4* (Fos et al. 2000, 2001). The involvement of *ARF8* and *IAA9* in fruit setting has recently been unrevealed. In the *Arabidopsis* wild type, *ARF8* acts as an inhibitor to stop further carpel development in absence of fertilization and the generation of signals required to initiate fruit and seed development (Goetz et al. 2006). The *fwf* mutant, identified in *Arabidopsis*, is parthenocarpic (Vivian-Smith et al. 2001). A mutation in the *ARF8* gene, which rendered it non-functional, is responsible for the parthenocarpic fruit development of the *fwf* mutant (Goetz et al. 2006). The identification of *ARF8* as a candidate for the parthenocarpy gene(s) *pat6*/*pat-8* (**Chapter 2**) enlarges the global scheme explaining this phenomenon in tomato (**Figure 2**). In addition, the anti-sense down regulation of the *IAA9* gene in tomato was found to trigger parthenocarpic fruit development (Wang et al. 2005). In this transformed plant, fruits started to set before fertilization, giving rise to parthenocarpy. A general scheme on the control of fruit setting and their respective mutation or down-regulation giving rise to parthenocarpy is presented in **Figure 2**.

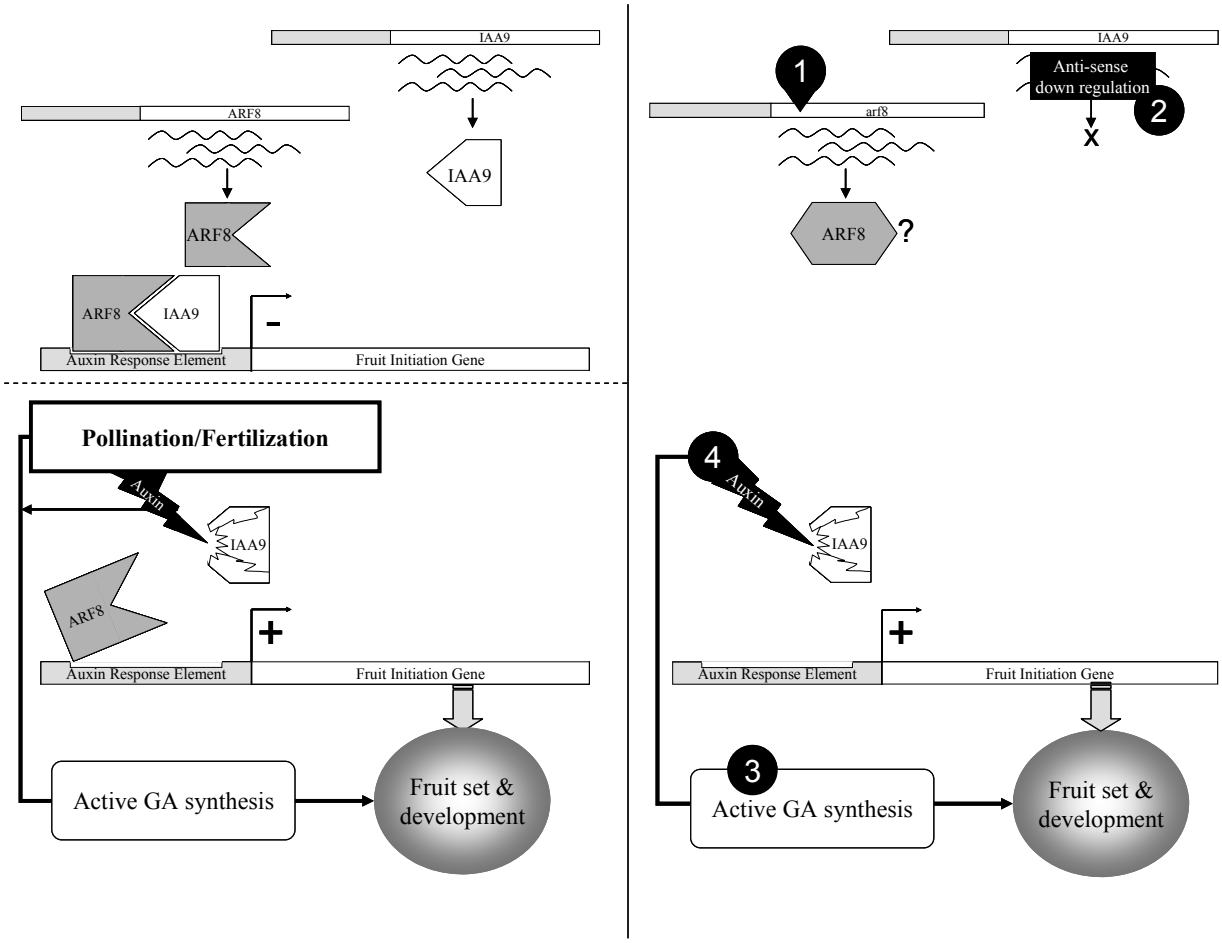

Figure 2: General scheme on the control of fruit set.
On the left side of the figure, the control of fruit set in wild type plants is presented: After synthesis of the ARF8 and IAA9 proteins (upper left), the ARF8/IAA9 complex is formed, which represses the expression of the fruit initiation gene. On the down left side, pollination and fertilization trigger the synthesis of Auxin which may degrade the IAA9 protein (Goetz et al. 2006). The fruit initiation gene becomes therefore unrepressed, which triggers fruit set. Meanwhile the Auxin increases the synthesis of active GA.
On the right side, the alteration points (white numbers in black circles) of this process of fruit set control, giving rise to parthenocarpy are presented:

1. Goetz et al. (2006) have shown that a mutation in the *ARF8* gene in *Arabidopsis* was responsible for the parthenocarpic fruit development in the *fwf* mutant. Such a mutation may either affect the expression of the *ARF8* gene or give rise to an aberrant protein which is not recognized by the *IAA9* protein.
2. Anti-sense down regulation of *IAA9* in tomato triggered parthenocarpic fruit development (Wang et al. 2005). In absence of *IAA9*, the *ARF8*/*IAA9* complex responsible for the repression of fruit initiation cannot be formed and therefore fruit set is not repressed in absence of pollination/fertilization.

3 & 4. GA and Auxin biosynthesis and sensitivity genes are candidate genes for parthenocarpy, as presented in **Chapter 1**.

Molecular control of anther dehiscence

Mature pollen is released from the anther by dehiscence, a program of cell destruction culminating in rupture of the stomium, creating a furrow separating each pair of anther locules (reviewed by Scott et al. 2004). The molecular control of anther dehiscence has been mainly investigated in *Arabidopsis*, where the major role of Jasmonic Acid was highlighted. At least seven mutants in the Jasmonic Acid pathway or the Jasmonic Acid sensitivity have been identified and studied in *Arabidopsis*. These mutants are presented in **figure 3**.

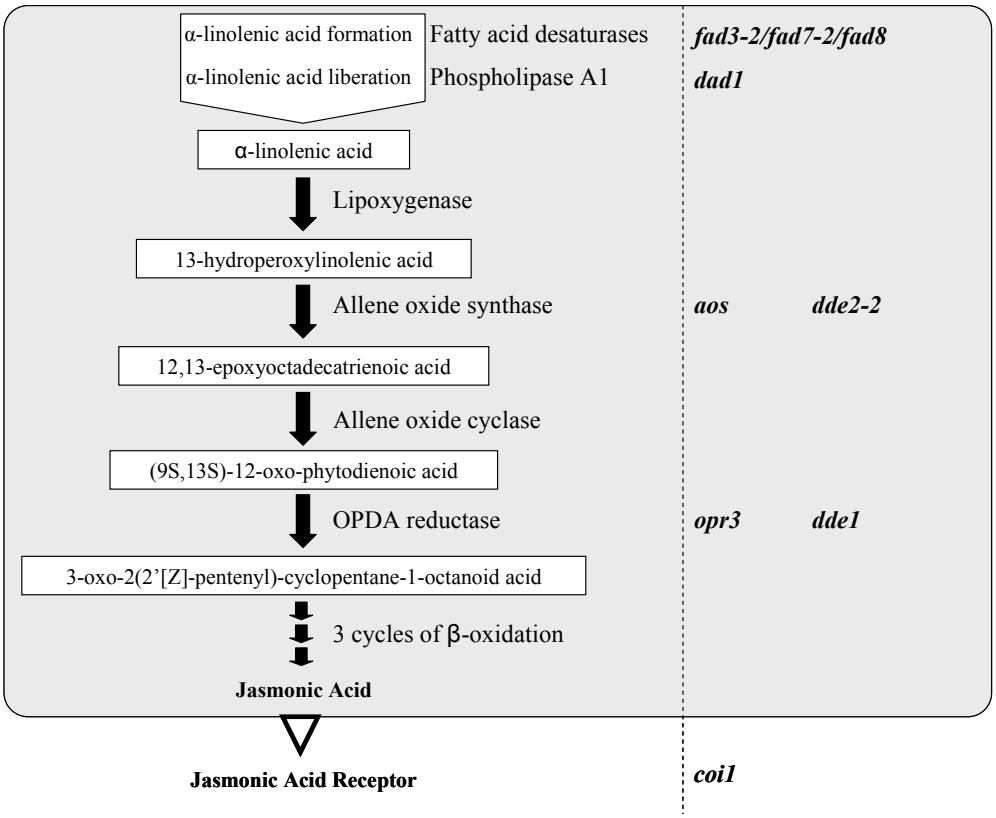

Figure 3: Jasmonic Acid biosynthesis pathway (left) and the respective mutants identified in *Arabidopsis* (right), used in the study of anther dehiscence.

In addition, ethylene was found to regulate the timing of anther dehiscence (Rieu et al. 2003). All above mutations only delay anther dehiscence and most of them are often associated with a reduction of pollen fertility which hampers their use in practical breeding. To our surprise, the *ps-2* gene was not directly involved in the JA or ethylene biosynthesis pathways, but the positional sterility is the result of a mutation in a polygalacturonase gene denoted *TDPG*. This gene is likely to be under the control of JA and ethylene (**Chapter 4**). The existence of an *Arabidopsis* homolog of *TDPG*, known as *ADPG*, which regulates silique

dehiscence and which is also expressed in the dehiscence line of anthers, may reveal a general action of this gene in anther dehiscence over the plant kingdom. The repression of this gene in other plant species is likely to produce functional sterile plants. Because in tomato the *ps-2* gene is known to procure a stable and complete functional sterility, with fertile pollen and without negative side effect, the repression of the *TDPG* homolog in other plant species may well give rise to a similar stable and complete functional sterility in plant species where no functional sterility gene has been identified.

In this study we found that *TDPG* was also expressed in fruit and that the most similar gene in terms of sequence and structure, was the *Tomato Fruit PG*. Both *TDPG* and *TFPG* may well have a similar action in the process of fruit maturation and ripening. Interestingly, anti-sense repression of *TFPG* has led to the production of fruit with longer shelf life, but the fruits did undergo ripening, which indicates that other genes act in concert with *TFPG* in this maturation process. *TDPG* may well be one of these actors. We do not know whether the introduction of *ps-2* gene in a tomato line will confer a longer shelf life to the fruits. To answer this question, the best approach would be to repress the *TDPG* gene in a standard tomato line, and compare the shelf life of the tomato fruits obtained on the non-transformed line and on the transformed line after hand pollination. Additionally the double repression of *TDPG* and *TFPG* in a single line would bring extra information to know whether *TDPG* and *TFPG* are the two only genes acting in the down-stream process of fruit maturation, or whether other cell degradation genes are also involved in this process.

Is there a biological explanation to the co-localization of *ps-2*, *pat-6* and *pat-8*?

In the present study we mapped the *ps-2* gene on Chromosome 4, between markers T0958 and T0635 (**Chapter 3**). As described above, the confidence intervals of *pat-6* and *pat-8* genes span the genetic interval T0958-T0635 (**Figure 1**). Though the co-localization of *ps-2* with *pat-6* and *pat-8* represents a certain difficulty for combining functional sterility with parthenocarpy, this may have a biological explanation. The wild type allele of *TDPG* is responsible for opening of the anther and therefore for the pollination event and the wild type allele of a parthenocarpy gene may be responsible for the non development of the fruit in absence of pollination/fertilization as proposed for the *ARF8* gene. Both gene actions may therefore be coordinated in order to have a pollination event occurring when the ovary is ready to develop into a fruit. This may represent a gene complex in which the genes are inherited as a unit. Another example of such a gene complex in tomato is the *style exertion 2.1*

locus. Style exertion is required to allow cross-pollination. For this, the style has to be longer than the stamen. The high resolution mapping of *se2.1* has revealed the presence of one major gene, controlling style length as well as closely linked genes controlling stamen length and stamen architecture (Chen et al. 2004). The co-inheritance of genes controlling style and stamen length favors cross pollination during the evolution of wild tomatoes. In our study, the co-inheritance of genes controlling pollination and genes prohibiting the development of fruit in absence of fertilization is likely to synchronize these two phenomena in the evolution of tomato species.

Candidate gene approach versus positional cloning

The isolation of a gene, in plants where the genome sequence is unknown, remains a long and challenging process. The most common approach to isolate a gene in such cases is positional cloning. Positional cloning includes several steps: after the mapping of the gene of interest, a fine mapping procedure to increase the resolution of the local genetic map is required. The development of molecular markers to saturate the gene region may become a very long and laborious task especially if the polymorphism level between the two parental lines is low. In addition this process of marker saturation needs the availability of sequence information near the gene of interest. Following the fine mapping of a gene, the construction of a physical map on the gene region can be time-consuming depending on the distance between the gene of interest and its closest molecular maker, used as starting point for BAC landing. If the starting point for "BAC walking" is more than say 500kb, the construction of the physical map is likely to be hampered by problems such as unspecific BAC end sequences which complicate further BAC walking as well as BAC orientation. In addition the construction of the BAC library itself, if required, is a very laborious process. The identification of the gene within all putative genes in the positive BAC often requires complementation. Therefore, before starting such a long and laborious process, it is wise to consider the potential alternatives to positional cloning.

A candidate gene approach may not appear at first as the easiest way to isolate a gene of interest. In the case of parthenocarpy, any gene related to the synthesis or sensitivity of the plant to Auxins and/or Gibberellins is a potential candidate, because the setting and development of fruit is mainly under the control of those two hormones. This may lead to a number of potential candidate genes that is too high. In addition, many genes in those pathways have not yet been isolated in tomato. The recent development and mapping of COS

markers in tomato, make it possible to investigate the microsynteny relationship with *Arabidopsis* for a specific region of interest. If such a microsynteny is identified, the position of the *Arabidopsis* candidate gene homologs can be compared to the microsyntenic region of interest. If a candidate gene is present in the region of interest the functionality and expression of this gene can be investigated. Eventually, the complementation of the mutant or line of interest with the wild type allele may confirm the action of the gene of interest. The constant development of databases and software greatly facilitates microsynteny analysis of regions of interest. The identification of a potential candidate gene may only require few hours.

However there are two major limitations to the use of this approach for gene isolation: Firstly, microsynteny between tomato and *Arabidopsis* is limited to few genome regions. Secondly, it is sometimes not feasible to consider every candidate genes. For instance, this approach was impossible for the isolation of the *ps-2* gene, because of the two previously mentioned limitations: No microsynteny was detected in the gene region and the only potential candidate genes that could have been considered were the JA synthesis and sensitivity genes which would have been the wrong choice. For the isolation of *pat-6*/*pat-8* gene, the microsyntenic relationship of the region of interest with the *ARF8 Arabidopsis* region makes the candidate gene approach worthwhile especially because the positional cloning of *pat-6*/*pat-8* is very complicated due to the difficulty to phenotype this trait. Positional cloning requires an accurate scoring of segregating plants for the trait of interest in order to locate precisely the gene of interest in a high resolution map. If few recombinant individuals are wrongly phenotyped (in the present case this could be plants carrying the parthenocarpy alleles and not producing any parthenocarpic fruit), the position of the gene in the high resolution map may change, which makes the isolation of the gene impossible. In a candidate gene approach to isolate the *pat-6*/*pat-8* gene, the isolation of the *ARF8* tomato homolog and its subsequent mapping in the tomato genome would bring extra information in favor or against *ARF8* as candidate gene, depending whether the tomato *ARF8* homolog co-segregates with *pat-6*/*pat-8* locus.

The future completion of the tomato genome sequencing will greatly facilitate the candidate gene approach. In addition, this will also ease the isolation of genes in closely related plants such as pepper and eggplant which share a genome wide synteny with tomato.

Complementation is not the only proof of gene function

In a gene cloning procedure complementation is considered as a must for final proof of gene function. Complementation is the introduction of the wild type allele of the isolated gene into the mutant plant. If the wild phenotype is recovered the inserted gene is considered as the gene of interest.

However the complementation of a gene may sometimes be very laborious or even impossible: In some plant species, the transformation is impossible or requires many trials and new protocols. In addition the success of the complementation is also dependent on the size and the structure of the gene to insert. In the present study, the genomic sequence of the *ps-2* gene, including the putative promoter and terminator, was evaluated at more than 10 kb which hampers the construction of the entry vector and the introduction of the vector itself in the plant. Complementation can be the bottle neck for the validation of the function of an isolated gene. The obvious question that researchers may pose themselves if they face such problems is whether complementation is really necessary to confirm that they have isolated the right gene. The answer lies in the accuracy of the high resolution map. If the recombinant population is large enough to have recombination between each putative gene of the positive BAC clone, then only one gene remains candidate. In addition, the phenotyping of the trait has to be accurate and clear, and any doubtful scoring should be discarded. In the present study, the isolation procedure of the *ps-2* gene fitted these criteria. Recombination was identified only few base pairs away from the SNP responsible for the mutant phenotype. In addition the phenotyping of the recombinant plants was performed using two different criteria: the ability of the anthers to release pollen and the absence of seed production. When both scores were not in accordance, the plants were discarded for further analysis. In addition, the nature of the gene identified corresponded to its expected physiological function. All this together can be considered as sufficient proof that the right gene has been isolated.

Concluding remarks

Parthenocarpy in tomato is a valuable trait to solve the problems of male and female sterility under harsh conditions. The combination of functional male sterility with parthenocarpy can solve the conflicting problem of seed production and at the same time allow a uniform seedless fruits production. The isolation of the *ps-2* gene will facilitate the introduction of positional sterility in tomato advanced breeding lines. It also represents a valuable tool to be use in the production of hybrid seeds. Homolog of the *TDPG* gene in other plant species may

also be repressed in order to introduce functional male sterility in species where no such sterility source is available. In addition, the mapping of a set of parthenocarpy genes in tomato brings new relevant tools for the development of parthenocarpic cultivars. Having markers available for the introduction of functional male sterility and parthenocarpy genes in hand, breeders can now focus on the performance of plants under harsh conditions. The development of a parthenocarpic tomato cultivar with great agronomic value may be achieved soon.

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SUMMARY

Parthenocarpy and functional sterility in tomato

Parthenocarpy is the development of the fruit in absence of pollination and/or fertilization. In tomato (*Solanum lycopersicum*), parthenocarpy is an interesting trait because it can improve fruit setting under harsh conditions. Facultative parthenocarpy is the most suitable form of parthenocarpy for practical use in tomato breeding programs. In this form of parthenocarpy the ovary, if fertilized, will develop into a seeded fruit and, if not fertilized, will develop into a seedless fruit. Auxins and gibberellins are considered as the key players in parthenocarpic fruit development. An increased level of these hormones in the ovary can be a substitute for pollination and trigger fruit development (reviewed in **Chapter 1**). The use of facultative parthenocarpy means that both fruit types (seeded and seedless) are often present on the same plant or even on the same cluster, thus affecting the uniformity of the fruit production.

The use of functional male sterility in combination with parthenocarpy can overcome this problem of uniformity of seedless fruit production but it still allows seed production by hand pollination when needed. The best functional male sterility gene in tomato is likely to be the *positional sterility-2* (*ps-2*) gene which confers non-dehiscent anthers, containing viable pollen.

The aim of this thesis was to study the genetics and biology of parthenocarpy and functional sterility in tomato, in order to develop breeding tools for the development of parthenocarpic tomato cultivars.

We characterized and mapped parthenocarpy in two different tomato lines, IL5-1 and IVT-line 1, both containing fragments of *Solanum habrochaites* (**Chapter 2**). In both lines parthenocarpy was under the control of two genes, named *pat-6* and *pat-7* in IL5-1, located respectively on Chromosome 4 and 5, and *pat-8* and *pat-9* in IVT-line 1, located respectively on Chromosome 4 and 9. It is not clear yet whether *pat-6* and *pat-8* are allelic. In addition, the

ARF8 tomato homolog is a likely candidate for *pat-6*/*pat-8* because of the microsyntenic relation between the *pat-6*/*pat-8* tomato locus and the *ARF8 Arabidopsis* locus. *ARF8* is known to act as an inhibitor for further carpel development in *Arabidopsis*, in absence of pollination and fertilization. Lesion in the *ARF8* sequence in *Arabidopsis* has led to the development of parthenocarpic siliques (Goetz et al. 2006). Mapping of the parthenocarpy genes in IL5-1 and IVT-line 1 allowed us to develop molecular markers closely linked to those genes, which can be used in molecular assisted selection.

We fine mapped the *ps-2* gene on the short arm of Chromosome 4 (**Chapter 3**). Because *ps-2* is the result of a spontaneous mutation in a cultivated tomato cultivar (and not coming from a wild tomato accession), its fine mapping in an interspecific mapping population did not lead directly to the development of universal markers for molecular assisted selection in tomato lines. After the successful construction of the physical map in the *ps-2* locus region, we isolated the wild type *PS-2* gene in a map-based cloning procedure (**Chapter 4**). We showed that the *ps-2* phenotype was the result of a single nucleotide mutation in a novel tomato polygalacturonase gene, named *TDPG* (*Tomato Dehiscence PolyGalacturonase*). This single mutation in *TDPG* affects one of the intron splicing recognition site, causing an alternative splicing during maturation of the pre-mRNA, which leads to an aberrant mRNA. We found that the expression of *TDPG* in the anther increases in parallel to the anther maturation process. In addition to the anthers, we found *TDPG* transcripts in maturing fruits, which suggests a role in fruit maturation process. The identification of the single nucleotide mutation in the *TDPG* sequence, responsible for the *ps-2* phenotype allowed us to develop a universal marker to assist to the introduction of this trait in tomato lines. The specific expression of the *Arabidopsis* homolog of *TDPG* in the anther dehiscence zone suggests a conserved mode of action over the plant kingdom, which means that the repression of *TDPG* homologs may be a potential way to introduce functional sterility in other species.

This study has led to the development of genetic tools for the production of parthenocarpic tomato cultivars. The combination of *ps-2* with parthenocarpy genes can solve the dilemma inherent to the seedless fruit production in tomato: the combination of uniform seedless fruit production with the possibility to produce seeds when needed.

SAMENVATTING

Parthenocarpie en functionele mannelijke steriliteit in tomaat

Parthenocarpie is het verschijnsel dat een vrucht zich ontwikkelt zonder de noodzaak van bestuiving en/of bevruchting. Voor tomaat (*Solanum lycopersicum*) is parthenocarpie een interessante eigenschap omdat het de vruchtzetting bevordert onder niet optimale condities. Facultatieve parthenocarpie is de meest geschikte vorm van parthenocarpie voor het gebruik in tomatenveredelingsprogramma's. Bij facultatieve parthenocarpie zal het vruchtbeginsel na bevruchting zich ontwikkelen tot een vrucht met zaden, maar als er geen bevruchting heeft plaatsgevonden ontstaat een zaadloze vrucht. Auxinen en gibberellinen zijn de belangrijkste spelers bij parthenocarpe vruchtontwikkeling. Een verhoogd niveau van deze hormonen in het vruchtbeginsel kan de noodzaak voor bevruchting omzeilen en zorgen voor vruchtontwikkeling (een literatuuroverzicht van de betrokken processen wordt gegeven in **Hoofdstuk 1**). Facultatieve parthenocarpie kan wel tot gevolg hebben dat beide type vruchten (met zaad en zonder zaad) aanwezig kunnen zijn op dezelfde plant en zelfs aan dezelfde cluster. Dit beïnvloedt de uniformiteit van de vruchtproductie doordat vruchten zonder zaad vaak in vorm en grootte kunnen verschillen van vruchten met zaad.

Het gebruik van functionele mannelijke steriliteit in combinatie met parthenocarpie voorkomt dit uniformiteitsprobleem met als bijkomend voordeel dat er ten behoeve van zaaizaadproductie en kruisingen nog steeds vruchten met zaad gevormd kunnen worden na handbestuiving. De beste functionele mannelijke steriliteit wordt in tomaat gevonden door de werking van het gen *positional sterility-2* (*ps-2*). In aanwezigheid van dit gen gaan de pollenzakjes niet open en komen er dus geen stuifmeel vrij, maar de pollenzakjes bevatten wel normale pollen. Bij het handmatig openen van deze pollenzakjes kom wel stuifmeel vrij.

Het doel van het onderzoek beschreven in dit proefschrift was het bestuderen van de genetica en biologie van parthenocarpie en functionele steriliteit in tomaat, met als doel

veredelingsmethodes te ontwikkelen die gebruikt kunnen worden voor de ontwikkeling van parthenocarpe tomatenrassen.

We hebben parthenocarpie gekarakteriseerd en de betrokken genetische factoren op de genetische kaart gelokaliseerd in twee verschillende tomatenlijnen, IL5-1 and IVT-line 1, waarvan bekend was dat ze beide chromosomale fragmenten bevatten van een wilde verwant van tomaat: *Solanum habrochaites* (**Hoofdstuk 2**). In beide lijnen wordt het niveau van parthenocarpie gecontroleerd door twee genen. Dit zijn *pat-6* en *pat-7* in IL5-1, gelokaliseerd op respectievelijk Chromosoom 4 en 5, en *pat-8* en *pat-9* in de IVT-lijn, gelokaliseerd op respectievelijk Chromosoom 4 en 9. Het is niet duidelijk of *pat-6* en *pat-8* hetzelfde gen zijn. De tomatenhomoloog van het *Arabidopsis* gen *ARF8* is een waarschijnlijke kandidaat voor *pat-6*/*pat-8* vanwege de microsyntenie tussen de *pat-6*/*pat-8* tomatenlocus en het *ARF8* locus. *ARF8* zorgt in *Arabidopsis* voor de remming van de ontwikkeling van de vruchtbladeren in *Arabidopsis* in afwezigheid van bestuiving en bevruchting. Een deletie in de *ARF8* sequentie in *Arabidopsis* leidt tot de ontwikkeling van parthenocarpe hauwtjes (Goetz et al, 2006). Het karteren van de parthenocarpie genen in IL5-1 en IVT-lijn 1 maakte het mogelijk moleculaire merkers te ontwikkelen die nauw gekoppeld zijn aan deze genen; deze merkers kunnen gebruikt worden in merker geassisteerde veredeling.

De positie van het *ps-2* gen voor functionele mannelijke steriliteit is nauwkeurig bepaald op de korte arm van Chromosoom 4 (**Hoofdstuk 3**). Omdat het *ps-2* allel het resultaat is van een spontane mutatie in een bestaand tomatenras (en dus niet afkomstig is van een wilde verwant van tomaat), leidde het nauwkeurig karteren in een interspecifieke karteringspopulatie niet direct tot bruikbare universele merkers voor merker geassisteerde selectie. Na de succesvolle ontwikkeling van een fysische kaart van het gebied rondom het *ps-2* locus, kon het wildtype *PS-2* gen via een op de kaart gebaseerde kloningsprocedure geïsoleerd worden (**Hoofdstuk 4**). We konden aantonen dat het *ps-2* fenotype het resultaat was van een mutatie van maar één enkele nucleotide in een nog niet bekend polygalacturonase gen (*TDPG*: *Tomato Dehiscence PolyGalacturonase*) in tomaat. De mutatie in *TDPG* zorgt, door het beïnvloeden van een van de intron splicing herkenningsplaatsen, voor een alternatieve splicing gedurende de ontwikkeling van het mRNA, wat leidt tot een afwijkend mRNA. We ontdekten dat de expressie van *TDPG* in het pollenzakje toeneemt tijdens de ontwikkeling van het pollenzakje. We vonden ook *TDPG* transcripten in rijpend fruit Dit suggereert een rol in het rijpingsproces van de vrucht. De identificatie van een mutatie, verantwoordelijk voor het *ps-2* fenotype, gebaseerd op een

enkele nucleotide in de *TDPG* sequentie, maakte het mogelijk een universele merker te ontwikkelen. Deze merker kan gebruikt worden voor het introduceren van deze eigenschap in andere tomatenlijnen. De specifieke expressie van de *Arabidopsis* homoloog van *TDPG* op de plek waar het pollenzakje opengaat, suggereert een geconserveerd mechanisme in het plantenrijk, wat betekent dat de onderdrukking van de expressie van *TDPG* homologen een potentiële weg is om functionele steriliteit in andere plantensoorten te introduceren.

Deze studie heeft geleid tot de ontwikkeling van de gereedschappen nodig voor de productie van parthenocarpe tomatenrassen. De combinatie van *ps-2* met parthenocarpie genen kan het dilemma oplossen dat inherent is aan zaadloze fruitproductie in tomaat: een combinatie van de productie van gelijkvormige, zaadloze tomaten met de mogelijkheid zaad te produceren wanneer dat nodig is.

RÉSUMÉ

Parthénocarpie et stérilité fonctionnelle chez la tomate

La parthénocarpie est définie comme le développement du fruit en l'absence de pollinisation et de fécondation. Chez la tomate (*Solanum lycopersicum*), la parthénocarpie représente un phénomène particulièrement intéressant permettant d'éviter les problèmes de nouaison du fruit qui occurrent lorsque les conditions climatiques sont défavorables. La parthénocarpie facultative est considérée comme la forme de parthénocarpie la plus appropriée pour une utilisation dans les programmes de sélection variétale de tomates. Sous cette forme de parthénocarpie, l'ovaire, si fécondée, se développera en fruit avec pépins. En revanche, s'il n'est pas fécondée, l'ovaire se développera en fruit sans pépin. Les hormones auxines et gibbérellines sont considérées comme les deux éléments clefs du développement parthénocarpique du fruit. Une augmentation de la quantité de ces deux hormones dans l'ovaire peut déclencher la nouaison et le développement du fruit en absence de pollinisation (**Chapitre 1**). L'utilisation de la parthénocarpie facultative résulte fréquemment en la double présence de fruits avec et de fruits sans pépins sur une même plante ou sur un même bouquet ; ce qui porte atteinte à l'homogénéité de la production.

Une utilisation combinée de la stérilité fonctionnelle et de la parthénocarpie facultative peut résoudre ce problème d'homogénéité de la production de fruits sans pépin tout en permettant de produire des semences, si nécessaire, en procédant à une pollinisation manuelle. Le gène *positional sterility-2* (*ps-2*) peut être considéré comme le gène de stérilité fonctionnelle le plus efficace car il bloque la déhiscence de l'anthère sans affecter la viabilité du pollen.

L'objectif de cette thèse est de comprendre les aspects génétique et biologique de la parthénocarpie facultative et de la stérilité fonctionnelle chez la tomate, afin de développer les outils moléculaires indispensables au développement de variétés de tomates sans pépin.

Nous avons caractérisé et cartographié la parthénocarpie dans deux lignées de tomates, IL5-1 et IVT-line 1, chacune contenant un fragment du génome de *Solanum habrochaites* (**Chapitre 2**). Dans chaque lignée, la parthénocarpie était sous le contrôle de deux gènes, nommés *pat-6* et *pat-7* pour la lignée IL5-1, localisés sur les Chromosomes 4 et 5, et *pat-8* et *pat-9* pour la lignée IVT-line 1, localisés sur les Chromosomes 4 et 9. La cartographie de ces quatre gènes de parthénocarpie nous a permis de développer des marqueurs moléculaires étroitement liés à chacun d'entre eux. Ces marqueurs peuvent désormais être utilisés dans la sélection variétale. Il est impossible à ce stade de déterminer si les gènes *pat-6* et *pat-8* sont alléliques ou non. L' utilisation de la micro-synténie entre la tomate et *Arabidopsis* nous a permis d' identifier l' homologue de la tomate au gène *ARF8* au niveau du locus *pat-6*/*pat-8*. En l'absence de pollinisation et de fécondation, *ARF8* agit comme un inhibiteur du développement du carpelle chez *Arabidopsis*. Une lésion dans la séquence du gène *ARF8* chez *Arabidopsis* est connue pour être responsable du développement parthénocarpique des siliques du mutant *fwf* (Goetz et al, 2006). L' homologue tomate du gène *ARF8* est donc un candidat très probable pour expliquer le locus *pat-6*/*pat-8*

Nous avons également cartographié le gène *ps-2* sur le bras court du Chromosome 4 (**Chapitre 3**). Du fait que le caractère *ps-2* est le résultat d'une mutation spontanée chez une variété de tomate cultivée (et non originaire d'une accession de tomate sauvage), la cartographie fine de ce gène dans une population interspécifique ne nous a pas directement conduit au développement de marqueurs moléculaires utilisables en sélection variétale. Après construction de la carte physique de la région du gène *ps-2*, nous avons pu isoler l'allèle fonctionnelle de ce gène par clonage positionnel (**Chapitre 4**). Nous avons ainsi pu mettre en évidence que le phénotype *ps-2* était le résultat d'une mutation n'affectant qu'un seul nucléotide de la séquence codante du gène en question. Il s'agit d'un gène de polygalacturonase que nous avons nommé *TDPG* (*Tomato Dehiscence PolyGlacturonase*). Cette mutation dans la séquence du gène *TDPG* est localisée dans une portion de séquence spécifique à un signal d'épissage, ce qui entraine un épissage alternatif de *ps-2* au moment de la maturation de l'ARN. Cet épissage alternatif entraine un décalage du cadre de lecture (les nucléotides sont lus trois par trois par le ribosome lors de la traduction) et de ce fait cause l'apparition prématurée d'un codon stop amenant à la production d'un ARN dit non-sens. Nous avons découvert que l'expression du gène *TDPG* dans les anthères augmentait de concert avec le processus de maturation de l'anthère. Des traces d'ARN de *TDPG* ont également été découvertes dans le fruit mûre de la tomate, ce qui nous amène à penser que ce

gène peut également être impliqué dans le processus de maturation du fruit. La découverte de la mutation dans la séquence du gène *TDPG*, responsable du phénotype *ps-2*, nous a permis de développer un marqueur moléculaire utilisable pour l'introduction du caractère *ps-2* en programmes de sélection variétale. L'expression spécifique dans la zone de déhiscence de l'anthère de la version homologue du gène *TDPG* chez *Arabidopsis*, nous amène à penser que le mode d'action de ce gène peut être généralisé à un grand nombre d'espèces végétales. Ce constat a pour implication la possibilité d'introduire la stérilité fonctionnelle dans d'autres espèces de plantes, par répression de la version homologue du gène *TDPG* chez ces espèces.

Cette étude nous a permis de développer les outils moléculaires indispensables au développement de variétés de tomates parthénocarpiques. La combinaison du gène *ps-2* avec les gènes de parthénocarpie peut résoudre le problème inhérent à la production de tomates sans pépin : l'homogénéisation de la production de fruits sans pépin associée à la possibilité de produire des semences si nécessaire.

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CURRICULUM VITAE

Benoit Gorguet was born on October 19th, 1979, in Cambrai (North of France). After the completion of his academic high school in the Institut d'Anchin, he studied agriculture at the Institut Supérieur d'Agriculture (ISA) in Lille (France) and graduated in November 2003.

In 2001 he went as an Erasmus student to Wageningen University to study plant breeding and prolonged his stay to complete the full MSc program. In January 2003 he obtained his MSc in Crop Sciences, with distinctions, from Wageningen University. He performed his MSc thesis at the department of plant breeding of Wageningen University, under the supervision of Dr. Rients Niks and Dr. Thierry Marcel, on partial resistance of barley to barley leaf rust.

In February 2003 he started his PhD study in the same department under the supervision of Dr. Pim Lindhout and Dr. Sjaak van Heusden on the topic: Parthenocarpy and functional sterility in tomato. The project was sponsored by Western Seed (The Netherlands). The outcome of this study is presented in this thesis.

From November 2007, he will be working as senior researcher at De Ruiter Seeds (The Netherlands).

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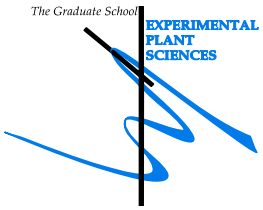
Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Benoit Gorguet
Date: November 16, 2007
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Unraveling the genetics of parthenocarpy in tomato	May 20, 2003
► **Writing or rewriting a project proposal**	
Unraveling the genetics of parthenocarpy in tomato	Feb, 2003
► **Writing a review or book chapter**	
Parthenocarpic fruit development in tomato (Plant Biology, 2005, 7:131-139)	2003
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day 2003 (Utrecht): attendance	Mar 27, 2003
EPS PhD student day 2005 (Nijmegen): attendance	Jun 02, 2005
► **EPS theme symposia**	
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting Lunteren 2004	Apr 05-06, 2004
NWO-ALW meeting Lunteren 2005	Apr 04-05, 2005
NWO-ALW meeting Lunteren 2006	Apr 03-04, 2006
► **Seminars (series), workshops and symposia**	
Flying seminars	2003-2007
Weekly seminars Plant Breeding	2003-2007
► **Seminar plus**	
► **International symposia and congresses**	
2nd Solanaceae Genome Workshop (Ischia, Italy)	Sep 25-29, 2005
3rd Solanaceae Genome Workshop (Madison, United-States)	Jul 23-27, 2006
4th Solanaceae Genome Workshop (Jeju, Korea)	Sep 9-13, 2007
Plant & Animal Genome XV Conference (San Diego, United-States)	Jan 13-17, 2007
► **Presentations**	
Oral presentations to Western Seed (2x per year)	2003-2007
Poster presentation at 3rd Solanaceae Genome Workshop (Madison, United-States)	Jul 23-27, 2006
► **IAB interview**	Jun 03, 2005
► **Excursions**	
Subtotal Scientific Exposure	*18.2 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Principles of ~Omics Data Analysis	Nov 7-10, 2005
Advanced Statistics (PE&RC)	Jun 26-30, 2006
Gateway to Gateway Technology (Wageningen)	Nov 20-24, 2006
► **Journal club**	
member of a literature discussion group at the Plant Breeding Group	2003-2007
► **Individual research training**	
Subtotal In-Depth Studies	*6.9 credits**

4) Personal development	*date*
► **Skill training courses**	
PhD Scientific Writing (CENTA, Wageningen)	Feb 13 - Apr 10, 2006
► **Organisation of PhD students day, course or conference**	
Organisation of the Lab-Trip of the Laboratory of Plant Breeding and PRI groups	Jun 01, 2006
► **Membership of Board, Committee or PhD council**	
Member of the board of EPS	2003-2007
Subtotal Personal Development	*6.1 credits**

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