Parthenocarpic fruit development in *Capsicum annuum*

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Abstract

Parthenocarpy (fruit set without fertilization) is a much desired trait in sweet pepper (*Capsicum annuum*) production as it minimizes yield irregularity, enhances total yieldandmakes theproduction possible under suboptimal environmental conditions. Beside this, parthenocarpyimproves the commercial value of the fruitsince parthenocarpic fruits are convenient for consumption, much wanted for minimal-processed food, and possess long shelf-life.Parthenocarpy has been widely studied for tomato and Arabidopsis but not for *C. annuum*.Physiological and morphological characterization of parthenocarpy in *C. annuum* is the main focus of this thesis with emphasis on finding evidence thattomato and Arabidopsis can be used as model plants to study fruit development in *C. annuum.*The series of physiological and morphological changes (i.e. pollen tube growth, vascular connection between ovule and carpel, cell division and cell expansion in carpel) that occurs in a post-fertilized ovary of*C. annuum*was similar to that reported in tomato and Arabidopsis. Similar to these two species, *C. annuum* showed a hierarchy between auxin and gibberellinwhere auxin acts upstream of gibberellin in fruit set, most likely by inducing gibberellin biosynthesis.These findings indicate that fruit set mechanisms in *C. annuum* are similar to that reported in tomato and Arabidopsis.Parthenocarpy was evident in most of the studied genotypes of *C. annuum*(n=24) suggesting that somedegree of intrinsic parthenocarpy is already present in *C. annuum*. External application of auxin and gibberellin on the stigma of emasculated flowers enhanced parthenocarpic fruit production.GA_3 did not significantly contribute to the final fruit size. GA_3 seems to play an important role in preventing flower and fruit abscission while auxin seems to be important for both fruit set and fruit development.

Almost all seedless fruits obtained either by only emasculation or emasculation followed by hormone application showed stronger growth of carpel like structures (CLS) compared to seeded fruits. Structural analogy of CLS with *bel1* mutant of Arabidopsis suggests that CLS are transformed from abnormal ovules.*Capsicum* genotypes with high parthenocarpic potential showed a stronger CLS development suggesting a relation between female sterility and parthenocarpy. The parthenocarpic potential appeared to be controlled by a single recessive gene. The CLS phenotype and parthenocarpy could not be linked to a single locus, suggesting that absence of fertilization induces parthenocarpic fruit development and allows CLS growth, which substitutes developing seeds in promoting fruit development.This thesis provides insight in the physiology and morphology and genetic basis of parthenocarpy in *C. annuum*.

Key words: Parthenocarpy, *Capsicum*, fruit set, hormones, cell division, cell expansion, auxin, gibberellin, temperature, carpel-like structures, genotype

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Chapter 1

General introduction

Introduction

Sweet pepper (*Capsicum annuum* L.) is an important vegetable crop worldwide. *C. annuum* is used as a salad, stuffing, cooked vegetable, pickled or processed and appreciated world wide for their flavor, aroma, color and as an important source of vitamin C, vitamin A (red stage), antioxidants, folic acid and fibers (Chakravarty *et al.*, 2009; Frank *et al.*, 2001; Palada *et al.*, 2006). Major problems experienced by sweet pepper growers are the strong week-to-week fluctuation/irregularity in yield plus the physiological disorder blossom-end-rot (BER) (Marcelis and Baan Hofman-Eijer, 1997). Irregular yield is caused by the fact that the presence of developing fruits induce flower or fruit abortion on the next few nodes (Marcelis *et al.*, 2004). Incidences of BER are caused by local deficiencies of calcium in young, rapidly expanding pepper fruit tissues (Bangerth, 1979; Rubio *et al.*, 2010). Seeds located in older fruits appear to play a dominant role in competition with flowers or fruits higher in the plant. Therefore, parthenocarpic fruits obtained by external application of auxin on the stigma of flowers results in more regular yield, minimizes the incidence of BER, improves the total yield and makes production possibleunder suboptimal environmental conditions (Heuvelink and Körner, 2001). Beside this, parthenocarpy increase the commercial value of the fruit since parthenocarpic fruits are easy to consume, much wanted for minimal-processed food, and possess high shelf-life (Gillaspy *et al.*, 1993; Gonzalez *et al.*, 2004; Habashy *et al.*, 2004). Despite of this, not much attention has been given to understand parthenocarpy in *C. annuum.* In this chapter, wesummarize the overview of various approaches that induce parthenocarpy in various crop species and possible application of each approach for inducing parthenocarpy in *C. annuum* is discussed.

Parthenocarpy

Parthenocarpy (*Parthenos*, virgin; *karpos*, fruit) is the natural or artificial induction of fruit development without pollination and fertilization. For various parthenocarpic plant varieties, an increased supply of phytohormones to the gynoecium from sources other than the developing seeds has been reported to be sufficient to induce fruit growth (Abad and Monteiro, 1989; García-Martínez, 1997; Gillaspy *et al.*, 1993). This observation suggests that parthenocarpic gene/s might primarily affect the hormone production, transport, and/or metabolism leading to sensitivity or higher hormone levels in the ovary capable of promoting fruit growth even in the absence of pollination and fertilization (Vivian-Smith *et al.*, 2001).

Parthenocarpy, like stenospermocarpy and apomixis are genetically controlled traits, in which fruit set are uncoupled from the series of events that occurs after pollination and fertilization (Fig. 1).In stenospermocarpy, though distinct from parthenocarpy, normal pollination and fertilization do occur and initiate fruit development, but early embryo abortion results in seedless fruits as recorded in grapes and watermelon (Ledbetter and Ramming, 1989). Apomixis is embryo and seed development without fertilization and as a result apomictic species also produce fruit in the absence of fertilization as recorded in citrus, blackberry,

mangoes and walnut (Hanna and Bashaw, 1987; Koltunow and Grossniklaus, 2003; Nybom, 1986).

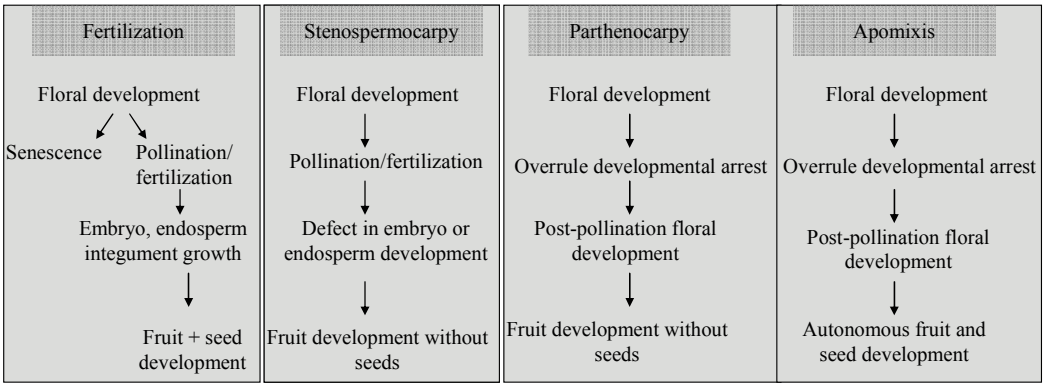

Figure 1 schematic representation of the developmental differences between fruit obtained by fertilization, stenospermocarpy, parthenocarpy, and apomixis.

Natural occurring parthenocarpy has been identified as obligate or facultative. Genotypes with obligate parthenocarpy are unable to produce viable seeds either in the presence or in the absence of fertile pollen as reported in satsuma mandarin (George *et al.*,1984). Plants with this condition are thought to be defective during female gametophyte development and require manual vegetative propagation. In contrast, facultative parthenocarpic plants produce seedless fruits only when pollination is prevented by growing conditions (e.g. temperature), or by manual manipulations (e.g. emasculation) or physiological barriers (e.g. self incompatibility) (Ho and Hewitt, 1986). Various parthenocarpic mutants of tomato (*pat 2*, *pat 3/4*),eggplants, squash, and pineapple are examples of facultative parthenocarpy (George *et al.*,1984).

Parthenocarpic fruits obtained either by growing plants at low night temperature or by emasculating flowers. Parthenocarpic fruit with shiny appearance and fruit weight similar to seeded fruits are the most desirable one for the commercial purpose. Usually parthenocarpic fruit production is accompanied with the production of knots (small, misshapen and undesirable fruits for commercial production) e.g. tomato and *C. annuum*(Asahira*et al.*, 1982; Rylski and Spigelman, 1982; Gorguet *et al.*, 2008). Production of seedless fruits without knots is a major challenge for breeders. Coupling parthenocarpy with self-incompatibility or male sterility can help to promote commercial seedless fruit production (Li, 1980; Yamamoto and Tominaga, 2002;Gorguet *et al.*, 2009).

There are various approaches to obtained parthenocarpy in diverse plant species. Genetic occurrence of parthenocarpy can be exploited by using classical, molecular or mutation breeding approaches.Though parthenocarpy has a genetic basis, it can also be induced artificially in non-parthenocarpic plants by external application of hormones or by altering the endogenous plant hormone within ovules or carpel tissues (Fig. 2).

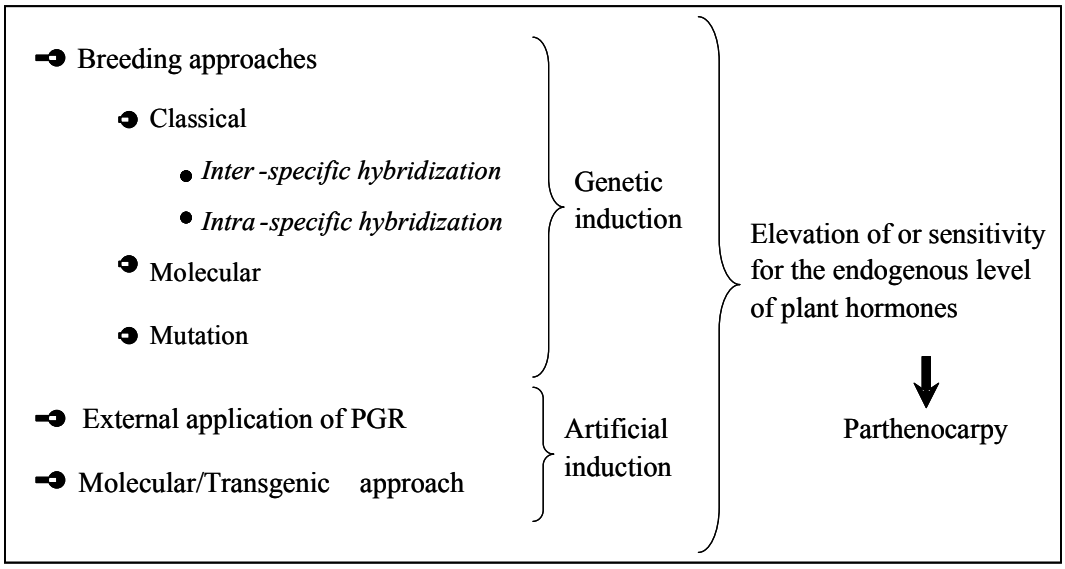

*Figure 2*Schematic representation of various approaches which influence endogenous level of plant hormones to induce parthenocarpic fruit set.

Parthenocarpy by breeding

Classical breeding

Classical or conventional breeding has been successfully applied to introduce parthenocarpy into economically important genotypes. Classical breeding comprises two fundamental steps; i) generating a breeding population that is segregating for the parthenocarpy trait of one parental genotype, and ii) selecting individual progeny from the segregating population that combine parthenocarpy with the desirable traits of the non-parthenocarpic parent. A breeding population is generated by using two types of crosses: inter- and intra-specific hybridization. Interspecific hybridization comprises variability in their offspring and is usually accompanied with sterility or semi sterility which helps in identifying genotypes that arise with strong parthenocarpic attributes or alleles(Gorguet *et al.*, 2008). Various parthenocarpic lines of tomatoes have been developed by using interspecific hybridization such as obligate parthenocarpy in aneuploid tomato developed from a cross between *Solanum esculentum* and *S. peruvianum*(Lesley and Lesley, 1941), IVT-line 1 was developed from a cross between *S. habrochaites and S. lycopersicum*(Zijlstra, 1985).

Altered ploidy through interspecific hybridization is a common approach to obtain parthenocarpic fruits in various crops such as banana, watermelon and citrus (Fortescue and Turner, 2005). Usually, triploid plants can not pass through meiosis, and are therefore sterile. Although the mechanisms of fruit initiation in triploid plants are largely unknown, the observations indicate that sterility is an important condition by which parthenocarpy becomes expressed. Several examples are given in Table 1 where ploidy alteration by interspecific hybridization results in parthenocarpic fruit set.

Table 1. Parthenocarpic fruit set in various species associated with ploidy level

Fruit crops	Species (Chromosome no.)	Other changes	Ploidy no.
Fig-common (Falistocco, 2009)	*Ficus carica* (2n=26)		triploid
Fig-sycomore (Condit, 1964)	*Ficus sycomorus*		triploid
Grape (Wakana *et al.*, 2007)	*Vitis vinifera* (2n=38)	Hermaphroditic, increased berry size	triploid
Apple (Dermen, 1965)	*Malus domestica(*2*n*=34, 51)	Aroma, loss of astringency, sweetness	triploid
Pear–European (Dermen, 1965)	*Pyrus communis (* 2*n*=34, 51)	Aroma, loss of astringency, sweetness,	triploid
Pear–Asian (Kadota and Niimi, 2004)	*Pyrus pyrifolia, P. bretscheiderii* *P. ussuriensies (*2*n*=34, 51	Aroma, loss of astringency, sweetness	triploid
Citrus (orange, mandarin, lemon, lime, pumello,grapefruit) (Habashy *et al.*, 2004; Mackiewicz *et al.*, 1998)	*Citrus spp*. (2*n*=18)	Nucellar embryony,	triploid
Persimmon Asian (Janick, 2006)	*Diospyros kaki (* 2*n*=90)	Loss of astringency	triploid
Loquat (Liang *et al.*, 2007)	*Eriobotrya japonica (*2*n*=34, 51)	Increased size	triploid
Pineapple (Collins, 1933b)	*Ananas comosus (*2n=50)		triploid
Banana (Fortescue and Turner, 2005)	*Musa paradisiaca*		triploid
Plantain (Fortescue and Turner, 2005)	*Plantago hawaiensis* (2n=33)		triploid
Tomato (Habashy *et al.*, 2004; Mackiewicz *et al.*, 1998)	*Lycopersicum esculentum*	Increase dry matter, TSS	triploid
Cucumber (Chen *et al.*, 2003; Habashy *et al.*, 2004; Mackiewicz *et al.*, 1998)	amphidiploid x diploid		triploid
Watermelon (Kihara, 1951)	*Citrullus lanatus* (2n=22) diploid male with autotetraploid female	High sugar content, more fruit/plant, thin rind	triploid

The use of interspecific hybridization to generate *de novo* parthenocarpy is not successful in most of the *Solanaceae* species e.g. tomato, potato and eggplant (Carputo *et al.*, 2000; Ehlenfeldt and Ortiz, 1995; Hanneman and Peloquin, 1968; Rick, 1945). Crossing between diploid plant and its colchicine-derived tetraploid prevents triploid progeny to be produced due to the imbalance of parental ploidy. This condition is known as a "triploid block" which results in no fruits or some fruits with sterile seeds which is due to the failure of normal endosperm development (Contolini and Hughes, 1989). Also, interspecific hybridization is not a successful approach to introduce parthenocarpy in *C. annuum*. Cross compatibility among cultivated and some wild species of *Capsicum* showed that *C. annuum* has a partial comparability with *C. chinense* and *C. frutescens*.However, thiscompatibility can not be

utilized for interspecific hybridization because crossing between these species results in partial sterile seeds, while seeds are required by farmers to grow plants (Fig. 3).

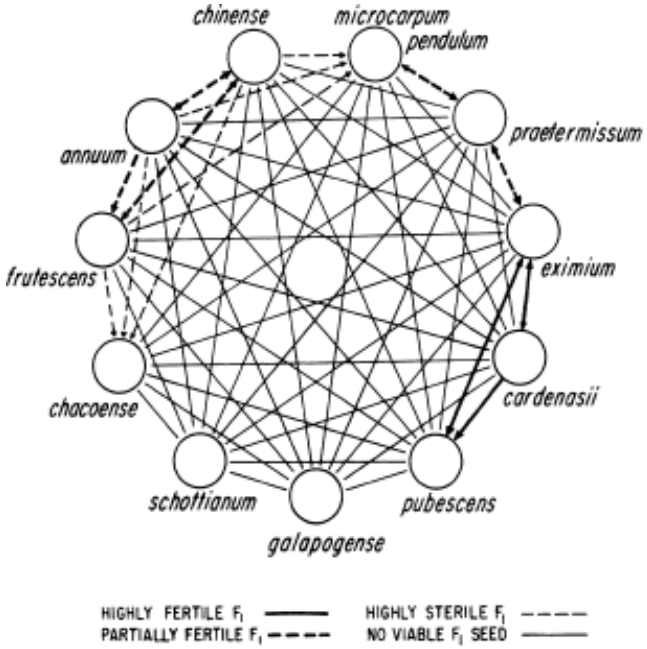

Figure 3 Cross compatibility of cultivated and some wild species of *Capsicum* (Lippert *et al.*, 1966)

Intraspecific hybridization is commonly used to generate new lines and cultivars because they integrate high variability in their offspring, and this can form the basis of future selections(Lifschitz *et al.*, 1993). The use of intraspecific hybridization for producing a facultative parthenocarpic line suitable for a hot and dry climate (normal fruit at moderate temperature) was first introduced in tomato (Hawthorn, 1937). After that, various other parthenocarpic lines have been generated by using intraspecific hybridization e.g. Severenien, Oregon T5-4, Oregon Cherry, Oregon 11, Line 75/79, Line P-26, Line P-31, Line RG and IVT-line 2 in tomato (Baggett and Frazier, 1978; Philouze Maisonneuve, 1978; Zijlstra, 1985), and 'AE-P' lines and 'Talina2/1' in eggplant (Kikuchi *et al.*, 2008). These genotypes were further utilized to generate segregating population and to study the genetics of parthenocarpy e.g. single (e.g. *pat*, *pat 2*) or double (e.g *pat3*/*pat4*) recessive gene/scontrol parthenocarpy in tomato (Gorguet *et al.*, 2008), group of recessive genes controlled parthenocarpy in eggplant (Tian *et al.*, 2003). Though use of intraspecific hybridization for introducing parthenocarpy has not been discuss beforein *C. annuum,* its application seems to be promising to understand the genetics behind parthenocarpy.

Classical breeding is relatively inexpensive and technically simple. However, there are sometimes limitations in the amount of genetic variation available within the crop (the gene-pool), and the approach requires large populations and multiple generations of selection to identify parthenocarpic plants that combine the best qualities of both parents. Moreover, with each cross, usually a large number of genes is transferred that may not be useful along with the desired ones in the target species (Beaver and Osorno, 2009; Lasley *et al.*, 1994). For minimizing these limitations as well as for the technical advancement,

molecular and mutation breeding approacheswere later introduced into the classical breeding.

Molecular breeding: molecular marker enhancesselection in classical breeding

Molecular breeding has beenintroduced not so long ago into classical breeding methodologies. The purpose is to maximize the probability of successful allele retention by reducing the need of a large breeding population and multiple generations of selection (Huang *et al.*, 2002; Ortiz and Vuylsteke, 1998; Ruttan, 1999). Through Marker Assistance Selection (MAS), DNA markers detect the presence of allelic variation in genes underlying a certain trait and thus increase the efficiency and precision in the selection process. For example, many lines are discarded after MAS early in the breeding program, and this reduces the total numbers of lines to be screened and thus minimizes space and time constraints. Replacement of phenotype by markers allows off-season and heterozygote selection possible and thus makes the overall process more cost-effective by growing many generations per year (Ribaut and Hoisington, 1998). Integration of MAS with Quantitative Trait Loci (QTL) analysis has provided more potential for increasing breeding efficiency (Doerge, 2002; Tanksley *et al.*, 1992). So far, only one parthenocarpy gene, *pat*, was mapped in tomato (Beraldi et al. 2004). Recently, four QTLs associated with parthenocarpy were identified and mapped in tomato (Gorguet *et al.*, 2008). The isolation of these QTLs will enhance not only our understanding about fruit set in tomato but also open possibilities to develop seedless fruits in other economically important species like *C. annuum*because of the high level of similarities within the *Solanaceae*.

Mutation breeding enhances genetic variability

A major constraint in classical breeding is the lack of natural genetic variation in the gene-pool, which can be overcome by mutation breeding. A mutation is defined as a heritable change in sporophytic (i.e. in the somatic cells) or gametophytic tissue (i.e. germ cell line from where gametes arise) that is not caused by normal genetic recombination or segregation. Induction and selection of specific mutations can now be achieved (Cooper *et al.*, 2008), but spontaneous mutations have historically been an important source of genetic variation.Spontaneous mutationsoccur naturally and are used in classical breeding approaches. Good examples of this are the parthenocarpic *sha-pat* mutants in the tomato line ‘Montfavet 191’ (Pecaut and Philouze, 1978). However, when the genetic variation is limited, mutations have been induced chemically, by radiation treatment, or by DNA insertion (transposon, T-DNA). Various radiation treatments, such as helium accelerated ions in tomato (Masuda *et al.*, 2004), soft–X-ray in watermelon (Sugiyama and Morishita, 2000), gamma and thermal neutron irradiation in grapefruit (Vanamala *et al.*, 2007) and gamma irradiation in *Citrullus lanatus*(Sugiyama and Morishita, 2001) and *Citrus* (Spiegel-Roy and Vardi, 1989), have been used successfully to generate parthenocarpic mutants. For chemical mutagenesis two major classes of compounds are available: alkylating agents, such as ethyl methane sulphonate (EMS), and ethyl ethane sulphonate (EES), and base analogs, such as 5-bromouracil and 2-aminopurine. EMS is the most common mutagen,as it is very effective andconvenient. It has been used to generate parthenocarpic mutants of Arabidopsis (*fwf*) and tomato (stock 2524: short anther mutant, *sha*) (Bianchi and Soressi, 1969; Soressi, 1970; Motto *et al.*, 1975; Vivian-Smith *et al.*, 2001). Transposon mutagenesis has generally been a cis-genesis approach where mutagenesis in the recipient

plant was induced by crossing with a transposon containing donor plant. The parthenocarpic mutants of apple have been identified where an already active and endogenous retro-transposon inserted into the intron region (intron 4 in Rae lime and intron 6 in Spencer seedless and Wellington Bloomless) and resulted in loss of function of MADS-box transcription factor gene *MdPI*(Yao *et al.*, 2001). Although mutation breeding is effective to obtain parthenocarpic fruits, its applicability has been limited in the condition where parthenocarpy is associated with pleiotropic or unfavorable genetic link characteristics (Huang *et al.*, 2002; Pandolfini *et al.*, 2002; Yao *et al.*, 2001).

Artificial induction of parthenocarpy

Artificial induction of parthenocarpic fruit set is useful to understand the role of plant hormones in fruit set and fruit development. Parthenocarpycan be induced artificially in non-parthenocarpic plants by external application of plant growth regulators or by altering the endogenous plant hormone levels within ovules or carpel tissues (Fig. 2), as has been shown in Arabidopsis, tomato, eggplant, watermelon, and citrus (Gillaspy et al., 1993; Vivian-Smith and Koltunow, 1999; Rotino et al., 1997; Carmi et al., 2003; Mezzetti *et al.,* 2004).

External application of plant growth regulators (PGRs)
A very effective way to induce parthenocarpy has been reported by exogenous application of PGRs to the stigma or the carpels. Auxins (IAA; or it synthetic analogs 2, 4-D and 4-chloro-IAA, NAA), gibberellins (primarily GA_1, GA_3, GA_4 and GA_{20}) and to a lesser extend cytokinins are known to induce parthenocarpy in several crops (Kojima *et al.*, 2003; Schwabe and Mills, 1981; Vivian-Smith and Koltunow, 1999). Also brassinosteroids have been reported to induce parthenocarpic fruits in cucumber, and the obtained fruit sizes are similar to pollinated fruits (Fu *et al.*, 2008). However, their application in tomato and Arabidopsis did not result in parthenocarpic fruits (Serrani *et al.,* 2007; Vivian-Smith unpublished observations). Exogenous application of auxin on the stigma of flowers before anthesis also resulted in parthenocarpic fruit set in *C. annuum* (Heuvelink and Körner, 2001), thoughthe underlying basis and relationship with other interacting plant hormones were not studied in this publication.
Commercial use of PGR has been recognized in various crops. For example, auxin (4-CPA: 4-Chlorophenoxyacetic Acid) application has been used to increase fruit set and -weight in Pepino (Ercan and Akilli, 1996), GA_3 has been applied to increase fruit bearing in cherries, and increases rachis length and berry size in grapes, whereas GA_4 has been used to promote fruit set in apple and pear trees (Ben-Tel, 1990; Facteau *et al.*, 1989). The down side of these practices is that PGRs can have negative effects on environment and human health (Osborne and Went, 1953; Pomeroy and Aldrich, 1943).

Molecular or transgenic approach
To overcome the negative effects of the use of PGRs, scientists have developed transgenic approaches that mimic their exogenous application. Expressionof the auxin biosynthesis genes *iaaM* (*tryptophan monooxygenase*) or *iaaH* (*indoleacetamide hydrolase*) from the *Agrobacterium* T-DNA under control of an ovule/placenta specific promoter has been reported to result in fertilizationindependent fruit set in tobacco (Bavrina *etal.*, 1999), egg

plant (Rotino *et al.*, 1997), tomato (Ficcadenti *et al.*, 1999), cucumber (Yin *et al.*, 2006), andstrawberry (Mezzetti *et al.*, 2004). Similarly, expression of the *Agrobacterium rhizogenes*-derived *rolB* gene, which confers sensitivity to auxins, under a fruit-specific promoter has been reported to induces obligate as well as facultative parthenocarpy in tomato (Carmi *et al.*, 2003). Other approaches have been designed to target the transcription factors (Aux/IAA and ARF) that are involved in auxin-responsive gene expression. Expression of the *Atarf8-4* mutant allele in tomato has resulted in plants display parthenocarpic fruit development (Goetz *et al.*, 2007). Moreover, parthenocarpic fruit set has been obtained in tomato by antisense silencing of *IAA9*(Wang *et al.*, 2005).

Success of the transgenic approach to obtained parthenocarpy in *C. annuum*is dependent upon an efficient and reliable transformation and regeneration protocol. Many members of the *Solanaceae* family are easy with regard to cell culture and regeneration e.g. petunia (Sitbon *et al.*, 1991), tobacco (Bavrina *et al.*, 1999), egg plant (Rotino *et al.*, 1997), and tomato (Ficcadenti *et al.*, 1999). In contrast, *C. annuum* is considered to be recalcitrant to regeneration due to the formation of sterile buds or shoot-like structures (Agrawal *et al.*, 1989; Gunay and Rao, 1978; Husain *et al.*, 1999; Wang *et al.*, 1991). Recently, efficient regenerations have been demonstrated in a wide range of important *C. annuum* genotypes (Balazs *et al.*, 2008). This recent established methods for transformation may open possibilities to obtain a transgenic *C. annuum* with parthenocarpic trait.

Transgenic parthenocarpic plants which are unable to disseminate by seed dispersion and crossing with male sterile plants will inhibit the chance of pollen contamination to the neighbor plant. Moreover, transgenic fruits are apparently similar to seeded fruits in size and morphological appearancei.e. tomato (Pandolfini *et al.*, 2002). Additionally, reduced processing costs, increased flavour (high soluble solid, high brix) and increased total yield has been reported in transgenic parthenocarpic plants compared to non-transgenic plants (Barg and Salts, 2000; Carmi *et al.*, 2003; Salts *et al.*, 1992). Although a transgenic approach can provide a valuable means of obtaining parthenocarpic varieties with additional benefits, difficulties to generate transgenic plants, and fear among the consumers has raised doubts about its commercial applicability (Acciarri *et al.*, 2002; Donzella *et al.*, 2000; Ficcadenti *et al.*, 1999; Rotino *et al.*, 1997).

Gap in knowledge

Thoughvarious approaches have been demonstrated to induce parthenocarpy in several crop species, their applicability in *C. annuum* is not well studied.None of the breeding approaches has been introduced to study the detail of parthenocarpy in *C. annuum*. No parthenocarpic mutants are identified nor are there any transgenic parthenocarpic plants generated in *C. annuum*. The physiology and genetics of parthenocarpic fruit growth in *C. annuum* is largely unknown. Availability of parthenocarpic mutants in *C. annuum* would facilitate extended studies of parthenocarpy to understand various physiological mechanisms involved in regulating fruit set and fruit growth. Mutagenesis by using reverse genetics is an effective approach to obtained parthenocarpic mutants. A particular gene's function can be determined by studying the phenotypes of individuals containing the alterations in the gene of interest (Sessions *et al.*, 2002). Few candidate genes are identified in Arabidopsis for regulating parthenocarpic fruit set (Goetz *et al.*, 2007). These genes can be targeted to be identified or captured in *C. annuum* by using the mutagenesis approach only when both species showed fundamental similarities for their fruit set mechanisms; however, this

comparison was never studied before. Parthenocarpic fruits can be obtained by growing plants at low night temperature (Rylski, 1974; Rylski and Spigelman, 1982). However, genetic variability among the genotypes for parthenocarpic fruit set and the effect of temperature on fruit size in different genotypes has never studied.Most of the *C. annuum* fruit which develop parthenocarpically, showed carpel-like structures (CLS) or internal proliferation (Cochran, 1934). In other species, such as Arabidopsis,CLS have been reported as abnormal structures arising from the placenta and have associated parthenocarpy (Payne et al., 2004). Though co-occurrence of parthenocarpy and CLS phenotype has been reported, their genetic basis and their interaction with each other are not known. External application of auxin induces parthenocarpy in *C. annuum* (Heuvelink and Körner, 2001); however, the effect of gibberellins, and the possible interaction between gibberellins with auxin, has also never been studied.

Scope and outline of the thesis

Experiments by Heuvelink and Körner (2001) has clearly demonstrates that parthenocarpy is a much desirable trait in *C. annuum* production because it can stabilize irregular fruit set. Physiological and developmental characterization of the parthenocarpic response in *C. annuum* is the main focus of this thesis with emphasis to find the evidences thattomato and Arabidopsis can be used as a model plant to understand the detail of fruit set and fruit growth in *C. annuum*.

Fruit-set involves a series of events that is well described in tomato and Arabidopsis but largely unknown in *C. annuum*. To check whether early fruit growth in *C. annuum* is the same as it is in tomato and Arabidopsis, we carefully described the physiological and morphological changes (pollen germination and tube growth, vascular development, endogenous IAA measurement, cell division and cell expansion in the carpel) in a post-pollinated ovary of *C. annuum*(**Chapter 2**). Genetic variations for parthenocarpic fruit set have been reported in various crops. To check whether *C. annuum* also exhibit genetic variation in their germplasm for parthenocarpic fruit set potential, and how the absence of seeds affects the fruit size in those genotypes, we evaluated genotypes of known parthenocarpic background in conjunction with a seeded cultivar as a control.This was done at normal and low night temperaturesto control fertility (**Chapter 3).** We found that seedless fruits obtained either by emasculation or by hormone application often contain CLS. Based on observations of Arabidopsis *bel1* mutants, where abnormal ovules develop into CLS, we studied the levels of aberrant *Capsicum*ovules and correlate that with CLS transformation and parthenocarpic fruit set in **Chapter 4**. Furthermore, in this chapter we describe the inheritance of parthenocarpy and CLS and their possible relation with each other. The role of auxin and gibberellin has been extensively studied in tomato and Arabidopsis and a model has been proposed to elucidate the role of hormones in fruit set. The same model for fruit set may or may not be applicable in *C. annuum*; therefore, detailed investigation is conducted to understand the role of auxin and gibberellin and their interaction for regulating fruit set, and their effect on fruit growth**(Chapter 5)**. In **Chapter 6**, the main achievements and limitations of this study are discussed. Furthermore, the prospects for obtaining parthenocarpic *C. annuum* accessions are discussed based on the results obtained in this thesis.

Chapter 2

Physiological and morphological changes during early fruit growth in *Capsicum annuum*

Abstract

Fruitset involves a series of physiological and morphological changes, which are already well described fortomato and Arabidopsis but largely unknown for sweet pepper (*Capsicum annuum*). The aim of this paper is to compare mechanisms of fruit set observed in Arabidopsis and tomato to *C. annuum.* To do this, we accurately timed the physiological and morphological changes such as *in vivo* pollen germination, pollen tube growth, andthe vascular connection between ovule and carpel. We also measured the endogenous auxin levels and evaluated the cell division and cell expansion in post-pollinated ovaries. The majority of pollen tubes arrived at ovules around 20 to 28 hours post pollination, indicating that most fertilization events occurred during that time. A vascular connection between ovule and replumwasalso observed in fertilized ovaries that undergo fruit development, and this connection was absent in non-fertilized ovaries that abort. This directs that vascular connection between ovule and replum is an early indicator for successful fruit development after pollination and fertilization. Evaluation of histological changes in the carpel of a fertilized ovary indicated that increase in cell number and cell expansion is comprised of early fruit growth. Cell division contributes moreduring early fruit growthwhile cell expansion continues into the later stages of fruit growth in *C. annuum*.The simultaneous occurrence of a peak in auxin concentration and a strong increase in cell diameterin the carpel of seeded fruits suggests that IAA directly stimulatesthe major increases in cell diameter at later stages of fruit growth.The series of physiological and morphological events observed during fruit set in *C. annuum*are similar to what has been reported for tomato and Arabidopsis. The results indicate that tomato and Arabidopsis are suitable model plants to understand details of fruit set mechanisms in *C. annuum.*

Introduction

Successful completion of pollination and fertilization is essential to trigger fruit set in most flowering plants (Gillaspy *et al.*, 1993). Fertilization induces the development of an ovary into a fruit, while failure of fertilization triggers senescence of floral organs and finally leads to flower abscission (Nitsch, 1970; Ozga *et al.*, 2002; Talon *et al.*, 1992). Fruit set involves a coordination between signaling pathways involving auxin, gibberellins, ethylene and cytokinin (Vriezen *et al.*, 2007). Several independent observations have indicated that auxin is important for fruit set and development. For example, auxin supplied either exogenously or ectopic expression of genes encoding enzymes of auxin biosynthesis induces fruit-set in tomato and Arabidopsis (Spena and Rotino, 2001; Vivian-Smith and Koltunow, 1999). An important question is how auxin signaling enables the coordinated developmental change from pistil/gynoecium into fruit. Prior to anthesis, carpel development is arrested and pollination/fertilization induces processes such as vascular development and differentiation within the carpel to assist the development of the pistil/gynoecium into a fruit (Gillaspy *et al.*, 1993). Modulations in the auxin signaling pathways have been reported to occur within 24 hours post-pollination in tomato, and to result in developmental changes and changes in carbon partitioning (Vriezen *et al.*, 2007). In Arabidopsis, pro-vascular strands are observed between fertilized ovule and carpel

margin to connect within 54 hours post-pollination, while they remain unconnected/undevoloped in unfertilized ovules(Fuentes and Vivian-Smith, 2009). This vascular connection has been reported to occur also in the absence of fertilization in the Arabidopsis parthenocarpic *fruit without fertilization (fwf*/*arf8*)mutant (Vivian-Smith, 2001). These findings support the idea that auxin signals might transfer from the ovule to the carpel triggering vascular connection and fruit set.

Sweet or bell pepper (*Capsicum annuum*) is an important vegetable fruit crop. In *C. annuum,* various physiological aspects have been studied including fruit set and flower abortion. Factors influencing these processes are the plant hormones auxin and ethylene (Heuvelink and Körner,2001; Huberman *et al.*, 1997; Wien *et al.*, 1989a), assimilate availability (Marcelis *et al.*, 2004), assimilate utilization and dominance of competing fruits (Aloni *et al.*, 1996), reduced metabolic activity due to for example heat stress (Aloni *et al.*, 1995), and cultivar susceptibility for flower abortion (Turner and Wien, 1994a; Turner and Wien, 1994b;Wubs *et al.*, 2009). Recently, a review summarized the effect of all these factors on flower or fruit abortionin *C.annuum*(Wubs *et al.*, 2009)

However, physiological and morphological changes that occur in a post pollinated ovary to regulate fruit set are not well studied in *C.annuum*. The aim of this paper is to investigate whether mechanisms of fruit set observed in tomato and Arabidopsis are also applicable to *C. annuum.* To do this, we accurately timed the physiological and morphological changes such as *in vivo* pollen tube germination and pollen tube growth in the ovule vicinity, and vascular connection between ovule and carpel. To understand the role of auxin in fruit set and fruit development, endogenous IAA levels were measured in seeded and seedless fruits obtained from pollinated and emasculated flowers, respectively. To check the contribution of cell division and cell expansion to fruit growth, we measured fruit diameter, cell number/number of cell layers or cell diameter in a developing ovary at early (0-72 hours post pollination) and at later (0-40 days after anthesis) stages of fruit growth. The results indicate that tomato and Arabidopsis are suitable model plants to understand details of fruit set mechanisms in *C. annuum.*

Material and methods

Plant material and growth conditions

Seeds of sweet pepper (*Capsicum annuum* L.) cultivar ‘Bruinsma Wonder’ were obtained from Plant Research International (PRI: 2004001). Four weeks after sowing, 20 seedlings were transplanted into pots of 2 litre size with potting soil and transferred into a climate room. Nutrient solution (Voogt and Bloemhard, 1993) was supplied regularly. Flower emasculation (removal of outer floral organs: petals and anthers) was performed 2 days before the expected date of anthesis to avoid accidental self-pollination. Two experiments were conducted where the first experiment includes: *in vivo* pollen tube growth, vascular development between ovule and carpel, and cell number and cell diameter in the carpel from 0-72 hours post pollination (HPP). The second experiment includes the measurement of endogenous IAA levels,number of cell layers and cell diameter in the carpel from 0- 40 days after anthesis (DAA).

In vivo pollen tube growth, vascular development, mesocarp cell number and cell diameter
Plants were grown in a climate room with a constant temperature of 24°C day/night temperature. From previous experiments we know that emasculation of flowers in this growth condition results in flower abortion (unpublished data). Plants were exposed to 16 hours day length (from 08.00 to 24.00) by using high pressure mercury lamps (Philips HPI 400 W) providing a minimum photon flux density of 100 µmol m^{-2} s^{-1} at plant level. Pots were arranged on a table of 1.20 meter height and were allowed to grow without any pruning. At anthesis, all emasculated flowers were hand pollinated by using fresh pollen from other flowers of the same plant and date and time of the pollination was registered.
To determine the *in vivo* pollen tube growth, flowers were collected at 0, 2, 4, 6, 8, 10, 12, 14, 15, 20, 28 HPP. For each stage, 5-15 flowers were harvested and fixed immediately in an acetic acid alcohol (1:3) solution for overnight. Flowers were rinsed three times with water, replaced with 10% sodium sulphite solution (w/v) and autoclaved at 121^{o}C for 5 minutes. After cooling down, flowers were again rinsed three times with water and finally replaced with decolorized aniline blue/glycerol (Decolorized aniline blue (DAB): mix 0.2 g of aniline blue and 4.6 g K_3PO_4, stored in dark overnight). Flowers were dissected to reveal the ovary, and observations of pollen tube growth on stigmas, and elongation into the style were made using an epifluorescence microscope (Leitz, Dialux 20EB). Pollen tubes contain callose (ß1-3 glucan) in their wall as well as in callose plugs that segment growing tubes. Callose plugs were highly fluorescent under the microscope and therefore the presence of callose plugs was used to locate the pollen tubes in the stylar tissue, placental tissue and near the ovule micropyle.
To evaluate vascular development in fertilized and unfertilized ovules, ovaries (n=3-5) obtained after pollination or emasculation were collected at 48 HPP. Same numbers (n=3-5) of ovaries from both treatments were allowed to grow on the plants to see whether they will set into fruit or abort. Collected ovaries were cleared by using Hoyers solution (Zhang and Somerville, 1997: 100g chloral hydrate; 5 ml glycerol; 30 ml H_2O). Cleared ovaries were dissected under a simple microscope and observed under a Zeiss Axioplan 2 fluorescence/DIC microscope.Photographs were taken with a Zeiss AxioCam MRc 5 digital colour camera. The vascular connectivity was observed also in a hot pepper cultivar (*Capsicum annuum*: “Fireflame”; F1 hybrid, De Ruiter Seeds, Netherlands), grown under the same growth conditions as Bruinsma Wonder. Cv. Fireflame has a simple anatomy than bell-shaped fruits. Ovules are concentrated along carpel margin that extends down to the style, which facilitates easy and clear observations of vascular connection compared to Bruinsma Wonder (ovules are concentrated around a centrally located axile type of placenta). To evaluate the vascular connection in ovaries that set into fruit without pollination and fertilization, 0.05% NAA or IAA (dissolved in water and mixed with heated lanolin (100^{o}C), stirred to a homogeneous paste and cooled to room temperature) were applied on the stigma of an emasculated flower (n=8-10)at anthesis (day-0) in cv. Bruinsma Wonder. However, none of the flowers treated with hormones set into fruit.
To evaluate cell division and cell expansion in the carpel, pollinated ovaries (n=3-5) were harvested at 12, 20, 30, 40, and 72 HPP. Length and diameter of each ovary was measured using digital vernier calipers. Horizontal and vertical cross sections of the carpel were mounted, stained with “safranin O” (stock solution: 2.5 g safranin O Certistain ® in 100 ml of 96% ethanol, working solution: 1:100 dilution in water), and observed under a Zeiss Axioplan 2 fluorescence/DIC microscope.Photographs were taken with a Zeiss AxioCam

MRc 5 digital colour camera. Cell diameter, measured by using Image tool version 3.0, was used as a measure of cell expansion and cell number was used as a measure of cell division. For each treatment, 20-30 cells were measured from 4-5 sections per fruit.To measure the diameter of individual cellswe used the same four to six layers in the mesocarp starting from layer 5 from the exocarp(box: Fig. 3A).From these data we estimated the total number of cells (Smith, 1950) using the assumption that carpel cell number = (fruit volume-placental cavity volume)/Average cell volume, where fruit volume was treated as a sphear and average cell diameter (calculated from the mesocarp) was used to calculate the average cell volume$\{4\pi/3\ (r)^3\}$.

Endogenous IAA levels and number of cell layers and cell diameter in the carpel

Plants of *C. annuum* cultivar 'Bruinsma Wonder' were grown in a climate room, as described above, at 20.4/20.2^{o}C day/night temperature. From previous experiments we know that emasculation of flowers in this growth condition results in parthenocarpic fruit set (approximately 30%) in cv. BruinsmaWonder (unpublished data). Plants were exposed to long day (from 06.00 to 22.00) by using fluorescence tubes (Philips TL 50W, color 84) with a light intensity of 200±2 µmol m^{-2} s^{-1}.The terminal flowers were removed from all plants at anthesis to support vegetative growth. The twin-branch system was applied, resulting in two stems per plant, with all side shoots restricted to one leaf and flower. Emasculated flowers were hand pollinated at anthesis (day 0) and ovaries (n=2-3) were collected at 0, 2, 4, 6, 8, 10, 15, 20, 30 and 40 DAA. The same numbers of ovaries were collected at the same time (DAA) for pollinated and un-pollinated flowers. After measuring fresh weight of each ovary, these were used for the measurement of endogenous IAA and for the evaluation of the number of cell layers and cell diameter in the carpel.

For the measurement of endogenous IAA, carpel and placenta were collected (10-20 mg fresh weight) from ovaries in 1.5 ml micro centrifuge tubes (Sarstedt) with conical bottom and stored at -80^{o}C. Endogenous level of IAA was quantified using an isotope dilution mass spectrometry technique (Edlund *et al.*, 1995). One to 6 ng [$^{13}C_6$] IAA (Cambridge Isotope Laboratories, Woburn, MA) was added to each sample as an internal standard. Analysis was performed by gas chromatography (GC)-selected reaction monitoring-mass spectrometry (MS), using a JMS-SX/SX102A instrument (JEOL, Tokyo).

For the evaluation of number of cell layers and cell diameter, rectangular pieces from the middle portion of the fruit carpel were cut in a transverse section from each stage except for 0 and 2 DAA where whole fruits were fixed by using paraffin method technique (Bunger-Kibler and Bangerth, 1983). Horizontal sections of paraffin-embedded samples were obtained using a rotary microtome. Transverse sections of 4-6 mm were mounted by aniline blue staining and observed under a light microscope (Leitz Aristoplan). Photomicrographs were then obtained using a camera (Nikon digital camera DXM1200) mounted on the microscope and analyzed by using Image tool (version 3.00). Cell diameter as a measure of cell expansion was determined by averaging diameter of 10–20 cells from 3-5 sections per fruit from exocarp, mesocarp (described above, box: Fig. 3A), and endocarp layers. The number of cell layers was counted in the mesocarp as a measure of cell division.

Statistical Analysis

Cell number and cell diameterat each developmental stage was tested by using one way ANOVA while IAA level was tested by using two way ANOVA (time as a second factor).

Mean separation was done by Student's t-test (LSD) based on the ANOVA mentioned above. Data processing and statistical tests were carried out with SPSS (Statistical Package for the Social Sciences) 15.0.

Results

Fertilization induces vascular connection between ovule and replum to initiate fruit set

To establish the approximate time of fertilization, pollen tube growth and their arrival at the ovule was observed after pollination. Pollen started their germination on the surface of the stigma already at 2 hours post pollination (HPP). However, none of the pollen tubes reached the base of the style up-to 8 HPP. Only a few pollen tubes reached the base of the style at 15 HPP (Fig. 1C), at which time the first pollen tubes were observed in the ovule vicinity. At 20 and 28 HPP many pollen tubes were observed close to the ovules, suggesting that the majority of ovules had been fertilized by that time (Fig.1D,E).

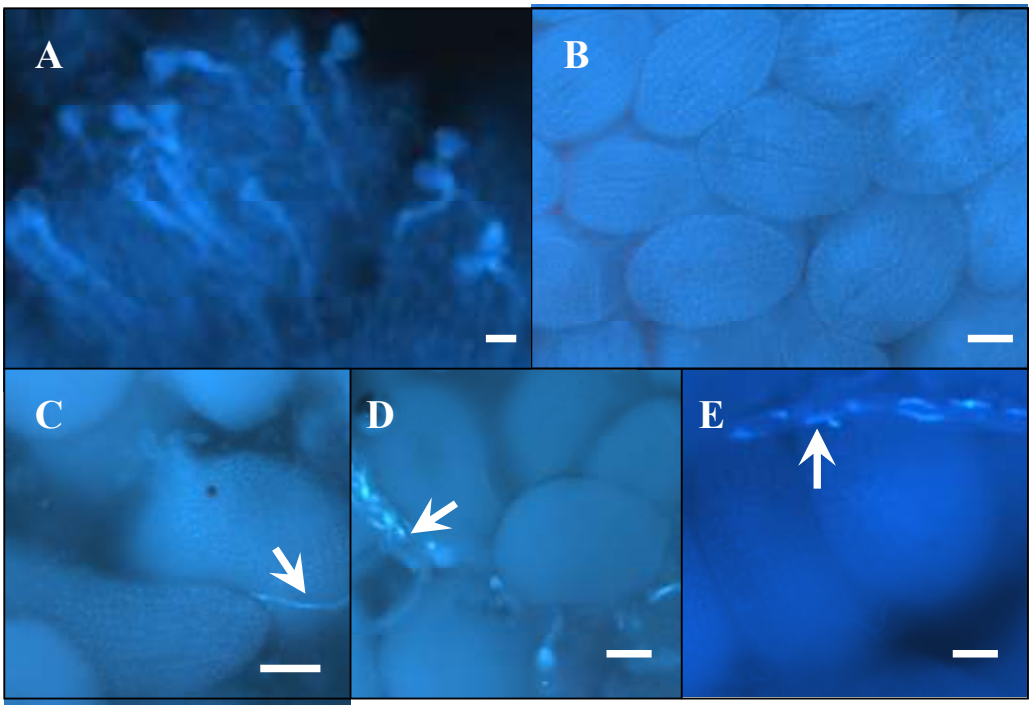

Figure 1In-vivo pollen tube growth in a pollinated ovary of *Capsicum annuum* cultivar 'Bruinsma Wonder'. A; Pollen germination on the stigma of emasculated flowers at 2 hours post pollination (HPP), B; No pollen tubes in the ovule vicinity at 12 HPP, C; A few pollen tubes in the ovule vicinity at 15 HPP (arrow head), and D,E; Many pollen tubes in the ovule vicinity at 20 HPP (D) and 28 HPP (E). The bright spots are callose plugs, indicating the position of pollen tubes (arrow heads). Scale bar: 100 µm (A), 50 µm (B-E).

Fertilization induced auxin signals might be transmitted from ovule to the carpel. Observation of vascular development could be used to infer such signaling, as it has been reported for Arabidopsis (Vivian-Smith, 2001). To test for the same processess in *C. annuum*, cleared fertilized and unfertilized ovules were evaluated under DIC

microscopy.Fertilized flowers set into fruit while unfertilized flowers aborted. At 48 HPP, vascular differentiation in fertilized flowers resulted in a vascular element that extends from the base of the ovule funiculus towards the replum vasculature in Bruinsma Wonder (Fig. 2A) and Fireflame at 48 HPP (Fig. 2C). In contrast, no vascular connection between funiculus and replum was observed in an unfertilized ovule (Fig. 2B, D-E) and also none of the unfertilized ovaries set into fruit. These observationssuggest that the vascular connection is an early indicator for successful fruit set after pollination and fertilization.

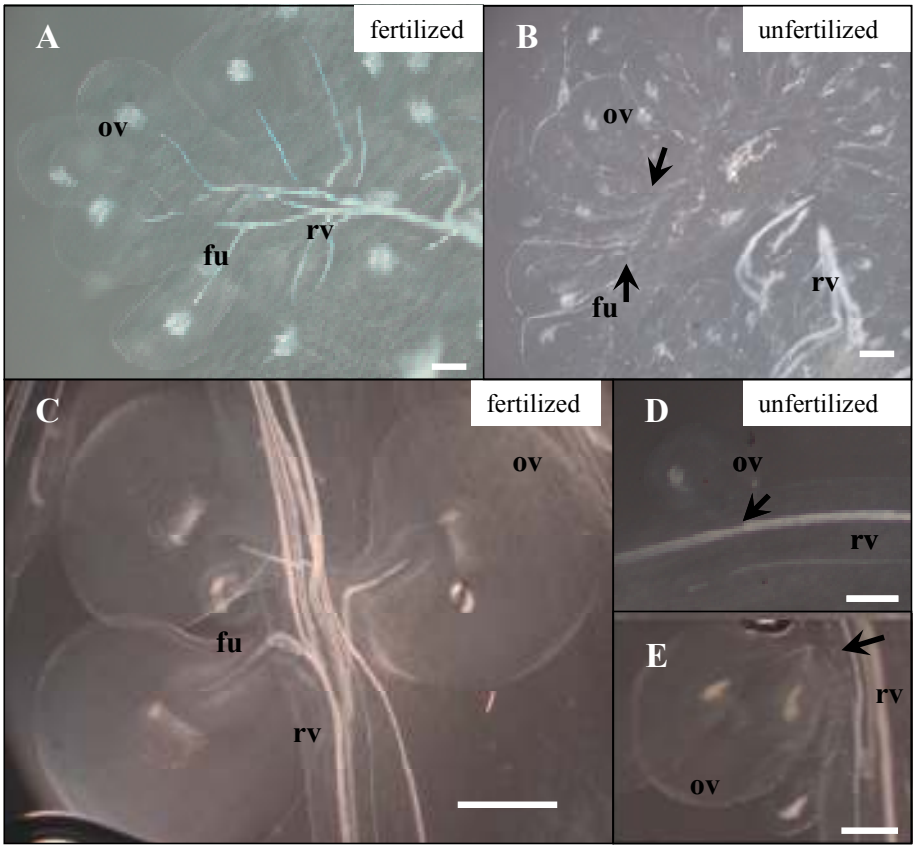

Figure 2 Vascular development in a fertilized and unfertilized ovule of *Capsicum annuum* at 48 hours post pollination. A-B; Vascular connectivity between funiculus and replum was present in fertilized ovules (A) but absent in unfertilized ovules (arrow head, B) of cultivar 'Bruinsma Wonder'. C-E; Vascular connectivity was present in fertilized ovules (C) but absent in unfertilized ovules (arrow head, D-E) of cultivar Fireflame. ov: ovule, rv: replum vasculature, fu: funiculus, Scale bar: 100 µm (A-B), 50 µm (C-E)

Cell number and cell diameter both contribute to early fruit growth

To know whether early fruit growth is due to cell division or cell expansion or a combination of both, we carefully recorded the fruit diameter, cell number and cell diameter in a post-pollinated ovary. The three distinct layers found in carpels of other plant species i.e. exocarp, mesocarp and endocarp could be clearly distinguished in *C. annuum* cv. Bruinsma Wonder. The exocarp being the outermost single cell layer, the mesocarp being multilayered and comprising vascular bundles, and the endocarp consisting of a

single layer of giant cells and a layer of small cells (Fig. 3A).In the exocarp and endocarp mainlyanticlinal cell divisions (perpendicular to the length of an existing cell) were observed, whereasthe orientation of cell division in the mesocarp was random (Fig. 3A: arrow). Cells in the exocarp and innermost endocarp remained small because of the cell division, while the mesocarp and giant-endocarp cells underwent significant cell expansion. A clear gradient in cell size was observed from exterior to the interior in mesocarp layers (broken arrow: Fig. 3A). The cells were smaller and flat towards the exterior (close to exocarp), more rounded and relatively isodiametric in the middle and around the vascular bundle, and approaching the interior they became larger and elongated (Fig. 3A).

Multilayered mesocarp was used to evaluate the contribution of cell division and cell expansion in fruit growth. From 12 to 30 HPP no difference in cell diameter (P=0.941), cell number (P=0.524) and fruit diameter (P=0.125) was observed. A significant increase in the cell diameter (P<0.001), cell number (P=0.009), and fruit diameter (P=0.009) was observed between 30 and 40 HPP (Fig. 3B).Between 12 and 72 HPP there was a significant positive correlation between fruit diameter and cell number (r^2=0.99), and between fruit diameter and cell diameter (r^2=0.99) (Fig. 3B), indicating that both cell division and cell expansion contribute to early fruit growth.The steepness of the curves indicates that cell division contributed more to increased fruit diameter than cell expansion.

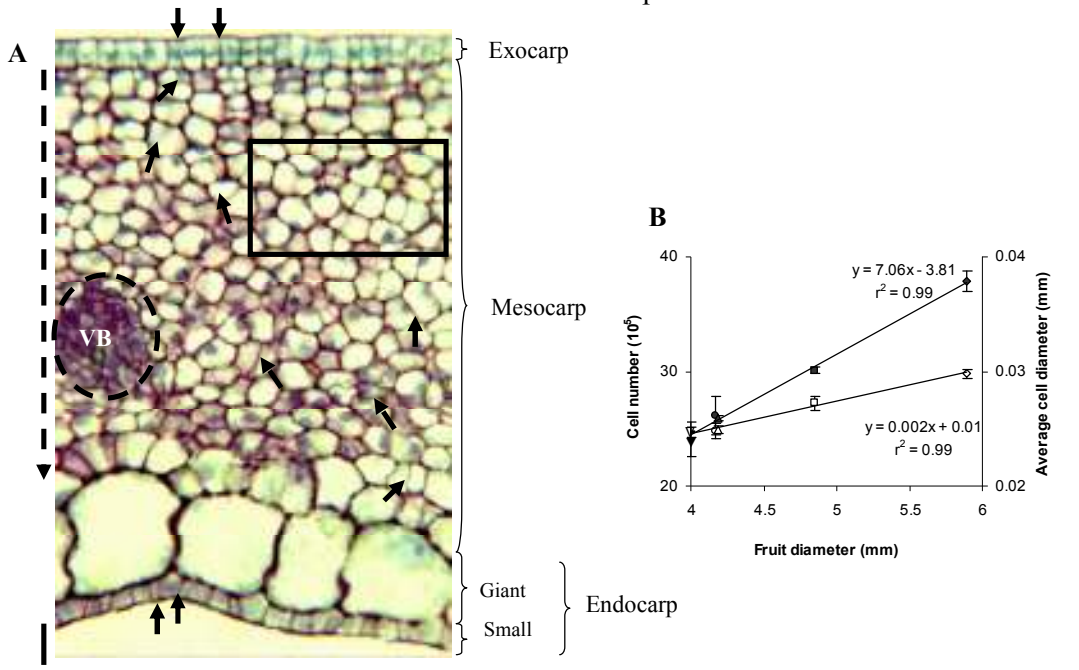

Figure 3 Histological observation in a carpel of *Capsicum annuum* cultivar 'Bruinsma Wonder' at 12, 20, 30, 40 and 72 hours post pollination A; Representative image of a horizontal cross section of the carpel with indication of an area (box) in which diameter of cells were measured, three distinct layers i.e. exocarp: single layered, mesocarp: multilayered, and endocarp: double layered (giant and small). VB: vascular bundle, Anticlinal cell division in exocarp and endocarp (vertical arrows), random orientation of cell division in mesocarp (random arrow head). Smaller to bigger cell size gradient from exterior (above) to interior (below) direction in mesocarp (broken arrow). Scale bar: 50 µm. B: Correlation between fruit diameter and cell number (closed symbols), and between fruit diameter and average cell

diameter (open symbols) at 12(▼,▽), 20(●, ○), 30(▲, Δ), 40(■, □) and 72(♦, ◊) hours post-pollination (n=3-5 ovaries).

Auxin is important in fruit set and fruit growth

To evaluate the role of IAA in fruit set and fruit growth, endogenous IAA levels were measured in the placenta and carpel of seeded and seedless fruits obtained from pollinated and emasculated flowers, respectively. The development of both seeded and seedless fruits comprised an initial phase of slow growth between 0 and 10 days after anthesis (DAA) followed by a rapid growth (10-40 DAA). The ovary weight in seeded and seedless fruits was the same at 0 DAA, but increased in seeded fruits at 4 DAA (P<0.001), whereas seedless fruit weight only significantly increased at 6 DAA (P<0.001) (Fig 4A). At 40 DAA,seedless fruits were more conical in shape while seeded fruits were blocky in shape and fruit weight of seedless fruits was significantly smaller than seeded fruit (P=0.012).In the placenta and in the carpel, the IAA level was higher ator around anthesis (0 DAA) compared to 10-12 DAA in both seeded and seedless fruits.A significant peak of IAA was observed in the carpel of seeded fruits at 20 DAA (P<0.001), whereas this peak was absent in seedless fruits (Fig. 4B, C).

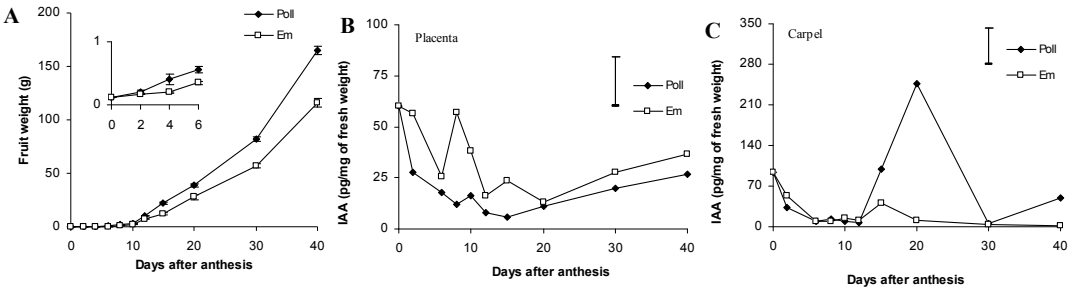

Figure 4 A; Fruit weight (g) (inset: fruit weight from 0-6 days after anthesis: DAA) and endogenous IAA level for*Capsicum annuum* cultivar 'Bruinsma Wonder' in B; Placenta and C; Carpel at 0, 2, 6, 8, 10, 15, 20, 30 and 40 DAA from seeded (closed symbols) and seedless (open symbols) fruits obtained from pollinated (Poll) and emasculated (Em) flowers respectively (n=2-3). Vertical bars represents interaction LSD(0.05) values.

To investigate the differences between seeded and seedless fruits at the cellular level, cell division (number of cell layers) and cell expansion (cell diameter) were quantified for both fruit types. The number of cell layers in the carpel was the same for seeded and seedless fruits at 0-12 DAA, while this was significantly higher in seeded fruits from 20 DAA onward, indicatingmore cell divisions in seeded fruits compared to seedless fruits. At 40 DAA, the mesocarp cell diameter was significantly higher in seeded fruits compared to seedless fruits (P=0.016), while no difference was observed in exocarp (P=0.840), giant endocarp (P=0.723) and small endocarp (P=0.742). A positive relation between mesocarp cell diameter and fruit diameter was observed in seeded (r^2=0.91) and seedless fruits (r^2=0.87) (Fig. 5C), indicating the contribution of cell expansion to later fruit growth. The increase in mesocarp cell diameter at 20 DAA coincided withthe peak in IAA levels in the carpel of seeded fruits (Fig. 5B, 4C), suggesting that IAA stimulates cell expansion to trigger a major increase in fruit growth.

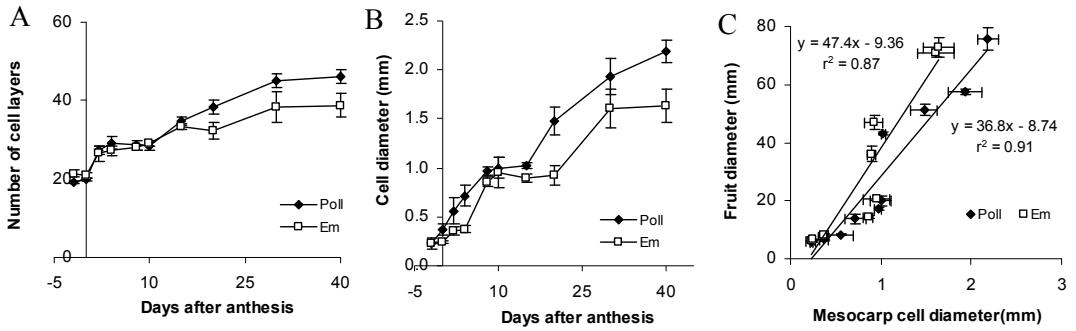

*Figure 5*Number of cell layers, cell diameterand fruit diameter in *Capsicum annuum* cultivar 'Bruinsma Wonder' (n=3-5). A; Average number of cell layers in the mesocarp, B; Average cell diameter in the mesocarp. C: Relation between fruit mesocarp cell diameter (mm) and fruit diameter (mm) in seeded (closed symbols) and seedless fruits (open symbols) obtained from pollinated (Poll) and emasculated (Em) flowers at respectively 0, 2, 6, 8, 10, 15, 20, 30 and 40 days after anthesis. Vertical and horizontal bars indicate standard error of mean.

Discussion

Fertilization induces vascular connection between ovule and carpel to initiate fruit set

After pollination, fruit set requires pollen germination and pollen tube growth in the stylar tissue towards the ovule and into the embryo sac to enable the fusion between male and female gametes (Dumas and Mogensen, 1993; Mascarenhas, 1993). Our study indicates that the majority of ovules are fertilized at 20 to 28 HPP at 24^{o}C of constant temperature. About the same time frame has been reported for fertilization in tomato at 20^{o}Cof constant temperature(Iwahori, 1966).After successful pollination and fertilization, endogenous IAA levels are reported to increase in the developing ovary e.g. in cocoa (Baker *et al.*, 1997), Arabidopsis (Dorcey *et al.*, 2009), melon (Lee *et al.*, 1997), and tomato (Sjut and Bangerth, 1981), suggesting that pollination and fertilization either induces *de-novo* auxin biosynthesis or hydrolyses auxin from its conjugates (Lemaire-Chamley *et al.*, 2005). At the same time, a vascular connection was observed between ovule and carpel in Arabidopsis (Vivian-Smith, 2001). The importance of auxin transport for vascular development(Scarpella *et al.*, 2006), suggests that the vascular connection between ovule and carpel is mediated by transport of ovule generated auxin to the carpel. This process also seems to occur in *C. annuum* where fruit set and vascular connection between funiculus carpel margin/placental tissue was observed in a fertilized ovary. Vascular connection were absent in unfertilized ovaries. The correlation between vascular development and fruit set was observed in both a sweet and a hot peppercultivar, i.e. Bruinsma Wonder and Fireflame, which strongly suggests vascular development as a determinant factor,which may sustained fruit growth in *C. annuum*after pollination and fertilization. Our results are in agreement with Arabidopsis, where a dependency of fruit development on the vascular connection between ovule and replum has been demonstrated(Fuentes and Vivian-Smith, 2009). In the Arabidopsis*fwf* mutant, the vascular development preceed or is precocious to parthenocarpic fruit development, whereas in the *ant fwf* double mutant, deficiencies in vascular development restricted parthenocarpic fruit development (Vivian-Smith, 2001).

This fertilization induced vascular biogenesis does not seem to be restricted to the ovule-carpel junction, but seems also to occur elsewhere in the flower:e.g. vascular biogenesis in the pedicel has been related with fruit development in citrus (Bustan *et al.*, 1995), apple (Drazeta *et al.*, 2004), and Prunus (Else *et al.*, 2004).
An important role for auxin in fruit set has been reported in tomato, Arabidopsis, and egg plant (Goetz *et al.*, 2006; Martinelli *et al.*, 2009). In *C. annuum*, we observed a comparatively high level of IAA at/around anthesis compared to 10-12 DAA in the carpel and placenta of seeded and seedless fruits (Fig. 4) suggesting a possible role of auxin in early fruit growth of *C. annuum*.

Both cell division and cell expansion contribute to early fruit growth in C. annuum
During fruit development in *C. annuum*, the exocarp and endocarp layers comprise of small but mitotically active cells, whereas a substantial increment in the cell size was observed in the mesocarp (Fig. 3A). Anticlinal divisions contribute toincrease in surface area and periclinal divisions contribute to increase in tissue thickness in *Vitis vinifera*(Considine and Knox, 1981). In *C. annuum*, mesocarp comprises both anticlinal and periclinal types of cell divisions, which contribute maximum to the fruit growth. The same pattern of cell division and expansion was reported in tomato and *Lagenaria leucantha*(Cheniclet *etal.*, 2005; Joubès and Chevalier, 2000; Varga and Bruinsma, 1976; Yu *et al.*, 2001).Agradient in cell size was observed in *C. annuum* mesocarp layers of the expanding ovary (Fig 3A: broken arrow). Interestingly, in tomato,genes controlling the cell expansion process(¶*TIP* and *PRP*) or its regulation (hormone synthesis, signaling,and response) are expressed along a gradient from the innerpart to the outer part of the fruit, resulting in a gradient in cell size (Lemaire-Chamley *et al.*, 2005).
Between 30 and 40 HPP the fruit diameter started to increase, and at the same time an observed increase in the mean cell diameter and increase in mesocarp cell number occurred (40 HPP), suggesting that early fruit growth is from the coordinated cell division and cell expansion. Our results are in agreement with Arabidopsis, tomato and pea where a combination of cell division and cell expansion promotes early carpel growth immediately after pollination and fertilization (Cheniclet *etal.*, 2005; Müller, 1961; Nitsch, 1970; Ozga *et al.*, 2002).
Cell division dominates early fruit growth in *C. annuum* (Fig. 3B) similar to what has been reported in tomato, *Lagenaria leucantha* and mandarin, where early fruit growth is primarily the result of cell division(Bertin, 2004; Yu *et al.*, 2001; Guardiola and Lazaro, 1987). Cell expansion dominated later fruit growth in *C. annuum* (Fig. 5B, C)similar to what has been reported in tomato,cucumber, and pear where after few weeks of early fruit growth, the cell division ceases and subsequent growth of the fruits is mainly supported by cell expansion and the formation of intercellular space(Gillaspy *et al.*, 1993; Marcelis and Baan Hofman-Eijer, 1993; Matsumoto *et al.*, 2008).

Cell expansion is important for later fruit growth
Fruit growth in *C. annuum* follows a sigmoid growth pattern: an initial phase with slow growth, an intermediate phase with high growth, and a last phase or ripening phase with no further growth (Nielsen *et al.*, 1991). In the same line of agreement, we found a sigmoid growth pattern though later stage of fruit growth was not included in the measurement (Fig. 4A). At 40 DAA, seedless fruits were smaller than seeded fruits, which seem to be due to

the limitation of both cell division and cell expansion as number of cell layers and cell diameter in mesocarp layers were significantly reduced in seedless fruits compared to seeded fruits (Fig. 5A, B). This result is in the agreement with tomato where fruit size is determined by both cell number and cell size (Ho, 1996).

It has been suggested thatauxin promotes cell expansionin fruits(Rayle and Cleland, 1992; Gillaspy *et al.*, 1993).Auxin presumably causes an increase in the extensibility of cell walls and induces uptake and retention of water and solutes (Masuda, 1969;Cleland, 1995). In seeded fruits of *C. annuum*, an increase in IAA level in the carpel and an increase in cell diameter were observed at 20 DAA (Fig. 4C, 5B). The concurrence of these two events suggests that IAA triggers a major increase in cell diameter filling the maximum fruit size. Similar results have been reported in tomato where an increase in auxin level (6-10 days after anthesis) coincides with the initiation of cell expansion and promotes fruit growth (Iwahory, 1967; Gillaspy *et al.*, 1993; Mapelli et al., 1978). While in seedless fruit, auxin peak was absent, growth spurt was delayed and final fruit size was smaller than seeded fruits. Auxin measurements were performed not at all the developmental stages and we may have missed increases in IAA level in seedless fruits just before the onset of growth (at or around 25 DAA).Moreover, we assumed that observed auxin peak might be smaller than peak observed in seeded fruits, which explain why seedless fruits are smaller than seeded fruits.

Conclusion

Fruit set and continuous vascular connectionbetween funiculus and replum was observed in fertilized ovaries while flower abortion and absence of vascular connection was observed in unfertilized ovaries. These results suggest thatvascular development is an early determinant of fruit set after pollination and fertilization in *C. annuum*.Increase in cell number and cell diameter both contribute to early fruit growth in *C. annuum*after pollination and fertilization. Increase in cell number is more prominent at early stage of fruit growth while increase in cell diameter is more prominent at later stages of fruit growth. These results are similar to what has been reported for tomato and Arabidopsis, which suggests that the available knowledge from tomato and Arabidopsis can be translated to *C. annuum*to understand details of fruit set mechanisms in this plant.

Chapter 3

Selection of sweet pepper (*Capsicum annuum* L.) genotypes for parthenocarpic fruit growth

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Abstract

Yield irregularity and blossom-end-rot are major problems in sweet pepper production, which can be reduced by parthenocarpy. However no commercial parthenocarpic cultivars are available. The purpose of this study was to find parthenocarpic genotype(s) of sweet pepper with the ability to produce good quality fruits without seeds. Eleven genotypes of sweet pepper were studied at normal (20/20°C D/N) and at low night temperature (20/10°C D/N), in two greenhouse compartments from January till May. Fruit length, diameter, fresh and dry weight and number of seeds per fruit were measured. A higher percentage of parthenocarpic fruits were observed for all genotypes at low night temperature compared to normal temperature. Within the genotypes which showed at least 60% parthencarpic fruit growth at low night temperature, two groups were distinguished, which showed a different expression of parthenocarpy in response to the environment. In one set of genotypes (Line 1 and Line 3) high expressivity for parthenocarpy was found irrespective of night temperature. In another set of genotypes (Gen A, Gen B, Gen C, Lamuyo A, Lamuyo B and Bruinsma Wonder), a high level of parthenocarpy was expressed at low night temperature only, which may be due to non-viable pollen at low night temperature. Further evaluation of the latter six genotypes resulted in one genotype (Bruinsma Wonder) for which the absence of seeds only marginally influenced shape and size of fruits. These selected genotypes

Introduction

Fruit harvest in sweet pepper shows a cyclic behavior: weeks of high production are followed by weeks of low production, which leads to irregular labor requirements and marketing problems. Besides this, high commercial loss has been reported in sweet pepper due to its susceptibility to blossom-end-rot (BER), a physiological disorder caused by local deficiency of calcium during the early phase of fruit development (Bangerth, 1979). Parthenocarpy is a physiological phenomenon where an ovary grows into a fruit without fertilization (Varoquaux et al., 2000). It has been proposed as a possible solution to reduce yield flushing and to minimize BER in sweet pepper (Heuvelink and Körner, 2001). These authors reported that auxin application on unpollinated flowers of sweet pepper resulted in seedless fruits and a more regular production than in non-treated plants.

Growth regulators inducing parthenocarpy have been reported for a large number of horticultural species (Schwabe and Mills, 1981). A high percentage of parthenocarpic fruit set was observed when sweet pepper plants were grown at low night temperature (8- 10°C) (Cochran, 1936). Low temperature may impair pollen fertility Polowick and Sawhney, 1985) causing hampered seed set (Rylski, 1973) and leading to the production of seedless fruits. The parthenocarpic fruits obtained either by hormone application or by preventing fertilization at low night temperature showed a deformed fruit shape (Bosland and Votava, 1999) and a reduced fruit size (Rylski, 1986). Genetically controlled parthenocarpy has been observed in tomato (Vardy et al., 1989), banana (Qrtiz and Vuylsteke, 1995) and cucumber (Rudich et al., 1977). A transgenic approach has been successfully applied for obtaining parthenocarpic fruits in eggplant and tobacco (Rotino et al., 1997). Parthenocarpy is a highly appreciated trait by consumers and process companies, not only because of easy processing (e.g. cut slices of sweet pepper) in a growing market (Gonzalez et al., 2004) but

also because of an improved shelf life, higher sugar and higher soluble solid content in fruit(Varoquaux et al., 2000). Male sterility can be used for the commercial cultivation of a parthenocarpic genotype (Shifriss and Eidelman, 1986), but first a sweet pepper genotype expressing parthenocarpic gene(s) along with marketable fruit appearance should become available. In this research, selected genotypes of sweet pepper were evaluated at normal and low night temperature to test their ability to produce parthenocarpic fruits. Seeded and seedless fruits of these genotypes were compared in order to find genotype(s) where the absence of seeds hardly influences the commercial quality of the fruits. This selected genotype(s) can be used as a candidate line in a breeding program for a parthenocarpic cultivar reducing yield flushing and minimizing the incidence of BER.

Materials and Methods

Seeds of ten genotypes and "Mazurka" (Table 1) as a standard cultivar of sweet pepper (*Capsicum annuum* L.) were obtained from commercial breeders and a Chinese colleague (Xue Linbao, Yangzhou University). These genotypes were expected to have the capacity to produce seedless fruits at low night temperatures. Seeds were sown on 23rd of November and seedlings of all genotypes were grown on Rockwool cubes with regular supply of nutrient solution.The crop was planted on Rockwool slabs (2.5 plants m^{-2}) on 23rd January 2006 in two compartments of a multispan Venlo-type glasshouse at Unifarm, Wageningen, The Netherlands and plants were pruned to two main stems. The genotypes were arranged in a randomized block design in both compartments, which consisted of three plots of 12 plants per genotype in each block (except Lamuyo B; 2 plots of 6 plants in each block) and Mazurka was planted in two rows on the borders. From 13th of February onwards, two different temperature set points were given: 20/20 ^{o}C D/N and 20/10 ^{o}C D/N, however realized low night temperature was not always strict at 10^{o}C. Average realized temperatures were 21^{o}C and 13^{o}C, for day and night, respectively. Normal night temperature setpoint of 20^{o}C was realized. All flowers were left to develop until fruit ripening or natural abortion. Ripe fruits were harvested and their length, diameter (maximum distance across the shoulders) and fresh weight were measured. Also, an evaluation was performed for presence of knots: fruits with at least 50% reduction in fresh weight compared to seeded fruits (average of fresh weight of seeded fruit at 20^{o}C) (Fig.1).Then fruits were cut transversely and observations were made for carpelloid structure and number of seeds was counted. Finally, fruits were dried in a ventilated oven for two hours at 70^{o}C followed by ten hours at 105^{o}C and again two hours at 70^{o}C to obtain the dry weight of individual fruits. The experiment ended on 31st May 2006.

Results

A higher percentage of parthenocarpic fruits were observed for all genotypes at low night temperature as compared to normal temperature (Table 1). For Line 1 and Line 3, percentage of parthenocarpic fruit was high at both low and normal night temperature, while substantial parthenocarpic fruit growth was observed for Lamuyo A, Lamuyo B, Gen A, Gen B, Gen C and Bruinsma Wonder only at low night temperature.

Table 1 Genotypes and their origin, percentage of seedless fruit and fruit with less than five seeds/fruit observed at low (10°C) and normal (20°C) night temperature. Total number of fruit used for the measurements ranged between 94-415, except for Lamuyo B (34-79 fruits).

Genotype	Origin	zero seeds/fruit (%)		<5 seeds/fruit (%)	
		10 °C	20 °C	10 °C	20 °C
Line 3	China	100	73	100	82
Line 1	China	96	49	97	91
Lamuyo A	De Ruiter Seeds	78	10	84	16
Lamuyo B	De Ruiter Seeds	70	0	89	3
Gen A	Vilmorin	64	2	83	6
Gen B	Vilmorin	70	10	88	15
Gen C	Vilmorin	63	7	83	31
Sirena RZ	Rijk Zwaan	51	9	74	12
Bruinsma Wonder	PRI (2004001)	66	13	89	24
Orlando	De Ruiter Seeds	9	2	22	2
Mazurka	Rijk Zwaan	20	5	33	7

Reduction in percentage of carpelloid growth was observed at low night temperature for all the genotypes except for Sirena RZ, Line 1 and Line 3. For Sirena RZ, observed percentage of carpelloid growth was low at both temperature regimes, while 80-90% fruits of Line 1 and Line 3 were with carpelloid growth (Table 2; Fig. 2). Percentage of knots was high for all the genotypes at low night temperature, however the highest percentage was observed for Sirena RZ (56%) and the lowest for Bruinsma Wonder (9%). For the other genotypes, it was between 20 and 40%.

Table 2 Percentage of fruits with carpelloid internal growth and average percentage of knots observed at low (10°C) and normal (20°C) night temperature for 11 genotypes of sweet pepper.

Genotype	% Carpelloid		% Knots	
	10 °C	20 °C	10 °C	20 °C
Line 1	82	82	42	15
Line 3	83	92	19	4
Lamuyo A	42	80	34	4
Lamuyo B	46	56	26	0
Gen A	35	69	25	1
Gen B	46	49	26	5
Gen C	44	66	41	8
Sirena RZ	32	26	56	9
Bruinsma Wonder	76	91	9	5
Orlando	3	41	23	2
Marzurka	21	27	20	4

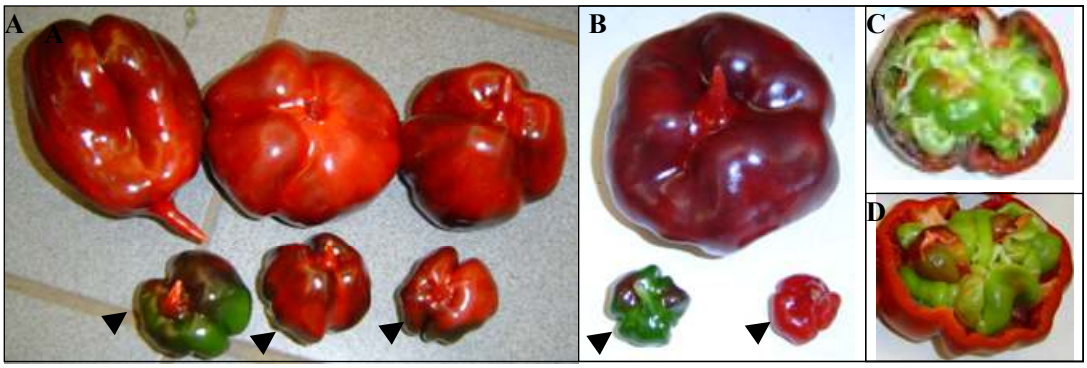

Figure 1 Shape and size of knot fruits compared to the normal fruit for Line 1 (A) and Line 3 (B). average length and diameter for normal fruits were 42 x 67 and 45 x 75 mm for Line 1 and Line 3 respectively and average length and diameter for knots were 18 x 32 and 13x 24 mm for Line 1 and Line 3 respectively (arrow head). Carpelloid growth observed in most of the parthenocarpic fruits of genotype Line 1 (C) and Line 3 (D)

Table 3 Genotypes which expressed substantial parthenocarpy at low night temperature were evaluated along with control cultivar for length (L) and length/diameter ratio (L/D) of seedless and seeded fruit at low night temperature (10^{o}C).

Genotypes	**Seedless**		**Seeded**	
	L(mm)	**L/D**	**L(mm)**	**L/D**
Lamuyo A	82	1.12	100	1.32
Lamuyo B	84	1.11	112	1.63
Gen A	81	1.17	98	1.32
Gen B	97	1.16	126	1.61
Gen C	55	0.70	67	0.86
Bruinsma Wonder	75	0.91	87	1.14
Marzurka	64	0.91	71	0.94

Five genotypes (Lamuyo A, Gen A, Gen C, Bruinsma Wonder and Mazurka) showed only a small difference in length/diameter ratio between seedless and seeded fruit at low night temperature (Table 3). Fresh weight of seedless and seeded fruit was the same for Bruinsma Wonder and almost the same for Gen C. Dry weight was the same for seedless and seeded fruit of Lamuyo B and Gen C and almost the same for Lamuyo A, Gen A and Bruinsma Wonder at low night temperature (Table 4).

Discussion

Expression of parthenocarpic fruit growth clearly showed genotypic variation. It was high for Line 1 and Line 3, suggesting the presence of parthenocarpic gene(s) expressed irrespective of night temperature. Most of the parthenocarpic fruit of Line 1 and Line 3 showed carpelloid growth inside the fruit: an internal growth abnormality also reported inArabidopsis thaliana by Vivian-Smith et al. (1999). These authors presumed that

Table 4 Average fresh weight (g) and dry weight (g) of seeded fruit (S) and ratio of fresh weight and dry weight between seedless and seeded fruit (SL/S) at low night temperature (10^{o}C), for genotypes which showed substantial parthenocarpy at low night temperature and control cultivar Mazurka.

Genotypes	Fresh weight (g/fruit)		Dry weight (g/fruit)	
	S	SL/S	S	SL/S
Lamuyo A	158	0.80	12	0.92
Lamuyo B	159	0.86	11	1.00
Gen A	153	0.74	12	0.92
Gen B	214	0.86	16	0.75
Gen C	133	0.92	11	1.00
Bruinsma Wonder	136	1.00	12	0.92
Mazurka	141	0.76	11	0.82

vascular strands, which are a good marker of auxin-induced fruit development, automatically joins and forms in such cases where the ovule is homeotically converted into a carpel-like structure. However, it is not yet clear whether carpelloid growth is a linked trait or a pleiotropic effect of parthenocarpic gene(s) or that some other physiological or molecular change leads to this malformation. A high percentage of knots or pseudo-fruits was found for all genotypes, except for Bruisma Wonder, at low night temperature (Table 2), where most likely the non-viability of pollen increased the percentage of unfertilized fruit. It has been reported that unfertilized fruit has poor sink strength (Nielsen et al., 1991) and deformed fruit shape (Rylski, 1973; Bosland and Votava, 1999) as compared to fertilized ones. This agrees with the high percentage of knots at low night temperature. Also Rylski (1986) reported that seedless fruits produced at low night temperature reached only half to one fourth of the weight of the corresponding fertilized ones.

Parthenocarpic fruit growth observed particularly in Gen A, Gen B, Gen C, Lamuyo A, Lamuyo B and Bruinsma Wonder at low night temperature (Table 1) is may be due to non viable pollen as reported by Rylski (1986). These genotypes were further evaluated and showed that absence of seeds has minimal effect on marketability (size and weight) of fruits. Lamuyo A, Gen A, Gen C and Bruinsma Wonder showed only a small difference and Mazurka showed no difference in size between seeded and seedless fruit (Table 3) and only a marginal difference for fresh weight between seeded and seedless fruit was observed for Bruinsma Wonder and Gen C at low night temperature (Table 4). These results confirm the findings of Picken (1984) that seeds are one of the factors for determining the shape and size of fruit but not the only one.

High expressivity of parthenocarpy was observed for Line 1 and Line 3; however, carpelloid growth appeared in these lines as a linked trait or pleiotropic effect of parthenocarpic gene(s) or as a result of some physiological or molecular changes. Bruinsma Wonder showed low percentage of knots and also minor differences in fruit weight and shape between seedless and seeded fruits at low night temperature. Therefore Bruinsma Wonder is candidate genotypes for further research and is proposed to be used in a breeding program for introgression and modulation of parthenocarpy in existing commercial pepper cultivars.

Chapter 4

Parthenocarpic potential in *Capsicum annuum* L. is enhanced by carpelloid structures and controlled by a single recessive gene

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Abstract

Parthenocarpy, or fruit set without fertilization, is a desirable trait in*Capsicum annuum*production because it can provide an enhanced fruit quality trait and stabilize irregular fruit set. We found that many *C. annuum*genotypes already show a certain level of parthenocarpy, and that the seedless fruits obtained from these genotypes often contain carpelloid structures (CLS). In this paper we study the levels of aberrant ovules and correlate CLS transformation with parthenocarpic fruit set. *C.annuum* gynoecia have an axial placenta where ovules develop in a gradient from top to bottom. The majority of the ovules were unitegmic and anatropous. However, we observed some abnormal ovules, abundant at the top and base of the placenta, with altered integument growth. As CLS arose from the placenta, abnormal ovule primordia transformed into CLS in analogy to the *Arabidopsisbell* mutant. Fruit weight was positively correlated with seed number, but in the absence of seeds, fruit weight proportionally increased with the CLS mass. *Capsicum* genotypes with high parthenocarpic potential showed synergistic CLS development though CLS were ubiquitously present at lower numbers in fruits of most genotypes. The parthenocarpic potential appeared to be controlled by a single recessive gene, but it was not linked to a mutation in *CaARF8*. Our results suggest that in most *C. annuum*genotypes, the absence of fertilization induces parthenocarpic fruit development and allows CLS growth, which can also substitute for developing seeds in promoting fruit development.

Introduction

Pollination and fertilization are required in most flowering plants to initiate the transition from a fully receptive flower to undergo fruit development. After fertilization the ovules develop into seeds and the surrounding carpels develop into the fruit, while in the absence of fertilization the ovules degenerate and the surrounding carpels stop growing (Fuentes and Vivian-Smith, 2009). The initiation of fruit set can be uncoupled from the fertilization trigger, and this result in the development of seedless or parthenocarpic fruits. This can be achieved by ectopic application or artificial overproduction of plant hormones (Fuentes and Vivian-Smith, 2009), or by mutating specific genes or altering their expression. In *Arabidopsis*, the *fruit without fertilization* (*fwf*) mutant that develops parthenocarpic fruit (Vivian-Smith et al., 2001) has a lesion in the *AUXIN RESPONSIVE FACTOR 8* (*ARF8*) gene (Goetz et al., 2006).Expression of an aberrant form of *ArabidopsisARF8* conferred parthenocarpy in Arabidopsis and tomato, indicating ARF8 as an important regulator in the control of fruit set (Goetz et al., 2007). Mapping of a parthenocarpic QTL in tomato further supports a role for *ARF8* in fruit set (Gorguet et al., 2008).

Ovules are complex structures that are found in all seed bearing plants. The ovule primordium develops both the protective integuments and the megagametophyte. Double fertilization of the egg cell and central cell occurs later in the mature ovule, and it is considered as a primary site from where fruit set is triggered (Berger et al., 2008; Fuentes an d Vivian-Smith, 2009). After fertilization, the integuments grow and expand to accommodate the developing endosperm, but they apparently have a role in coordinating the growth of both fruit and seeds(Fuentes and Vivian-Smith, 2009). Various *Arabidopsis* mutants have been identified where ovules show disrupted integument growth, such as *aintegumenta* (*ant*; lack inner and outer integuments), *aberrant testa shape* (*ats*; contains

single integument), *inner no outer* (*ino*; outer integument elongates opposite to the ovule primordium), *short integuments1* (*sin1*; both integuments are short), *BELL* (*bel1*),and *apetala2* (*ap2)*(Rayet al., 1994;Lang et al., 1994; Leon-Kloosterziel et al., 1994; Modrusan et al., 1994; Elliott et al., 1996; Baker et al., 1997). In the latter two loss-of-function mutants ovule integuments are converted into carpelloid structures (CLS) (Rayet al., 1994;Modrusan et al., 1994; Pinyopich et al., 2003). Interestingly, two of these mutants have been reported to affect parthenocarpic fruit development of the *Arabidopsis fwf* mutant. Firstly, the *ats-1/kan4-1* loss-of-function mutantion enhances the *fwf* parthenocarpic phenotype, suggesting that modification of ovule integument structure influences parthenocarpic fruit growth (Vivian-Smith et al., 2001). Secondly, parthenocarpic fruit development was also enhanced in the *bel1 fwf-1* double mutant, and at the same time a higher frequency of CLS was observed compared to the *bel1* single mutant (Vivian-Smith, 2001). This suggests that on the one hand CLS enhance parthenocarpic fruit development, and that on the other hand CLS development is enhanced in the absence of seed set (Vivian-Smith, 2001).

Parthenocarpy is a desired trait in *Capsicum annuum*, sometimes also known as sweet pepper, as it is expected to minimize the yield fluctuation and to enhance the total fruit production (Heuvelink and Körner, 2001). Research into the developmental and genetic basis for parthenocarpy in *C. annuum* is limited. Several *C. annuum* genotypes have been identified that show tendenciesfor facultative parthenocarpic fruit development (Tiwari et al., 2007). Seedless fruit from these facultative genotypes display a high frequency of CLS development at low night temperatures (Tiwari et al., 2007). To understand the relationship between parthenocarpy and CLS, we investigated ovule development and the occurrence of abnormal ovules in *C. annuum* genotypes possessing a range of high (Chinese Line 3), moderate (Bruinsma Wonder) and low (Orlando) potential for parthenocarpic fruit set. Our results show that parthenocarpy in *C. annuum* can promote carpelloid ovule proliferation and the appropriate genetic background enhancesthe transformation of ovules which can further stimulate seedless fruit growth. Five selected genotypes that differed most in their parthenocarpic fruit development and CLS growth were evaluated to identify a possible correlation between these two traits. Through genetic analysis with crosses between Line 3 and contrasting parents we linked the parthenocarpic potential of this genotype to a single recessive gene. Sequence analysis showed that the parthenocarpic potential already present in *C. annuum* genotypes is not caused by a mutation in*CaARF8*.

Materials and Methods

Greenhouse conditions

The genotypes and greenhouse conditions used in different experiments are summarized in supplementary Table S1. In all the experiments, seeds were transferredon rockwool cubes with regular supply of nutrient solution (Voogt and Bloemhard, 1983). Seedlings were transplanted on rockwool slabs at a density of 2.5 plants m^{-2} in a compartment of a multispan Venlo-type glasshouse or in an air conditioned glasshouse, Wageningen, The Netherlands. Supplemental lighting by high pressure sodium lamps (Philips, SON-T, 600 W) for 16 hours (from 06.00 to 22.00) provided a minimum photon flux density of 125 µmol m^{-2} s^{-1} at the crop level. The terminal flower was removed from all plants at anthesis to support vegetative growth.

Occurrence of parthenocarpy among C. annuum genotypes

C. annuum(Table, 1) genotypes were selected on the basis of their blocky appearance and seed number (supplementary table S1: Exp 1). In total, 70-150 emasculations were performed in each genotype using 10 plants per genotype and fruit set was evaluated when fruits were ripe.

Genotype effect on number and weight of carpelloid structures

Five genotypes: Parco, California Wonder 100 (CW), Riesen v. Californien (RVC), Bruinsma Wonder (BW), and Line 3 were arranged in one row of 8 plantsat two temperatures (20/18^{o}C, 16/14^{o}C D/N) (supplementary table S1: Exp 2). Two treatments (induced pollination or prevent-pollination) were completely randomized within the row. Induced pollination was done by vibrating the stem two times per week. Prevent-pollination was done by applying lanolin paste on the stigma of the flowers (Wien and Zhang, 1991). In genotypes Parco, RVC, CW and Line 3, flowers were given the treatments till three fruits per plant were obtained. In genotype BW, two flowers (one on main branch and one on a side branch) were treated at each of 20 nodes. Mature red fruit were harvested and their length, diameter and fruit fresh weights were recorded. Those seedless fruits that reached minimum of 50% of the weight of seeded fruit were considered as parthenocarpic and were used in our analysis while remaining were considered as knots. The number of seeds and number of Carpelloid structures (CLS) was counted in each fruit and each CLS was weighted.

Ovule development in C. annuum

Line 3 and BW were inbred lines with high and medium potential to set parthenocarpic fruits (Tiwari et al., 2007) while Orlando (OR) was a fourth-generation inbred line developed from Orlando-F_1 (De Ruiter seeds) (supplementary table S1: Exp 3).Flowers were collected at 3-4 days before balloon stage. Pericarp was removed and morphological analysis of ovule development was conducted in the laboratory by using a field-emission cryo-scanning electron microscopy (SEM) (Jeol 6300F), equipped with an Oxford CT 1500HF cyro system (Nijsse et al., 2004).

Correlation of abnormal ovule development with reduced seed set and enhanced development of carpelloid structures

Two set of experiments were conducted(supplementary table S1: Exp 4). In first experiment, genotypes Line 3 and OR were used to evaluate the occurrence of CLS.Flowers were tagged at 2 days before anthesis and allowed to pollinate naturally. Developing ovaries were harvested at 2 days of interval, dissected and evaluated for the presence of CLS by visual inspection. With the same set of genotypes, percentage of aberrant ovules and number of seeds was evaluated. Flowers (n=6) were collected randomly at or around the anthesis stage.After removing the carpel, ovules were scraped smoothly in a water medium on a clean slide and the frequency of abnormal ovules was observed under optical microscope (Leitz Aristoplan).Seedset was counted when fruits reached the maturity (red) in both genotypes.In second experiment, genotypes Line 3 and BW were used. To evaluate the female fertility, plants were grown at day/night temperature of 14/16^{o}C (low), 18.20^{o}C (normal) and 22/24^{o}C (high) and pollination was induced by vibrating the main stem two

times per week. Number of seeds was counted when fruits reached the maturity (red) in both genotypes.

Pollen viability and germination

Pollen grains of BW were collected from normal temperature (20/20^{o}C day/night) and low night temperature (20/10^{o}C day/night) in morning time (9.00-10.00 PM) (supplementary table S1: Exp 5). To test the pollen viability,hydrated pollens were dissolved in FDA solution(Heslop-Harrison and Heslop-Harrison, 1970) and scored under fluorescence microscope. Pollen which fluoresced brightly under fluorescence was scored as viable.For pollen germination, the hanging drop technique was employed following published procedures (Deng and Harbaugh, 2004)with some modifications. A liquid medium containing0.25 mM MES (pH5.9 KOH), 15% (w/v) PEG 4000, 2% (w/v) sucrose, 700 ppm $Ca(NO_3)_2$, 100 ppm H_3BO_3, 200 ppm $MgSO_4$, 100 pm KNO_3) was used.Germination was considered only when germinating tube was larger or equal to the size of the pollen.Viability and germination percentages were determined, using 10-12 replicates of about 20-40selected grains.

Relation between parthenocarpy and carpelloid structures

Genotype BW with moderate potential for parthenocarpy was used in the experiment (supplementary table S1: Exp 6). To obtain seeded fruits, flowers were tagged at anthesis and allowed to pollinate naturally. To obtained seedless fruit, flowers were emasculated two days before the expected date of anthesis and stigmas were cover with the lanolin paste or lanolin paste containing 0.05% 1-Naphthaleneacetic acid (NAA) or Gibberellic acid (GA_3)(Wien and Zhang, 1991). Fifteen plants per treatment were used. On each plant, two flowers (one on main branch and one on a side branch) were treated at each of 20 nodes. All the fruits were harvested at mature red stage and their length, diameter and fruit fresh weights were recorded. Criteria to define parthenocarpic fruit and knot were the same as mentioned earlier (Exp.2). The number of seeds and number of CLS was counted in each fruit and each CLS was weighted.

Inheritance of parthenocarpy and its relation with carpelloid structures

In order to understand the genetics of parthenocarpy and a possible association with CLS, genetics of both traits were evaluated in cross progenies of Line 3 (supplementary table S1: Exp 7). Line 3 was used as a parthenocarpic parent (Pp) and Lamuyo B, ORF2#1, and Parco as non-parthenocarpic parents (Pn). F2 progenies were obtained for all three crosses, and also a backcross with Line 3 in the cross with Lamuyo B. The flowers (15-20) were emasculated prior to anthesis and tagged. All the fruits were harvested at the mature red stage. Length, diameter and fruit fresh weights were recorded for individual fruits. In each fruit, CLS were counted and the CLS mass was weighed. All three crossing population were evaluated and mono- or digenic-models were tested to understand the genetics behind parthenocarpy and CLS growth.

Sequence analysis of CaARF8

Young leaf material from Line 3, BW and OR (supplemental table S1: Exp 8)was collected for DNA extraction. Primers for PCR amplification were designed against the pepper, tomato or potato*ARF8* EST sequences available from the Sol Genomics Network (SGN),

http://solgenomics.net/ (supplemental table S2). SEFA PCR was used to amplify non transcribed regions. PCR products were cleaned with the Invitek MSB® Spin PCRapace. 120ng of PCR product per reaction was sent with the appropriate sequencing primer (12 pmol) to ServiceXS, Leiden, The Netherlands. Resulting chromatograms were manually trimmed and checked for calling errors. Contigs were build by using Contig Express of the Invitrogen Vector NTI suite Version 10.

Statistical analysis

Experiments and their statistical treatment are listed in supplementary Table S1. For experiment 3 and 4, one way analysis of variance (ANOVA) was used, and treatment effects were tested at 5% probability level using F-test. For experiment 5, effect of each treatment on each genotype at each temperature was tested separately by using one way analysis of variance (ANOVA). Mean separation was done by student's t-test. Data processing and statistical tests were carried out with SPSS 15.0. The inheritance of parthenocarpy was tested by using chi square distribution with 1 degree of freedom at 0.05 level of significance to test the null hypothesis that parthenocarpy was controlled by a single recessive gene. The CLS inheritance was tested using a chi square distribution, with different mono- or digenic models.

Results

Parthenocarpy is widely present in Capsicum annuum L. genotypes

To test whether parthenocarpy is widely present in *C. annuum*, twelve genotypes which had not been evaluated for their parthenocarpic potential were tested by emasculating flowers (Table 1). We included Bruinsma Wonder (BW), which was already known for its moderate levels for parthenocarpy (Tiwari et al., 2007). All genotypes except Parco set seedless fruit after emasculation, indicating a wide occurrence of parthenocarpy in *C. annuum*genotypes (Table 1). Additionally, CLS were reported in most parthenocarpic fruit from the *C. annuum*genotypes studied (Tiwari et al., 2007), and here we investigate the origin and effect of these structures on fruit initiation.

Table 1 Parthenocarpic potential in thirteen genotypes of *Capsicum annuum*. The accession numbers are from the Center of Genetic Resources, the Netherlands (CGN), The number of emasculated flowers is indicated. The percentage of flowers that set parthenocarpic fruit was categorized in three levels (>30%, 1-30% and 0%)

Genotype	**Accession Number**	**Number of emasculated flowers**	**Parthenocarpy fruit set (%)**		
			>30%	1-30%	0%
Yellow Belle	CGN22851	78	√		
Neusiedler Ideal; Stamm S	CGN21562	66	√		
Spartan Emerald	CGN16846	137		√	
Wino Treib OEZ	CGN23270	110	√		
Florida Resistant Giant	CGN16841	75	√		
California Wonder 300	CGN19189	141		√	
Sweet boy	CGN23823	58	√		

Emerald Giant	CGN21493	73	√			
Keystone Resistant Giant	CGN23222	82	√			
Green King	CGN22122	69	√			
Bruinsma Wonder	CGN19226	88	√			
Parco	CGN23821	149			√	
Riesen v.Kalifornien	CGN22163	79	√			
	De Ruiter				√	
Lamuyo B*	Seeds					
	De Ruiter			√		
Orlando*	Seeds					

*referred from (Tiwari et al., 2007)

Number and weight of carpelloid structures is influenced by genotype

To study whether a positive relation between CLS development and parthenocarpy occurs in most of the genotypes of *C. annuum*, we tested five different genotypes, each showing a different potential for parthenocarpic fruit set, at two different temperatures: 20/18^{o}C D/N as a normal temperature with 16/14^{o}C D/N as a low temperature. Previous analysis showed that parthenocarpy is enhanced when plants are grown at low temperature (Tiwari et al., 2007). Pollen viability and pollen germination were significantly reduced at low temperature ($P<0.001$) compared to normal temperature (Figure S2), suggesting that the reduced fertility might enhance the occurrence of parthenocarpy. For non-pollinated flowers (pollination was prevented by applying lanolin paste on the stigma), only those seedless fruits that reached at least 50% of the weight of seeded fruits (i.e. only fruits of at least 76 g) were considered as true parthenocarpic fruit and met the criteria for our analysis, while remaining fruits were considered as knots(Tiwari et al., 2007). Knots are seedless fruit-like organs characterized by small size and a pale color(Gorguet et al., 2008).

At normal temperature parthenocarpic fruit set and CLS growth were clearly genotype dependent (Fig. 1), and we observed a strong positive correlation between CLS weight and number and the percentage of parthenocarpic fruit produced. The CLS weight was significantly higher in non-pollinated flowers (Fig. 1A, B). After preventing pollination, Line 3 showed the highest parthenocarpy (89% of fruits were seedless, excluding knots), and produced the highest number (10 ±1.16) and weight (17 ±2.6 g) of CLS per fruit. In contrast, Parco showed lowest parthenocarpy (56%) with the lowest number and weight of CLS per fruit (1.6 ±0.37 and 2.8 ±0.7 g, respectively; Fig. 1A-B). Also after hand pollination and a positive relation between CLS production and seedlessness was observed (Fig. 1C-D).

Evaluation of the same five genotypes at a low temperature regime showed increased levels of parthenocarpy and decreased CLS growth; however, the correlation between parthenocarpy and CLS was still observed. After preventing pollination, Line 3 showed highest parthenocarpy (88%) with high number (4±1.1) and weight (11±2.2 g) of CLS, in contrast to Parco where the lowest parthenocarpy (71%) was observed together with a low number (1±0.44) and weight (2±1.15 g) of CLS (Fig. 1E-F). A positive correlation between the presence ofnaturally occurring parthenocarpicfruit and CLS was also observed in pollinated flowers (Fig. 1G-H). In conclusion, under different temperature conditions and after treatments of both pollinationand where pollination was prevented a positive

correlation was observed between the percentage of parthenocarpic fruits and both number and weight of CLS.

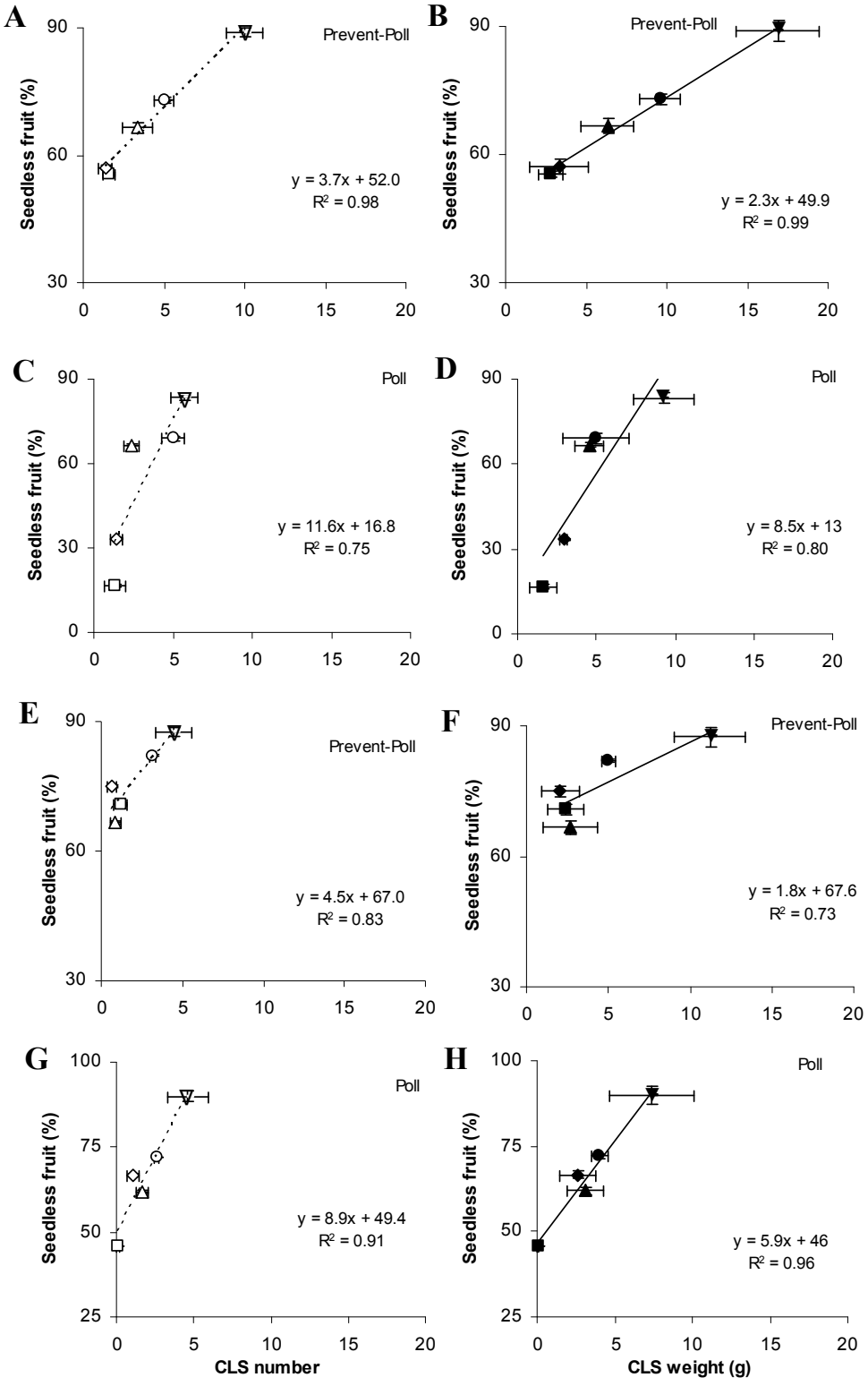


*Figure 1.*Genotype-specific evaluation of the percentage of seedless fruits and carpel like structure (CLS) development. A-H: Correlation between the percentage of seedless

fruit (only those fruits were counted that reached at least 50% of the weight of seeded fruits) and the mean CLS number (unfilled symbol) and weight (g) (filled symbol) per fruit in the genotypes Parco (n=18-24) (■,□), California Wonder (n=18-24) (♦,◊), Riesen v. Californien (n=18-24) (▲,Δ), Bruinsma Wonder (n=92-146) (●,o), and Line 3 (n=18-24) (▼, ∇), at normal 20/18°C D/N (A-D) and low 16/14°C D/N (E-H) temperatures following hand pollination (Poll; C,D,G,H), or prevention of pollination by applying lanolin paste on the stigma at anthesis (Prevent-Poll; A,B,E,F). The regression lines are based on the means of the five *Capsicum annuum* genotypes.

The occurrence of abnormal ovule development in C. annuum

To study the basis of both parthenocarpic and CLS proliferation we checked for deviations in ovule development in specific *Capsicum* genotypes. *C. annuum* has an axillar placenta, where ovules develop in a gradient from top to bottom as shown in genotype OR, BW, and Line 3 (Fig. 2A-C). Normally the ovule primordium initiates as finger-like protrusion from the placental tissue, which differentiates over time into three main proximal-distal elements: respectively the funiculus, the chalaza and the distally-located nucellus. The funiculus is a small stalk-like structure that connects the ovule to the placenta. The chalaza is characterized by the presence of a single integument (unitegmic), which gradually covers the nucellus leaving an opening at the micropylar end. At anthesis, the micropylar end orients towards the placenta resulting in an anatropous ovule (Fig. 2D-F).

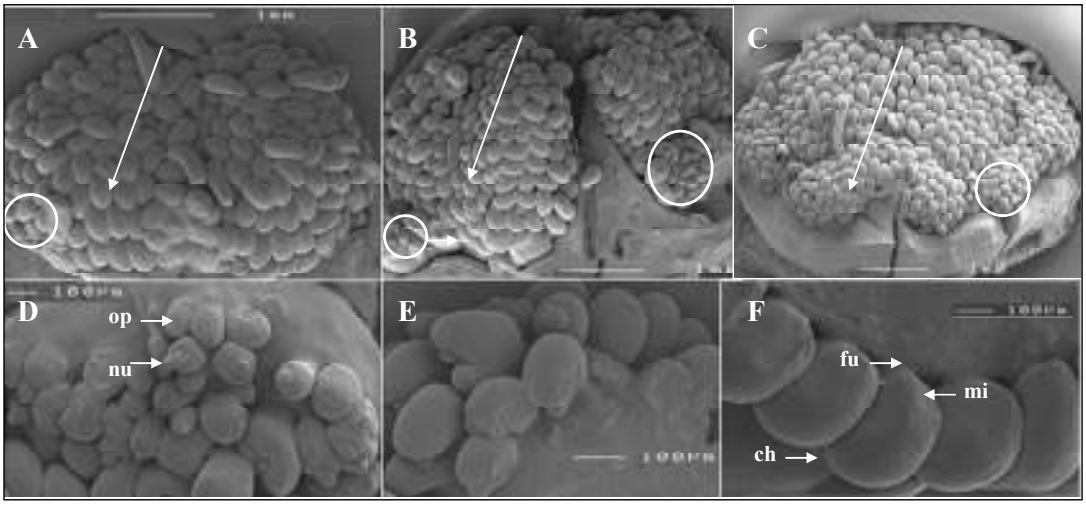


Figure 2. Cryo-scanning electron microscopy images of ovule development in *Capsicum annuum*. A-C, Comparison of genotypes Orlando (A), Bruinsma Wonder (B), and Line 3 (C) grown at 20/18°C D/N. Gradient of ovule development from top to bottom (arrow head; small circle: undeveloped ovules) Bar = 1 mm. D,E, Ovule primordia (op) initiated from the placenta (arrows), and differentiated in nucellus (nu), chalaza (ch) and funiculus (fu), integument development (E) and development of the micropyle (F). F, Single integument (unitegmic) ovules with micropylar end (mi) situated near the base of the funiculus and oriented towards the placenta (anatropous). Bar = 100 μm.

*Capsicum annuum*genotypes Orlando (OR), BW, and Line 3 all contained abnormal ovules, which were most abundant at the top and base of the placenta. Ovule abnormalities were detected after integument growth was initiated. Various types of integument abnormalities were observed. For example integument development expanded transversally or longitudinally to form CLS (Fig. 3A, B). In some cases the funiculus continued to grow and the nucellus expanded, forming excessively long ovules in which the integument failed to cover the nucellus (Fig. 3B). In other cases the integument failed to cover the nucellus, as the integument-like structure did not proliferate from the distal but rather from the more proximal end (Fig. 3C). Ovule primordia were also observed to be transformed into amorphic or staminoid tissues (Fig. 3D). Others lost the normal anatropus development and took on a "hairdryer" phenotype, reminiscent of the *superman* phenotype (Gaiser et al., 1995; Fig. 3E) or only differentiated into a funiculus lacking distal elements (Fig. 3F).

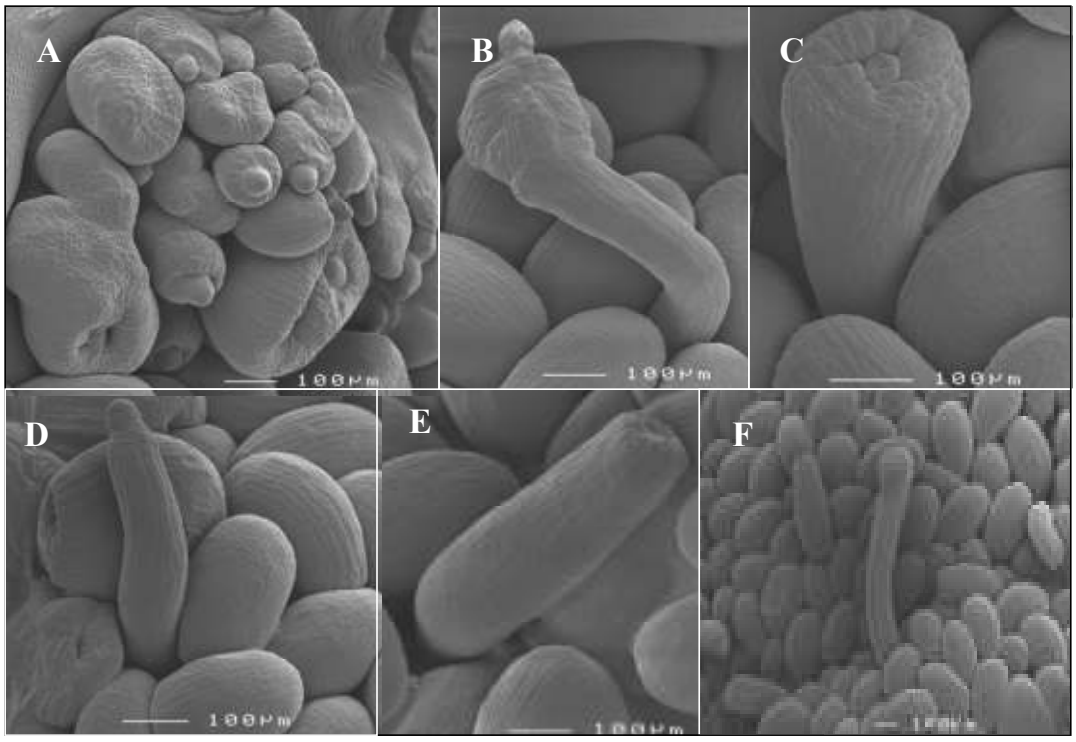


Figure3. Cryo-scanning electron microscopy images showing abnormal ovule development as observed in *Capsicum annuum* genotypes Orlando, Bruinsma Wonder and Line 3 grown at 20/18^{o}C D/N. A-F, Abnormalities detected in the three genotypes were excessive integument growth (A), or CLS proliferation of integuments and or the incomplete coverage of the nucellus (B), integuments failing to cover the nucellus (C). In some, ovule structures the integuments partially recurved (D) or were absent (E). Some ovule primordia lacked chalaza and nucellus specification (F). Bar = 100 μm.

Abnormal ovule development correlates with reduced seed set and enhanced development of carpelloid structures

To test the effect of aberrant ovule development on seed set and CLS growth, we quantified the number of aberrant ovules in genotypes Line 3 and OR by evaluating six gynoecia per

genotype and 20-30 ovules per gynoecium, and we quantified the seed number by evaluating fruits in Line 3 (n=5) and OR (n=55). The percentage of aberrant ovules was significantly higher in Line 3 compared to OR (14% versus 6%, *P*=0.001), while the number of seeds was lower in Line 3 compared to OR (21 versus 79, *P*=0.040) (Fig. 4A). CLS growth was first observed within a week after anthesis in Line 3, and after 2 weeks in OR, suggesting a precociousness in growth and development in Line 3.

To evaluate a possible role of reduced female fertility as a cause of reduced seed set in Line 3, we quantified the number of seeds in Line 3 and BW at low, normal and high night temperature. Pollination was done by vibrating the main shoot two times per week. Previously, 20°C was reported as an optimum temperature for flowering and fruit setin *C. annuum*, and a temperature below 16°C was reported to increase the percentage of seedless fruit (Rylski and Halevy, 1972; Pressman et al., 1998). Therefore we contrasted 20/18^{o}C D/N with 16/14^{o}C D/N as a low temperature and 24/22^{o}C as a high temperature. The number of seeds was always lower in Line 3 compared to BW at low (0 versus 34±1.5), normal (18±2.8 versus 54±5.1) and high temperature (44±2.8 versus 101±5.5) (Fig. 4B). Thus, in Line 3 the high number of abnormal ovules correlated with a precocious occurrence of CLS and a lower seedset, suggesting that the ovule semi-sterility might also be in part related to the parthenocarpic potential in Line 3.

In all three tested genotypes (OR, BW and Line 3), CLS were observed as internal green abnormal structures arising from the placenta. The CLS had an extrusive growth from the placenta (Fig. 4C-F), and varied in size and phenotype from small to big, and from mildly (Fig. 4D) to severely deformed (Fig. 4E), respectively. Most of the time the CLS remained green even after ripening of the fruits and stayed firmly attached to the placenta. Only occasionally, red colored CLS were observed in a ripe fruit. The size and weight of CLS increased with time and in some fruits the carpel margin boundaries were split as CLS continued to grow outside the fruit (Fig. 4F).

Correlation between CLS, seed number and parthenocarpic fruit size and the effect of phytohormones

We used the genotype BW that has moderate parthenocarpic potential (Tiwari et al., 2007), to test the relationship between CLS growth and seed set, and the effect of phytohormone application on CLS proliferation. In seeded fruits a positive correlation was observed between fruit fresh weight and seed number up to about 100 seeds (Fig. 5A). For emasculated flowers, only those fruits that reached at least 50% of the weight of seeded fruits were considered as parthenocarpic fruit and were used the analysis. Emasculated flowers treated with or without hormones (NAA, GA_3), resulted in seedless fruits. More than 90% of both seeded and seedless fruits showed CLS on their placenta. The average number of CLS was the same in seeded and seedless fruits (*P*=0.382), but the average weight of CLS was significantly higher in parthenocarpic fruits (*P*<0.001) (Fig. 5B). Again, with various treatments a positive correlation between seedless fruit (%) and CLS weight was observed (Fig. 5B). Seedless fruit weight, excluding CLS structures,also increased proportionally with the internal CLS mass (Fig. 5C-E), indicating a strong synergistic effect between the presence of CLS structures and seedless fruit growth.

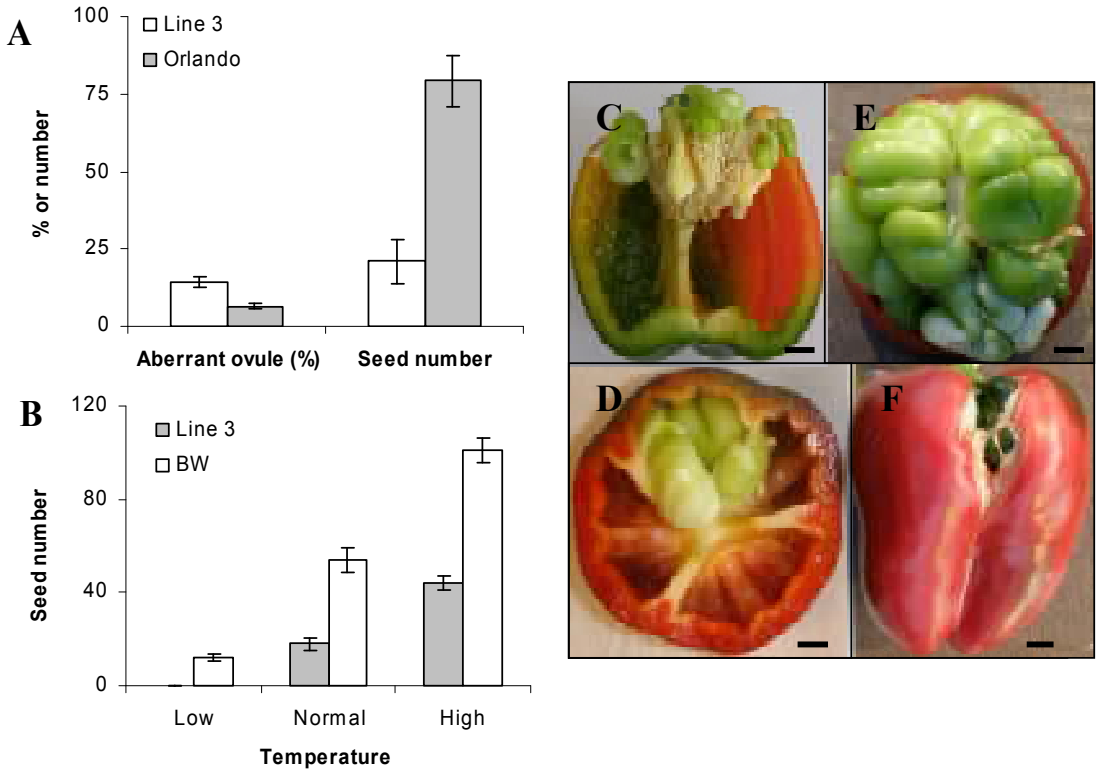


Figure 4. Genotype-dependent seed set and aberrant ovule frequencies, and phenotypes of carpelloid structures in *Capsicum annuum*. A: percentage of aberrant ovules (6 gynoecia per genotype), and average seed number in genotypes Line 3 (n=5) and Orlando (n=55), B: Average seed number in genotype Line 3 (n= 18 at low and normal, and 269 at high temperature) and Bruinsma Wonder (BW, n=146 at low, 92 at normal and 167 at high temperature) grown at day/night temperature of 16/14^{o}C (low), 20/18^{o}C (medium) and 22/20^{o}C (high). Data are expressed as mean ± standard error of the mean. C-F: structure and position of CLS in fruits. CLS developing at the basal placental position in seeded fruits (C), or in seedless fruits showing minor (D) or strong (E) CLS growth, or extreme CLS growth resulting in a split at the fruit valve (F). Scale bars: 1cm.

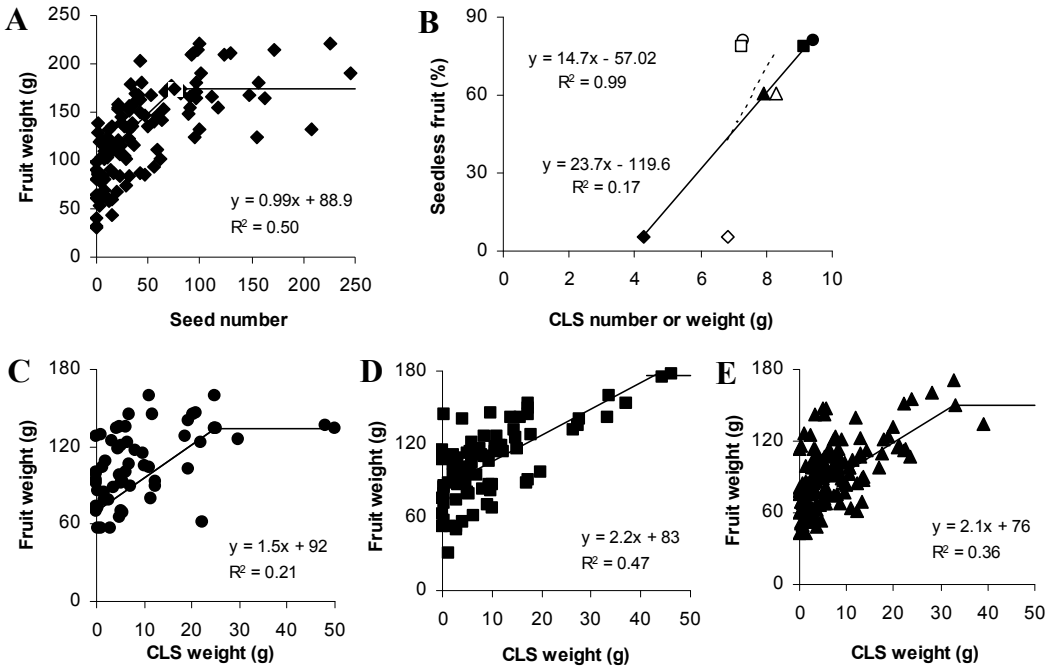


Figure 5 Relationship between fruit weight, seed set and CLS development in *Capsicum annuum* genotype 'Bruinsma Wonder'. A: A positive correlation between fruit weight (in grams) and seed number up to about 100 seeds (n=101). B: positive correlation between percentage of seedless fruit and CLS weight (closed symbols, solid line, R^2=0.99) but not with CLS number (open symbols, dashed line, R^2=0.17). Fruits obtained from untreated flowers (♦, ◊), emasculated flowers (●, o), or emasculated flowers that were treated with NAA (■, □) or GA_3 (▲, Δ). C-E: Positive correlation between fruit weight (excluding CLS weight) and CLS weight in fruits obtained from C: emasculated (n=57), D: NAA treated (n=84), or E: GA_3 treated (n=139) flowers. Only fruits of at least 76 g were considered as parthenocarpic and were used for our analysis.

Inheritance of parthenocarpy and the relationship with CLS

To study the genetic basis and inheritance of the parthenocarpic potential in *C. annuum*, the parthenocarpic genotype Line 3 was crossed with the non-parthenocarpic parents Lamuyo B, ORF_2#1 (a male sterile plant selected from an F_2 population) and Parco. Since Line 3 is a small fruited genotype (Supplemental Fig. S1; with an average fruit weight of 121 g) and Lamuyo B is a large fruited genotype (average weight of 208 g for seeded fruit; Tiwari et al., 2007), fruit size traits segregated independently upon crossing. This precludedfruit size as the sole criterion to distinguish fruit from knots as discussed earlier. Instead, we took the appearance of fruit as the criterion to distinguish true seedless fruit of small size from knots. Supplemental Fig. S1 shows the shiny appearanceof normal fruit versus the dull appearance of knots. In the F_2 analysis, a plant was considered parthenocarpic when emasculated flowers all produced seedless fruits showing a shiny appearance. In all three F_2 populations parthenocarpic plants were observed in 1:3 ratios. Furthermore when the F_1 of Line 3 x Lamuyo B was backcrossed with Line 3, parthenocarpy was observed in a 1:1 ratio. This data supports that parthenocarpy present in Line 3 is controlled by a single

recessive gene (Table 2).The same F_2 plants were evaluated for the occurrence of CLS. We used two different criteria to distinguish CLS from non CLS plants: (i) a less stringent one where plants were scored as having the CLS trait if all the true seedless fruits contained at least one CLS; plants with no seedless fruits were excluded from the analysis; and (ii) a more stringent one by which plants were scored as having the CLS trait if more than 75% of all the true seedless fruits contained at least one CLS; plants with less than 2 seedless fruits were excluded from the analysis. However, taking either criterion into consideration, no mono- or digenic-models fitted an observed CLS/non CLS segregation.

Table 2. F_2 population analysis for parthenocarpy in crosses of Line 3 x Lamuyo B, Line 3 x OR F_2#1 and Line 3 x Parco, tested by chi-square distribution assuming monogenic recessive inheritance. (O: observed, E: expected, P: probability)

Crossing	Generation	Expected ratio	Total	Parthenocarpic			
				O	E	X^2	*P*
Line 3 x Lamuyo B	F2	1:3	42	10	10.5	0.03	0.86
	F1 x Line 3	1:1	41	20	20.5	0.02	0.88
Line 3 x OR F_2#1	F2	1:3	62	17	15.5	0.19	0.66
Line 3 x Parco	F2	1:3	24	5	6	0.22	0.64

Ninety-four percent of the fruits of Line 3 and 40% of OR F_2#1 contained CLS. Both the average number ($P<0.001$) and the weight ($P=0.011$) of CLS per seedless fruit was higher in Line 3 than in OR F_2#1 at 21/19^{o}C D/N temperature. This agrees with the results described above that genotypes with a higher potential for parthenocarpy produced more CLS.

Parthenocarpic potential in C. annuum is not caused by a mutation in CaARF8

Similar to tomato and *Arabidopsis*, a mutation in the *ARF8* gene might lead to parthenocarpic phenotypes in Line 3. Sequence analysis was performed for 7508bp of *CaARF8*(-1816 in the promoter to the 3'UTR) in Line 3, BW and OR (supplemental Fig. S2). No differences in the sequence of this gene was observed between the three genotypes (Supplemental Fig. 2, 3), indicating that the parthenocarpy is not caused by mutations in the coding region of*CaARF8*.

Discussion

Most C. annuum genotypes have parthenocarpic potential

As an initial step in our attempt to establish parthenocarpy in *C. annuum*, we tested several genotypes for their potential to set seedless fruits following emasculation. In line with our previous findings (Tiwari et al., 2007), most *C. annuum* genotypes developed seedless fruits following emasculation (Table 1), suggesting that somedegree of intrinsic parthenocarpy is already present in these genotypes. Genetic variation for the strength of parthenocarpic fruit development was observed, which may occur due to genotypic differences in endogenous auxin and/or gibberellins contents in the ovaries or placenta. Genotypes with high potential for parthenocarpy could contain higher levels of hormones compared to those with

lesserpotential (Fos et al., 2003). Intriguingly, however, we also observed that the genotype with the highest parthenocarpic potential (i.e. Line 3) showed reduced female fertility and seed set, and developed significantly more aberrant ovules as compared to the genotype for which no seedless fruit development was observed (OR). Pollination at different temperatures could not rescuecomplete seedset in Line 3 compared to BW, supporting the hypothesis that reduced female fertility is associated with enhanced parthenocarpy in Line 3. This hypothesis is corroborated by our previous observation that the expression of parthenocarpy was most prominent in Line 3 (100%) and Lamuyo B (70%) at low night temperature, which leads to further reductions in male fertility (Figure S2), while this was reduced in Line 3 (73%) and not detectable in Lamuyo B (0%) at normal night temperature (Tiwari et al., 2007).Mazzucato and coworkers (1998)also reported reduced fertility fromaberrant ovules and aberrant anther development as perhaps an associated or causal phenotype with parthenocarpy in the tomato*pat* mutant (*pat* allele). Precocious CLS growth was observed in Line 3 compared to OR, suggesting that Line 3 contains traitsleading to precocious parthenocarpic and CLS transformation well before fertilization. Similar to this, Mapelli and coworkers (1979)and Vivian-Smith and coworkers(2001)reported that parthenocarpic fruit development is characterized by autonomous and precocious onset of ovary development.

Number and CLS mass is influenced by genotype

CLS development was observed in all *C. annuum* genotypes tested, which is in agreement with Lippert and coworkers (1966)who reported that CLS are evident in a wide range of *Capsicum* varieties, but are most commonly observedproliferating in bell or blocky type accessions where there is an axial placenta. We found that the number and weight of CLS was genotype dependent (Fig. 1A-H). Interestingly, also for the *Arabidopsisbel1* mutant the severity of CLS is ecotype dependent (Modrusan et al., 1994).

CLS proliferation is most prominent in genotypes that possess a high potential for parthenocarpic fruit set (i.e. Line 3), suggesting that both traits synergistically interact withoneanother. Previous observations in tomato, where the down regulation of *TM29* (*SEPALLATA* homolog) transcription factor resulted in similar synergistic phenotypes of carpelloid tissue proliferation and parthenocarpy. Likewise the*Arabidopsis knuckles* mutant that was defective in the *MAC12.2* gene, developed reiterating carpelloid tissue from ovule primordial and triggered parthenocarpy(Ampomah-Dwamena et al., 2002; Payne et al., 2004). In both mutants CLS developed ontogenically from the placental tissue. The co-occurrence of CLS and parthenocarpy points to a possible regulatory link between both traits (Payne et al., 2004).

In most flowering plants,flowers consist of sepals(first whorl), petals(second whorl), stamens(third whorl), and pistils(fourth whorl) (Immink et al., 2010). In the Arabidopsis*fwf-1/arf8-4* mutant, the third whorl organs have an inhibitory effect on parthenocarpic silique development, (Vivian-Smith et al., 2001). In the male sterile *pop1/cer6-1* background, the*fwf-1/arf8-4* parthenocarpy mutation only induces strong silique growth when the stamens are removed. This need of emasculation is negated when the*pop1/cer6-1-fwf-1/arf8-4* double mutant is combined with the*ats-1/kan4-1*mutant,which has a lesion in ovule integument development (Vivian-Smith et al., 2001). This suggests that the inhibitory signal derived from the stamens, in the third whorl, acts through the ovule integument (fourth whorl) to retard parthenocarpic silique development in*fwf-1/arf8-*

4 (Vivian-Smith et al., 2001).In *C. annuum*, we observed parthenocarpic fruit set was enhanced by CLS development, which potentially is governed partially by third whorl identity regulators. These observations suggest that the third and fourth whorl prevent fruit initiation possibly by a shared pathway, and might explain why emasculation or CLS development can stimulate fruit set in the absence of fertilization.

Inheritance of parthenocarpy and relation between parthenocarpy and CLS

The expression of parthenocarpy in the *C. annuum* genotype Line 3 is facultative, producing seeded and/or seedless fruits depending on growth conditions and some semi-sterility. We studied the inheritance of parthenocarpy in Line 3 at normal temperatures by using emasculation, and found that the parthenocarpic potential in Line 3 is linked to a single recessive gene (Table 2). Recessive mutations inducing facultative parthenocarpy have been reported before in tomato,citrus and Arabidopsis(Yamamoto et al., 1995; Gorguet et al., 2008; Vivian-Smith et al., 2001). Mutations in Arabidopsis*ARF8* gene can provide parthenocarpy), but it can also be obtained when defective forms of *ARF8* are expressed in Arabidopsis and tomato (Goetz et al., 2007). In *C. annuum*no changes in the *CaARF8*coding sequence were detected in different *C. annuum* genotypes, excluding that the occurrence of parthenocarpy is caused by a mutation in this gene.

In our F_2 analysis no simple inheritance pattern was observed for CLS growth, and no genetic relation could be observed between CLS and parthenocarpy. The main reason for this is that all parental genotypes used in the three crosses showed at least some degree of CLS development (92% fruits with CLS in Line 3, 56% in Lamuyo B, 46% in OR:Tiwari et al., 2007) . In order to study CLS inheritance, a parental genotype completely devoid of CLS growth would be desirable.The ubiquitous nature of CLS, but synergistic interaction with parthenocarpy, suggests a non-mendelian form of inheritance. Perhaps this is already fixed, but it has an incomplete penetrance or CLS growth may be a result of certain pleiotropic effects associated with parthenocarpy.

Abnormal ovule development and reduced seed set, enhanced CLS development and parthenocarpic fruit size

C. annuum has an axillar placenta where ovules develop in a gradient from top to bottom. The majority of the ovules are anatropous and unitegmic, as is characteristic for the *Solanaceae* family (McAbee et al., 2005). Deviations in normal ovule development were observed mainly at the top and base of the placenta (Fig. 2), which might be due to abnormal integument growth. A similar pattern of deviations was reported in *Arabidopsis* and petunia where abnormal integument growth resulted in an abnormal ovule mainly at the top and the base of the placenta (Palser et al., 1989; Angenent et al., 1995).Stunted integuments in some *Solanaceae* may have a genetic basis since Angenent and coworkers (1995) suggested that reduced resource availability may lead to the aberrant ovule growth. The genotype Line 3 contained a high fraction of aberrant ovules and also contained high CLS growth compared to OR. Although our data can not exclude that some CLS arise as *de novo* structures directly from the placenta, the majority result from homeotic ovule primordia conversions. CLS are generally observed as internal green abnormal structures arising from the placenta, e.g. in *Arabidopsis* and *Capsicum*(Murthy and Murthy, 1962a; Payne et al., 2004). The CLS in *Capsicum* have a placental origin and somewhat resemble a normal ovule with an abnormal uninucleate embryo sac (Cochran, 1934b). Furthermore,

Cochran showed that CLS histologically resemble carpel tissue. Altogether these results strongly suggest that CLS are the result of aberrant ovule development.

An inverse relation between the percentage of aberrant ovules and seed number was observed when comparing the genotypes Line 3 and OR (Fig. 4A), suggesting that fertility is reduced in Line 3. This might explain why induced pollination even at normal and high night temperature did not improve the seed set in Line 3 compared to BW (Fig. 4B). Moreover it may be the reason why the parthenocarpic potential is higher in Line 3 as compared to other genotypes. Reduced fertility might allow more time for the expression of the parthenocarpic potential.

In general, fruit weight was positively correlated with seed number, and in the absence of seeds fruit weight proportionally increased with the CLS mass (Fig. 5A-F). This suggests that CLS growth imitates the growth signals that normally occur only after pollination and fertilization, and that CLS support fruit growth by mimicking the role of developing seeds. In the absence of fertilization, CLS can utilize all the available assimilates and thus grow prominently. These observations explain a likely relation between CLS, seeds and fruit growth.

Conclusion

Based on our findings we postulate a model indicating the role of fertility, aberrant ovules and CLS growth in parthenocarpic fruit set and development (Fig. 6). CLS development positively reinforces fruit growth, particularly in genotypes showing parthenocarpic potential.Abnormal ovules may convert into CLS, however, growth of CLS only becomes prominent in the absence of fertilization. In agreement with this model, the Line 3 genotype showed reduced fertility and developed more CLS. Upon fertilization, normal seed development prevailsand at the same time induces fruit set and development, possibly suppressing CLS proliferation. Facultative parthenocarpy is widely present in *C. annuum* genotypes, and the absence of fertilization allows the parthenocarpic potential to be expressed, and at the same time induces CLS development, possibly following homeotictransformation of abnormal ovules.CLS development supports parthenocarpic fruit growth by increasing the fruit's sink strength in a similar way as seeds do in seeded fruits.

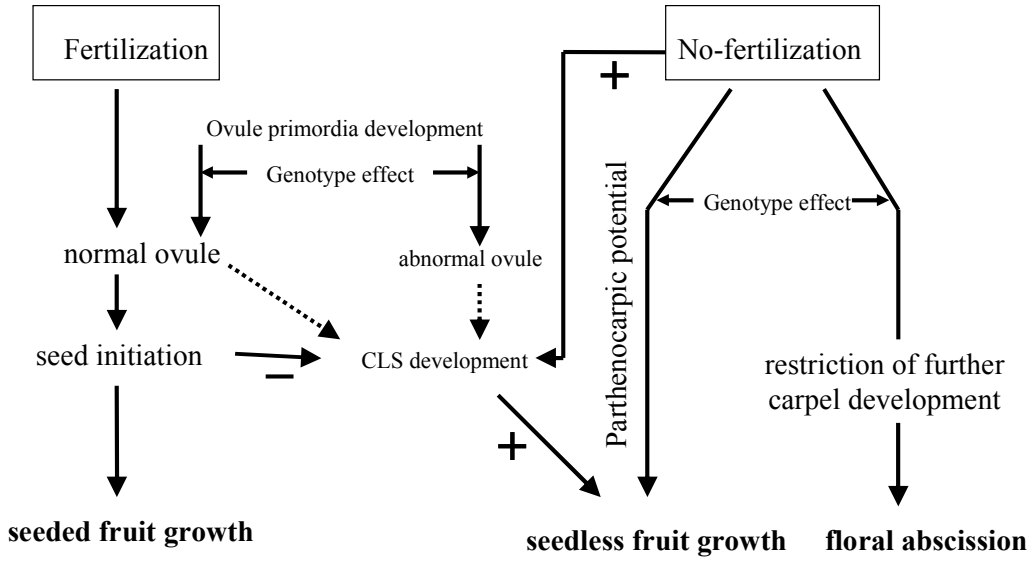


*Figure 6.*The proposed model indicating the role of carpelloid structures (CLS) in parthenocarpic fruit development. Genotypes have genetic potential for parthenocarpic fruit set, which becomes only expressed in the absence of fertilization. Abnormal ovules may convert into CLS, however, CLS growth only becomes synergistically connected with fruit initiation in the absence of fertilization/seed initiation. The CLS mimic the role of seeds and support parthenocarpic fruit growth. (solid lines represent our experimental findings and dashed lines represent likely routes).

Supplemental data files

Supplemental Figure S1

*Figure S1.*Fruit characteristics used in the segregation analysis. A-B:Fruit shape and size of genotype Line 3, C-E:seedless fruit of shiny appearance and pointy bottom (C), and large depression on bottom (D), and small size (E); F- H:small size knots of partial dull appearance (F), big(G), and small (H) knots of fully dull appearance; I: seeded fruit. Plants were grown at 21/19^{o}C D/N temperature. Scale bars: 1cm (A-I).

Supplemental Figure S2

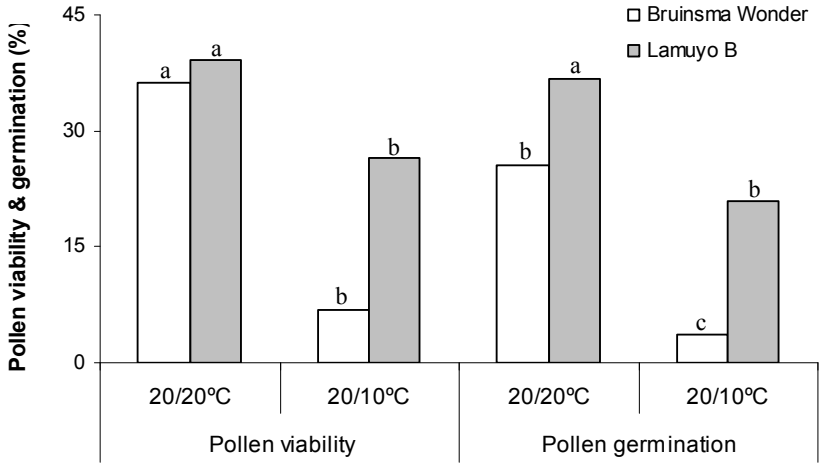
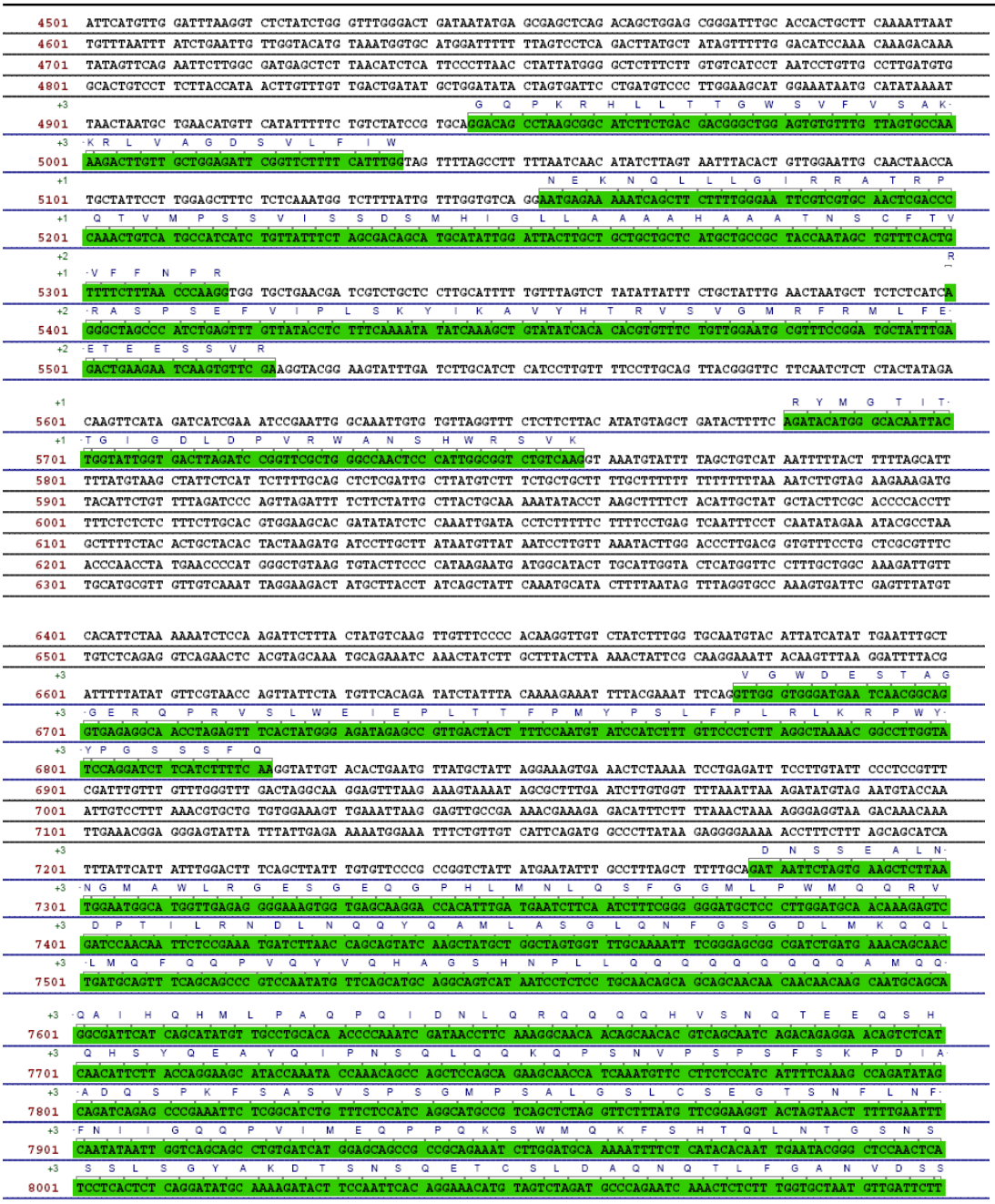
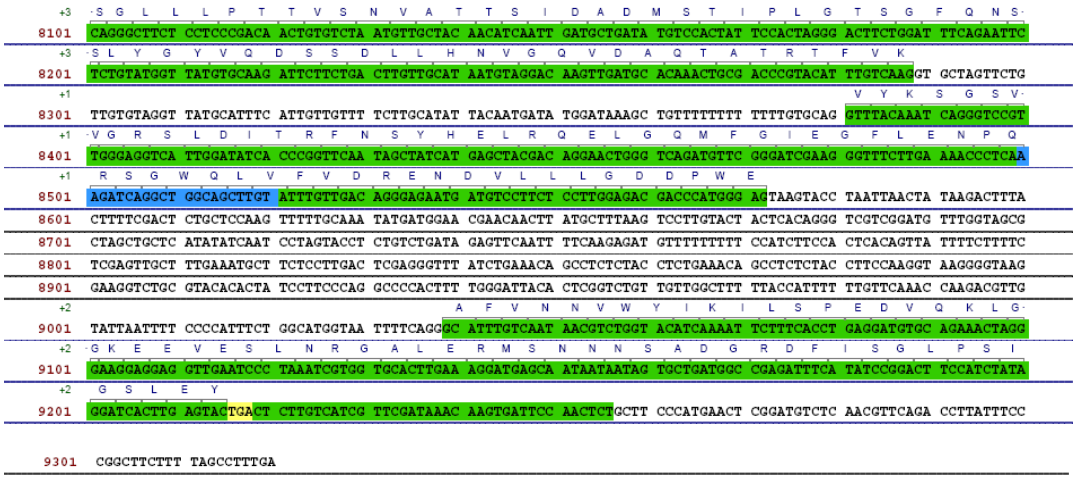


*Figure S2.*Pollen viability and germination for genotypes Bruinsma Wonder and Lamuyo B grown at normal (20/20^{o}C) and low (20/10^{o}C) day/night temperature.Different

letters above the bars represent different classes that are significantly different according to the LSD-test (P = 0.05). n=5-7 replicates.

*Supplemental Figure S3.Capsicum annuumARF8*genomic sequence in genotypes Line 3, Orlando and Bruinsma Wonder. Exons are marked green, dark grey or light gray, depending on their correspondence to our cDNA clone, the Arabidopsis coding sequence or a Solgene EST, respectively, the translation start is marked yellow the miRNA167 binding site is marked blue.

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+3 S L Y G Y V Q D S S D L L H N V G Q V D A Q T A T R T F V K
8201 TCTGTATGGT TATGTGCAAG ATTCTTCTGA CTTGTTGCAT AATGTAGGAC AAGTTGATGC ACAAACTGCG ACCCGTACAT TTGTCAAGGT GCTAGTTCTG
+1 V Y K S G S V
8301 TTGTGTAGGT TATGCATTTC ATTGTTGTTT TCTTGCATAT TACAATGATA TGGATAAAGC TGTTTTTTTT TTTTGTGCAG GTTTACAAAT CAGGGTCCGT
+1 V G R S L D I T R F N S Y H E L R Q E L G Q M F G I E G F L E N P Q
8401 TGGGAGGTCA TTGGATATCA CCCGGTTCAA TAGCTATCAT GAGCTACGAC AGGAACTGGG TCAGATGTTC GGGATCGAAG GGTTTCTTGA AAACCCTCAA
+1 R S G W Q L V F V D R E N D V L L L G D D P W E
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8601 CTTTTCGACT CTGCTCCAAG TTTTTGCAAA TATGATGGAA CGAACAACTT ATGCTTTAAG TCCTTGTACT ACTCACAGGG TCGTCGGATG TTTGGTAGCG
8701 CTAGCTGCTC ATATATCAAT CCTAGTACCT CTGTCTGATA GAGTTCAATT TTCAAGAGAT GTTTTTTTTT CCATCTTCCA CTCACAGTTA TTTTCTTTTC
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8901 GAAGGTCTGC GTACACACTA TCCTTCCCAG GCCCCACTTT TGGGATTACA CTCGGTCTGT TGTTGGCTTT TTACCATTTT TTGTTCAAAC CAAGACGTTG
+2 A F V N N V W Y I K I L S P E D V Q K L G
9001 TATTAATTTT CCCCATTTCT GGCATGGTAA TTTTCAGGGC ATTTGTCAAT AACGTCTGGT ACATCAAAAT TCTTTCACCT GAGGATGTGC AGAAACTAGG
+2 G K E E V E S L N R G A L E R M S N N N S A D G R D F I S G L P S I
9101 GAAGGAGGAG GTTGAATCCC TAAATCGTGG TGCACTTGAA AGGATGAGCA ATAATAATAG TGCTGATGGC CGAGATTTCA TATCCGGACT TCCATCTATA
+2 G S L E Y
9201 GGATCACTTG AGTACTGACT CTTGTCATCG TTCGATAAAC AAGTGATTCC AACTCTGCTT CCCATGAACT CGGATGTCTC AACGTTCAGA CCTTATTTCC
9301 CGGCTTCTTT TAGCCTTTGA

Supplemental Table S1. The set of genotypes, their parthenocarpic fruit set potential, their origin, temperature set point and realized temperatures, cultivation method (one or two branch pattern system), and start month and end of the experiments

Genotypes	**Parthenocarpic potential**	**Origin**	**Temperature °C (D/N) (realized)/ Glasshouse compartment**	**Branch pattern**	**Experiments (start-end)**
Exp. 1: Occurrence of parthenocarpy among *C. annuum* genotypes					
Yellow Belle, Neusiedler Ideal; Stamm S, Spartan Emerald, Wino Treib OEZ, Florida Resistant Giant, California Wonder 300, Sweet boy, Emerald Giant, Keystone Resistant Giant, Green King, Bruinsma Wonder, Parco, Riesen v.Kalifornien	unknown	Centre for Genetic Resources, Netherlands	20/20 (22.6/20.5)/ multispan venlo-type	2	Jan-May (2008)
Exp. 2: Number and weight of carpel-like structures is influenced by genotypes					
Orlando	Low	Centre for Genetic Resources, Netherlands	20/18 (20.5/18.6) 16/14 (17.2/14.5)/ air-condition	2	March-July (2009)
Parco	Low			1	
California Wonder 100 (CW)	Low			1	
Riesen v. Californien (RVC)	Medium			1	
Bruinsma Wonder	Medium			2	
Line 3	High			1	
Exp. 3: Ovule development in *C. annuum*					
Orlando (OR)	Low*	De Ruiter seeds	20/18 (20.5/18.6)/ air-condition	2	March-July (2009)
Bruinsma Wonder (BW)	Medium*	Plant Research International: 2004001		2	
Line 3	High*	Xue Lin Bao Yangzhou University, China		1	
Exp. 4: Correlation of abnormal ovule development with reduced seed set and enhanced development of carpel-like structures					
Line 3, Orlando	High, Low		21/19 (22.5/20.0)/ multispan venlo-type	2	Sep-Jan (2009-2010)
Line 3, Bruinsma Wonder	High, Medium		16/14 (17.2/14.5) 20/18 (20.5/18.6) 24/22 (24.6/22.6)/ air-condition	2	March-July (2009)

Exp.5:Pollen viability and germination					
Bruinsma Wonder, Lamuyo B			20/10 (20.5/10.4) 20/20 (20.4/20.3) Climate room	2	Sep-Dec(2006)
Exp. 6:Relation between parthenocarpy and carpel-like structures					
Bruinsma Wonder	Medium		20/20 (20.6/20.5)/ air-condition	2	Oct-Jan (2008-2009)
Exp. 7: Inheritance of parthenocarpy and its relation with carpel-like structures					
ORF2#1 (OR-MS)	Zero	De Ruiter Seeds	21/19 (22.5/20.0)/ multispan venlo-type	2	Sep-Jan (2009-2010)
Lamuyo B	Low			2	
Parco	Low			2	
Line 3	High			2	
Exp 8: Sequence analysis of *ARF8* genes					
Line 3	Medium		24ºC constant Climate cell	Multi-branch	2008-2009
BW	Zero				
Orlando (F2 generation: ORF2)	Zero				

*referred from (Tiwari et al., 2007)

Supplemental Table S2. Primer sequences used to amplify *Capsicum annuum CaARF8*and *CaBEL1* gene sequences.

Primer	Sequence
CA_SGN-U205478 -87F	TTAGGGTTTGAATTGGGTTTTG
CaARF8 RP#1-5'UTR (U98922)	TCATGGGAAGCAGAGTTGGA
CABw8 int#1	GAGTTGGGTATTCCTAGCAGGC
CABw8 int#11	GAGTTGGAGCCCGTATTCAA
CaBw8 int#14	GTGTCAATCAAGGCATCCACT
CaBw8 int#21	TGAGAGGGGAAAGTGGTGAG
CaBw8 int#23	TCAACTGTGGTGGCAAGTTC
CABw8 int#27	CGTGAGTTCTGACCTCTGAGACAAGC
CABw8 int#29	GGCTGGAGTGTGTTTGTTAGTGC
CABw8 int#31	CCATGCCATTCCATTAAGAGCTTCAC
CABw8 int#6 5' to miR167 F	GGGCTTCTCCTCCCGACA
CABw8 int#7 5' to miR167 F	CAAATCAGGGTCCGTTGG
CABw8 int#9 1528g F	AGCAGGATAGTGGCTGGTGT
ARF8 SEFA Seq 1	CGAAGGCAGTGGCTTGAGTC
ARF8 SEFA Seq 5	CAGGTCTAGCTTGGTCGGGTG
SlARF8 SP 1-1 R	CACTGTGACCCTGAGGAAAGTAAACCACTCGACTCCCTAC

Chapter 5

Auxin-induced parthenocarpic fruit set In *Capsicum annuum L* requires downstream gibberellin biosynthesis

Submitted as:
Tiwari A, Offringa R, Heuvelink E.Auxin-induced parthenocarpic fruit set in *Capsicum annuum* L requires downstream gibberellin biosynthesis.

Abstract

A hierarchical scheme for the central role of the plant hormones auxin and gibberellins in fruit set and development has been established for the model plants Arabidopsis and tomato. In the fruit crop Capsicum annuum, the importance of auxin as an early signal in fruit set has also been recognized, however, the effect of gibberellins and their interaction with auxin has not yet been studied. The aim of this paper is to determine the role of gibberellinand the hierarchy between auxin and gibberellin.We applied gibberellin (GA_3) alone or in combination with auxin or with the gibberellin biosynthesis inhibitor paclobutrazol (PCB) on stigmas of emasculated flowers. GA_3 application enhanced parthenocarpic fruit set, whereas application of PCB reduced fruit set. The effect of PCB treatment could be counteracted by co-application of GA_3 but not by auxin. These results indicate that in C. annuum, like in Arabidopsis and tomato, auxin is the major inducer of fruit set that acts in part by inducing gibberellin biosynthesis. Interestingly, GA_3 does not significantly contribute to the final fruit size, and instead it seems to play an important role in preventing flower and fruit abscission, a major determinant of production loss in C. annuum. At the same time GA_3 together with auxin seems to balance cell division and cell expansion during fruit growth. In addition, we may have identified a parallel pathway that contributes to the C. annuum fruit size involving a fertilization-induced invertase activity that seems to be required to elevate the hexose levels during the rapid growth phase of the fruit.

Introduction

Fruit set and fruit development is a genetically controlled developmental program existing in most flowering plants (Gillaspy *et al.*, 1993). Usually the trigger of fruit set dependson the successful completion of pollination and fertilization, indicating the importance of pollination/fertilization and seed-derived signalsin fruitsetand subsequent development (Fuentes and Vivian-Smith, 2009). Fruit development is important for plant reproduction, as it provides a suitable environment forseed maturation and seed dispersal. Though seeds are important for plant reproduction, in most economically important fruit crops, e.g. citrus, grape, melon and pineapple, they are considered as a nuisance, as they decrease productivity and need to be removed during processing or before consumption (Gillaspy *et al.*, 1993).Fruit set without fertilization or parthenocarpy provides a solution to these problems (Gillaspy *et al.*, 1993).An important additional advantage of parthenocarpic plants is that they show fruit set and fruit development under environmental conditions that are unfavourable for successful pollination and fertilization(Gorguet *et al.*, 2008). For several fruit crops, parthenocarpy has been obtained by breeding methods that have relied on mutagenesis (e.g. tomato) or alteration of the ploidy level (e.g. banana and watermelon) (Spena and Rotino, 2001). However, for many other crops genetic parthenocarpy is not available yet. In some species such as tomato and eggplant, external application of plant growth regulators has been proven to be effective for seedless fruit production in the absence of fertilization (Gillaspy *et al.*, 1993).

Auxin and gibberellins are two classes of plant hormones that are most frequently used to obtain parthenocarpic fruits (Dorcey *et al.*, 2009;Gillaspy *et al.*, 1993;Heuvelink and

Körner, 2001; Ozga and Reinecke, 2003; Serrani *et al.*, 2008; Vivian-Smith and Koltunow, 1999). External application of auxin (Alabadi *et al.*, 1996; Koshioka *et al.*, 1994; Ramin, 2003; Serrani *et al*., 2007) and ectopic expression of genes encoding enzymes of auxin biosynthesis (Pandolfini *et al*., 2002) induce fruit-set in tomato. Similar to auxin, external application of gibberellin induces fruit-set in several species (Dorcey *et al.*, 2009; Fos *et al.*, 2000; Fos *et al.*, 2001; George *et al*., 1984; Serrani *et al*., 2007).The application of both hormones on emasculated flowers at the optimal concentration can promote fruit development to the extent observed in seeded fruits e.g. tomato, pea (Ozga and Reinecke, 2003; Serrani *et al.*, 2008; van Huizen *et al*., 1996).

Several studies indicate that auxin and gibberellin application induces different changes in morphology, histology and sugar metabolism during fruit development. For example, in tomato auxin-induced fruits are bigger than GA_3-induced fruits, whereas in Arabidopsis the reverse is true (Serrani *et al*., 2007; Vivian-Smith and Koltunow, 1999). Auxin stimulates cell division in tomato and cell expansion in Arabidopsis, while gibberellins stimulate cell expansion in tomato and cell division in Arabidopsis(Bunger-Kibler and Bangerth, 1983; Serrani *et al*., 2007; Vivian-Smith and Koltunow, 1999). Enhancement of sugar accumulation at the site of hormone application is correlated with fruit growth in a number of species (Beny *et al*., 1986; Daie, 1987; Stutte and Gage, 1990). For example, post-bloom application of GA_3 increases seedless grape berry size by increasing invertase enzyme activity and the hexose content compared to untreated fruits (Perez *et al*., 2000)

A hierarchical scheme for the role of auxin and gibberellin in fruit set has been established for the model plants Arabidopsis and tomato, where auxin signals are mediated in part through the downstream gibberellin pathway (Dorcey *et al.*, 2009; Serrani *et al.*, 2008). The importance of IAA in parthenocarpic fruit set has been recognized in the vegetable crop sweet pepper(*Capsicum annuum*)(Heuvelink and Körner, 2001). However, the effect of gibberellins on parthenocarpy induction and the interaction between auxin and gibberellin has not yet been studied in *C. annuum*. To improve our understanding of the hormonal control of fruit set and development in *C. annuum*,we studied the hierarchy between auxin and GA_3 in fruit set, and determined their effect on cell division, cell expansion, sugar concentration and sugar metabolism.

Materials and methods

Plant materials and growth conditions

Seeds of sweet-pepper (*Capsicum annuum* L.) cultivar ‘Bruinsma Wonder’ were obtained from Plant Research International in Wageningen (PRI: 2004001), and of cultivar ‘Riesen v.Californien’from ‘The Centre for Genetic Resources’, Wageningen, The Netherlands. Previous experiments have shown that both cultivars have a medium potential for parthenocarpic fruit set (unpublished data). Four weeks after sowing, seedlings were transplanted on rock-wool cubes with a regular supply of nutrient solution. Four weeks after transplantation, plants were transferred on Rockwool slabs at a density of 2.5 plants m^{-2} in a compartment of a multispan Venlo-type glasshouse in Wageningen, The Netherlands (latitude 52° N). Nutrient solution was prepared according to Voogt and Bloemhard (1993) and was supplied by trickle irrigation. Supplemental lighting by high pressure sodium lamps (Philips, SON-T, 600 W) for 16 hours (from 06.00 to 22.00) provided a minimum photon flux density of 125 μmol m^{-2} s^{-1} at the crop level. The terminal flower was removed from all

plants at anthesis to support vegetative growth. The twin-branch system was applied, resulting in two stems per plant, with all side shoots restricted to a single leaf and flower.

Hormone application

Flowers were emasculated (removal of petals and anthers) 2 days before expected date of anthesis to avoid self pollination. The stigma of an emasculated flower was covered with lanolin paste at anthesis (day-0), mixed with water (1:1) without (control) or with 0.05% hormone (dissolved in water and mixed with heated lanolin (100^{o}C), stirred to a homogeneous paste and cooled to room temperature) by using a spatula. Seeded fruits were obtained by natural pollination of non-emasculated flowers. Treatments (natural pollination: Poll, emasculation: Em, Indole-3-Acetic Acid: IAA, Naphthalene Acetic Acid: NAA, Gibberellin: GA_3, and Paclobutrazol: PCB) either alone or in combinations were applied to two flowers at each node (one flower on main branch and one flower on side branch) on both branches.

For cultivar Bruinsma Wonder, 6 plants per treatment were used, and flowers up-to 10-15 nodes were treated in two experiments.For the first experiment, the number of treated flowers were n=110 for Poll, n=80 for Em, n=74 for Em+IAA, n=84 for Em+GA_3, n=70 for Em+ (IAA+GA_3).For the second experiment,the number of treated flowers were n=30 for Em, n=11 for Em+IAA, n=12 for Em+GA_3, n=43 for Em+PCB, n=52 for Em+PCB+IAA and n=48 for Em+PCB+GA_3.Temperature set point was 20/20^{o}C while realized temperature was 22.4/20.7^{o}C D/N. The treatments started in March and experiment ended in June.

For cultivar Riesen v.Californien, six plants were used for each treatment in two experiments. For the first experiment, flowers were treated until six fruits per plant were obtained. The remaining flowers were removed. Fruits were set at 1st, 2nd or 3rd and sometimes up-to 4th node positions. Treatments were: Poll, Em, Em+IAA, Em+NAA, Em+GA_3, Em+ (IAA+GA_3), Em+ (NAA+GA_3). After harvesting of these fruits, new flowers on the same plants were used to test the effect of the gibberellins biosynthesis inhibitor (PCB). Lanolin paste containing PCB was applied on the stigma of emasculated flowers either alone or in combinations of hormones: Em+PCB(n=15), Em+PCB+NAA(n=13), Em+PCB+GA_3(n=15). GA_3 was applied on flowers that were earlier (1-2 days) treated with Em+PCB+NAA (n=11). Fruits were harvested at 4-5 weeks after treatment and fruit set was calculated. Temperature set point was 20/20^{o}C while realized temperature was 22.3/21.2^{o}C D/N. The treatments started in September and experiments ended in December.

Fruits were harvested from both cultivars (i.e. Bruinsma Wonder, Riesen v.Californien) when they were completely red and their length, diameter, and fresh weight were measured. Fruits with a fresh weight less than 50% of the average weight of fruits obtained after natural pollination were considered as knots and the remaining fruits were considered as normal fruits. In all experiments, knots were included only for the determination of fruit set and excluded for all other measurements i.e. fruit weight, percentage fruit with carpel-like structures (CLS). Number of days required from anthesis till fruit maturity was calculated in all fruits of cv. Riesen v.Californien.

Measurement of cell division and cell expansion, and sugar concentration and invertase enzyme activity

Plants of *C. annuum* cultivar 'Bruinsma Wonder'were grown between December and July, as described above, in an air conditioned greenhouse at 20.4/20.2^{o}C day/night temperature. Five plants were used per treatment and only one flower per node was given the treatment: Poll, Em, Em+IAA, Em+GA_3, Em+ (IAA+GA_3), and Em+ (IAA+GA_3). Fruits were harvested at 5, 20 and 60 days after anthesis (DAA) with 3-5 fruits for each stage and for each treatment. Length, diameter and fresh weight were measured. The same fruits were used for the evaluation of number of cell layers and cell diameter in the carpel (only at 60 DAA), and for the measurement of sugar (glucose, fructose and sucrose) concentrations and invertase enzyme activity.

To evaluate cell division and cell expansion, horizontal cross sections of the carpel were mounted, stained with "safranin O" (stock solution: 2.5 g safranin O Certistain ® in 100 ml of 96% ethanol, working solution: 1:100 dilution in water), and observed under a Zeiss Axioplan 2 fluorescence/DIC microscope.Photographs were taken with a Zeiss AxioCam MRc 5 digital colour camera. Cell diameter was measured using Image tool version 3.0. For each treatment, 20-30 cells were observed from 4-5 sections per fruit. The number of cell layers was counted in mesocarp layers as a measure of cell division. Cells diameter was measured in the mesocarp area, 5-6 layers below exocarp as a measure of cell expansion.

The remaining carpel was freeze dried by using 'Edwards Modulyo EF4-174 Freeze Dryer'. Freeze dried samples were divided into two parts where one part was used to evaluate the sugar concentration (glucose, fructose and sucrose) and the other part was used to measure soluble and insoluble invertase enzyme activity by using the methods described by Helder and Vreugdenhil (1999).

Statistical Analysis

The effect of auxin (IAA, NAA), GA_3, and PCB on fruit set and fruit size was tested in a one way ANOVA. GA_3 treatment and emasculation were excluded from this ANOVA due to the large variation as a result of their small sample size only in cv. Riesen v.Californien. Mean separation was done by Student's t-test (LSD) based on the ANOVA mentioned above. Change in the number of cell layers/cell diameter or sugar concentration/ invertase enzyme activity was also tested in a one way ANOVA. Data processing and statistical tests were carried out with SPSS (Statistical Package for the Social Sciences) 15.0.

Results

Capsicum annuum fruit set is induced by auxin and requires downstream gibberellin biosynthesis

To study the hierarchical order of the action of auxin and gibberellin in fruit set in *C. annuum*, auxin (IAA) and the gibberellin (GA_3) were applied separately or in combination on stigmas of emasculated flowers of cultivar 'Bruinsma Wonder'. Under our growth conditions, following emasculation,already 34% of the flowers in this cultivar developed into fruits, of which one third remained too small to be considered as normal fruits (below 50% of the weight of seeded fruits) and were called knots. Hormone application enhanced fruit set, and this effect was stronger for GA_3 (60%) and IAA+GA_3 (63%) application than for IAA application (46%) (Fig.1A). The percentage of knots was reduced by application of IAA but not by application of GA_3 and IAA+GA_3 compared to only emasculation (Fig. 1A). Fruits of Bruinsma Wonder often contained internal out-growths that partially or fully

occupied the fruit cavity andwere named carpel-like structures (CLS), as these out-growths most likely resultedfrom homeotic conversion of aberrant ovules, (Fig. 2B-E). The CLS development was clearly inhibited in the presence of seeds, as the number of CLS-containing fruits increased from 40% in seeded fruits to 75% in seedless fruits. The average weight of parthenocarpic fruits (excluding knots) obtained either by emasculation or by application of auxin and GA_3 on emasculated flowers (149g ± 5.8 for IAA, 123g ± 3.9 for GA_3, 137g ± 3.8 for IAA+GA_3) was smaller than that of seeded fruits (167g ± 5.2, P<0.001; Fig. 1F). Fruits obtained after GA_3application were significantly smaller than auxin-induced fruits (P<0.001). Simultaneous application of auxin and gibberellin did not increase the fruit weight more than IAA (P=0.056)(Fig. 1F). These results indicate that IAA enhances both fruit set and fruit weight and reduces the number of knots, whereas GA_3 enhances fruit set but results in smaller fruit size at maturity.

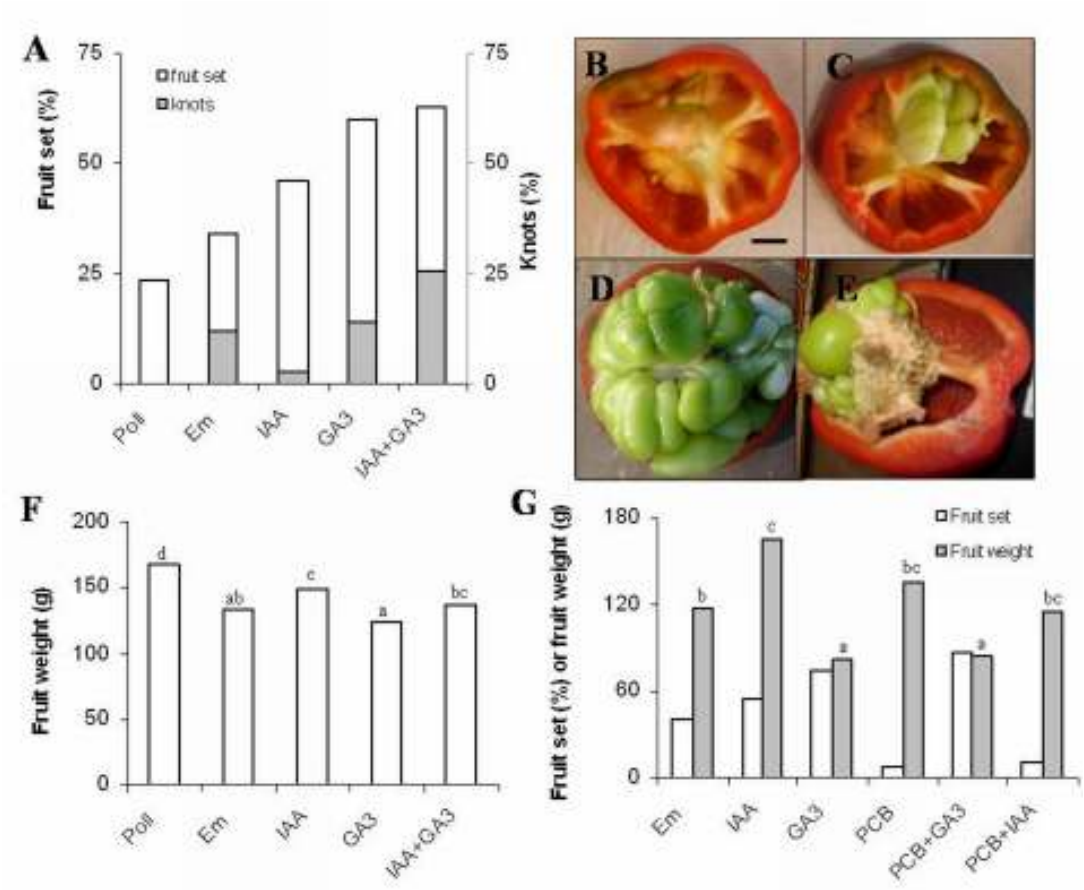

Figure 1 The effect of auxin and GA_3 on seedless fruit set and development and carpel-like structures (CLS) in *Capsicum annuum* cultivar 'Bruinsma Wonder'. A; Percentage of fruit set including percentage of knots. B-E; Structure and position of CLS in seedless fruit with minor (B), medium (C) and strong (D) growth, and in seeded fruits at the base of placenta (E), Scale bar: 1cm for B-E. F; Average weight (g) of fruits obtained from untreated (Poll; n=26) or emasculated flowers (Em; n=83), or from emasculated flowers that were treated with IAA (n=34), GA_3 (n=50),IAA+GA_3 (n=44). G; Percentage of fruit set and fruit weight in fruits obtained after emasculation (Em, n=12) or after emasculation followed by IAA

(n=4), GA_3 (n=5), paclobutrazol (PCB) (n=3), PCB+GA_3 (n=19) or PCB+IAA (n=5) application. Different letters above the bars represent different classes that are significantly different according to Student's t-test ($P < 0.05$). Fruits with a fresh weight less than 50% of the mean weight of natural pollinated fruits were considered knots. Knots are included for the measurement of fruit set and excluded for other measurements.

To test whether auxin-induced fruit set is dependent on *de novo* gibberellin biosynthesis, like it is in Arabidopsis and tomato, we applied auxin and gibberellin in combination with the gibberellin biosynthesis inhibitor paclobutrazol (PCB). PCB application alone reduced fruit set from 30% to 7%. Fruit set was improved to 88% when PCB was applied in combination with GA_3, but not when applied in combination with IAA (12%) (Fig. 1G). These results indicate that auxin-induced fruit set is mediated by the induction of gibberellin biosynthesis. The fruit weight after IAA and GA_3 application was in accordance with the previous observation (Fig. 1F) where GA_3-induced fruits were smaller than IAA-induced fruits. The fruit weight obtained after PCB treatment was comparable to that obtained after emasculation or emasculation followed by IAA treatment. These observations lead to a model where auxin acts upstream of gibberellin in fruit set, most likely by inducing gibberellin biosynthesis, and is important for full fruit development (reduced number of knots), while GA_3 is required for fruit set by reducing flower abscission. As such, it is difficult to conclude that GA_3 reduces fruit size, as the smaller fruit size might be caused by the increased number of fruits per plant.

GA_3 enhanced fruit set results in smaller fruits due to reduced growth rate

We subsequently analysed whether our observations in cv. Bruinsma Wonder could be reproduced in genotype Riesen v.Californien. In addition, to check whether reduced fruit weight after GA_3 application was caused by the higher number of fruits per plant or by their reduced capacity to attract assimilates, only six fruits were allowed to set per plant by removing the subsequent flowers.Like with Bruinsma Wonder (Fig.1), emasculation in Riesen v.Californienresulted in fruit set (41%) and hormone application enhanced the percentage of fruit set, where the effect of GA_3 was higher (87%) than IAA (57%). GA_3 application increased the percentage of knots (73%), whereas this was reduced after NAA (21%) and IAA (22%) application compared to emasculation only (Fig. 2A). More than 50% of seeded and seedless fruits contained CLS. The placental origin of CLS and the variability in their size was the same as observed for Bruinsma Wonder (Fig. 1B-E). The average weight of parthenocarpic fruits was less than that of seeded fruits ($P<0.001$) (Fig. 2B). NAA- and IAA-induced fruits were comparable in weight (Fig. 2B), but fruit set after NAA application was higher than after IAA (Fig. 2A). Application of GA_3 resulted in fruit size smaller than IAA- or NAA-induced fruits ($P=0.036$) and comparable to those obtained from untreated emasculated flowers. Simultaneous application of IAA and GA_3 resulted in fruits with a similar weight as IAA-induced fruits ($P=0.252$) (Fig. 2B). No difference in growth period (number of days required from anthesis till fruit maturity) was observed between GA_3-induced and auxin-induced fruits or between GA_3-induced and pollination/fertilization-induced fruits (Fig. 2C). As the fruit load per plant was kept constant (n=6), the competition between the fruits would be lessthan in Bruinsma Wonder (Fig. 1) where all flowers up-to 10-15 nodes were treated. This suggests that smaller fruit

size after GA_3 application occurs because of reduced growth rate and not because of competition between fruits.

To test whether the hierarchy between auxin and gibberellin is also the same as observed in cv. Bruinsma Wonder, we applied auxin and gibberellin in combination with the gibberellin biosynthesis inhibitor PCB. PCB application alone reduced fruit set from 47% to 7%. The fruit set was improved to 91% when PCB was applied in combination with GA_3 but not when applied in combination with NAA (10%). Application of GA_3 on NAA and PCB-treated flowers resulted in increased fruit set (91%) (Fig. 2D). These results confirm our previous observation in Bruinsma Wonder that auxin can initiate fruit set, result in full fruit development, and strongly suggest that auxin-induced gibberellin biosynthesis is required for fruit set.

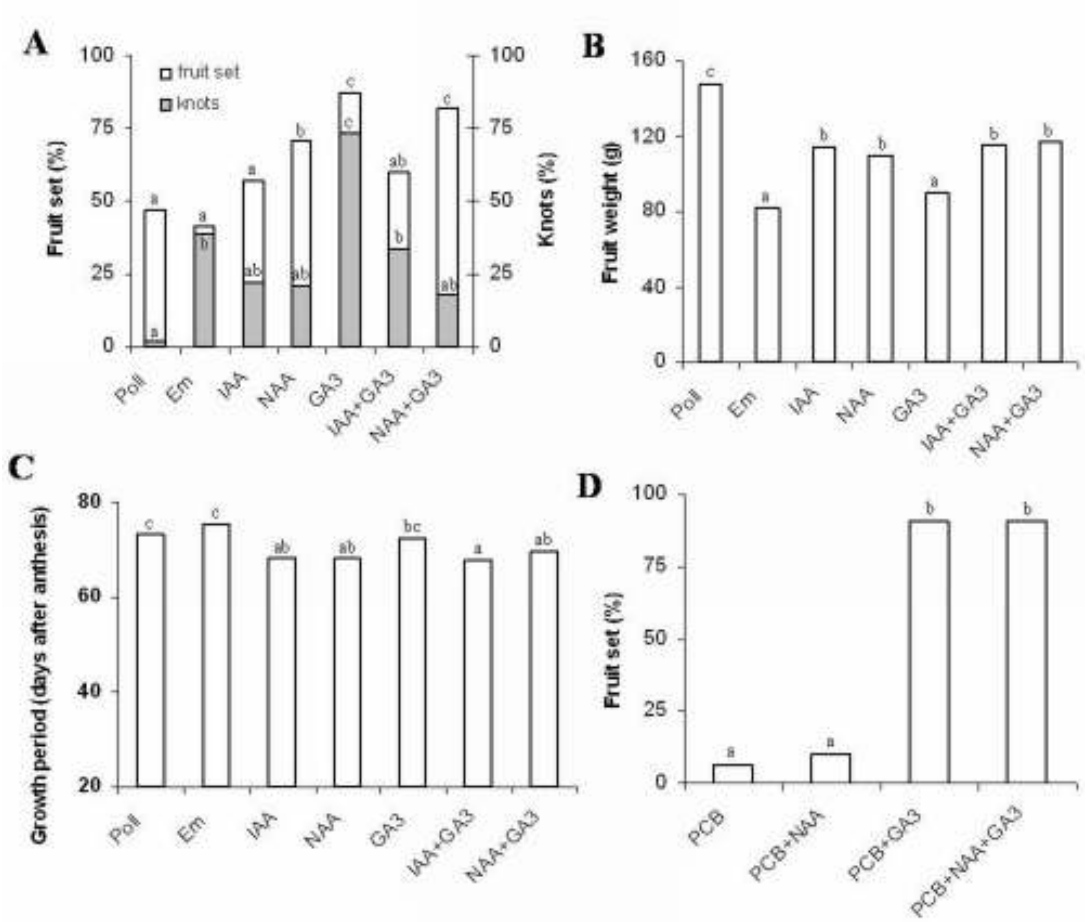

*Figure 2*Evaluation of fruit set and fruit development in *Capsicum annuum* cultivar 'Riesen v.Californien' under constant fruit load conditions. A; Percentage of fruit set including percentage of knots. Fruits which obtained fresh weight less than 50% of the average weight of natural pollinated fruits were considered as knots. Knots are included for the measurement of fruit set and excluded for other measurements. B; Average fruit weight (g) obtained after pollination and fertilization (Poll; n=17), emasculation (Em; n=11), or emasculation followed by IAA (n=21), NAA (n=23), GA_3 (n=8),IAA+GA_3 (n=21),or NAA+GA_3 (n=27) application.Six plants with six fruit per plant were used for each treatment. C;

Growth period (from anthesis till ripening) for all the fruits obtained from different treatment (same as Fig 2B). D; Percentage of fruit set after application of paclobutrazol (PCB) on stigmas of emasculated flowers, either alone (n=15) or in combination with NAA (n=13), GA_3 (n=15), or NAA+GA_3 (n=11). Different letters above the bars represent different classes that are significantly different according to the LSD-test (P=0.05).

GA_3 stimulates cell division and auxin stimulates cell expansion during fruit growth

To clarify the effect of GA_3 and auxin on cell division and cell expansion, we compared the histology of seeded and seedless fruit of cv. Bruinsma Wonder at 60 DAA. The carpel section showed a single layer of exocarp cells, multiple layers of mesocarp cells and two layers of (giant and small) endocarp cells (Fig. 3A). The number of cell layers in the mesocarp was used as a measure of cell division (Fig 3A: broken arrow). The average number of cell layers in the mesocarp was 22 (±0.6) in fruits obtained after emasculation and 27 (±0.4) in fruits obtained after pollination. The number of cell layers in GA_3-induced fruits (26±0.4) was not different from fruits obtained after pollination (*P*=0.313), whereas IAA-induced fruits (23±0.8; *P*=0.012) developed significantly less cell layers (Fig. 3B). Because a gradient in cell size was observed from the exterior to the interior of the mesocarp, we used the same four to six layers (starting from layer 5 from the exocarp) to measure the diameter of individual cells (Fig. 3A, box; Fig. 3C, arrow). The mean cell diameter was the same in fruits obtained after pollination or IAA application (*P*=0.585), while this was significantly smaller in fruits obtained after GA_3 (*P*=0.001) application (Fig. 3B). The simultaneous application of IAA and GA_3 resulted in a cell diameterintermediate of auxin and gibberellin. These data indicate that during fruit growth auxin stimulates cell expansion, whereas GA_3 rather enhances cell division. This together with the observation that auxin-induced fruits in general are bigger than GA_3-induced fruits suggest that cell expansion is an important determinant of the final fruit size in *C. annuum*.

Hexose content during the exponential growth phase as a possible determinant of fruit weight

To test whether the smaller size of seedless fruits could be correlated with a lower hexose content or invertase activity, we measured the glucose, fructose and sucrose concentrations and the acid invertase enzyme activity in seeded and seedless fruits (Em, Em+IAA, Em+GA_3, Em+IAA+GA_3) at 5, 20 and 60 DAA forcv. Bruinsma Wonder. The criteria for the selection of sugars and enzyme activity were based on the finding by Nielsen *et al.*, (1991)that acid invertase enzyme activity is important at early and late stages of fruit development, and glucose, fructose and sucrose are major carbohydrates in seeded fruits of *C. annuum*.

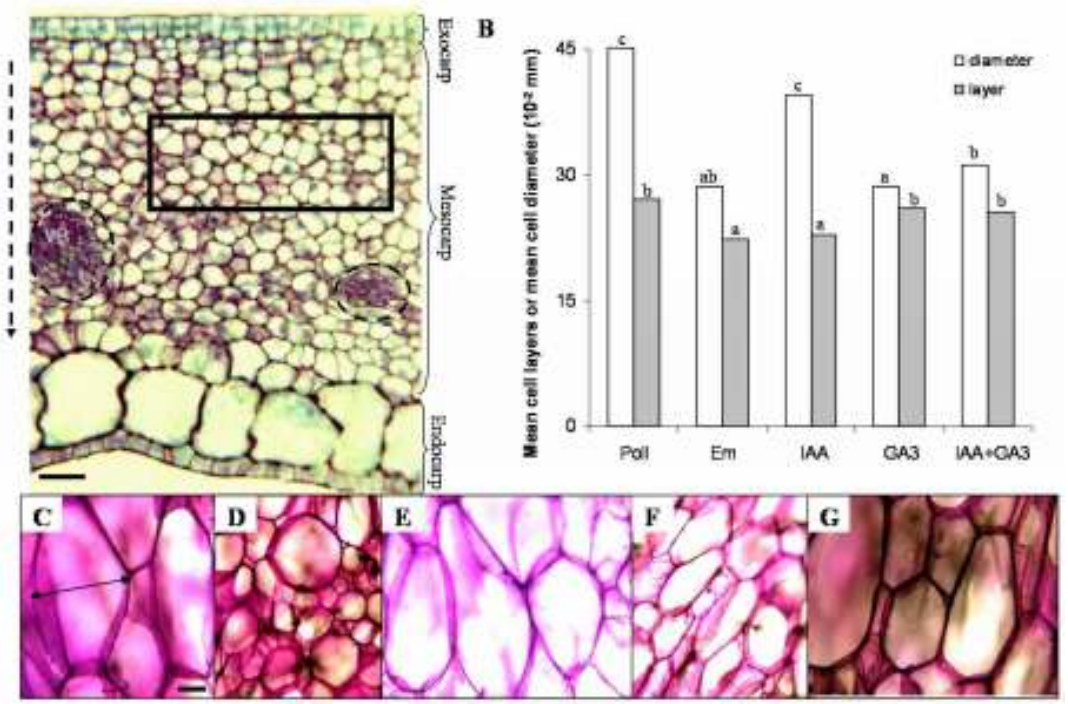

Figure 3 Analysis of cell division and -expansion in mesocarp layers from fruits *Capsicum annuum* cultivar 'Bruinsma Wonder' at 60 days after anthesis. A, A representative image of a horizontal cross section of the carpel with the indication of an area (box) from where diameter of cells were measured and differentiation of layers into exocarp (outer single layer), mesocarp (multilayered) and endocarp (two layered). VB = vascular bundle. B, Average number of cell layers and average cell diameter from fruit obtained after pollination (Poll), emasculation (Em), and emasculation followed by IAA, GA_3 or IAA+GA_3 application (n=3-5). Different letters above the bars represent different classes that are significantly different according to the LSD-test (P=0.05). C-G; Detail of a horizontal cross sections of the mesocarp in mature fruits, showing parts of the region where cell size was measured in transverse direction (arrow). Fruits were obtained from pollinated (C), or emasculated flowers (D) or from emasculated flowers treated with IAA (E), GA_3 (F), or IAA+GA_3 (G). Scale bars: 50 μm (A) or 20 μm (C-G).

Differences in fruit weight were already visible at 5 DAA, where pollinated and auxin treated flowers resulted in the same fruit weight, while flowers treated with GA_3 resulted in a reduced fruit weight compared to seeded fruits (P=0.010). As observed earlier in Fig. 1 and 2, fruits obtained after pollination at 20 DAA were bigger than fruits obtained after emasculation or after emasculation followed by IAA or GA_3 or IAA+GA_3 treatment (P<0.001). IAA and IAA+GA_3 application resulted in fruits of similar weight (P=0.633), whereas GA_3 application resulted in the smallest fruit size (P=0.029). Similar differences in fruit weight were observed at 60 DAA (Fig. 4A).

In all fruits, the hexose concentration increased (up to 9-fold) whereas the sucrose concentration decreased (up to 4-fold) in the period between 5 and 20 DAA. At 5 DAA the

glucose and fructose concentrations did not differ significantly (P=0.289), but at 20 DAA the fructose concentration was significantly lower than the glucose concentration (P<0.001) (Fig. 4B, C). Interestingly, at 20 DAA both the glucose and fructose concentrations were higher in seeded fruits compared to most seedless fruits (glucose: emasculated: P=0.002, IAA: P=0.009, IAA+GA_3: P=0.014), except for the glucose concentration in GA_3-induced fruits (P=0.213) (Fig. 4B, C). At this time, the total hexose concentration was also higher in seeded fruits (P=0.005) compared to seedless fruits. A positive relation between fruit fresh weight and hexose content was observed at 20 DAA (r^2=0.82) for all treatments (Fig. 4E), suggesting hexose as a possible determinant for fruit weight. In the nearly mature fruits at 60 DAA, glucose and fructose were the dominant carbohydrates stored in the fruit representing more than 80% of the total soluble sugar (Fig. 4B, C). Now the glucose concentration was clearly higher in seedless fruits compared to seeded fruits (P=0.001) (Fig. 4B), while no clear difference was observed for the fructose and sucrose concentration (Fig. 4C-D).

The soluble invertase enzyme activity decreased while fruit fresh weight increased at between 5 and 20 DAA. The enzyme activity was slightly higher at 60 DAA compared to 20 DAA in GA_3 and IAA+GA_3 induced fruits, and this increase in activity was also reflected in an increase in glucose and fructose levels, indicating that hexose accumulation at maturity is due to increase soluble invertase enzyme activity (Fig. 4F). Higher insoluble invertase enzyme activity was observed in seeded fruits compared to seedless fruits at 5 DAA (P=0.021) (Fig. 4G). A clear positive relationship was observed between insoluble invertase enzyme activity at 5 DAA and hexose concentration at 20 DAA (Fig. 4H), suggesting an important role for this enzyme activity and the resulting increase in hexose concentration in seeded fruit growth. The stronger reduction in hexose levels in seeded fruits at 60 DAA can be attributed to dilution by stronger growth, or to the fact that in seeded fruits these assimilates accumulate in the seeds, which are not included in the tissue samples for these measurements.

Discussion

To test the role ofgibberellinin fruit set and to investigate the hierarchy between auxin and gibberellin we studied the effect of GA_3 (the most commonly used gibberellin) and auxin (IAA, NAA) on fruit set and fruit growth in *C. annuum* cv. Bruinsma Wonder and Riesen v.Californien. External application of GA_3 and auxin on the stigma of emasculated flowers enhanced parthenocarpic fruit set compared to only emasculation, indicating that these phytohormones can trigger fruit set. However, application of these two hormones could not reproduce the exact fruit size and shape as observed following fertilization. This suggests that either seeds have other roles in stimulating fruit size besides being a source of gibberellin and auxin (Gillaspy *et al.*, 1993; Mapelli *et al*., 1978; Sjut and Bangerth, 1982), or perhaps the method of ectopic application may never be sufficient to reproduce the normal endogenous signalying pathways that trigger fruit initiation and development. The majority of seedless fruits showed CLS development, suggesting that the absence of fertilization stimulates CLS development. This observation is in line with previous reports (Tiwari *et al*., 2007) where CLS were prominent in seedless fruits, while less or no CLS were reported in seeded fruits.

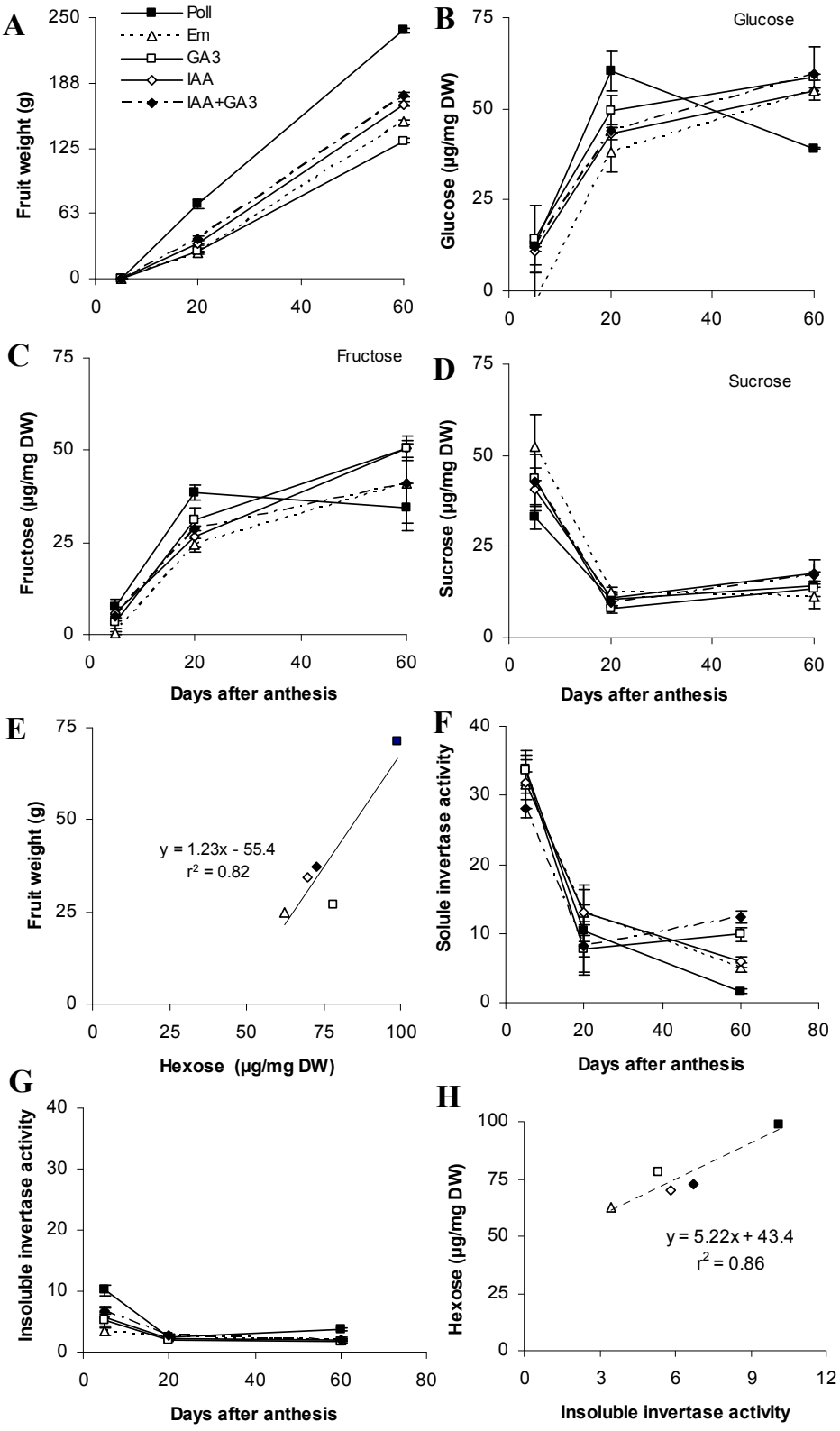

Figure 4 Fruit weight, sugar concentration, and invertase enzyme activity from *Capsicum annuum* cultivar 'Bruinsma Wonder' at 5, 20 and 60 days after anthesis (DAA), A; Fresh weight (g) of fruits obtained after pollination (Poll), emasculation (Em), or

emasculation followed by GA_3, IAA or IAA+GA_3 treatment. B-D; Sugar concentration expressed as µg per mg dry weight in fruits obtained after different treatments for glucose (B), fructose (C) and sucrose (D). E; A positive relation between fruit fresh weight (g) and hexose concentration at 20 DAA. F;G, Soluble (F) and insoluble (G) invertase enzyme activities expressed as µmol g per dry weight per hour. H; A positive relation between insoluble invertase enzyme activity at 5 DAA and the total hexose concentration at 20 DAA. For B-H use legends as in A. Vertical bars represent SEM (n=3-5).

Auxin-induced parthenocarpic fruit set requires downstream gibberellin biosynthesis to reduce abscission

The IAA- or NAA-induced fruits were comparable in size, but NAA induced fruit set was higher, which could be due to its longer lasting effect (more stable synthetic hormone) compared to IAA (unstable natural hormone) (Paciorek *et al.*, 2005; Staswick, 2009). In contrast to Arabidopsis (Vivian-Smith and Koltunow, 1999), but similar to tomato (Serrani *et al*., 2007), we found that fruits obtained after GA_3 application were smaller than fruits obtained after auxin application. This smaller size is a consequence of a reduced growth rate, as the time required from anthesis until mature ripe fruit did not differ between GA_3 and auxin-induced fruits (Fig. 2C). A likely reason for smaller fruit size after GA_3 application compared to auxin application is that gibberellin alone does not whereas auxin does trigger the first steps of fruit initiation, and that at the same time auxin stimulates gibberellin biosynthesis, as has been observed in tomato through the transcriptional activation of *GA 20-oxidases*(Marti *et al.*, 2007; Olimpieri *et al.*, 2007; Serrani *et al*., 2007).
Fruit set was reduced by simultaneous application of gibberellin biosynthesis inhibitor paclobutrazol (PCB) both in cv. Bruinsma Wonder and Riesen v.Californien (Fig. 1G, 2D). Subsequent application of gibberellin could overcome the inhibitory effect of PCB and resulted in normal fruit set. These experiments nicely demonstrated that also in *C. annuum* auxin acts upstream of gibberellin biosynthesis during fruit initiation. These observations are in agreement with both tomato and Arabidopsis where fruit set in auxin-induced fruits requiresdownstream gibberellin signalling(De Jong *et al*., 2009; Frigerio *et al.*, 2006; Serrani *et al*., 2007; Serrani *et al.*, 2008; Vivian-Smith and Koltunow, 1999).
Gibberellin had no additional effect on fruit growth, as treatment with GA_3 alone did not increase fruit size compared to emasculation only, and since combined treatment with auxin and GA_3 did not result in bigger fruits as compared to auxin treatment alone (Fig. 1F, 2C). This result is in contrast to Arabidopsis, tomato and pea where simultaneous application of gibberellin and auxin resulted in fruit size larger than auxin-induced fruits, and more similar to fruit induced by pollination and fertilization(Bunger-Kibler and Bangerth, 1983; Serrani *et al*., 2007; van Huizen *et al*., 1996; Vivian-Smith *et al.*, 2001). A likely mechanism by which GA_3 enhances fruit set in *C. annuum* is by preventing floweror fruit abscission, as in *C. annuum* this process is easily induced by environmental and growth conditions (Pressman *et al.*, 1998). Gibberellins are well-recognized as antagonists of abscisic acid in the control of fruit growth in citrus (Mahouachi *et al.*, 2005). By contrasting ABA, gibberellin-treated ovaries might stay longer on the plant and thus have a higher chance that the fruit development program is initiated. For many of such ovaries, however, the auxin levels are not sufficiently induced to trigger full fruit development, and thus these ovaries result in knots.In tomato these knots are also known as misshapen fruits, pseudo-fruits, puffs or nuts

(Carmi *et al.*, 2003; Serrani *et al*., 2007). GA_3-induced fruits in tomato show poorly developed locular tissue that lacks jelly inside the cavity. Although these fruits have not been named as knots, their characteristics do not support them as being normal fruit (Serrani *et al*., 2007).

GA_3 stimulates cell division and auxin stimulates cell expansion during fruit growth

The coordinated action of auxin and gibberellin is required for normal fruit development in tomato (Bunger-Kibler and Bangerth, 1983), blueberry (Cano-Medrano and Darnell, 1997), citrus (Guardiola *et al*., 1993; Vercher and Carbonell, 1991), rape (Srinivasan and Morgan, 1996), watermelon (Sedgley *et al*., 1977), pea (Vercher *et al*., 1987),and Arabidopsis(Vivian-Smith and Koltunow, 1999). Our results show that this is also the case for *C. annuum*, as $GA_3$is required for fruit set and stimulates mesocarp cell division and auxin stimulates mesocarp cell expansion, whereas application of both hormones or fertilization stimulates both cell division and cell expansion (Fig 3B). These observations are similar to Arabidopsis (Vivian-Smith and Koltunow, 1999), but in contrast to tomato where GA_3stimulates mesocarp cell expansion and auxin stimulates mesocarp cell division(Bunger-Kibler and Bangerth, 1983; Serrani *et al.*, 2008). Although both hormones are required for normal fruit development, fruit weight obtained after simultaneous application of auxin and gibberellin were not similar to fruits obtained after fertilization (Fig. 1F, 2B), suggesting that not only hormones but also other factors might be involved in regulating the final fruit size.

Hexose content during the exponential growth phase as a likely determinant of fruit weight

Hexose accumulation at the early stage of rapid fruit growth has been shown to enhance the final fruit size in tomato and grapes (Ho, 1984; Perez *et al*., 2000).Also in *C. annuum* hexose was observed as a major accumulating sugar both in seeded and seedless fruits at 60 DAA (Fig. 4B, C) (Nielsen et al, 1991), similar to seeded fruits of red fruited tomato (*S. lycopersicum, S. chmielewskii, S. pimpinellifolium*), where mature fruits accumulate glucose and fructose as a main carbohydrates(Kortstee *et al.*, 2007).In seeded fruitsat 20 DAA the hexose concentrations peak, whereas this peak was reduced in seedless fruits, which might reduce fruit growth and result in smaller size of seedless fruits compared to seeded fruits.

Invertase enzyme activity is responsible for the cleavage of sucrose into hexose. During fruit development the soluble invertase enzyme activity was highest at 5 DAA, decreased at 20 DAA, and then was elevated again at 60 DAA; we did not observe a difference between seeded and seedless fruits.However, as measurements were performed only at three developmental stages (5, 20, 60 DAA), we may have missed increases in invertase activity in seeded fruits just before the onset of growth. Strikingly, the high soluble invertase activity at 5 DAAwas followed by an increase in glucose and fructose levels at 20 DAA, suggesting that hexose accumulation at maturity might be due to high soluble invertase enzyme activity at the start of fruit development. These results are similar to what has been previously reported for seeded fruits of *C. annuum* and also for red fruited tomato, where an increase in hexose concentration was in accordance with acid invertase activity (Kortstee *et al.*, 2007; Nielsen *et al*., 1991). The higher hexose levels in seeded fruits compared to seedless fruits at 20 DAA is not related to their difference in soluble invertase enzyme activity. Insoluble invertase enzyme activity was higher in seeded fruits compared to

seedless fruits at 5 DAA and this might results in the higher hexose levels at 20 DAA (Fig. 4H).

Conclusion

Our results clearly demonstrate that external application of auxin (IAA, NAA) and GA_3 on stigmas of emasculated flowers enhances parthenocarpic fruit set compared to fruits obtained after emasculation. The hierarchy between auxin and gibberellin in fruit set in *C. annuum*appears similar to that reported in Arabidopsis and tomato where GA_3 biosynthesis acts downstream of auxin. In *C. annuum*, GA_3 seems to have an essential role in promoting fruit set while auxin seems to be important for both fruit set and fruit development. Application of auxin or GA_3 resulted in fruit size smaller than seeded fruits, which is due to a reduced cell division or cell expansion, respectively. Simultaneous application of auxin and gibberellin could also not reproduce the exact fruit size and shape of seeded fruits, suggesting that seeds have other roles in stimulating fruit size besides being a source of auxin and gibberellin. Our data suggest that fertilization triggers other auxin- and gibberellin-independent pathways, such as early insoluble invertase activity thatmight be required to elevate the hexose levels during the rapid growth phase leading to larger fruit size of seeded fruit than seedless fruit.

Chapter 6

General discussion

Introduction

The aim of this thesis has been to investigate the physiological and developmental changes that occur during parthenocarpic fruit set in sweet pepper (*Capsicumannuum*)and providing an analysis as to whether *C. annuum* can be comparatively used as a model with those developed already for tomato and Arabidopsis fruit development..From a literature survey, we know thatparthenocarpy has been studied in a wide range of plant species including the model plant Arabidopsisand the fruit crop tomatobecause of its agronomical and commercial benefits. This trait is desirable also for *C. annuum* production because it minimizes yield fluctuation and enhances total yield. Beside this,parthenocarpyfruits are easy to consume, much wanted for minimal-processed food (cut slices of fruit), and have a high shelf-life (Gonzalez *et al.*, 2004). Despite of this need, parthenocarpyhas beenlargely unstudied in *C.annuum.*Nobreeding approach has been successfullyapplied to obtain parthenocarpic cultivars in*C.annuum.* Likewiseinter- or intra-specific hybridization has also never been used to introduce parthenocarpy into*C. annuum*. Parthenocarpic mutants have not yet been described nor any transgenic parthenocarpic plants generated in *C. annuum*.We do not know whether, physiological and molecular mechanisms which regulate fruit set in *C. annuum* are the same as those reported for the model plant Arabidopsis and tomato. Low night temperature induces parthenocarpy in *C. annuum*, however, differences among genotypes for their parthenocarpic fruit set potential and the effect of temperature on fruit size is also not studied in different *C. annuum* genotypes. Though co-occurrence of parthenocarpy and carpel-like structures (CLS) phenotype has been reported, their genetic basis and their synergistic interaction have not been reported until now. External application of auxin induces parthenocarpy in *C. annuum*, however, the role of gibberellin and the hierarchy between auxin and gibberellin was not known until results from this thesis came to light.

In this thesis, we investigated the physiological and morphological changes that occur in a post pollinated ovary to understand the mechanism of fruit set. Then, we evaluated the genetic variation among the *C.annuum* genotypes for their parthenocarpic fruit set potential. Furthermore, parthenocarpic genotypes were evaluated at low night temperature for their fruit size. The morphological and genetical basis of parthenocarpy and CLS was investigated and hormonal regulation of fruit set was studied.

Fruit set mechanisms are conserved among *C. annuum*, tomato and Arabidopsis

Plant hormones play a central role in inducing fruit set leading to both seeded and seedless fruits (García-Martínez and Hedden, 1997; Gillaspy *et al.*, 1993). Fruit set in seeded fruits of Arabidopsis and tomato follows a series of events including pollen germination, pollen tube growth, transmission of growth signal from ovule to carpel and initiation of cell division and cell expansion leading to fruit growth (Abad, Monteiro, 1989; García-Martínez, 1997; Gillaspy *et al.*, 1993; Gillaspy *et al.*, 1993;Vivian-Smith, 2001; Fig. 1). In *C. annuum,* the combination of cell division and cell expansion contributes to early fruit growth where cell division was prominent during the early stage and cell expansion was prominent during the later stage of fruit growth. A hierarchy between auxin and gibberellin was observed for fruit set where auxin signals are mediated through gibberellin pathways (Chapter 2, and 5). These observations in *C. annuum* are similar to that reported in tomato and Arabidopsis (Abad, Monteiro, 1989; García-Martínez, 1997; Gillaspy *et al.*, 1993;Dorcey *et al.*, 2009; Goetz, 2006; Vivian-Smith *et al.*, 2001; Dorcey *et al.*, 2009; Goetz *et al.*, 2007; Serrani *et al.*, 2008), which indicates a

conservation of fruit set mechanism among these species. In this scenario, the molecular, genetic and physiological observations in tomato and Arabidopsis can used as future framework to relate to what happen in *Capsicum*during fruit set and fruit development.

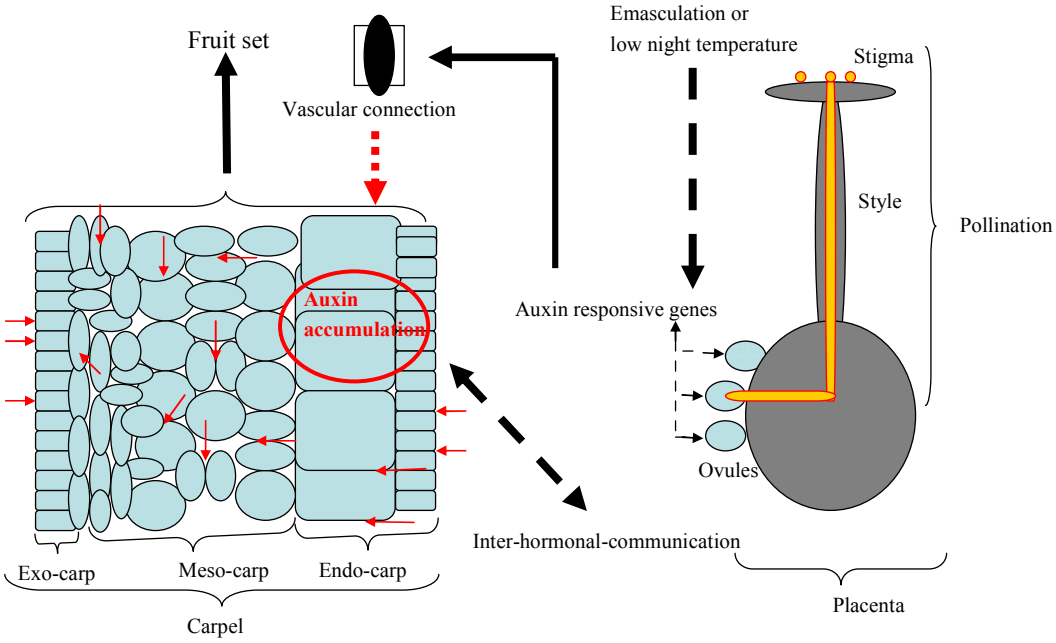

Figure 1 Model indicating a series of events that occurs after pollination and fertilization in *Capsicum annuum* similar to that already described tomato and Arabidopsis. Fertilization stimulates endogenous auxin synthesis or auxin de-conjugation (in the ovule), which in turn induces auxin responsive genes. Transport of auxin from ovules to carpel mediates vascular connection between ovule and carpel, and induces cell division and cell expansion in different layers of the carpel (in combination with other hormones) resulting in an increase in fruit diameter (early fruit growth). (Vertical arrows in exocarp and endocarp layers represent anticlinal division and random arrows in mesocarp represent random orientation of cell division). Autonomous accumulation of endogenous plant hormones might trigger fruit set even in the absence of pollination and fertilization (i.e. when either performed by emasculation or by growing plants at low night temperature), following the same series of physiological events that occurs after fertilization.

However, beside these similarities, there are differences for example mature fruits of *C. annuum* obtained after auxin and gibberellin applications are morphologically similar to tomato and histologicaly similar to Arabidopsis (Chapter 5). These differences might be because of the involvement of various factors such as environmental conditions, genotypic constitution, epistatic and pleiotropic influences of genes that regulate the optimal performance of hormones and subsequently affect the normal fruit growth (Duca, 2003). Also possible that ectopic phytohormone might not trigger all the same processes that occur after pollination/fertilization due to difference in their transport, application and concentration.

Intrinsic parthenocarpic potential in*Capsicum annuum*

Usually, absence of fertilization leads to ovule degeneration and abscission of the surrounding carpels (Bertin, 1995). However, we found fruit set in most of the studied *C.annuum* genotypes after preventing the pollination and fertilization, which might be because of their genetic potential for parthenocarpic fruit set (Chapter 3, Chapter 4). *C. annuum* is one of the most domesticated species in the world(Pickersgill, 1997) and has been under intense selection and domestication for thousands of years. Certain traits might have already under selection and narrowed the variability, consequently resulting in presence of parthenocarpy among *C. annuum* genotypes that we observed in chapter 3.

Emasculation or growing plants at low night temperature are two effective means to prevent pollination and fertilization in *C. annuum*. Emasculation or removal of outer floral organs is a common practice to assess the parthenocarpic fruit set potential, which has also been applied in tomato and Arabidopsis (Philouze and Maisonneuve, 1978a; Vivian-Smith, 2001; Vivian-Smith, Koltunow, 1999). Parthenocarpic fruits can be obtained either by increasing the concentration of phytohormones or the alteration of phytohormones signalling in fruits by other means than seed development (Abad, and Monteiro, 1989; García-Martínez and Hedden, 1997; Gillaspy *et al.*, 1993). This implies that emasculation not only assures the failure of pollination and fertilization but will probably influence the endogenous plant hormone level and signalling. Outer whorl organs might have an inhibitory effect on fruit set as reported in the *fruit without fertilization* (*fwf*) mutant of Arabidopsis where physical removal of floral organs surrounding the carpel resulted in stronger parthenocarpic fruit set (Vivian-Smith et al., 2001). Genes that express or regulate the signaling from outer whorl organs to the carpel development are not yet identified, and it would be interesting to explore these in future because this will help to understand the inter-floral communication and their possible involvement in fruit set.

Low night temperature-induced parthenocarpic fruits are most likely due to poor pollination and fertilization as temperature affects the release of pollen, pollen viability, or pollen tube growth (*in- vitro* and *in- vivo)*(Iwahori, 1966; Rylski *et al.*, 1994; Charles and Harris, 1972; Rudich *et al.*, 1977; Maestro and Alvarez, 1988)e.g. in tomato and *C. annuum* (Bianchi, 1969; Tiwari, 2007; Chapter 4).Thus, autonomous accumulation of plant hormones after preventing pollination and fertilization leads to parthenocarpic fruit set and might follow the same series of physiological and morphological changes that occurs after pollination and fertilization (Fig. 1).

Auxin is a main signal for initiation of fruit development while gibberellins are mainly involved in fruit set

Both auxin and gibberellins were found to be effective in enhancing parthenocarpic fruit set in *C. annuum*, similar to what has been reported in various crops of commercially grown fruits (Martinelli *et al.*, 2009; Park, 2010; Pharis and King, 1985). Simultaneous application of both hormones resulted in fruit weight smaller than seeded fruits suggesting that seeds are needed for full fruit development because seeds have also other roles in stimulating fruit size besides being a source of gibberellin and auxin (Gillaspy *et al.*, 1993; Mapelli *et al.*, 1978; Sjut and Bangerth, 1982).

Auxin is an effective hormone to induce fruit set and fruit size

Early fruit growth is more due to cell division than cell expansion (Chapter 2) and the role of GA_3 is also recognized for stimulating cell division (Chapter 5) suggesting the importance of gibberellin in fruit set. Importance of auxin has been recognized in early and later stage of fruit growth (Chapter 2, Chapter 5). External application of auxin results in higher fruit set and larger fruit size (Chapter 5) suggesting that molecular insertion of genes that induce endogenous level of auxin couldalso is used to obtain parthenocarpic fruits in *C. annuum*. The molecular insertion of auxin biosynthesis gene (e.g. *iaaM*) influences the endogenous level of auxin and results in parthenocarpic fruit set e.g. in strawberry, grape, tomato and eggplant (Ficcadenti *et al.*, 1999; Rotino *et al.*, 1997). Fruits obtained by using transgenic approach are apparently similar to seeded fruit in size and morphological appearance i.e. tomato (Pandolfini *et al.*, 2002). The transgenic approach to obtained parthenocarpic plant in *C. annuum* is not successful yet because of the difficulties to regenerate the transformed *C. annuum*. Recently, stable transformation and efficient regeneration has been demonstrated in a wide range of important *C.annuum* genotypes (Balazs *et al.*, 2008), which could help to obtain a transgenic parthenocarpic *C.annuum* in the future.

Hormone-induced parthenocarpic fruits reduce yield fluctuation

Auxin-induced parthenocarpic fruits resulted in more regular fruit set compared to the natural pollinated fruits (Heuvelink, Körner, 2001). Similar to this, we found regular fruit set in auxin and gibberellin-induced fruits compared to seeded fruits (unpublished data). Introduction of the parthenocarpy trait would alter dominance and assimilate partitioning in favor of developing fruit because fruits with no seeds have no inhibitory effect on set and growth of later developing fruits (Marcelis, HofmanEijer, 1997). There is good evidence that translocation of auxin down the pedicel prevents the formation of an abscission layer and therefore abscission is prevented (Jinu, 2009). Under stress conditions, ethylene is generated, which both reduces the polar auxin transport down the pedicel, and causes the formation of the abscission layer (Wien, Zhang, 1991). However, it is not clear whether this model also applies to the abortion of flowers/fruits as a result of competing earlier-formed fruits on the plant.High expression of genes comprise both ethylene biosynthesis genes and ethylene signal transduction pathway has been reported from the transcriptome analysis of tomato ovaries (Pascual *et al.*, 2009), suggesting a possible positive reinforcement role of ethylene in fruit set. It would be interesting to investigate how auxin interacts with ethylene to regulate fruit set and to validate this model under different conditions i.e. fruit load, stress condition.

Hormonal responses influenced by the environmental condition

External application of hormones on the stigma of emasculated flowers resulted in parthenocarpic fruit set only when light intensity was sufficient and temperature was moderate. Hormone treated flowers aborted when light intensity was low and temperature was suboptimal for fruit set (Chapter 2, Chapter 5), which suggests a strong interactive relationship with the ability to attract assimilates or perhaps determine a sufficient sink threshold. Also, (Osborne and Went, 1953) reported NAA induced parthenocarpic fruit set under moderate temperature and high light intensity, whereas NAA treated flowers aborted under high day/night temperature and low light intensity. This observation suggests a possible interaction between the environmental conditions and hormonal response for regulating fruit set.

Fruit set was high at low night temperature; however, the majority of fruits were small, misshapen and with an elongated or pointed blossom endin *C. annuum* (Rylski, Spigelman, 1982). The highest fruit set was also obtained when gibberellins were applied on the stigma of flowers compared to pollinated or auxin treated flowers, however, the majority of fruits were knots (small and dull colour; Chapter 5), citrus(pointed stylar ends, big shoulders, thick rind, less juice and dull colour) (Coggins et al., 1960), tomato (poorly developed locular tissue with undeveloped jelly inside the cavity) (Serrani *et al*., 2007). Flower morphology and fruit development in response to low night temperature and gibberellin application was similar in *C. annuum* (Sawhney and Mills, 1983b; Chapter 3, Chapter 5). GA_3 actsantagonistically to ABA e.g. in citrus and tomato (Mahouachi *et al.*, 2005; Nebenfuhr, 2000; White *et al.*, 2000), and ABA content is known to reduce at low night temperature (Singh and Sawhney, 1998), supporting the idea that low night temperature increases the endogenous level of gibberellin to induce fruit set.

Carpelloid structures (CLS) are an unnecessarytrait but they enhance parthenocarpic fruit growth

Origin and nature of CLS

CLS are a widely present trait in *C. annuum* genotypes; however, abundant CLS growth was only evident in seedless fruits obtained either by emasculation or by hormone application. When all the flowers up-to 10-15 nodes were treated with auxin or gibberellins, no difference in CLS weight was observed between both treatments (Chapter 4); however, when only six fruits per plant were obtained, CLS growth was stronger only in gibberellin-induced fruits. This result suggests that inter-fruit competition might be involved in regulating the CLS development. Enhanced CLS growth in gibberellin-induced fruits indicates the need of cell division for CLS proliferation.CLS in *C. annuum*appear to be transformed from abnormal ovules in analogy with what occurs in the Arabidopsis *bel1-1* mutant (Brambilla *et al.*, 2007; Reiser *et al.*, 1995; Western, Haughn, 1999). Therefore, like seeds, CLS might create sink strength by producing high levels of plant hormones. Various types of ovule defects were found in parthenocarpic fruits of *C. annuum* and Arabidopsis *bel1* mutant suggesting that structure of the ovule is an important factor in enabling parthenocarpic fruit development in both crop species. The Arabidopsis *bel1* and *knuckles* mutants the length of parthenocarpic fruits is correlated with the total number of CLS produced in the carpel (Vivian-Smith, 2001). Similarly, *C. annuum* showed a positive correlation between fruit weight (excluding CLS) and CLS weight. This strongly suggests a possible relation between parthenocarpy and CLS development (Chapter 4).

Genetics of CLS

C. annuum germplasm showed a genetic variation for CLS growth, however, no genotype was found without CLS (Chapter 4). Genetic analysis showed no simple Mendelian inheritance for CLS development; however, the role of epigenetic (inherited changesin phenotype caused by mechanisms other than changes in the underlying DNA sequence) and Quantitative Trait Loci (a single phenotypic trait that is determined by many genes or in a quantitative manner) can not be ruled out. Perhaps, parthenocarpy and CLS are not genetically linked (Chapter 4), which suggests that seedless fruits of *C. annuum* without CLS can be obtained in future by using appropriate breeding techniques.

Breeding aspect of parthenocarpy

Even though the parthenocarpy trait is widely present in *C. annuum* genotypes, evaluation of this trait is difficult because screening is hampered by the difficulty to evaluate the plant for such a trait. Plants grown at low night temperature or flowers emasculated or treated with hormones not only resulted in parthenocarpic fruits, but also set many knots. These types of fruits were undesirable for the commercial production (Chapters 3, 4 and 5). The same problem has been experienced in tomato breeding where knots are described as a seedless fruit like organ characterized by dull appearance (Gorguet *et al.*, 2008).A selection criterion to distinguish true fruits from knots is crucial for the success of a breeding program in *C. annuum*. Therefore, to be accurate in phenotyping, we considered a very strict and defined criterion for knotsand parthenocarpic fruits.Knots were defined as those seedless fruits which were small in size, irregular in shape, dull in appearance and obtained fruit weight below 50% of the average weight of seeded fruits while remaining fruits were considered as parthenocarpic fruits. In a segregating population, where parents having different fruits size were crossed for example Chinese Line 3, which is asmall fruited genotype with an average fruit weight of 121 g was crossed with Lamuyo B, which is a large fruited genotype with an average weight of 208 g for seeded fruit at normal temperature (Chapter 3).In this scenario, seedless fruits of small size can be a parthenocarpic fruit and a seedless fruits of larger size can be a knot depending upon parental combination.Therefore, the criterion of fruit weight was not included to distinguish parthenocarpic fruits from knots.

Among all tested genotypes, Chinese Line 3 possesses the highest potential for parthenocarpic fruit set (Chapter 3). Despite the difficulty of producing seed and the drawback of the smaller fruit size, the higher fruit set make Chinese Line 3 of considerable practical interest. Parthenocarpy in Chinese Line 3 is controlled by a single recessive gene (Chapter 4). This finding will provide an opportunity to understand the physiological and molecular aspects of parthenocarpy in *C. annuum*. Follow-up research using Chinese Line 3 can now be directed at the characterizing, mapping and eventually cloning the parthenocarpic gene. This would allow future breeding to improve the fruit size and introduce parthenocarpy.

Parthenocarpic expression is accompanied by male or female sterility

We hypothesized that precocious ovary development in facultative parthenocarpic genotypes of *C. annuum* is due to genetic stimuli, however, parthenocarpy can be expressed only when it is combined with aberrations in male or female fertility (Chapter 5). The occurrence of parthenocarpy together with male or female sterility has been reported in several parthenocarpic mutants/plants, e.g. the *pat* mutant of tomato showed parthenocarpy as well as aberrant anthers and CLS bearing external ovules. Antisense down-regulation of the*TM8* MADS-box gene in this mutant caused deformation of the pistil and complete male and female sterility besides an extremely high incidence of parthenocarpy (Lifschitz *et al.*, 1993). A high level of parthenocarpy was observed in Chinese Line 3 and also additional hand pollination did not increase the number of seeds in Line 3, suggesting that parthenocarpy in this genotype is accompanied by a large degree of female sterility in agreement with a higher number of abnormal ovules (Chapter 3, 4).

Combining male sterility with parthenocarpy

Various factors such as high or low temperature, day length, plant growth regulators, low humidity or solar UV light have reported reduced gametophytic fertility in diverse plant

species (Aylor, 2003; Ohnishi *et al.*, 2010; Wang *et al.*, 2004) E.g. reduced pollen viability at low and high night temperature has been reported in *C. annuum* and tomato(Aloni *et al.*, 2001; Erickson and Markhart, 2002; Shaked *et al.*, 2004; Chapter 4), use of plant growth regulators such as Maleic hydrazide has been reported as a chemical emasculating agent in eggplant and coriander (Kalidasu *et al.*, 2009; Sidhu *et al.*, 2005).

Delayed anther dehiscence can also be an effective alternative to avoid the chance of pollination and fertilization because the pollen is released after the pistil/stigma is receptive to pollen. Specific hormones such as jasmonic acid and ethylene can be applied effectively on the stigma to reduce or delay or diminish the fertility of anthers without having abnormal growth phenotypes (Huang *et al.*, 2003; Rieu *et al.*, 2003).Cytoplasmic Male Sterile lines (CMS) or functional sterility can also be combined with parthenocarpy to obtain commercial seedless fruit production in *C. annuum*.

Conclusion

The physiological and morphological changes that occur during fruit set and fruit growth of *C. annuum* was similar to that has been reported in tomato and Arabidopsis. This indicates a possibility to use Arabidopsis as a model plant to study molecular, physiological and developmental aspects of fruit development in *C. annuum* and vice versa.Emasculation or low night temperature results in parthenocarpic fruits; however, fruit set percentage was enhanced by external application of auxin and gibberellins.Co-occurrence of parthenocarpy and carpelloid structures(CLS) indicates a synergistic relation between them. Parthenocarpyis controlled by a single recessive gene but CLS phenotype could not be linked to a single locus. Our results clearly indicate that CLS proliferation becomes prominent in the absence of fertilization and CLS mimic the role of seeds and support parthenocarpic fruit growth.This thesis provides insight in the physiology and genetic basis of parthenocarpy in *C. annuum*.

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Summary

Problems in fruit set and retention are a major cause of yield irregularity insweet pepper (*Capsicum annuum*) production.Yield fluctuationi.e. the alternation of weeks with large numbers of ripe fruits with weeks with almost no ripe fruits, causes major yield losses. The fluctuating yield pattern for *C. annuum* is hardly influenced by planting date or other cultivation measures. Therefore all growers are in roughly the same phase and prices are low in high yield periods, and high in low yield periods. Yield fluctuations also result in strong fluctuations in labour demand in the greenhouses. Parthenocarpic fruit set has been reported as a possible solution to minimize this yield fluctuation. However, parthenocarpy has not been studied extensively in *C. annuum*. This project aims at physiological and morphological characterization of parthenocarpic fruit set in *C. annuum*and finding evidence that tomato and Arabidopsis can be used as model plants to study fruit development in *C. annuum*.

In **Chapter 1**, a literature review showed that parthenocarpic fruit setin various crops can be obtained by genetic alterations or artificial induction. Genetic alteration includes classical, molecular or mutation breeding approaches which result in a heritable change in the genotypic constitution to induce parthenocarpy. Artificial induction of parthenocarpy in non-parthenocarpic plants includes external application of plant growth regulators or alteration of the endogenous plant hormone levels within ovules or carpel tissues. Artificial induction is a useful tool to understand the role of plant hormones in fruit set and fruit development.Details of various approaches were summarized and the oppertunities each approach offers to obtained parthenocarpy in *C. annuum* is discussed in **Chapter 1**.

In **Chapter 2**, we investigate whether mechanisms of fruit set reported in Arabidopsis and tomato also occur in *C. annuum.* To do this, we accurately timed the physiological and morphological changesthat occur in a post-pollinated ovary. Results suggest vascular connection between ovule and replum as an early indicator for successful fruit development after fertilization.Cell division was moredominant during early fruit growth, whereas cell expansion was continued to the later stages of fruit growth in *C. annuum*. The importance of auxin has been recognized at early and later stages of fruit growth. The series of physiological events observed during fruit development in *C. annuum* is similar to what has been reported for tomato and Arabidopsis, suggesting a conservation in fruit set mechanisms between these three species (**Chapter 2**).

Usually absence of fertilization induces flower abortion; however, in most of the studied genotypes of *C. annuum,* some fruit set was observed even after preventing fertilization (**Chapter 3, 4**).To evaluate how the absence of seeds influences the fruit size in different genotypes, various *C. annuum* genotypes which were known for their parthenocarpic potential were evaluated at low night temperature as well as normal night temperaturetogether with standard genotypes as controls (i.e. Mazurka).Parthenocarpic fruits were defined as those seedless fruits which were shiny in appearance and obtained fruit

weight above 50% of the average weight of seeded fruits while remaining fruits were considered as knots. Most of the *C. annuum* genotypes showed facultative parthenocarpy, which is the most suitable form of parthenocarpy for practical use in 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. The fraction of parthenocarpic fruits was higher at low night temperature due to non-viable pollen. Substantial reduction in fruit weight was observed for seedless fruits in all the tested genotypes except for 'Bruinsma Wonder'where only 10% reduction in weight was observed for seedless fruit compared to seeded fruits **(Chapter 3)**.

Furthermore, to check the presence of parthenocarpy within the *C. annuum* germ-plasm randomly selected genotypes (n=11) were grown at normal temperature and their parthenocarpic fruit set potential was evaluated by emasculating flowers. Parthenocarpy was evident in most of the genotypes suggesting that somedegree of intrinsic parthenocarpy is already present in *C. annuum*(**Chapter 4**).

Despite this wide occurrence of parthenocarpy in *C. annuum*, the genetic basis for this desirable trait has not been studied. Though expression of parthenocarpy was genotype and environment dependent, we could study the inheritance of parthenocarpy by taking a criterion that a plant was considered parthenocarpic when all seedless fruits resulting from emasculated flowers showed a shiny appearance. Other criteria of fruit weight could not be considered because segregating population that was used in the experiment was generated by crossing parents of having different fruits size. Fruit size segregates upon crossing and results in small, medium or large in size depending upon parental combination. In this scenario, a small size fruit can be a true fruit and a larger fruit can be a knot. Therefore, fruit appearance was the only used criteria to distinguish true parthenocarpic fruits from knots. Screening results showed that parthenocarpy was controlled by a single recessive gene (**Chapter 4**).

Though emasculation resulted in parthenocarpic fruit set, the percentage of fruit set was enhanced when emasculated flowers were treated with hormones (i.e. auxin: IAA, NAA, and gibberellin: GA_3)**(Chapter 5)**.Auxin acts upstream of gibberellin in fruit set, most likely by inducing gibberellin biosynthesis, and is important for full fruit development (reduced number of knots), while GA_3 is required for fruit set by reducing flower abscission.Application of auxin and GA_3 resulted in fruit size smaller than seeded fruits, which is due to the limitation of cell division and cell expansion, respectively. At the cellular level fruits obtained after pollination and fertilization were very similar to fruits obtained after simultaneous application of auxin and gibberellin. This suggests that the action of both hormones tightly balancing cell division and cell expansionis required for normal fruit development in *C. annuum*.However, fruit size obtained after simultaneous application of auxin and gibberellincould not reproduce the exact fruit size and shape as observed following pollination and fertilization, suggesting that seeds have also other roles in stimulating fruit size besides being a source of gibberellin and auxin. In addition, we may have identified a parallel pathway involving a fertilization-induced invertase activity that seems to be required to elevate the hexose levels during the rapid growth phase andthat contributes to the *C. annuum* fruit size**(Chapter 5)**.

The majority of seedless fruits obtained either by emasculation or hormone application on the emasculated flowers contained stronger growth of CLS compared to seeded fruits (**Chapter 4**).The structural analogy of CLS with *bel1* mutant of Arabidopsis suggested that

CLS are transformed from abnormal ovules. Our results clearly showed that CLS is a genotype dependent trait; however, growth of CLS becomes stronger only in the absence of fertilization, indicating fertility as an important determinant of CLS development.Upon fertilization, normal seed development prevails, which suppresses CLS growth, and at the same time induces fruit set and development.CLS development supports parthenocarpic fruit growth by increasing the fruit's sink strength in a similar way as seeds do in seeded fruits. A positive correlation between parthenocarpy and CLS growth was observed in **Chapter 4**.Assuming that parthenocarpy and CLS might be linked traits, we studied the inheritance of CLS using the same segregating population which was used to study the inheritance of parthenocarpy.From the obtained data, we find neither a simple Mendelian segregation for the CLS development, nor a genetic association between CLS and parthenocarpy. We assume that CLS might be a pleiotropic effect of parthenocarpy. However, involvement ofepistasis or incomplete penetrance or other more complex genetics can not be excluded (**Chapter 4)**.

Finally,in **Chapter 6**, the main achievements and limitations of this study are discussed. Furthermore, the prospects for obtaining high yielding parthenocarpic cultivars in*C. annuum* are discussed on the basis of the present results. Also, suggestions for future research are presented.

Samenvatting

Problemen met vruchtzetting en vruchtretentie zijn de belangrijkste oorzaak van een onregelmatig oogstpatroon bij paprika (*Capsicum annuum*). Fluctuaties in opbrengst, dat wil zeggen de afwisseling van weken met veel rijpe vruchten met weken met bijna geen rijpe vruchten, zorgen voor grote verliezen in financiële opbrengst. Het tijdstip van planten of andere teeltmaatregelen hebben nauwelijks invloed op het fluctuerende oogstverloop van *C. annuum*. Daarom zitten de gewassen van alle telers in ongeveer dezelfde fase en zijn de prijzen laag in perioden met veel oogst, en hoog in perioden met weinig oogst. Oogstfluctuaties resulteren ook in sterke fluctuaties in arbeidsbehoefte in de kassen. Parthenocarpe vruchtzetting is beschreven als een mogelijke oplossing om deze oogstfluctuaties te minimaliseren. Echter, in *C. annuum* is parthenocarpie niet uitgebreid bestudeerd. Dit project heeft als doel om fysiologische en morfologische aspecten van parthenocarpe vruchtzetting in *C. annuum* te karakteriseren en aan te tonen dat tomaat en Arabidopsis als modelplant gebruikt kunnen worden om vruchtontwikkeling in *C. annuum* te bestuderen.

Hoofdstuk 1, is een literatuuroverzicht van het verkrijgen van parthenocarpe vruchtzetting in diverse gewassen door genetische aanpassingen of door kunstmatige inductie. Onder de genetische mogelijkheden vallen klassieke, moleculaire en mutatieveredeling. Deze methoden resulteren in een vererfbare verandering in de genetische samenstelling, dieparthenocarpe vruchtzetting mogelijk maakt. Kunstmatige inductie van parthenocarpie in niet-parthenocarpe planten kan door externe toediening van plantengroeiregulatoren of door verandering van de endogene plantenhormoonniveaus in de eicellen of het vruchtblad (carpel). Kunstmatige inductie biedt mogelijkheden om de rol van plantenhormonen in vruchtzetting en –ontwikkeling te bestuderen en te begrijpen. Detailinformatie over de diverse technieken zijn samengevat en de mogelijkheden die elke techniek biedt om tot parthenocarpe vruchtzetting in *C. annuum* te komen worden bediscussieerd in **Hoofdstuk 1**.

In **Hoofdstuk 2**wordt het onderzoek beschreven naar de overeenkomsten en verschillen in vruchtzettingsmechanisme tussen *C. annuum* en Arabidopsis en tomaat. De fysiologische en morfologische veranderingen die plaatsvinden in een eicel vanaf bestuiving zijn nauwkeurig bestudeerd. De resultaten duiden erop dat een vasculaire verbinding tussen de eicel en het replum een vroege indicator is voor succesvolle vruchtontwikkeling na bevruchting. Celdeling is het belangrijkst tijdens de vroege groei van de vrucht, terwijl celstrekking vooral in de latere stadia van de groei van de *C. annuum* vrucht een grote rol speelt. Het belang van auxine is vastgesteld in zowel vroege als late stadia in de vruchtgroei. De opeenvolging van fysiologische gebeurtenissen die tijdens de vruchtontwikkeling in *C. annuum* waargenomen zijn, is vergelijkbaar met wat er voor tomaat en Arabidopsis gerapporteerd is. Dit duidt erop dat de vruchtzettingsmechanismen tussen deze drie soorten geconserveerd zijn.

Normaal gesproken zal in afwezigheid van bevruchting de bloem verwelken en van de plant afvallen; echter in de meeste van de bestudeerde *C. annuum* genotypes kon enige vruchtzetting worden waargenomen, zelfs na verhindering van bevruchting door bijvoorbeeld emasculatie (**Hoofdstuk 3, 4**). Om te bestuderen hoe de afwezigheid van zaden de vruchtgrootte in de verschillende genotypes beïnvloedt, zijn diverse *C. annuum* genotypes waarvan de potentie tot parthenocarpe vruchtzetting bekend was, geëvalueerd bij zowel lage nachttemperatuur als normale nachttemperatuur met standaardrassen (bijv. Mazurka) als controle. Parthenocarpe vruchten zijn gedefiniëerd als zaadloze, glimmende vruchten die een vruchtgewicht van meer dan 50% van het gemiddelde gewicht van vruchten met zaden hebben, terwijl overige vruchten als knopen beschouwd worden. De meeste *C. annuum* genotypes vertoonden een facultatieve parthenocarpie, welke de meest bruikbare vorm van parthenocarpie voor veredelingsprogramma's is. In deze vorm van parthenocarpie zal de eicel, indien bevrucht, zorgen voor de ontwikkeling van een vrucht met zaden en, indien de eicel niet bevrucht wordt, ontwikkelt zich een zaadloze vrucht. De fractie parthenocarpe vruchten was hoger bij lage nachttemperaturen vanwege niet-levensvatbaar stuifmeel. Substantiële reductie in het vruchtgewicht van zaadloze vruchten werd in alle geteste genotypes waargenomen, behalve in 'Bruinsma Wonder'. In dat ras waren zaadloze vruchten slechts 10% lichter dan vruchten met zaden (**Hoofdstuk 3**).

Vervolgens zijn, om de aanwezigheid van parthenocarpie binnen het *C. annuum* germplasm te onderzoeken,elf willekeurig geselecteerde genotypes geteeld bij normale temperatuur en is de potentie voor parthenocarpie bepaald door emasculatie van bloemen. Parthenocarpe vruchtzetting kwam voor bij de meeste genotypes, wat suggereert dat een zekere mate van intrinsieke parthenocarpie reeds aanwezig is in *C. annuum* (**Hoofdstuk 4**).

Ondanks het wijdverspreid voorkomen van parthenocarpie in *C. annuum* is de genetische basis voor deze gewenste eigenschap niet bestudeerd. Hoewel expressie van parthenocarpie afhankelijk is van het genotype en de omgeving, konden we de overerving van parthenocarpie bestuderen door een criterium te hanteren waarbij een plant parthenocarp beschouwd werd als alle zaadloze vruchten die voortkwamen uit geëmasculeerde bloemen een glimmend uiterlijk hadden. Andere criteria zoals vruchtgewicht konden niet meegenomen worden vanwege de uitsplitsende populatie die in het experiment gebruikt werd, gegenereerd door ouders met verschillende vruchtgroottes te kruisen. Vruchtgrootte splitst uit bij kruisingen en resulteert in kleine, gewone en grote vruchten afhankelijk van de oudercombinaties. In dit scenario kan een kleine vrucht een echte vrucht zijn en een grotere vrucht kan een knoop zijn. Daarom is alleen het uiterlijk van de vrucht als criterium genomen om echte parthenocarpe vruchten van knopen te onderscheiden. Resultaten van deze screening lieten zien dat parthenocarpie in *C. annuum* gereguleerd wordt door een enkel recessief gen (**Hoofdstuk 4**).

Hoewel emasculatie resulteerde in parthenocarpe vruchtzetting, werd het percentage vruchtzetting versterkt als geëmasculeerde bloemen werden behandeld met hormonen (bijv. auxine: IAA, NAA en gibberelline: GA_3) (**Hoofdstuk 5**). Auxine werkt upstream van gibberelline bij vruchtzetting, waarschijnlijk door de gibberelline biosynthese te induceren, en is belangrijk voor volledige vruchtontwikkeling (gereduceerd aantal knopen), terwijl GA_3 nodig is voor vruchtzetting door abscissie van bloemen tegen te gaan. Toediening van auxine en GA_3 resulteerde in een vruchtgrootte kleiner dan vruchten met zaden, welke wordt veroorzaakt door zowel minder celdeling als minder celstrekking. Op cellulair niveau waren vruchten die verkregen werden na bestuiving en bevruchting vrijwel gelijk aan vruchten die

verkregen werden door gelijktijdige toediening van auxine en gibberelline. Dit suggereert dat de werking van beide hormonen die een strikte balans tussen celdeling en celstrekking handhaven nodig is voor normale vruchtontwikkeling in *C. annuum*. Het feit datvruchten verkregen na gelijktijdige toediening van auxine en gibberelline kleiner blijven dan vruchten ontstaan na bestuiving en bevruchting, duidt erop dat zaden ook andere rollen vervullen bij het stimuleren van vruchtgrootte naast het zijn van een bron van gibberelline en auxine. Daarnaast hebben we waarschijnlijk een parallelle pathway geïdentificeerd, die betrokken is bij een bevruchtingsgeïnduceerde invertase activiteit die nodig lijkt te zijn om hexose niveaus te verhogen tijdens de snelle groeifase en die bijdraagt aan de *C. annuum* vruchtgrootte.

Het merendeel van de zaadloze vruchten verkregen door emasculatie of hormoontoediening aan geëmasculeerde bloemen bevatte sterk uitgegroeide carpelloide structuren (CLS); dit in tegenstelling tot zaadhoudende vruchten (**Hoofdstuk 4**). De structurele analogie met CLS in de *bel1* mutant van Arabidopsis suggereert dat CLS ook in *C. annuum* zijn getransformeerd vanuit abnormale eicellen. Onze resultaten laten zien dat het optreden van CLS een genotype-afhankelijke eigenschap is. De uitgroei van CLS wordt alleen sterker in de afwezigheid van bevruchting, dit geeft aan dat vruchtbaarheid een belangrijke determinant van CLS ontwikkeling is. Na bevruchting zal normale zaadontwikkeling de overhand hebben, welke CLS groei onderdrukt, en gelijktijdig vruchtzetting en -ontwikkeling induceert. CLS ontwikkeling ondersteunt parthenocarpe vruchtgroei door de “sink strength” van een vrucht te vergroten op een vergelijkbare manier zoals zaden dit doen in vruchten met zaden. Parthenocarpie en CLS groei vertoonden en positieve correlatie. |Er vanuit gaande dat parthenocarpie en CLS gekoppelde eigenschappen kunnenzijn, bestudeerden we de overerving van CLS door gebruik te maken van dezelfde uitsplitsende populatie welke gebruikt was om de overerving van parthenocarpie te bestuderen. De resultaten duidden echter noch op een eenvoudige Mendeliaanse uitsplitsing voor CLS ontwikkeling, noch op een genetische associatie tussen CLS en parthenocarpie. We nemen aan dat CLS een pleiotropisch effect van parthenocarpie kan zijn. Echter, betrokkenheid bij epistasie of niet-volledige penetratie of andere complexere genetica kan niet uitgesloten worden.

Tot slotte worden in **Hoofdstuk 6** de belangrijkste behaalde resultaten en beperkingen van dit onderzoek bediscussiëerd. Daarnaast worden de vooruitzichten voor het verkrijgen van een parthenocarpe *C. annuum* cultivar met hoge opbrengst op basis van de gepresenteerde resultaten besproken. Tevens worden suggesties voor toekomstig onderzoek gepresenteerd..

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Curriculum Vitae

Aparna Tiwari was born on 1st January 1979 in Varanasi, India. She received her bachelor degree (B.Sc.) in Agricultural sciences from the Institute of Agricultural Sciences, Banaras Hindu University (B.H.U), Varanasi, India in 2001. She continued her Master of Science from the same university (B.H.U); however, she stopped there after one year and started her new M.Sc. program from the Wageningen University in 2003. She performed her M.Sc. thesis at the department of plant breeding of Wageningen University, under the supervision of Dr. Pim Lindhout and Dr. Benoit Gorguet, on the topic of 'Gibberellin biosynthesis gene expression study in tomato'. In November 2005 she obtained her M.Sc. in Plant Sciences, with specialization in Plant Breeding and Genetic Resources.

In January 2006, she started her PhD study in the department of Horticultural Production chains group under the supervision of Prof. Olaf van Kooten, Dr. Ep Heuvelink and Dr. Remko Offringa and work on 'Parthenocarpic fruit development in *Capsicum annuum*' under the project of "New tools and strategies for fruit breeding in sweet pepper; project number LPB 6822)". The result of this study is presented in this thesis.

List of publications

Papers in refereed journals

Gorguet, B.J.M.; Eggink, P.M.; Ocaña, J.; Tiwari, A.; Schipper, E.H.; Finkers, R.;Visser, R.G.F.; Heusden, A.W. van (2008) Mapping and characterization of novel parthenocarpy QTLs in tomato Theoretical and Applied Genetics 116 (6). - p. 755 - 767.

Conference proceedings

Tiwari, A.; Dassen, J.H.A.; Heuvelink, E. (2007) Selection of sweet pepper (Capsicum annuum L.) genotypes for parthenocarpic fruit growth Acta Horticulturae (761).- p. 135 - 140.

PE&RC PhD Education Certificate

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With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (5.6 ECTS)

- Approaches for obtaining parthenocarpic fruits: a review (2010)

Post-graduate courses (1.5 ECTS)

- Bioinformatics: a user approach; EPS (2010)

Deficiency, refresh, brush-up courses (3 ECTS)

- Greenhouse technology; Farm Technology (2006)
- Basic statistics; PE&RC (2006)

Competence strengthening / skills courses (7.2 ECTS)

- Scientific writing: WGS (2006)
- Interpersonal communication; WGS (2008)
- Project and time management; WGS (2009)
- Career perspectives; WGS (2009)
- Techniques for writing and presenting a scientific paper; WGS (2010)
- Talent classes (branding yourself, marketing yourself effectively; NWO (2010)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)

- PE&RC Weekend (2006)
- PE&RC Day: the scientist agenda – who pulls the strings? (2006)
- PE&RC Day: collapse (2007)
- PE&RC Day: accelerate scientific progress: expect the unexpected (2008)
- PE&RC Day: intelligent communication: on the origin of communication (2009)

Discussion groups / local seminars / other scientific meetings (6.7 ECTS)

- Frontier literature of plant physiology (FLOP) (2006-2010)

International symposia, workshops and conferences (7.5 ECTS)

- International horticultural congress; Seoul (2006)
- 11th International symposium on plant bio-regulators in fruit production; Bologna (2009)
- 9th IPMB Congress; St. Louis (2009)

Supervision of a MSc student (10 days)

- Effect of temperature on fruit set pattern and potential fruit size of sweet pepper (*Capsicum annuum* L.)

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