CARLA ELISA ALVES BASTOS

HORMONAL BASES OF GROWTH AND YIELD IN TOMATO (*Solanum lycopersicum*)

Thesis submitted to Federal University of Viçosa, as part of the requirements for obtaining the *Doctor Scientiae* degree in Plant Physiology.

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“Mas é preciso ter manha
É preciso ter graça
É preciso ter sonho, sempre
Quem traz na pele essa marca
Possui a estranha mania
De ter fé na vida”

(Milton Nascimento e Fernando Brant)

I offer to my family

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CONTENTS

ABSTRACT

BASTOS, Carla Elisa Alves, D.Sc., Universidade Federal de Viçosa, March 2017. **Hormonal bases of growth and yield in tomato (*Solanum lycopersicum*)**. Adviser: Agustin Zsögön. Co-adviser: Dimas Mendes Ribeiro.

Tomato hybrids exhibit superior phenotypic performance in both optimal and stressful conditions. This superiority is believed to result from the interaction between the genomes of the parents, altering the transcriptional pattern of genes associated with energy production, metabolic rates, stress responses and hormonal signaling, culminating with higher biomass production and increased yield. QTL analyses indicate that a large number of genes contribute to the heterotic phenotype. The understanding of the physiological bases of heterosis is of interest since it could be targeted by plant breeding programs which can increase yield traits of crops. However, the molecular and physiological mechanisms explaining heterosis are still poorly understood. Therefore, this work was conducted to analyze the hormonal balance of tomato hybrids and to identify the physiological changes that result in the strongest phenotypic and agronomic traits in hybrids plants. The results suggest that the optimization of auxin and ethylene concentrations results in structural changes in leaves and consequently in the photosynthetic assimilation rate, which led to more vigorous vegetative growth of the hybrid plants in to increased fruit production.

RESUMO

BASTOS, Carla Elisa Alves, D.Sc., Universidade Federal de Viçosa, março de 2017. **Bases hormonais do crescimento e da produtividade de tomateiro (*Solanum lycopersicum*).** Orientador: Agustin Zsögön. Coorientador: Dimas Mendes Ribeiro.

Híbridos de tomateiro exibem uma performance fenotípica superior à dos parentais, seja em condições ideais ou estressantes. Essa superioridade acredita-se resultar da interação entre o genoma dos parentais, alterando o padrão transcricional de genes associados à produção de energia, taxas metabólicas, respostas a estresses e sinalização hormonal, que culminam com maior produção de biomassa e produtividade dos híbridos. Análises de QTL apontam que um grande número de genes contribui para a formação do fenótipo heterótico. O entendimento das bases fisiológicas da heterose é de interesse para aumentar a produtividade de plantas cultivadas, contudo, os mecanismos moleculares e fisiológicos que explicam a heterose são ainda pobremente conhecidos. Diante disto, este trabalho propôs analisar o balanço hormonal de híbridos altamente produtivos de tomateiro e identificar quais as alterações fisiológicas e moleculares que resultam nas alterações fenotípicas e agronômicas observadas nestes híbridos. Os resultados encontrados sugerem que a otimização nas concentrações de auxina e etileno resultam em alterações estruturais nas folhas e em consequência na assimilação fotossintética, as quais suportam o crescimento mais vigoroso das plantas híbridas tanto na performance vegetativa quanto na produção de frutos.

INTRODUCTION

Heterosis, or hybrid vigor, is a genetic phenomenon generated by cross-fertilization between strains, races or species of plants and animals (SCHNABLE & SPRINGER, 2013; CHEN, 2013) resulting in a progeny of greater vigor, growth and adaptation to stress (BIRCHLER *et al.*, 2010; CHEN, 2013; FENG *et al.*, 2015). That cross-fertilization was beneficial to the performance of plants was first observed by Charles Darwin in 1876, but the theoretical framework for this phenomenon was laid out only in 1914, when G. H. Shull introduced the concept of *heterosis* (GOFF, 2011; CHEN, 2013; FENG *et al.*, 2015). Upon the introduction of the first maize hybrids in the 1930s, and to the present day, the impact of heterosis in agriculture has been on the rise, representing increases in yield of up to 50% in crops as diverse as maize (*Zea mays*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongenata*) (LIPPMAN & ZAMIR, 2007; FU *et al.*, 2014).

Heterosis in F_1 hybrids occurs due to alterations in the gene expression pattern resulting from the combinations between parental alleles (GROSZMANN *et al.*, 2014, 2015). Three hypotheses are classically used to explain this interaction: dominance, overdominance and epistasis (GOFF, 2011; SCHNABLE & SPRINGER, 2013; CHEN, 2013; FENG *et al.*, 2015).

The hypothesis of dominance, or complementation, posits that heterosis results from the complementation of deleterious recessive alleles by superior alleles that are combined in the hybrid to form a heterozygous individual for that locus (SCHNABLE & SPRINGER, 2013). Dominance is the most widely theory accepted, although it presents some problems. First, crop plants are artificially selected for maximum performance, ergo deleterious alleles are reduced in crops with greater homozygosity, what contradict that dominance is the major answer for heterosis (LIPPMAN & ZAMIR, 2007). Second, dominance model cannot explain why allopolyploids have higher heterosis than autopolyploid, and tetraploids have higher heterosis than diploids (BIRCHLER *et al.*, 2010a; CHEN, 2013).

Overdominance postulates that allelic interactions in a single heterozygous gene or several overdominance *loci* of small effect contribute cumulatively to heterosis; and epistasis suggests that the interaction of favorable alleles located at different *loci* in the parental generates an improved hybrid phenotype (BIRCHLER *et al.*, 2010; SCHNABLE & SPRINGER, 2013; YAO *et al.* 2013). Epistasis is particularly difficult to resolve because cannot be easily explained by quantitative genetic models (CHEN, 2010).

Particular interest has been placed on overdominance, as it is believed that specific *loci* can trigger a better phenotypic performance of hybrids and thus as few as only one *locus* in heterozygosis would be sufficient to generate a heterotic hybrid in F_1 generation, hence "single-gene overdominance" (RÉDEI, 1962; LIPPMAN & ZAMIR, 2007; KRIEGER *et al.*, 2010). Various examples of single-gene overdominance have been described in plants, such as the *erecta* and *angustifolia locus* in Arabidopsis (RÉDEI, 1962) and *purple plant locus* in maize (HOLLICK & CHANDLER, 1998). Besides, recently a case of single gene overdominance was shown in *single flower truss* (*SFT*) (KRIEGER *et al.*, 2010; JIANG *et al.*, 2013), but it is possible that it involves a greater gene dosage balance on regulatory networks in hybrid (BIRCHLER *et al.*, 2006, 2010; YAO *et al.*, 2013), a mechanism that does not involve allelic or *loci* interaction but contributes to heterosis.

All three modes of gene interaction described above have been observed in heterotic plants (HUA *et al.*, 2013; MEYER *et al.*, 2010), but the interpretation of these effects on the final phenotype remains complex and largely undefined, despite decades of research (BIRCHLER *et al.*, 2010; KAEPPLER, 2012; SCHNABLE & SPRINGER, 2013). Recently, new complementary approaches that explain heterotic phenotypes have been proposed, such as variations in the presence of regulatory RNAs (CHEN, 2013; ZHANG *et al.*, 2014) and in DNA methylation patterns in hybrids (SHEN *et al.*, 2012; CHEN, 2013), both factors having the potential to alter gene expression in many hybrids species.

A common morphological phenotype in plant hybrids is the increase in biomass production, which many experiments showed to be related to an increase in organ size, mainly related to cell proliferation (FUJIMOTO *et al.*, 2012; GROSZMANN *et al.*, 2014, 2015), in the number or size of mesophyll cells (FUJIMOTO *et al.*, 2012; GROSZMANN *et al.*, 2015) and in leaf area (FLINT-GARCIA *et al.*, 2009; MEYER *et al.*, 2010). Besides morphological changes, hybrid heterosis can be evidenced by higher photosynthetic capacity (FUJIMOTO *et al.*, 2012; OFFERMANN & PETERHANSEL, 2014), increases in seedling viability and number (MEYER *et al.*, 2012), nutritional efficiency, and abiotic tolerance and metabolite contents (STEINFATH *et al.*, 2010; MEYER *et al.* 2012). These phenotypic characteristics are partially explained by the increase in the expression of genes related to carbohydrate metabolism, stress mitigation, circadian rhythm control and sensitivity to nutritional status (GOFF, 2011; GROSZMANN *et al.*, 2014, 2015), suppression of genes related to the basal defense pathways (GROSZMANN *et al.*, 2015) and alteration in the expression of genes that regulate the production and accumulation of auxin, brassinosteroid, gibberellin and salicylic acid (GUO *et al.*, 2010; SHEN *et al.*, 2012; LI *et al.*, 2013; GROSZMANN *et al.*, 2015).

The spontaneous tomato mutant *epinastic* (*epi*) (*Solanum lycopersicum* cv. VFN8), whose genetic basis has not yet been unveiled, shows a phenotype characterized by strong leaf epinasty, dark green leaves, erect growth, branched root system, thickened stem and petioles, reduced anthocyanin content and overproduction of ethylene in the apical part of the plants (FUJINO *et al.*, 1988*a*,*b*, 1989; URSIN & BRADFORD, 1989). Through what is believed to be a block in ethylene signaling pathway, *epi* plants show a constitutive ethylene response (BARRY *et al.*, 2001). Negi et al, (2010) further verified greater transport of 3-indoleacetic acid (AIA) in both roots and hypocotyls in *epi* mutants compared to wild-type, suggesting that ethylene stimulates localized auxin biosynthesis and/or changes in auxin transport, leading to auxin accumulation in the tissues.

During introgression of the *epi* mutation from its original background VFN8 to cv. Micro-Tom (MT) (CARVALHO *et al.*, 2011), we observed vigorous vegetative growth when *epi* was in heterozygosity. The F_1 generation obtained from the cross between *epi* and wild type (MT) plants presented leaf epinasty growth restricted to the youngest leaves and higher biomass production. This work is based on the hypothesis that physiological and morphoanatomical traits altered by ethylene and auxin-mediated effects lead to more vigorous growth and an increase in tomato yield. Thus, the aims of this work were: a) to confirm the existence of heterosis for vegetative and reproductive characteristics in hybrids of *epi*; b) to characterize the phenotype of the hybrid plants at the anatomical, morphological and physiological levels; and c) to determine auxin and ethylene levels in tissues of hybrids and their parents.

This thesis is divided in four chapters. In the first, we explore the genetic bases of heterosis in hybrid and we describe morphology and anatomy of hybrid and their parents. In the second chapter, we evidence aspects related to growth and development of hybrids, specially related to hormonal levels. The third chapter elucidates physiological aspects involved in vigorous growth in hybrids and in chapter four we demonstrate that the *epi* allele is a promising alternative to improve agronomical aspects in commercial tomatoes varieties.

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CHAPTER 1: THE *epinastic* MUTATION IN HETEROZYGOSITY PROMOTES HYBRID VIGOR IN TOMATOES

INTRODUCTION

Heterosis, or hybrid vigor, is a genetic phenomenon whereby the hybrid progeny of different strains, races or species of plants or animals results in increased vigor or performance (BIRCHLER *et al.*, 2010; SCHNABLE & SPRINGER, 2013). Charles Darwin described the phenomenon importance in the late 19th century, but it was only in 1914 that Shull coined the term ‘heterosis’ and properly framed it conceptually (GOFF, 2011). Large-scale cultivation of hybrid crops was introduced in the 1930s and has been growing steadily since, providing yield increases of 15 to 50% in cereals crop, e.g. maize (*Zea mays*), rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*), and in horticultural crops, e.g. eggplant (*Solanum melongenata*) and tomato (*Solanum lycopersicum*) (BARRY *et al.*, 2001; FU *et al.*, 2014; LIPPMAN & ZAMIR, 2007). In spite of considerable research efforts, the biological explanation for heterosis is remarkably unclear (SCHNABLE & SPRINGER, 2013).

The improved growth, performance or yield in hybrid plants is assumed to be related to altered gene transcription profile resulting from particular interactions of parental alleles (BIRCHLER *et al.*, 2010; GOFF, 2011). These interactions have been defined by three classical hypotheses of gene activity: dominance, overdominance, and epistasis (GOFF, 2011; GROSZMANN *et al.*, 2014; SHEN *et al.*, 2012).

The hypothesis of dominance suggests that heterosis results from the reciprocal complementation of deleterious recessive alleles from either parental (SCHNABLE & SPRINGER, 2013), which causes a masking of recessive alleles; overdominance postulates that the existence of allelic interactions at specific heterozygous *loci* results in a synergistic effect on vigor that surpasses both homozygous forms (LIPPMAN & ZAMIR, 2007) and, finally, epistasis suggests that *interlocus* allelic interaction leads to improved performance (GREAVES *et al.*, 2015). According to the overdominance model, heterozygosis in only one gene would be sufficient to cause a heterotic phenotype, and a single gene or single *locus* is considered a distinct type of heterosis (GOFF, 2011; LIPPMAN & ZAMIR, 2007). Because none appears to be a general mechanism to explain the complexity of heterosis, new models are often proposed.

Evidence for single *locus* overdominance is debated, but there are examples of it in crop and non-crop species: the *erecta* mutant in *Arabidopsis thaliana* provides heterozygous hybrids with greater fertility (Rédei, 1962) and *single flower truss* is the first and so far only single overdominance gene described for yield (GOULET; RODA; HOPKINS, 2017; KRIEGER; LIPPMAN; ZAMIR, 2010). Besides, from an agricultural perspective, single gene can result in heterosis in quality aspects when in heterozygosity, as the hybrids of *ripening-inhibitor* (*rin*), *non-ripening* (*nor*) and *alcobaça* (*alb*) mutants, exploited in the tomato industry to produce long shelf-life tomatoes (LIPPMAN & ZAMIR, 2007).

The spontaneous *epinastic* (*epi*) mutation in tomato has been studied for years but its molecular nature has not been discovered (FUJINO *et al.*, 1988a, 1988b; FUJINO; BURGER; BRADFORD, 1989; URSIN & BRADFORD, 1989). *epi* has been mapped to a 1Mb-region in chromosome 4 (BARRY *et al.*, 2001). The *epi* phenotype points strongly to an ethylene-related process, with a strong leaf epinasty, compact growth and thickening stem, however there is evidence that this may not be the primary lesion.

Despite the close relatedness of *epi* mutant and WT, during introgression of *epi* mutation from the original background (tomato cultivar VFN8) to cv. Micro-Tom (MT), we observed indication of hybrid vigor for vegetative and reproductive traits. Therefore, we characterized the F_1 generation from crosses between *epi* and wild-type. Here, we demonstrated that *epinastic* hybrid on F_1 generation shows heterosis for vegetative and yield-related traits in different genetic backgrounds. Hybrid plants show epinasty restricted to youngest leaves and normal branching pattern. We propose that hybrid heterosis be based on single gene and showed evidences to overdominance.

MATERIAL AND METHODS

Plant material and growth conditions

For this work, seeds of tomato (*Solanum lycopersicum* cv. MT, cv. VFN8) were germinated and plants grown in 3 L pots to cv. MT and 6 L to cv. VFN8 with commercial substrate fertilized with 4,5 g L^{-1} of NPK (4-14-8) and lime (5 g L^{-1}) in a greenhouse located at Federal University of Viçosa, Brazil. Plants were watered regularly and fertilized weekly with a foliar solution (Biofert®).

Two independent alleles of *epinastic* in cv. MT were utilized in this study, one of them we called *epi-2* and the other *epi*. The *epi-2* allele was generated by EMS-mutagenesis, as described previously (PINO-NUNES *et al.*, 2009) and the *epi* allele was derived by an introgression of *epi* gene of original background (cv. VFN8) after a series of backcrosses, resulting in an isogenic line (CARVALHO *et al.*, 2011). The reciprocal hybrids were obtained through the crosses between *epi* and WT, where pollen of *epi* mutant, in MT and VFN8 background, was collected and used to pollinize flowers emasculated before anthesis to prevent accidental self-pollination of WT and *epi*, respectively.

In the experiments, we used F_1 hybrid seed. Maternal effects can play a significant role in fitness of hybrids, thus in this experiment, we selected seed of similar size at the beginning of the experiment to decrease possible maternal effects on seed size, germination, growth, factors which are often influenced by the female parental (ROACH & WULFF, 1987). Here, we used the nomenclature +/+ to refer to a homozygous normal plant (MT and VFN8), *epi/epi* to refer to homozygous *epinastic* mutant*,* +/*epi* to a hybrid when MT or VFN8 was a female parent and *epi*/+ to a hybrid when mutant *epinastic* was the female parent.

Plant biometric parameters

Leaves, stems and roots of the genotypes were collected in paper bags and oven-dried at 60°C for 72 h to dry mass measurement by analytical scales. Additional measurements were determined in specific phases of growth: plant height, number of flowers and fruits per plant, total soluble solids content in fruits (Brix) and frequency of green and ripe fruits. Total soluble solids content was assessed using a digital refractometer (PR-101, Atago, Tokyo, Japan) in fruits from at least 6 different plants per genotype. Shoot diameter was determined at the height of the fourth internode 65 days of germination (DAG), using a digital pachymeter (Western Ferramentas, São Paulo, SP, Brazil). To characterize plant architecture, we verified the presence of leaves, lateral branching, leaf primordium or none across all axillary leaf buds on the main stem in all genotypes. Allometric relationship between dry roots and dry shoot were performed as described by Poorter *et al.* (2000, 2012).

Leaf anatomy

Sections of the terminal leaflet of fully-expanded leaf were hand cut from the widest part of the leaf using a razor blade and storage in methanol. Afterwards, the sections were mounted on a microscope slide, and cleared using a solution of 85% (w/v) lactic acid

(KALVE *et al.*, 2014). Slides were placed in water bath at 95°C overnight. Sections were viewed using a photomicroscope (Zeiss Scope A1) and images captured using an AxioCam 105 color digital camera (Zeiss). Measurements of stomatal index and density, pavement cell form and size were performed on AnatiQuanti (UFV, Viçosa) and ImageJ (University of Wisconsin-Madison).

For cross section analyses, the terminal leaflet of full-expanded leaf was hand cut from the widest part of the leaf using a razor blade and storage in formaldehyde-acetic acid-ethanol (FAA) 70% solution for 24h. Leaf tissue samples were then transferred to ethanol 70% for 24h. Tissues samples were dehydrated in a progressively increasing ethanol concentrations (of 70% to 100%) and later embedded in historesin (Leica), sectioned by ultramicrotome Leica (3 μm) and stained with toluidine blue 5%. Cross sections were viewed using a photomicroscope (Olympus AX70) and images captured using a AxioCam Hrc digital camera (Zeiss). Four subsamples for each cross-section were selected for measurement of upper and lower epidermis, palisade and spongy parenchyma thickness. Total leaf thickness was also determined in five random places for each cross-section of the leaf. The mean values of all the anatomical measurements were calculated per leaf sample, and subsequently for each genotype. Measurements of traits in transverse section were performed on ImageJ (University of Wisconsin-Madison).

Stem anatomy

Stem cross-section and longitudinal micrographs were obtained from section in the fourth internode in 90-old-days plants. Stem were fixed in 70% FAA until we mounted the sections on a microscope slide. Sections were cut by hand and stained with 1% safranin/Astra blue. Slides were examined on a photomicroscope (Zeiss Scope A1) and mages captured using a AxioCam 105 color digital camera (Zeiss).

Statistical analyses

All experiments in this study were performed on completely randomized design, with the number of replicates varying in each experiment. Means of each trait measured were analyzed by *t* test, when we compared two genotypes, or by Tukey's test, when we compared three genotypes.

RESULTS

Vegetative traits show heterosis in tomato *epinastic* hybrid

An enlarged and vigorous plant was found in a EMS-mutagenized population of tomato cv Micro-Tom (MT) plants. Self-fertilization, however, yielded strongly epinastic plants (Fig S1). An allelic test with the *epinastic* (*epi*) mutant introgressed into MT from its original background (cv VFN8), showed the EMS mutant to be a new allele of *epi,* which we call *epi-2* (Table S1).

Reciprocal F_1 hybrids of MT and *epi-2* exhibited significant growth vigor in many characters, including leaf size (Fig. 1a, 1b, 1c and 1d), plant height (Fig. 1a) and dry mass (Fig. 1c). Total dry mass, leaf dry mass and root dry mass were increased 93.8%, 124% and 400%, respectively, in +/*epi-2* in relation to +/+ and 75%, 64% and 482%, respectively in *epi-2*/+ in relation to +/+ (Fig. 1b). In general, both reciprocal hybrids showed significant increase in height until first inflorescence, length of two internodes and number of leaves in relation to +/+, in spite of some of these traits not being different from the *epi-2/epi-2* parental (Table 1). For some traits, +/*epi-2* was significantly different from *epi-2*/+, among them stem dry weight, total dry weight and plant height until first inflorescence.

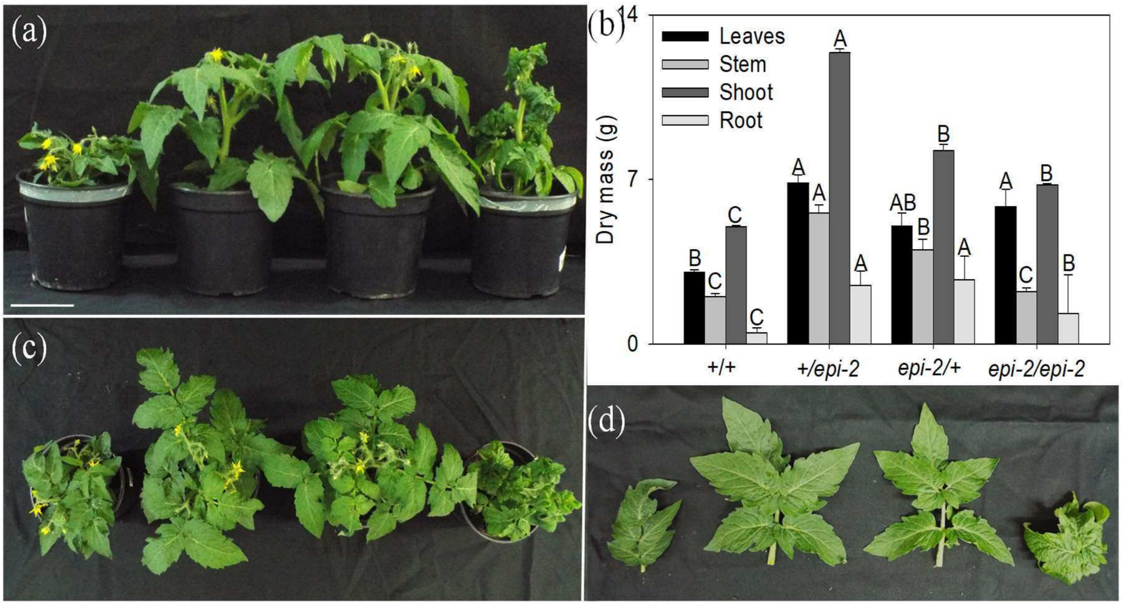

Figure 1: *epinastic-2* hybrids in cv. MT are more vigorous than both parents. Representative 70-days-old plants of parental (+/+ and *epi-2*/*epi-2*) and reciprocal hybrids (+/*epi-2* and *epi-2*/+). (a) Side view of representative plants, (b) Dry mass of leaves, stem, shoot and root (n=6; means ± S.E, same letters do not differ by Tukey's test at 5% in each tissue), (c) Top view of representative plants (d)

Fifth fully-expanded leaf aspect. Left to right: +/+, +/*epi-2*, *epi-2*/+ and *epi-2*/*epi-2*. Scale bar=10 cm.

Table 1: Plant and internodes height and number of leaves of +/+, +/*epi-2, epi-2*/+ and *epi-2/epi-2*. Measurements calculated from 5 to 10 samples three days before anthesis of the first flower. Values are mean ± S.E.M. Same letters do not differ by Tukey's test at 5%. Internode 1 equals the distance between 3rd and 4th leaves and internode 2 between the 4th and 5th leaves

Property	**+/+**	**+/*epi-2***	***epi-2*/+**	***epi-2/epi-2***
Internode 1 (cm)	1.19±0.12 B	1.91±0.16 AB	2.38± A	1.913±0.1 AB
Internode 2 (cm)	1,78±0.05 B	3.09±0.21 A	3.10± A	2.5±0.12 AB
Height until 1st inflorescence	5.05±0.36 C	12.30±0.40 B	15.98± A	13.78±0.31 B
Number of leaves until 1st inflorescence	4.63±0.24 C	5.70±0.15 B	6.50± AB	7.25±0.15 A

Plant architecture also was different between hybrids and parental. Plant architecture refers to the organization of the plant body and includes branching pattern, shape and position of leaves and flower organs (REINHARDT & KUHLEMEIER, 2002). Interestingly, reciprocal hybrids (+/*epi-2* and *epi-2*/+) presented more profuse side branching than +/+ and *epi-2/epi-2* during vegetative stage (Fig. 2).

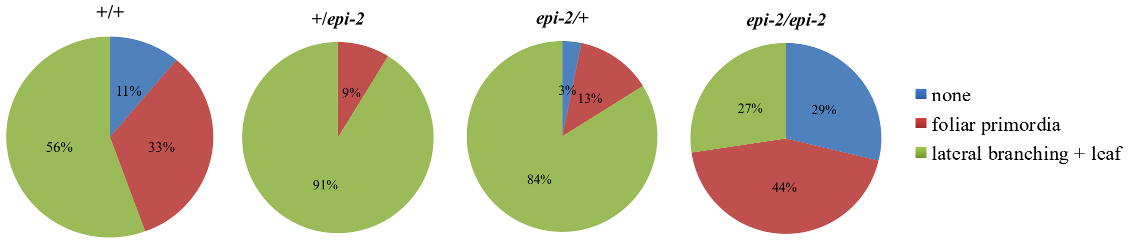

Figure 2: Increased side branching in *epi-2* hybrids. Branching pattern along leaves in the main stem of +/+, +/*epi-2*, *epi-2*/+ and *epi-2/epi-2*. Measurements obtained from 5 to 10 samples before anthesis of the first flower in each genotype.

Hybrid plants from *epinastic* mutant of both alleles, *epi* and *epi-2,* were enlarged and all phenotypically similar regardless whether MT or either mutant was used as pollen donor or receptor. Because the *epi* mutants have a reduced flower set (data not shown), all the next results presented here for heterozygotes are using the original *epi* mutation crossed with wild-type as pollen receptor (+/*epi*). For simplicity, the next results presented here for heterozygotes are using the original *epi* mutation.

Crosses between +/+ and *epi/epi* produced F_1 hybrid combination that shows heterosis at some points during vegetative development (Fig. S2). The differences between +/+ and +/*epi* started in the vegetative stage and continued until fruit set (Fig. 3). After two weeks of plant growth we observed significant differences in leaves (Fig. 3b), roots (Fig. 3c) and total dry mass (Fig. 3d), while the differences in total leaf area of plants arised only during the third week of growth (Fig. 3a).

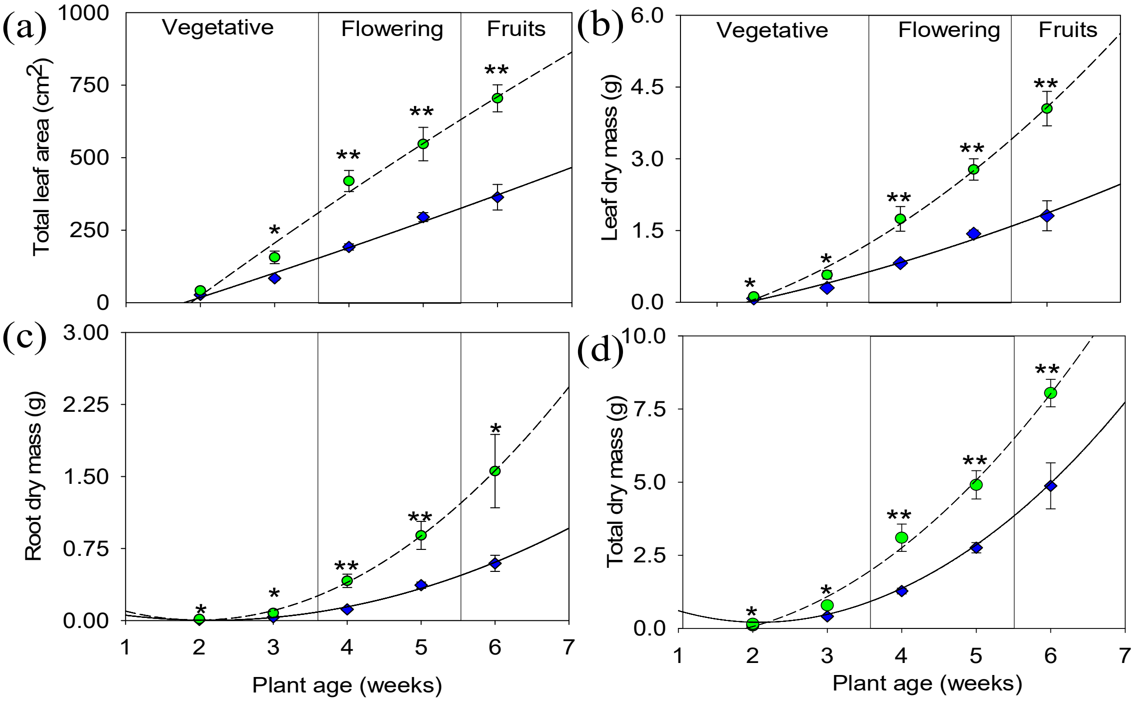

Figure 3: Plant dry mass and leaf area are increased across all phenological stages in +/*epi* plants. Values of (a) total leaf area, (b) leaf dry mass, (c) root dry mass and (d) total dry mass of +/+ (blue diamond) and +/*epi* (green circle). The genotypes differ to 5% (*) or 1% (**) by t test (n = 7, means ± S.E.M).

Higher leaf area was established in +/*epi* hybrid at beginning of development (Fig. 3a) and at 45 DAG we observed that the leaflets are less overlapping in +/*epi* than in +/+ (Figs. 4a and 4b). Besides, hybrid +/*ep*i plants are taller than +/+ and their leaves point downward but the epinastic phenotype is restricted to younger leaves, older leaves showed a normal blade appearance (Fig. 5a).

We verified considerable side branching in +/*epi* plants and thus broke down the leaf area and dry mass contributed by either leaves on the main stem (Fig. 4c) or in side branches (Fig. 4d). In both cases, principal stem and branching, we visualized that +/*epi* showed higher leaf dry mass and leaf area than +/+.

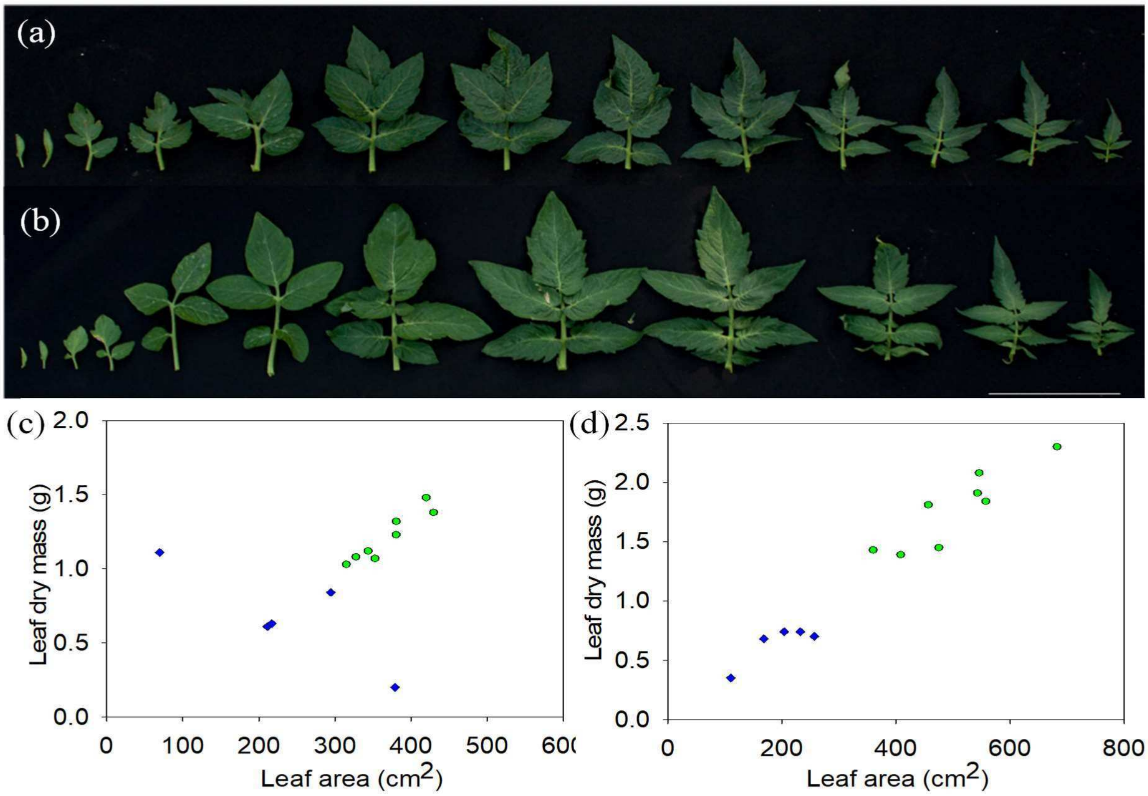

Figure 4: Leaf area is increased in +/*epi* with a large contribution from side branches. Representative leaf series in 45 DAG of (a) +/+ and (b) +/*epi*. The relationship between dry mass and leaf area broken down between (c) leaves in the main shoot and (d) leaves in side branches (*ie* derived from axillary meristems). Scale bar=10 cm.

Growth of plants is generally resulted from a balance between biomass invested in shoot and in roots. According Poorter *et al.* (2012) one way to demonstrate carbon allocation is to analyze broad allometric relationships between shoot and roots. Although differences in leaf size and biomass of roots and shoots occurs between +/+ and +/*epi,* there was no drastic changes in development of hybrids in relation to maternal parent, in other words, carbon allocation is not the major cause of higher growth in +/*epi* (Fig. 5).

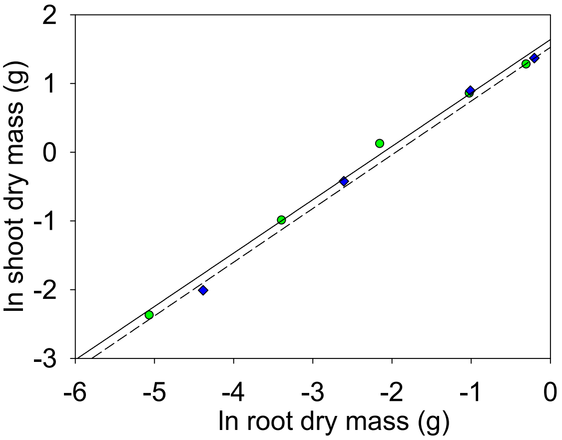

Figure 5: +/+ and +/*epi* shows similar carbon allocation between shoot and roots. Allometric relationship between +/+ (blue diamond) and +/*epi* (green circle), (n=7).

The heterotic effect on vegetative traits caused by *epi* in heterozygosity was also found in crosses between *epinastic* mutant with other backgrounds. In cv. VFN8, we showed higher shoot and total dry mass in +/*epi* than both parental (Fig. S3). In fact, the differences in vegetative and growth traits were more evident in cv. MT than in VFN8 background. Besides, crosses between *epi* and MT plants harboring a functional allele of the *SELF-PRUNING* (*SP*) gene, an indeterminate tomato plant, exhibited greater increase in dry mass of leaves, stem and root. (Fig. S4).

Reproductive traits showing heterosis in tomato *epinastic* hybrid

Classical tomato heterosis is driven predominantly by QTL that control reproductive traits (KRIEGER; LIPPMAN; ZAMIR, 2010). To determine if +/*epi* shows heterosis for reproductive traits, we examined the contributions of chronological time to flowering, number of leaves and plant height until first inflorescence, total number of flowers per plant and fruit weight (Fig. 6).

Previous reports on heterosis in plants suggested that delays in the flowering time could contribute to increased vegetative biomass in hybrids (FUJIMOTO *et al.*, 2012; JIANG *et al.*, 2013). In our study, time to flowering in the hybrid +/*epi* (32 DAS) is intermediate between +/+ (30 DAS) and *epi*/*epi* (35DAS), but the same height and number of leaves is produced before appearance of the first inflorescence (Figs. 6a and 6b) in both genotypes, +/*epi* and +/+. Reproductive growth is considerably boosted in +/*epi* plants, as evidenced by increased numbers of both flowers and fruits per plant (Fig. 6c), but without significant differences in the number of flowers per inflorescence. Fruit weight per plant is increased more than 200% in +/*epi* plants compared to parental, although the ratio of green to red yield is not altered (Fig. 6d).

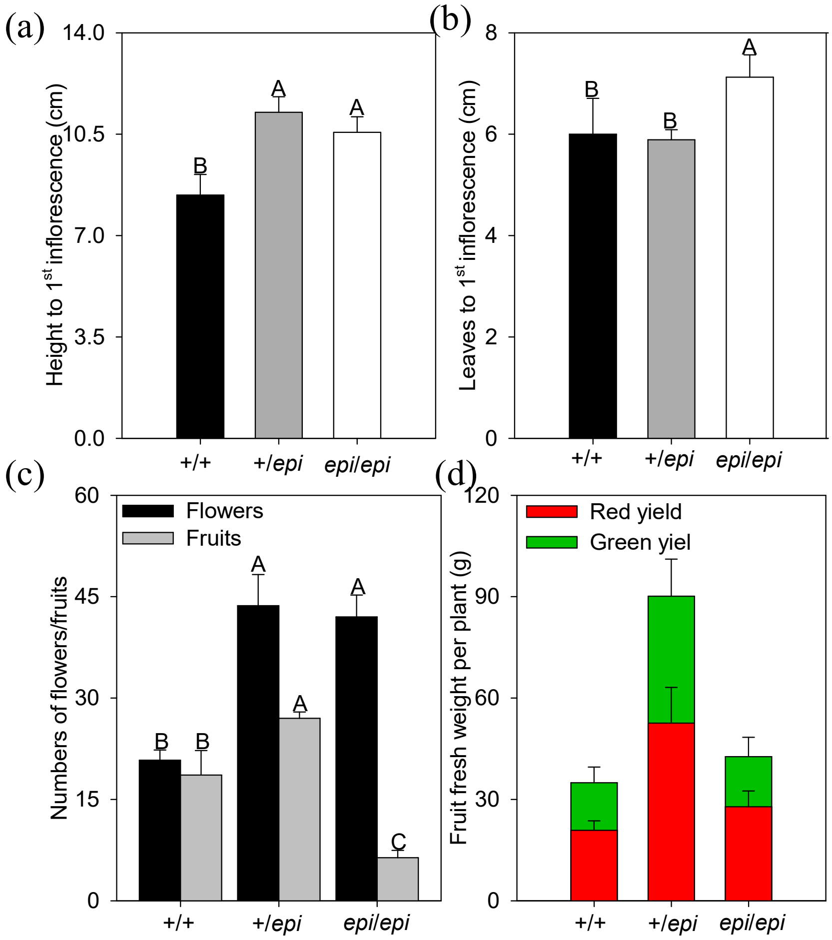

Figure 6: Flower and fruit development is enhanced in the +/*epi* hybrid. (a) Plant height until first inflorescence, (b) Number of leaves on the main stem until first inflorescence, (c) Number of flower and fruit and (d) Yield broken down in green (unripe) and red (ripe) yield, of fruits harvested 90, 95 and 100 days after germination. Values above each column are the ratio of green/red yield (n=10; means ± S.E.M.). Same letters do not differ by Tukey's test at 5%.

To describe the growth of reproductive tissue, the ratio of reproductive dry mass to vegetative dry mass was calculated. The proportion of +/*epi* vegetative dry mass appeared conserved in relation to +/+, while *epi/epi* showed increase in vegetative dry weight in relation to reproductive dry weight at the time of harvest (Fig. 7).

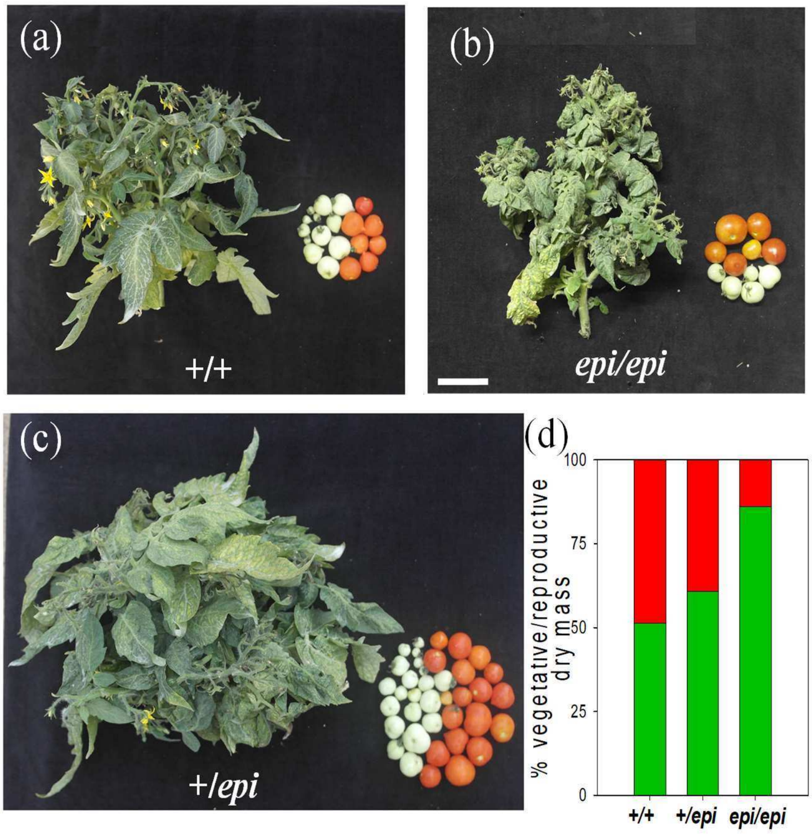

Figure 7. The vegetative-to-reproductive balance is not altered in +/*epi* hybrid. Representative plant and total fruit yield from (a) +/+, (b) *epi*/*epi* and (c) +/*epi*. Scale bar=2cm. (d) Partitioning of vegetative (leaves, stem, roots) and reproductive (green and red fruits) dry mass.

Tomato breeding, specially to the processing tomato industry, seeks varieties with high total fruit yield and high sugar content (Brix value) (GUR & ZAMIR, 2004; KRIEGER; LIPPMAN; ZAMIR, 2010). We observed that hybrid +/*epi* achieved more fruit yield than their parental (Fig. 5c) and, such is a typical relationship between fruit yield and sugar, the Brix values were significantly lower in hybrid and in the *epinastic* parent. However, Brix yield is considerably higher in +/*epi* genotypes (Table 2).

Table 2. Brix × yield is increased in +/*epi* hybrid. Measurements determined from 5 red fruits per plant (n=8) and Brix × yield calculated using red yield values. Values are mean ± S.E.M. Same letters do not differ by Tukey's test at 5%

	+/+	+/*epi*	*epi*/*epi*
Brix	5.34±0.17 A	3.75±0.10 C	4.55±0.17 B
Brix × yield	188.46±26.93 C	342.54±48.42 A	203.60±20.34 B

Higher leaf area in the +/*epi* hybrid is mainly due to an increase in cell number

The large increase in leaf area must ultimately be related to differences at the cell level, through two processes: cell division and cell expansion, that normally occur simultaneously to maintain cell homeostasis (GONZALEZ *et al.*, 2010). To analyze the extent to which cell proliferation and/or cell expansion contribute to enlarged leaves in +/*epi*, the abaxial and adaxial surface area and circularity index were analyzed in five leaflets for +/+, +/*epi* and *epi/epi*. The measurements were carried out at 75 DAG. The pavement surface area in both sides of the leaf did not differ between +/+ and +/*epi* and were lower in *epi/epi* (Fig. 8d). In relation to circularity index, cell appearance in *epi/epi* was abnormal, but in +/*epi* hybrid had a normal shape, very similar to +/+ plant (Figs. 8a, 8b, 8c and 8e).

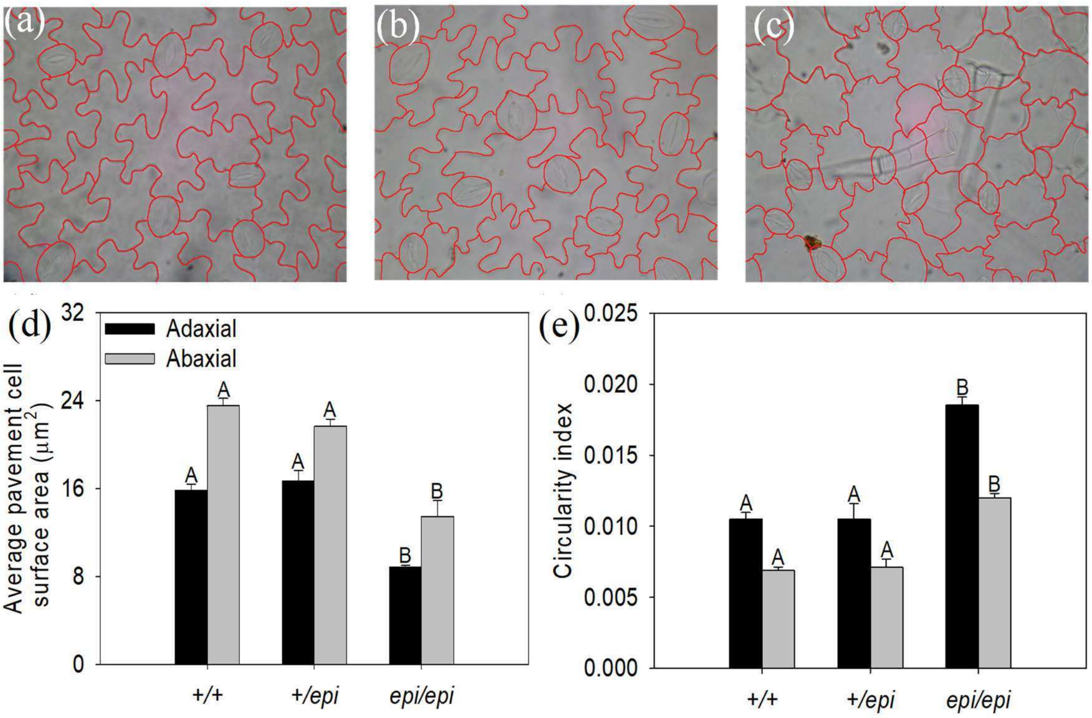

Figure 8: Larger leaf area in +/*epi* hybrid is not explained by cell size differences. Representative micrographs of the abaxial epidermis of a fully-expanded leaf of (a) +/+, (b) +/*epi*, and (c) *epi/epi*. (d) Abaxial and abaxial cell surface area in +/+, +/*epi* and *epi/epi,* and (d) Circularity index in +/+, +/*epi* and *epi/epi*. Cell outlines were drawn in red for clarity. Scale bar=10µm. The genotypes differ to 5% (*) by t test (n = 5, means ± S.E.M).

+/*epi* hybrids shows leaf and stem alteration

Differences in anatomical composition of the leaves, including packing of mesophyll cells, thickness of mesophyll and leaf air space, have the potential to affect leaf functioning

(DE LA RIVA *et al.*, 2016; POORTER *et al.*, 2009). Because ethylene is considered a signal to plant growth as a regulator of cell expansion (LOVE *et al.*, 2009), we hypothesized that *epi* in heterozygosity could change anatomical traits in leaves. In this sense, we did not find significant differences in total thickness, mesophyll thickness, spongy parenchyma thickness or epidermis thickness, but only in palisade parenchyma thickness (Table 3).

Table 3: Leaf anatomical traits in +/+ and +/*epi*. Measurements obtain in the fifth fully-expanded leaf in the principal stem at 70 DAG. Values are mean ± S.E.M (n=4).

Transversal section leaf measurements (µm)	+/+	+/*epi*	P-value
Total thickness	222.53±2.50	236.61±11.55	0.4029
Mesophyll thickness	177.41±18.97	204.19±9.72	0.2395
Palisade parenchyma	**77.08±2.19**	**96.20±1.60**	**0.0061**
Spongy parenchyma	100.32±3.62	107.99±2.78	0.6578
Adaxial epidermal	15.65±0.72	16.32±0.72	0.5683
Abaxial epidermal	11.44±0.62	13.34±0.53	0.06

We next analyzed the considerable difference in stem size between genotypes. The considerable increase in stem dry mass of +/*epi* hybrid is accompanied by a proportionally higher stem diameter, measured at the fourth internode (Figs. 9a, 9b and 9c). Microscopic analyses of transverse and longitudinal cross-sections of the stem showed the highly-lignified xylem to be the main tissue responsible for this difference.

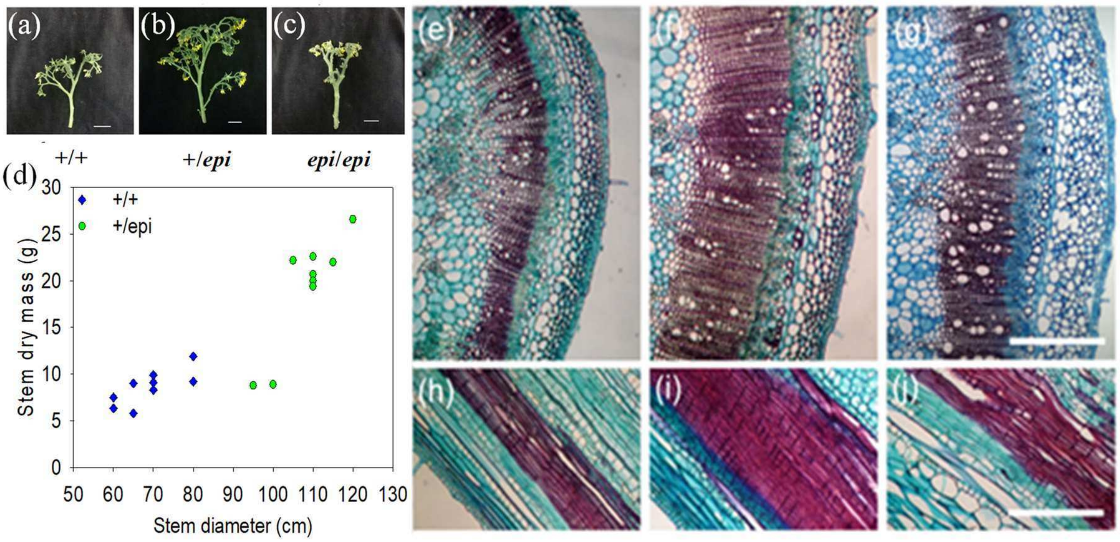

Figure 9: Increased stem growth and xylem development in +/*epi* plants. Representative stems of 90-day-old plant of (a) +/+, (b) +/*epi* and (c) *epi/epi*. Scale bar=5 cm. (d) Relationship between stem dry mass and stem diameter, measured at the fourth internode (n=7,8,9 for +/+, +/*epi* and *epi/epi*, respectively). Each data point represents one individual. Representative micrographs of transverse (e-g) and longitudinal (h-j) cross-sections of the stem at the fourth internode stained with Safranin (lignin, red) and Astra Blue (cellulose, blue). Scale bar=500 µm.

DISCUSSION

Heterosis is a phenomenon that increase performance and yield of F_1 hybrids derived from crosses between related but divergent genotypes (GOFF, 2011; GREAVES *et al.*, 2015). It is clear that interaction between alleles and epialleles from parental results in plant heterosis, but variation in an individual phenotype may be resulted not only by environment and genotype, but also by maternal effects (KIRK; VRIELING; KLINKHAMER, 2005; ROACH & WULFF, 1987). In the case described here, because we decreased maternal effect on design experiment and because both hybrids showed better fitness than the best parent, we can exclude the possibility of heterosis to be exclusively caused by the maternal effect, that is, *epinastic*-driven heterosis is predominantly caused by allelic interaction.

In our study, the parental +/+ and *epi/epi* have similar genomes with only one genetic difference, a mutation in the *epi* gene. Overdominance is one of the three classical model to explain the molecular causes of heterosis. In this model, theoretically only a single heterozygous gene is needed to achieve heterosis (KRIEGER; LIPPMAN; ZAMIR, 2010; LIPPMAN & ZAMIR, 2007). To confirm if +/*epi* heterosis is caused by overdominance, we confirmed that overdominance co-occurred with heterozygosity for two alleles, *epi* and *epi-2*, which we considered independent (Fig. 1, 3 and 4). It was suggested that overdominance single-gene in tomato increases yield (JIANG *et al.*, 2013; KRIEGER; LIPPMAN; ZAMIR, 2010; SHALIT *et al.*, 2009). Unfortunately, because we do not know the identity of the *epi* gene we could not measure expression of this gene in homozygous and heterozygous plants.

The F_1 hybrids between +/+ and *epi*/*epi,* that are isogenic lines, showed different patterns of growth in their life cycle. Both hybrids, +/*epi-2* and *epi-2*/+, had sizeable increases in biomass exceeding the better parent (Figs. 1 and 2; Table 1). Despite showing vigorous growth, we found differences in growth between the reciprocal hybrids and this data are consistent with reports that different combinations of parents can produce F_1 generation with different levels of heterosis in some traits (CHEN, 2013; GROSZMANN *et al.*, 2014). It seems reasonable to assume that epigenetic differences between parental may contribute to the different performance between reciprocal hybrids, since *epi/epi* genotype is in an elicited stadium given the high production of ethylene, which is considered a stress hormone.

Alleles responsible by heterosis must be robust and increase agronomic performance of a range of genetic backgrounds besides shows substantial potential for broad agricultural application (KRIEGER; LIPPMAN; ZAMIR, 2010). The heterosis caused by *epinastic* in heterozygosity was verified in the backgrounds MT, VFN8 and MT-SP (*self-prunning*) (Figs. 1, 4; S2, S3). Then, *epinastic* mutation can be an evidence to a relatively simple molecular mechanism to heterosis shared across backgrounds.

Hybrid seeds obtained from the cross with +/+ as a female parent and *epi/epi* as a pollen donor is easier since the flower set is larger in WT than in *epi* mutant, thus we performed others experiments to identify growth characteristics of hybrids and parents using the genotype +/*epi.* We identified heterosis in +/*epi* hybrid in early stages of development, in two-weeks-old plants and because of this, we concentrated our analyses of the hybrid to the early period of development before the transition to the reproductive developmental phase.

Vegetative and reproductive heterosis in +/*epi* hybrids

In this study, the biomass in the hybrid +/*epi* compared to the maternal parent generated increase in more than 100% in total dry biomass, considering the dry weight of leaves, stems and roots. The increase in the dry weight of leaves and stem is associated with higher number of lateral branches of +/*epi*, since the number of leaves in the main stem did not change between +/+ and +/*epi*. Regarding the total number of leaves, the increase of the individual leaf area also contributes to the increase of the total leaf area and total dry weight, because leaf area growth determines light interception. Both characters, branching pattern and increase in leaf area are directly or indirectly controlled by phytohormones, specially auxin.

Lateral bud activation is resulted from both, increase in auxin biosynthesis in apical meristem and increase in polar auxin transport (MÜLLER & LEYSER, 2011). Reports have found that ethylene inhibits polar auxin transport in shoot tissues (GROSZMANN *et al.*, 2015; MORGAN & GAUSMAN, 1966; NEGI *et al.*, 2010). In *epi/epi* plants this factor is combined which results in erect phenotype and lower lateral branching, but in +/*epi* we verified more lateral branches than in *epi/epi* and +/+, thus we hypothesize that there is some involvement of increased in auxin transport or level to determine +/*epi* hybrid phenotype. The influence of phytohormones in heterosis was previously reported in hybrids of Arabidopsis, maize, tomato, among other species, with special emphasis on the role of auxin, brassinosteroids and salicylic acid (GREAVES *et al.*, 2015; GROSZMANN *et al.*, 2014). Among these, the altered auxin responses appear to be common in hybrids of various plant species, as maize (GUO *et al.*, 2010), rice (ZHANG *et al.*, 2014) and Arabidopsis (GROSZMANN *et al.*, 2015; SHEN *et al.*, 2012).

The optimality theory argues that preferential partitioning of growth between the shoots and the roots is given by which part of the plant currently acquires the most limiting resource (LOHIER *et al.*, 2014). In our experiments plants were grown under abundant supply of water and nutrients, thus the increased shoot-to-root dry mass ratio in *epi*/+ and +/*epi* plants appears to be not a consequence of resource supply, rather an intrinsic result of the higher overall growth rate. True shifts in partitioning are difficult to distinguish from differences in biomass distribution arising from size differences. However, our allometric analyses strongly suggest that the allocation patterns are not altered in *epi*/+ plants, as both genotypes diverge along the same developmental trajectory, with almost exactly the same partitioning patterns.

Structural anatomy changes in leaves and stem in +/*epi* hybrid

Leaves drive important roles in plant function and in this sense leaf anatomical traits are key to implications for photosynthesis and posterior adaptation to environment (KALVE; DE VOS; BEEMSTER, 2014; TIAN *et al.*, 2016). Many studies correlated mesophyll characteristics with photosynthesis rate specially in determining photosynthetic capacity by both altering gas exchange variables (PEGUERO-PINA *et al.*, 2017; TOMÁS *et al.*, 2013) and chlorenchyma density (TIAN *et al.*, 2016). Mesophyll (palisade and spongy) are the leaves tissue likely associated with photosynthetic capacity, the palisade parenchyma closely associated with chloroplasts amount and spongy parenchyma closely to gas transportation (KENZO *et al.*, 2004; TIAN *et al.*, 2016). In this study, higher palisade parenchyma thickness was found in +/*epi* hybrid, which we can associated to increase photosynthesis per leaf are and per leaf, essential to support biomass production of higher shoots and roots in this genotype.

In relation to stem size, notably there was an increase in vascular thickness in the +/*epi* hybrid in relation to +/+, what we associated with higher plant diameter. A "canalization of auxin flow" theory provided a mechanism of specification of vascular tissue auxin-mediated (SCHUETZ; SMITH; ELLIS, 2013). In this model, channels drive auxin transport from surrounding cells, resulting in an auxin concentration that induces vascular tissue formation. The accumulated of auxin within developing procambial was experimentally confirmed, but while it is considered sufficient to trigger specification of vascular tissue and xylem differentiation, other authors say that endogenous ethylene stimulates cell division in the cambial meristem mediated through ethylene receptors, resulting in xylem growth by increased in cambial cell division (LOVE *et al.*, 2009). Considering the synergism between auxin and ethylene, recent studies suggest that ethylene can increase localized biosynthesis of auxin and/or can alter auxin transport (IKEDA *et al.*, 2009; NEGI *et al.*, 2010), triggering higher localized concentration of auxin. Thus, we can suppose that auxin and ethylene are mediators of cambial growth in the +/*epi* hybrid, probably related to a more favorable auxin/ethylene ratio in hybrids.

Increased cell number is a key for a higher leaf area in +/*epi*

The regulation of organ size is still poorly understood but must include a complex spatial and temporal coordination of cell division and expansion, resulting in a certain number of cells which determines mature organ size (GONZALEZ *et al.*, 2010; KALVE *et al.*, 2014; KALVE; DE VOS; BEEMSTER, 2014). Analysis of pavement surface area in leaf epidermal cells revealed no differences between +/*epi* and +/+, but how leaf blade area in +/*epi* is higher than in +/+, we can conclude that cell number is the contributor to larger leaf area in hybrid. Larger leaf size associated to cell number is a common heterotic phenotype in hybrids as suggested by various studies (BLUM *et al.*, 2013; GOFF, 2011; GONZALEZ, VANHAEREN, INZÉ, 2012; GROSZMANN *et al.*, 2015; HE *et al.*, 2010).

In the leaf development, it is known that cell division has a large influence on final leaf size, since when cell division ceases, the cell continues expanding until the final leaf size (KALVE; DE VOS; BEEMSTER, 2014). This transition from cell division to cell expansion is caused by changes in cell cycle and prolonging the cell proliferation period in hybrids and non-hybrids, resulting in a greater leaf size dues increase in cell number, mainly to auxin (GONZALEZ *et al.*, 2010; KALVE; DE VOS; BEEMSTER, 2014). Auxin induces the expression and action of a DNA-binding protein *ANT* (*AINTEGUMENTA*) and *AUXIN REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*), whose overexpression increases growth through prolonging the cell proliferation period in Arabidopsis, and in a transcriptional profile of Arabidopsis hybrids, the genes *ARABIDOPSIS VACUOLAR H+-PYROPHOSPHATASES* (*AVP1*), *NAC1* and *ARGOS* were upregulated, what suggest that growth vigor of F_1 hybrids was at least partially due higher auxin signalization. Taken together, our results of increasing in cell number on +/*epi* fully-expanded leaf, we have more than one evidence to an auxin role in growth of *epi* hybrids.

CONCLUSION

epi gene in heterozygosity on F_1 generation shows heterosis to vegetative dry mass and yield traits. We suggest that heterosis might result from overdominance in single gene, although more studies are needed to prove that other molecular changes linked to *epi* gene are not involved in the effects described here. Our results provide a new potential gene to be explored in plant breeding since it drives heterosis in reproductive traits in different tomato genetic backgrounds. From a physiological point of view, increased in plant growth is associated with a higher number of leaves on the main stem and increased side branching. We demonstrate insights about increases in cell number and in palisade parenchyma thickness which can increase photosynthesis rate and help plants to support growth. In the next chapters, we evaluated growth and development of +/*epi* hybrids and we study metabolism and gas exchange to support our hypothesis that there is a fine-tuning balance between ethylene and auxin levels in hybrids, which causes structural alteration on leaves and leads to increased growth.

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SUPPLEMENTAL INFORMATION OF CHAPTER 1

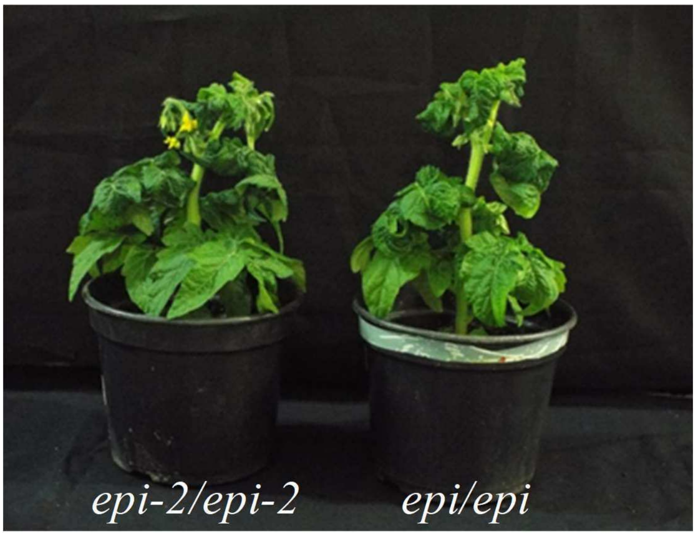

Figure S1. The *epi-2* phenocopies the *epi* allele. Representative 40-day-old plants of *epi-2/epi-2* (left) and *epi/epi* (right). Both genotypes show severely epinastic leaf growth and compact growth habit.

Table S1. Complementation test of allelism between MT *epinastic* (BC_6F_5, introgressed from cv VFN8) and the EMS mutant phenocopying *epinastic*. Results of 3 independent crosses for each pair of parental genotypes.

		Phenotype of F_1	
	Replicate #	Wild-type	*epinastic*
epinastic x EMS mutant	1	0	24
	2	0	15
	3	0	16
EMS mutant x *epinastic*	1	0	26
	2	0	31
	3	0	18

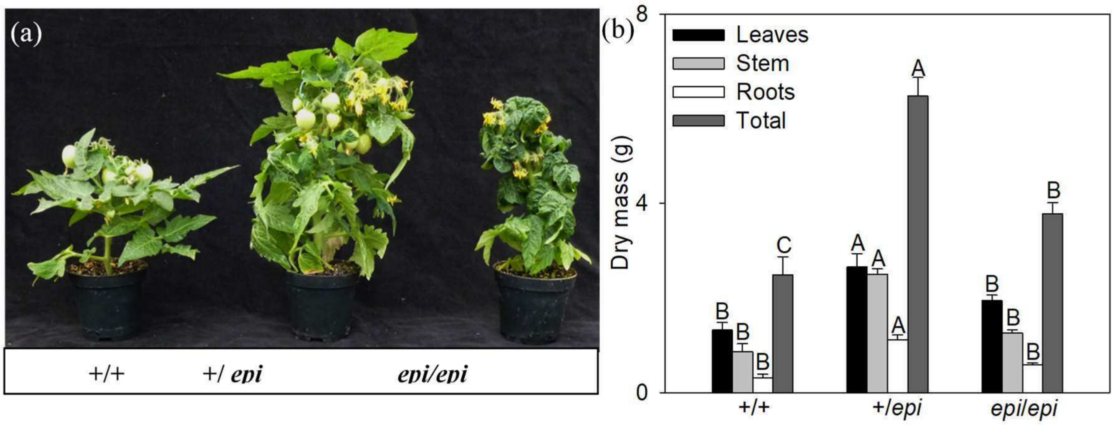

Figure S2: +/*epi* hybrid leads to increased plant growth in tomato cv. Micro-Tom (MT). (a) Representative 90-day-old plants of +/+ parent (MT), a hybrid (+/*epi*) and *epi/epi* parent. (b) Dry mass of plants of the same age broken down between leaves, stem and roots. Same letters do not differ between the same organ by Tukey's test at 5%. All values are mean±SEM (n=8).

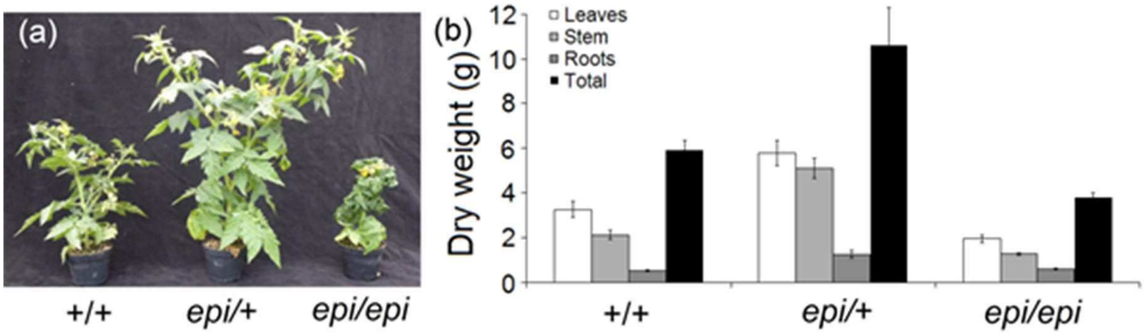

Figure S3: Heterozygosis at the *epinastic* locus leads to heterosis in indeterminate tomatoes +/*epi* hybrid in SP-MT. (a) Representative 90-day-old plants of +/+ parent, a hybrid (+/*epi*) and *epi/epi* parent. (b) Dry mass of plants of the same age broken down between leaves, stem and roots. Same letters do not differ between the same organ by Tukey's test at 5%. All values are mean ± S.E.M (n=8).

CHAPTER 2 – FINE-TUNING OF AUXIN-ETHYLENE BALANCE DRIVES HETEROSIS IN *epinastic* TOMATO HYBRID

INTRODUCTION

F_1 hybrids can show better performance than their parents in yield (KRIEGER; LIPPMAN; ZAMIR, 2010; TOLLENAAR; AHMADZADEH; LEE, 2004), vegetative biomass (BLUM *et al.*, 2013; GOFF, 2011; GROSZMANN *et al.*, 2014) and fitness in adverse conditions, such as low nutrient supply, diseases and low water availability (BLUM *et al.*, 2013; KAEPPLER, 2012), in a phenomenon called heterosis. Heterosis has an important role in crop yield, since hybrids have been explored in agriculture breeding since the 1930s to increase cereals and horticultural production (FENG *et al.*, 2015; SCHNABLE & SPRINGER, 2013). The advantage exhibited by F_1 hybrids is normally lost in the next generation (SCHNABLE & SPRINGER, 2013; WANG *et al.*, 2015), because of this, the biological pathways involved in heterosis are of interest to identify pathways and mechanisms that will allow engineering non-hybrid plants and fix heterosis in the subsequent generations (OFFERMANN & PETERHANSEL, 2014).

Typically, heterotic phenotype in F_1 hybrids plants is characterized by increases in plant height, dry biomass production and larger leaf area compared to either parent (FENG *et al.*, 2015; KAEPPLER, 2012; TOLLENAAR; AHMADZADEH; LEE, 2004), a process probably related to hormonal changes, especially auxin, gibberellin, brassinosteroids (GREAVES *et al.*, 2015; GROSZMANN *et al.*, 2014, 2015; ROOD *et al.*, 1988), the most classical hormones related to plant growth (GROSZMANN *et al.*, 2015). Besides these, changes in florigen dosage response, does not alter plant size, but increases yield through of the optimization of plant architecture and the vegetative-to-reproductive balance (JIANG *et al.*, 2013; KRIEGER; LIPPMAN; ZAMIR, 2010).

Networks and genome-wide studies in hybrids plants and allopolyploids have identified many alterations in biological pathways involved in light responses, hormonal signaling, carbohydrate metabolism, nutrient assimilation and stress responses (BARANWAL *et al.*, 2012; CHEN, 2010, 2013). An emerging model to explain the bases of heterosis established that alterations in one or more key regulator genes in hybrid plants can mediate the expression of many genes associated to changes in plant development and physiology (CHEN, 2010, 2013). A study related external photoperiod and the internal circadian clock with hormone gene expression in Arabidopsis showed that circadian clock indirectly modifies growth by mediating hormone transcript levels during the day period (MICHAEL *et al.*, 2008).

Association between heterosis and hormones has been reported in crop (LI *et al.*, 2013; ROOD *et al.*, 1988) and non-crop hybrids (GROSZMANN *et al.*, 2014, 2015; SHEN *et al.*, 2012). In Arabidopsis, intraspecific hybrids, a profile of genes related to increasing in leaf size together with a set of circadian clock genes that have been implicated in heterosis was described. In F_1 hybrids, 26 genes with different expression in relation to either parent were detected, including hormone regulation genes. Reduction in the expression of ORGAN SIZE RELATED 1 (OSR4), a cellular response to ethylene stimulus, and increased expression of genes connected to auxin response, such as AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS) and AUXIN-RESPONSIVE FAMILY PROTEIN (SAUR) were shown (GROSZMANN *et al.*, 2014). Other studies in Arabidopsis hybrids confirm the involvement of auxin in increased leaf area and plant size through both mechanisms, alteration in auxin biosynthesis and in auxin response by changes in auxin transport, since genes involved in flavonoid synthetic pathways, a repressor of auxin transport, were downregulated in F_1 hybrids (GROSZMANN *et al.*, 2015; SHEN *et al.*, 2012).

Given the involvement of hormones in heterotic phenotypes and because mutant *epinastic* is an ethylene overproducer (FUJINO; BURGER; BRADFORD, 1989; URSIN & BRADFORD, 1989), we decided to verify the relationship between ethylene and auxin, and the heterotic larger plant size in tomato hybrids from *epi* and Micro-Tom. Our results suggest that the balance between auxin and ethylene contributes to stimulate cell proliferation in hybrids plants, leading to increases in leaf area.

MATERIAL AND METHODS

Plant material and growth conditions

For this work, seeds of +/+, +/*epi* and *epi*/*epi* in the cv. Micro-Tom (*Solanum lycopersicum* cv. MT) background were germinated and plants grown in 3 L pots with commercial substrate fertilized with 4.5 g L^{-1} of NPK (4-14-8) and lime in a greenhouse located at Federal University of Viçosa, Brazil. Plants were watered regularly and fertilized weekly with a foliar solution (Biofert®). The *epi* mutant in MT background is an introgression of the *epi* gene from the original VFN8 background after a series of backcrosses, resulting in a near-isogenic line (CARVALHO *et al.*, 2011). The reciprocal hybrids were obtained through the crosses between *epi* and MT, where pollen of *epi* and MT were collected and used to pollinize emasculated flowers of MT and *epi*, respectively. Here, the name + /+ refers to the female parent (MT), *epi/epi* to the male parent and +/*epi* to a hybrid.

Plant biometric growth parameters

The relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), specific leaf area (SLA) and leaf weight ratio (LWR) was estimated during vegetative stage, between 7 and 42 days after sowing, according to Poorter *et al.*, (2012a, 2012b), using the follow equations:

$$\mathrm{RGR} = (\ln M_2 - \ln M_1) / (t_2 - t_1) \tag{1}$$

$$\mathrm{NAR} = [(M_2 - M_1) / (t_2 - t_1)] * [(\ln A_2 - \ln A_1) / (A_2 - A_1)] \tag{2}$$

$$\mathrm{LAR} = A / M \tag{3}$$

$$\mathrm{SLA} = A / Mf \tag{4}$$

$$\mathrm{LWR} = Mf / M \tag{5}$$

where:

$M_2 - M_1$ is the difference between dry shoot mass of plants harvested in two successive times (time 1 and time 2);

$T_2 - t_1$ is the number of days between the two harvest;

$A_2 - A_1$ is the difference between the total leaf area of the plants in two successive times;

A is the total leaf area;

M is shoot dry mass;

Mf is leaves dry mass

To perform growth analyses, leaves, stems and roots of the genotypes were collected in paper bags and oven-dried at 60°C for 48 h to dry mass measurement by analytical scales. Total leaf area was determined on principal and lateral stems a scanner and the ImageJ software (University of Wisconsin-Madison).

Seed size analyses

Seeds of +/+, +/*epi* and *epi/epi* in the backgrounds Micro-Tom (MT), VFN8 and MT and MT *self-prunning* were separated in four groups, in a total of 60 to 100 seeds to determine seed size and seed mass. To genotypes where the number of seed was lower than 100, the values were expanded to 100. To determine seed mass, groups of seeds had their mass measured in analytical balance and to determine seed area, photographs of the same groups were analyzed on ImageJ software (University of Wisconsin-Madison).

Auxin and ethylene determinations

Ethylene content measurements were analyzed as described previously (MELO *et al.*, 2016). Briefly, expanding leaves and stem apex of approximately 100 individuals were collected from plants growing inside sealed magenta boxes flushed with ethylene-free air for 5 min and incubated for about 24 h. After incubation period, 1 mL gas samples were withdrawn with a gas-tight syringe and injected into a Trace Ultra gas chromatograph (Thermo Electron) fitted with a flame ionization detector and an RT-Alumina Plot column (Restek).

Endogenous IAA was determined by gas chromatography-tandem mass spectrometry-selective ion monitoring, in a protocol described by Melo *et al.* (2016). In this case, approximately 200 mg fresh weight of expanding leaves and apex stem (typically 100 individuals) were used to performed analyses. Endogenous levels of IAA were calculated based on extracted chromatograms at mass ratio/charge 244 and 250.

The auxin/ ethylene ratio was calculated by the quotient between the endogenous levels of IAA and ethylene measured at the apex stem. The results were normalized with respect to +/+.

Auxin sensitivity assays

To evaluate the sensitivity of genotypes to auxin, two experiments were performed: sensitivity of hypocotyls to auxin and rhizogenesis in cotyledon explants. In the first experiment, the genotypes +/+, +/*epi* and *epi/epi* were cultivated in a green house and, after 10 days after germination, hypocotyl plants were sectioned (10 mm) and conditioned for 24 h in solutions with increasing doses of IAA (0, 0.1, 1.0, 10 and 100 μM). After incubation, hypocotyl thickening was measured in ImageJ. In auxin sensitivity experiment in rhizogenesis, cotyledon explants were cultured in vitro in MS medium plus NAA concentrations 0, 0.1 and 0.5 μM. The material was incubated in a growth room with photoperiod of 16 h, 25 ºC for 11 days, from which the percentage of adventitious roots of the explants was evaluated.

GUS staining

Transgenic lines carrying synthetic auxin-responsive promoter *DR5,* Arabidopsis cytokinin-responsive promoter *ARR5* and *EBS* fused with GUS reporter were crossed with MT and *epinastic* in the MT background. Seeds of this crosses were cultivated in MS medium in a growth room with photoperiod of 16 h, 25ºC. Seedlings of 11 days old plants, (three per genotype), were incubated with GUS staining solution (100 mM phosphate buffer, 10 mM EDTA, 0.1% Triton X-100 and 1mM X-Gluc) at 37°C overnight. After GUS staining, seedlings were decolorized using several washes in ethanol series (50% until 95%). For *DR5::GUS* analyses, three seedlings per genotype were treated with auxin (IAA 20 mM for 3 h) or mock solution before GUS staining (VITHA, 1995).

Statistical analyses

All experiments were performed on completely randomized design, with the number of replicates varying in each experiment. The experimental units were one plant per pot. All data were subjected to analyses of variance (ANOVA) and means of each trait measured were analyzed by *t*-test at 5% level of significance or Tukey's test. The Pearson correlation coefficient was used to examine relationship between relative growth rate (RGR) and its components.

RESULTS

+/*epi* hybrids show higher growth rate

Total plant biomass is controlled by several factors (VANHAEREN et al, 2016). First, seed size can affect growth of adult plants to which they give rise after germination (LEISHMAN *et al.*, 2000). Second, germination rate can delay or accelerate plant development, and third, plants can differ in relative growth rate (THOLEN; VOESENEK;

POORTER, 2004). To discriminate between these three possibilities, we studied if there are some differences in each factor between +/+ and +/*epi.*

Seed mass and seed area in +/*epi* hybrids was not higher than +/+ and *epi/epi* in different genetic backgrounds (VFN8, MT and SP) (Table 1). About timing of germination, the plants +/*epi* showed lower time to germinate 50% of seeds than +/+ and *epi/epi* parental, there was less than two days of difference in the time of germination (data not shown). In our experiments, time to germination is not a reliable parameter to relate with plant growth because *epi/epi* is an overproducing mutant of ethylene, a hormone that can delay germination. After transplantation of the seedlings to pots, we observed the largest differences in plant growth starting in the vegetative stage and on until the reproductive stage (Chapter 1). Plant growth was assessed by leaf area, dry mass production of leaves, roots and total and plant height (Chapter 1). Classical growth analyses often are performed in the vegetative stage (KENZO *et al.*, 2004), thus we considered RGR during the vegetative stage. Increases of about 30% in RGR in +/*epi* hybrid were observed in relation to the +/+ parent. To examine in greater detail RGR, we broke down its components into SLA, NAR, LWR and LAR.

Table 1. Seed mass and size in +/+, +/*epi* and *epi/epi* in different genetic backgrounds. Measurements calculated from 60-100 samples and expanded to 100 seeds. Values are mean ± S.E.M. Same letters do not differ by Tukey's test at 5%.

Genotype	Mass of 100 seeds (g)	Area of 100 seeds (cm^2)
VFN8 (+/+)	0.31073 ± 0.005172 A	0.09 ± 0.002244 A
VFN8 x *epinastic* (+/*epi*)	0.31856 ± 0.01384 A	0.09 ± 0.003543 A
epinastic (epi/epi)	0.26213 ± 0.01051 B	0.08 ± 0.003438 B
MT (+/+)	0.29811 ± 0.002204 AB	0.07 ± 0.001888 A
MT x *epinastic* (+/*epi*)	0.32820 ± 0.022786 A	0.07 ± 0.003094 A
epinastic (epi/epi)	0.26427 ± 0.006088 B	0.06 ± 0.002479 A
MT SP	0.26880 ± 0.002954 A	0.07 ± 0.001973 A
SP x MT *epinastic*	0.28087 ± 0.008567 A	0.07 ± 0.002265 A
MT *epinastic*	0.26427 ± 0.006088 A	0.06 ± 0.002479 A

When examining components that determine RGR, we found that NAR was significantly greater in +/*epi* than in +/+ (>60%) (Fig. 1b). A higher NAR results from higher net photosynthesis and it was the component that best related to RGR in both genotypes (Pearson correlation of 0.93 to +/*epi* and 0.82 to +/+). The biomass allocation to leaves, expressed as LWR and SLA, was higher in +/+ compared to +/*epi* (Fig. 1c and 1e) and we did not find differences in LAR between genotypes (Fig. 1d). We conclude that in +/*epi* hybrids, higher biomass arises mainly from increase in photosynthetic capacity, rather than carbohydrate allocation whereas no contribution of seed size, seed mass and germination time were observed Because of this, total biomass is higher in +/*epi* plants (Fig. 1f).

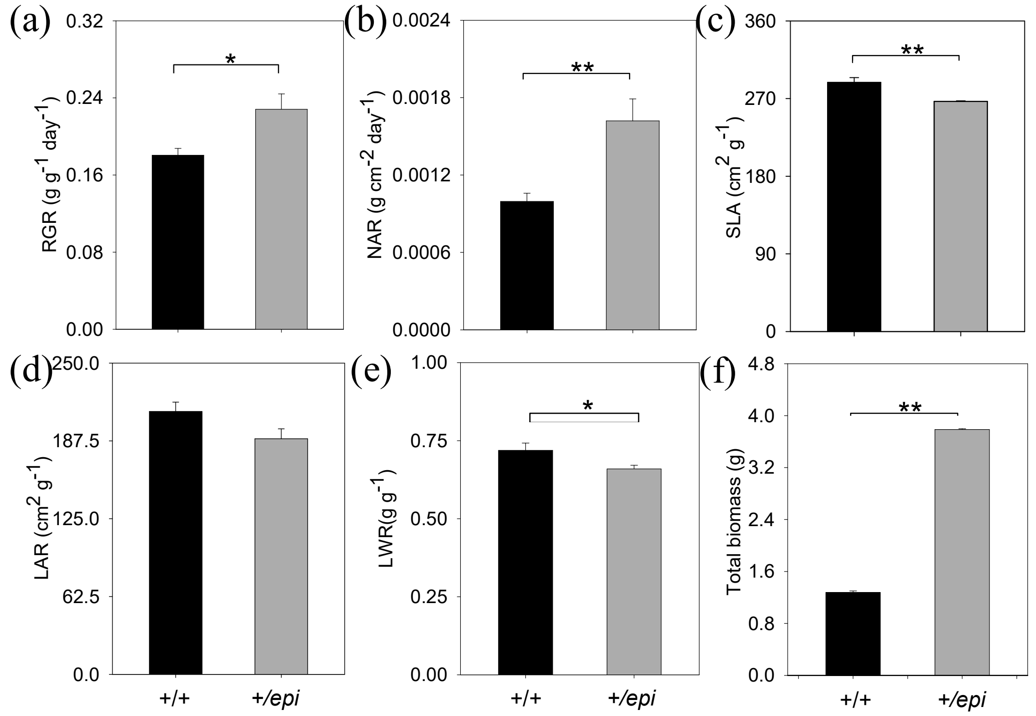

Figure 1: +/*epi* hybrids show higher relative growth rate than the wild-type parent. (a) Relative Growth Rate (RGR), (b) Net Assimilation Rate (NAR), (c) Specific Leaf Area (SLA), (d) Leaf Area Ratio, (e) Leaf Weight ratio of +/+ and +/*epi* plants during vegetative phase (2-7 weeks after germination) and (f) Total plant biomass (shoot + root) of +/+ and +/*epi* plants 7 weeks after germination. The genotypes differ among themselves to 5% (*) or 1% (**) by t test (n = 7, means ± S.E.).

Ethylene and auxin characterization in +/*epi* hybrid

Hormonal balance in plants modules growth since many genes involved in hormone biosynthesis and/or signaling modify leaf size (GONZALEZ *et al.*, 2010). It is known that *epi/epi* mutant show an altered production of ethylene, thus we decided to compare levels of ethylene and auxin in hybrid in relation their parents, because previous studies stablished closely relationship between auxin and ethylene in plants (PIERIK *et al.*, 2006; MUDAY *et al.*, 2012). Levels of IAA and ethylene in +/*epi* hybrid and their parents, +/+ and *epi/epi*, were realized in two plant tissue: apical stem and expanding leaves. In the stem apex, +/*epi* hybrid showed intermediary level of ethylene, high levels were verified in *epi/epi* and lower in +/+ (Figs. 2a and 2b), for IAA level, +/*epi* and *epi/epi* exhibited indistinct values and both are higher than +/+ plant (Figs. 2a and 2c). In relation to expanding leaves, level of ethylene was higher in *epi/epi* and no differences to IAA were visualized (Figs. 2b and c).

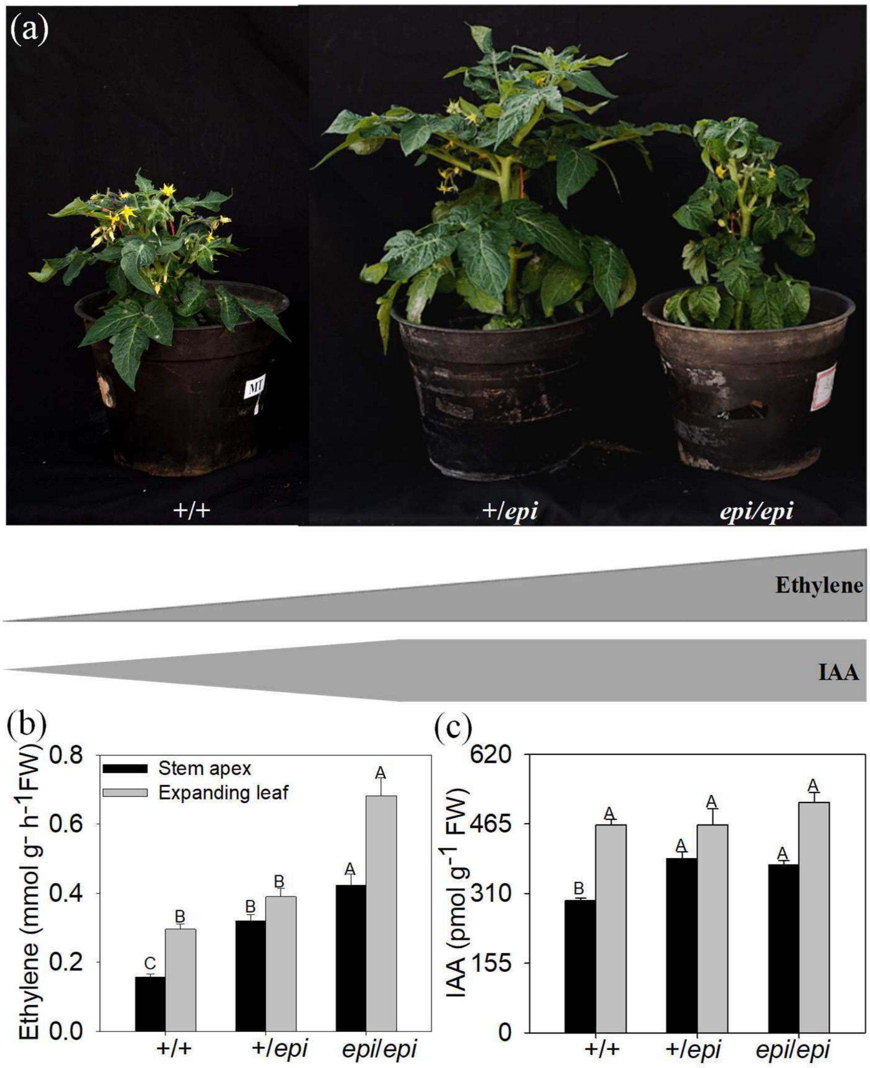

Figure 2: The +/*epi* hybrid has increased ethylene and IAA levels in the stem apex. (a) Evolution of ethylene and auxin levels in the stem apex in +/+, +/*epi* and *epi/epi*, (b) Ethylene determination and (c) IAA determination in the stem apex and expanding leaves in seedlings of +/+, +/*epi* and *epi/epi*. Same letters do not differ between in the same tissue by Tukey's test at 5%. All values are mean ± S.E.M. (n=100 seedlings).

Auxin is a hormone with potential to stimulate growth in many species, while ethylene has a dual role in plants, with potential to inhibit or stimulate growth (PIERIK *et al.*, 2006; LOVE *et al.*, 2009). In the +/*epi* hybrid, vigorous growth was accomplished possibly due to increases in auxin and ethylene levels in the stem apex in relation to wild-type (+/+). We therefore decided to calculate the ratio between auxin and ethylene in all three genotypes to estimate if different auxin:ethylene ratios could promote the production of larger leaves and increased plant size. This balance in +/*epi* hybrids was intermediary between +/+ and *epi/epi*, with a higher ratio in +/+ and lower value to *epi/epi* (Fig. 3).

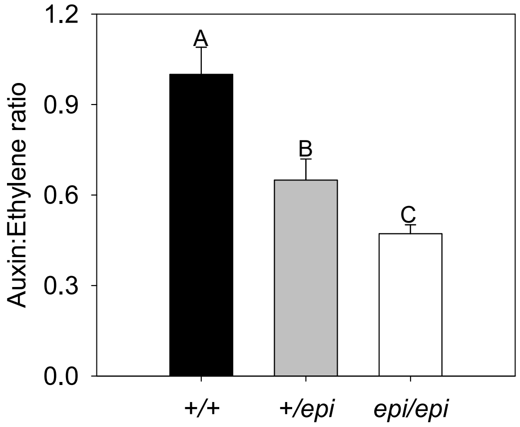

Figure 3: Intermediate auxin:ethylene ratio in +/*epi* hybrid in relation to their parents. Relationship between levels of auxin and ethylene in the stem apex of +/+, +/*epi* and *epi/epi*. Same letters do not differ between in the same tissue by Tukey's test at 5%. All values are mean ± S.E.M. (n=100 seedlings).

+/*epi* shows higher auxin sensitivity and expression of auxin in seedlings

We demonstrated that +/*epi* hybrid has increased level of auxin in apical stem (Fig. 2c), but besides higher auxin levels, auxin transport and/or sensitivity can determine phenotype observed in +/*epi*, such as larger cell number, more lateral branching and increase in xylem development (Chapter 1). In this sense, we applied different concentrations of NAA (0; 0.1 and 0.5 μM) in cotyledon explants to verify rhizogenesis. The +/*epi* hybrids showed significant high number of roots per explant (Figs. 4b and 4c), what is closely related to sensitivity to NAA. In relation to hypocotyl, we observed more elongation in +/+ compared to +/*epi* and *epi/epi* with increased levels of IAA (Fig. 4e).

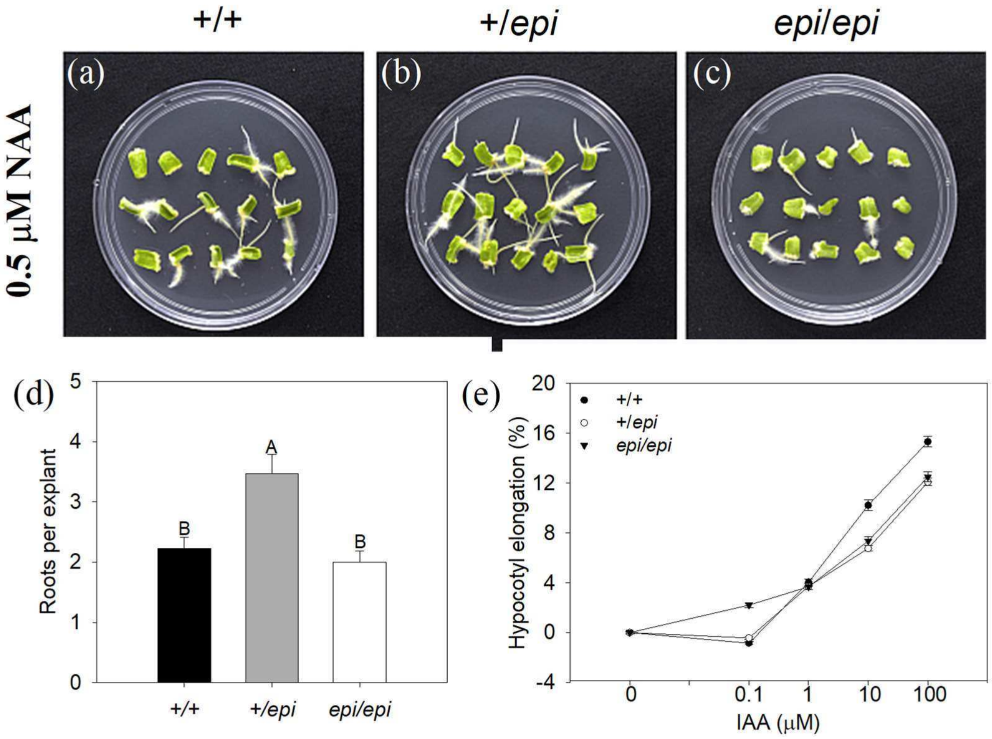

Figure 4: +/*epi* hybrids shows higher auxin sensitivity to rhizogenesis but not to hypocotyl elongation. (a-c) Representative plates from cotyledon explants incubated for 11 days in basal medium supplied with 0.5 mM NAA in +/+, +/*epi* and *epi/epi*, (d) Number of roots per explant in cotyledon explants in a medium supplied with 0.5 mM NAA (n=4 Petri dishes with 15 explants each) and (e) Hypocotyl elongation after incubation in crescent IAA concentration (n=10). The elongation of the hypocotyl is given as percentage of initial length after 24 hours of incubation in the solution containing the indicated concentration of indole acetic acid (IAA). Same letters do not differ between in the same tissue by Tukey's test at 5%. All values are mean ± S.E.M.

To confirm auxin response in +/*epi* hybrids, we crossed the *DR5::GUS* auxin reporter line with MT and the *epi* mutant to further analyze the hybrids in two situations: without application of external auxin (-IAA) and with exogenous auxin (+IAA). The intensity of GUS staining in the +/*epi* line showed an increase in response to exogenous IAA in relation to +/+, and without auxin increment we verified strong signal in hypocotyl, while no signal was detected in +/+ plants. This is consistent with our hypothesis of increased auxin sensitivity in +/*epi* hybrids (Fig. 5).

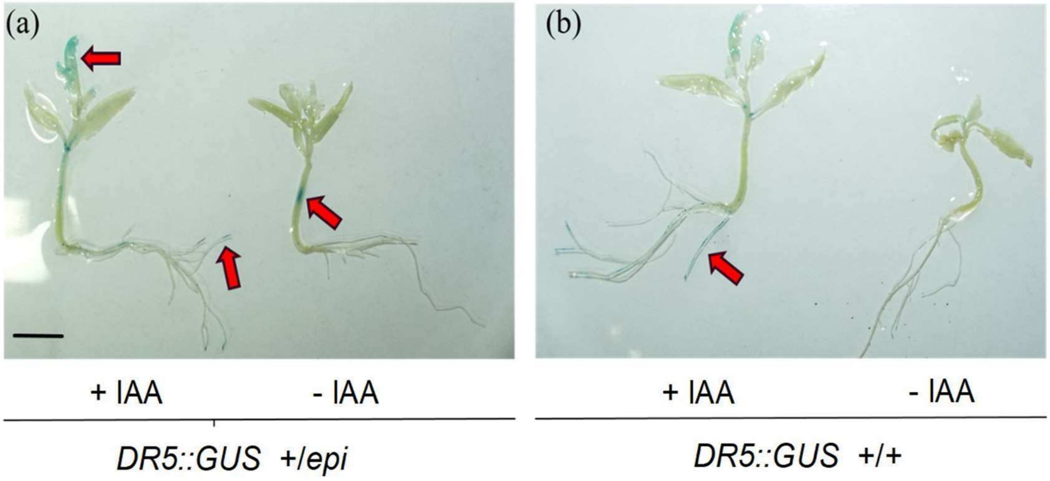

Figure 5: Increased auxin sensitivity in +/*epi* hybrids. Representative GUS staining of *DR5*::*GUS* in (a) +/*epi* and (b) +/+ seedlings without and with treatment with exogenous IAA. Seedlings of +/+ and +/*epi* were incubated for 3h in a mock or 20 µm IAA solution before GUS assay. Arrows point to GUS precipitate. Bar: 1cm.

Besides changes in auxins levels and sensitivity, we decided to investigate the intensity of ethylene and cytokinin response in seedlings from crosses between MT and *epi* with *EBS::GUS* and *ARR5::GUS*. In +/*epi* hybrid we found more intense *GUS* reporter expression in response to cytokinin and ethylene than in +/+ (Fig. 6). Strongest signal in *EBG::GUS* crossed with *epi* mutant in relation to +/+ was found, an expected result since *epi* mutant is an ethylene overproducer. On the other hand, high cytokinin levels in *epi* mutant has not previously been related, thus the result pertaining *ARR5::GUS* provides a potential novel avenue to explore the hormonal effects of *epi*.

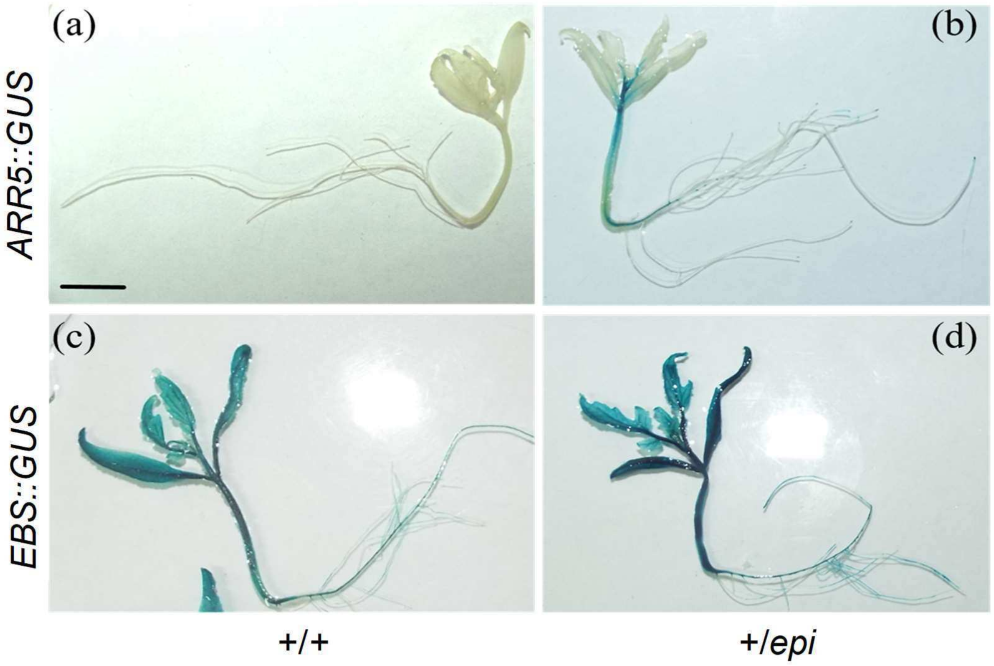

Figure 6: Ethylene and cytokinin expression pattern is increased in +/*epi* hybrid. Representative GUS staining of (a-b) *ARR5::GUS* and (c-d) EBS*::GUS* in +/+ and +/*epi* seedlings. Bar = 1cm.

DISCUSSION

Understanding the regulation of plant growth is an important aim in biology, especially in crop systems, since it is the basis for adaptive growth in diverse environmental conditions. Plant growth includes coordination between many regulatory pathways, such as sugar (LEÓN & SHEEN, 2003; LASTDRAGER *et al.*, 2014) and hormone signaling (VASEVA *et al.*, 2016). *epi* hybrids exhibit increases in RGR associated mainly to increases in NAR and structural changes (SLA), so we resolved to explore RGR in function to hormonal alterations.

Auxin biosynthesis and signaling pathways are well established (VANNESTE & FRIML, 2009). Auxin can be synthesized from tryptophan and transported to specific sites (LJUNG, 2013). Perception of auxin by cells is accomplished through F-box protein TIR1 (TRANSPORT INHIBITOR RESPONSE 1) protein (DHARMASIRI *et al.*, 2005). Once Aux/IAAs are degraded, the release of auxin response factor (ARFs) activates auxin-responsive genes leading to the auxin response (BARGMANN & ESTELLE, 2014). In the absence of auxin, auxin-responsive genes are not transcribed because repressors called

AUX/IAAs block the activity of ARFs (WOLTERS & JÜRGENS, 2009). Effectiveness of auxins as regulatory molecules depends of biosynthesis, conjugation (with sugars or amino acids), metabolic degradation and polar transport, all mechanisms involved in local concentration of auxin. In this study, we found increase in IAA levels in +/*epi* hybrid in the major site of auxin biosynthesis in plant, the apical meristem.

Plant hormone action is often organ-specific, but different cell types show different response to hormones (VASEVA *et al.*, 2016). In tomato plants three cell types with different responses to auxin and ethylene were identified, and in the first studies with the *epi* mutant Ursin (1989) demonstrated that *epi* possesses cell types different to +/+, because cell elongation occurs in response to auxin and ethylene, while in *epi/epi* the same cells elongate even if ethylene biosynthesis is blocked (FUJINO *et al.*, 1988*a*, 1989; URSIN & BRADFORD, 1989). The greater response to auxin in +/*epi* hybrids to rhizogenesis and in *DR5::GUS* assays seems to bear out the idea of individual cell types responding differently to hormones in a mechanism mediated by *epi*.

Mutant tomato plants with lesions in auxin signaling transduction components display altered plant phenotypes and growth. Down-regulation of Aux/IAA gene in tomato mutant *entire* and suppression of IAA9 expression by antisense RNA strongly increase auxin sensitivity in tomatoes, culminating in phenotypes characterized by increased lateral shoot number and plant height, similar traits observed in +/*epi* hybrids (WANG *et al.*, 2005). Moreover, the up-regulation of many auxin-responsive genes, such as AVP1, (GONZALEZ *et al.*, 2010), ARGOS and ANT, is associated with growth increase. Together, these results emphasize the importance of auxin signaling to increase plant vigor.

Ethylene biosynthesis occurs from methionine, that is converted to SAM (S-adenosylmethionine), and next to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, and finally to ethylene by ACC oxidase. Ethylene biosynthesis in vegetative tissues is triggered in response to environmental stimulus (LIN *et al.*, 2009). The most classical ethylene functions in plants are related to foliar abscission and senescence, and fruit ripening (PECH *et al.*, 2012). However, less information exists relating ethylene to vegetative growth, although believed that ethylene can affect positively vegetative growth through its role in the cell expansion and proliferation (PIERIK *et al.*, 2006). Subtle increases in ethylene levels have the potential to stimulate endoreduplication in cucumber hypocotyls, growth of intercalary meristems in flooded rice and cell division in the cambial meristem of *Populus* (LOVE *et al.*, 2009). Pierik *et al.* (2006) proposed a biphasic model to explain differential responses to ethylene, with low levels promoting growth and high levels inhibiting it. In our study, ethylene concentration at the stem apex shows an intermediary level between WT plants and the ethylene overproducing mutant, thus we hypothesize that this increase, associated to auxin accumulation in the stem apex promotes an optimal auxin:ethylene ratio, leading to increased plant growth.

Auxin-ethylene crosstalk is well described in the literature, since high levels of auxin stimulate ethylene production in plants in an effect mediated by gene transcription, mainly from the ACC oxidase family, and, conversely, it has been proposed that ethylene can also positively regulate auxin concentration in plant tissues (MUDAY; RAHMAN; BINDER, 2012). Negi et al (2010) verified greater transport of AIA in both roots and hypocotyl, in *epi*/*epi* in relation to +/+, suggesting that ethylene stimulates localized auxin biosynthesis and/or alters auxin transport. In agreement with this study, we observed that the *epi* allele in heterozygosity also increase auxin content in stem apex and sensitivity to auxin. Interestingly, besides the relation between ethylene and auxin, +/*epi* hybrids also show an increment in cytokinin levels, as revealed by the analysis of the *ARR5::GUS* cytokinin reporter lines (Figure 6).

Auxin and cytokinin ratios appear to coordinate many process in plant development that are in agreement with phenotypic changes observed in +/*epi* hybrids, (NOGUE *et al.*,2000; SWARUP *et al.*, 2007; SWARUP & PÉRET, 2012). Moreover, cytokinin increases auxin levels and transport, since cytokinin inhibits the formation of the inactive conjugate IAA-aspartate (YIP; YANG, 1986) and modulates auxin transport by regulating PIN transporters (EL-SHOWK *et al.*, 2013). In relation to ethylene, cytokinin shows the ability in increase stability of ACC synthase but, on the other hand, ethylene-responsive transcription factor EIN3 represses ARR5 (EL-SHOWK *et al.*, 2013). In seedlings of +/*epi* crossed to various *GUS* reporter lines, we verified higher expression of auxin, ethylene and cytokinin, an increase which has potential to increase plant growth. Unfortunately, we could not at this time measure cytokinin levels, thus possible changes in the levels of this hormone in +/+ and +/*epi* remain the matter of speculation.

CONCLUSION

We have shown that +/*epi* hybrids showed an intermediary auxin:ethylene ratio in stem apices compared to both parents. This ratio could perhaps represent the expression of an optimal balance between ethylene and auxin to promote more vigorous plant growth. We also showed that +/*epi* hybrids are more responsive to external auxin, probably by cell-specific changes to auxin perception. Together, these results could account for the increases in leaf cell number and thus in leaf area in +/*epi* hybrids.

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CHAPTER 3 - HETEROSIS IN TOMATO HYBRIDS BETWEEN MICRO-TOM AND MUTANT *epinastic* IS ASSOCIATED WITH INCREASED PHOTOSYNTHESIC RATES

INTRODUCTION

The superiority of hybrids has been exploited in agriculture since the 1930s when Shull performed the first experiments with hybrid maize (BLUM *et al.*, 2013; GOFF, 2011; GOULET; RODA; HOPKINS, 2017). Thenceforth, the use of heterosis in plant breeding is one of the most successful strategies, but the underlying molecular and physiological mechanisms that explain heterotic phenotypes are still poorly defined (GOFF, 2011; OFFERMANN; PETERHANSEL, 2014).

Various genetic mechanisms have been put forward to explain heterosis: dominance, overdominance and epistasis (GOFF, 2011; SCHNABLE & SPRINGER, 2013). Advances in genetic and genomic tools allowed new mechanisms to be proposed, such as those involving changes in methylation sites in hybrids (SHEN *et al.*, 2012) and the involvement of interference RNA and small RNA (GREAVES *et al.*, 2015; VENU *et al.*, 2014). Approaches to understand the molecular basis for heterosis include quantitative genetic methods for mapping heterotic quantitative trait loci (QTL) (MEYER *et al.*, 2010), comparative whole-genome studies (SHEN *et al.*, 2012), transcriptional profile, and proteome-based studies (VALE *et al.*, 2016) and, recently, a potential role for the study of metabolism in hybrids has been suggested, because of its central role in growth and development (MEYER *et al.*, 2012).

Increases in growth rate and yield, both traits common phenotypes in hybrid plants, result from metabolic changes, given that an increase in growth rate must be underpinned by changes in metabolism (BLUM *et al.*, 2013; KAEPPLER, 2012; SULPICE *et al.*, 2013). This higher performance in hybrids of several plant species is apparently not associated with specific metabolic pathways, which makes it hard to understand the physiological mechanisms that drive heterosis (KAEPPLER, 2012). Heterotic phenotypes in plants, it is a consensus, show an increase in organ size, associated with higher rates of cell division and differentiation (GROSZMANN *et al.*, 2015; KALVE; DE VOS; BEEMSTER, 2014). These larger organs are considered strong sinks and, therefore, require physiological alterations to increase the carbon and energy supply (BLUM *et al.*, 2013). In this sense, increased photosynthesis and/or alteration in the carbohydrate allocation are considered crucial elements to growth maintenance in the hybrids (BLUM *et al.*, 2013; OFFERMANN; PETERHANSEL, 2014).

Heterosis at the photosynthetic rate is expected to provide substrate for plant hybrid growth and biomass production (BLUM *et al.*, 2013), either through morphological changes in leaves, where photosynthesis take place, or changes in the carbon cycle (OFFERMANN & PETERHANSEL, 2014). In this sense, heterosis can be due to delayed leaf senescence, alterations in plant architecture to increase light interception (GARG *et al.*, 2006), increases in the photosynthetic rate associated to biochemical (FUJIMOTO *et al.*, 2012), structural or diffusive changes and increase in total leaf area (MEYER *et al.*, 2010). However, changes in photosynthetic parameters may not occur in all heterotic hybrid plants. For instance, hybrids between *sft* and WT, that show heterosis in yield, results from flowering delay and optimization of the plant architecture via changes in the florigen pathway (JIANG *et al.*, 2013; KRIEGER; LIPPMAN; ZAMIR, 2010). In addition to changes in photosynthesis, the increase in the performance of hybrid plants in several environments is partially explained by the increase in the expression of genes related to carbohydrate metabolism, stress mitigation, circadian rhythm control and sensitivity to nutritional status (BLUM *et al.*, 2013; GOFF, 2011) and the suppression of genes related to the basal defense pathways (GROSZMANN *et al.*, 2015). Another frequent feature changed in the expression of genes are related to the production, accumulation and transport of phytohormones (GROSZMANN *et al.*, 2015; SHEN *et al.*, 2012).

The discovery of physiological metabolic changes in hybrid plants can provide news target to enhance productivity in many crops, since hybrids can guide to identify targets for improvement of biological system (OFFERMANN & PETERHANSEL, 2014). Here, we are interested in explaining heterosis of tomato hybrids between cv. Micro-Tom and *epinastic*, an ethylene overproducing mutant, with respect to biomass and yield. Increases in biomass of a plant system reflects changes in physiology and biochemical parameters. Therefore, it is tightly linked to gas exchange reactions and metabolic state of the plant, manifested in the metabolite levels. Higher growth in hybrids is partially explained by increase in photosynthetic rate, associated with higher stomatal conductance and increased chlorophyll content.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of tomato cv MT (*Solanum lycopersicum* cv. MT) were germinated and plants grown in 3 L pots with commercial substrate fertilized with 4.5 g L^{-1} of NPK (4-14-8) and lime in a greenhouse located at Federal University of Viçosa, Brazil. Plants were watered regularly and fertilized weekly with a foliar solution (Biofert®). *epinastic* mutant in cv. MT was derived by an introgression of *epi* gene of original background VFN8 after a series of controlled backcrosses, resulting in a near-isogenic line (CARVALHO *et al.*, 2011). Hybrids were obtained through the crosses between *epinastic* and WT, where pollen of *epi* was used to pollinate emasculated flowers of WT. In results, hybrid plants we called +/*epi* (maternal parent WT and *epi* mutant as a pollen donor), +/+ to a WT genotype and *epinastic* mutant which we called *epi/epi*.

Measurements of leaf gas exchange and chlorophyll parameters

Gas exchange analyses were performed on the last newest fully expanded leaf of non-flowering plants using a portable infrared gas analyzer (IRGA) (Li 6400XT, Li-Cor, Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chamber and using 1000 µmol photons m^{-2} s^{-1} and 10% blue light. Measurements were obtained using the terminal leaflet of third fully-expanded leaf in five or six samples in plants of 37 days after germination (DAG) for each genotype. The measurements of net CO_2 assimilation rate (A_N), stomatal conductance (g_s), internal carbon concentration (C_i) and fluorescence parameters photochemical quenching (qP), non-photochemical quenching (NPQ), electron transport rate (ETR), actual PSII photochemical efficiency (ϕPSII), maximum chlorophyll fluorescence ratio (Fv'/Fm') were performed on attached leaves in greenhouse conditions; block temperature was set for 25 °C; CO_2 concentration of about 400 µmol mol^{-1} and humidity was kept around 70 % in a light-adapted state (from 8h a.m. until 11h a.m.). Dark respiration (R_D) and Fv/Fm were determined at the same leaves of photosynthesis analyses and environmental conditions but in a dark-adapted state (2 h after sunset). More details about gas exchange and chlorophyll parameters measurements can be obtained in (MARTINS *et al.*, 2014).

Estimation of mesophyll conductance (g_m), maximum rate of carboxylation (V_{cmax}) and maximum rate of carboxylation limited by electron transport (J_{max})

Photosynthetic light-response curves (A/C_i curves) were measured using 1000 µmol photons m^{-2} s^{-1} of irradiance, flow rate of 100 µmol s^{-1} and block temperature of 25°C. Measurements were started at ambient CO_2 concentration (C_a) of 400 µmol mol^{-1} and thereafter decreased in step changes until 100 µmol mol^{-1}. From there, it was increased until 1600 µmol mol^{-1}, as previously described (LONG; BERNACCHI, 2003). A/C_i curves were obtained using the fifth fully-expanded leaf from four different 40 days-old plants per genotype. We estimated maximum rate of carboxylation (V_{cmax}), maximum rate of carboxylation limited by electron transport (J_{max}), triose-phosphate utilization (TPU) and mesophyll conductance (g_m) according Sharkey *et al.* (2007).

Metabolic analyses in leaves tissue

As metabolism is sensitive to changing environmental conditions, our experiment was designed to keep environmental influences to a minimum by identifying the heterotic trait in early age of plants. Terminal leaflet of third fully-expanded leaf in 38 DAG were harvested in the middle of the light period. The samples were stored in N_2 liquid and dry-freezing until analyses. To quantify pigments, free amino acids, protein, soluble sugars and starch, samples were ground followed by ethanolic extraction (50% to 99% ethanol). In the supernatant, we quantified chlorophylls, soluble sugars and amino acids, and in the pellet, we quantified starch and protein in a methodology described by Cross *et al.* (2006). To perform metabolic profile analyses we used the same leaflet of biochemical analyses, adopting the extraction proposed by Lisec *et al.*, 2006 and metabolites identification in a gas chromatograph accoupled in a mass spectrometer (GC-MS). All metabolite levels were normalized through the mass.

Statistical analyses

All experiments were performed on completely randomized design, with the number of replicates varying in each experiment. The experimental units were one plant per pot. All data were subjected to analyses of variance (ANOVA) and means of each trait measured were analyzed by t-test at 5% level of significance.

RESULTS

Stomatal conductance, photosynthesis rate and dark respiration are higher in +/*epi* hybrids in cv. MT

The photosynthesis rate (*A*) per unit area and photosynthesis rate per mass were higher, 11% and 31%, respectively, in +/*epi* hybrid than in +/+ parental in saturating light intensity of 1000 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} at 37 DAG (Fig. 1a and 1b). The +/*epi* hybrid also showed increased stomatal conductance (g_s) (27%) (Fig. 1c) and in dark respiration (*R*d) (61%) (Fig. 1d). Although we observed clear differences between +/+ and +/*epi* in their g_s, we noticed unaltered stomatal index and stomatal density in both genotypes (Fig. 2a and 2b).

The parameters maximum photochemical efficiency of PSII in light (Fv'/Fm') and in dark (Fv/Fm), photochemical quenching (qP), non-photochemical quenching (NPQ), electron transfer rate (ETR), internal CO_2 concentration (C_i) and actual PSII photochemical efficiency (ϕPSII) did not show differences between both genotypes (Table 1), then we can conclude that photochemical phase of photosynthesis is similar between +/+ and +/*epi*. In others words, the increase in photosynthetic capacity in +/*epi* hybrids was not caused by a higher photochemical efficiency. Another independent experiment was carried out to measure gas exchange and chlorophyll a fluorescence in cv. MT, in a different season, and the same pattern of response between the +/*epi* and parent +/+ was observed for gas exchange (Table S1).

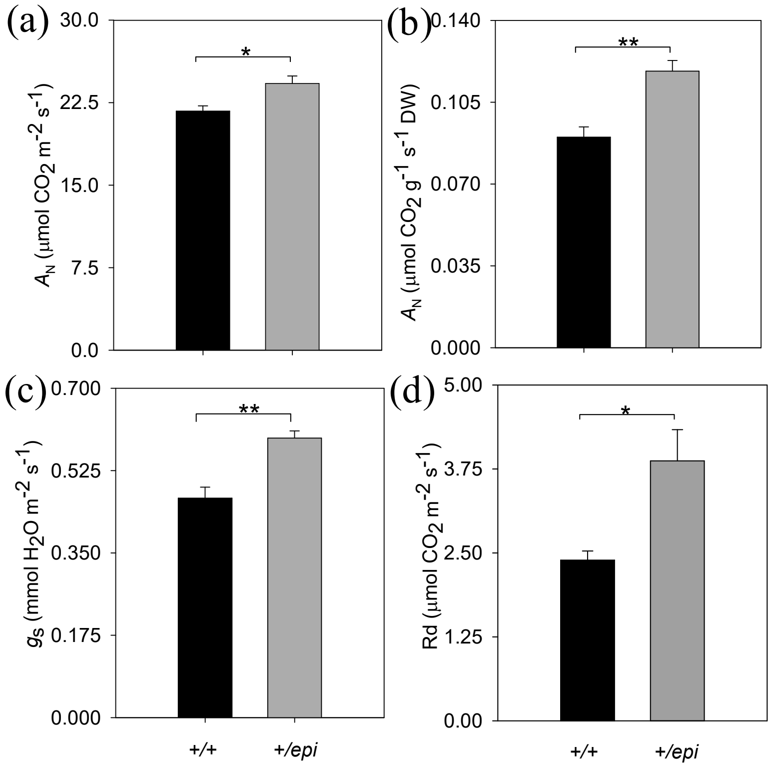

Figure 1: Gas exchange parameters in +/+ and +/*epi*. (a) Net CO_2 assimilation rate per area; (b) Net CO_2 assimilation rate per mass; (c) Stomatal conductance; (d) Dark respiration rate of +/+ and +/*epi* hybrid at 37 days-old plants. Values are mean ± S.E.M. * is a significant value at 5% level of significance, **is a significant value at 1% level of significance and NS is not significant by t test.

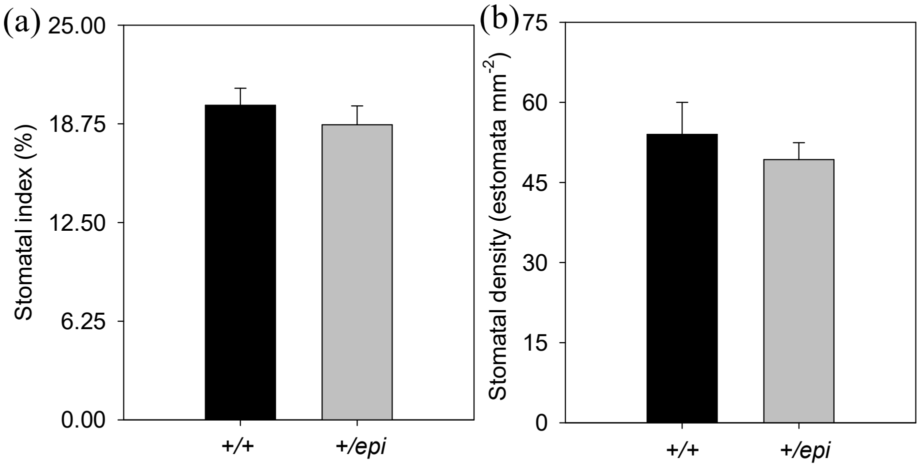

Figure 2: Stomatal characteristics are not affected in +/*epi* hybrid. (a) Stomatal index and (b) Stomatal density in +/+ and +/*epi* hybrid at 40 DAG. Values are mean ± S.E.M. * is a significant value at 5% level of significance, **is a significant value at 1% level of significance and NS is not significant by t test.

To further explore the causes for an increase in photosynthesis rate, we investigated if the higher CO_2 assimilation by +/*epi* is exclusively caused by increased physical diffusion through stomata. We performed A/C_i curves to estimate the effect of mesophyll CO_2 conductance (g_m), the maximum rate of Rubisco-mediated carboxylation (V_{cmax}), the maximum rate of carboxylation limited by electron transport (J_{cmax}) and TPU (triose-phosphate utilization) (Fig. 3). We did not find differences between V_{cmax}, J_{cmax} and TPU between +/+ and +/*epi* (Table 1), that is, plant carboxylation rates in both genotypes did not show differences in carboxylation limited by Rubisco, neither in ribulose biphosphate (RuBP) regeneration controlled by electron transport rate and triose-phosphate utilization, thus we conclude that there are no differences in Calvin cycle capacity between genotypes.

Table 1: Photosynthetic characterization of +/+ and +/*epi*. Values presented are means ± S.E.M obtained using the third fully-expanded leaf from 5-6 different plants per genotype to punctual analyses and fifth fully-expanded leaf from 4 different plants to parameters derived from A/Ci curve. Significant values $P<0.05$ by t test.

Parameters	+/+	+/*epi*	**P-value**
C_i	311.16 ± 5.001	323.41 ± 8.447	0.2474
C_c	131.92 ± 0.54	133.44 ± 8.73	0.9028
$F_{v'}/F_{m'}$	0.647 ± 0.007	0.662 ± 0.007	0.1909
F_v/F_m	0.8341±0.001	0.8245±0.009	0.3453
ϕPS2	0.421 ± 0.008	0.431 ± 0.013	0.5244
ETR	183.87 ± 3.426	188.13 ± 5.478	0.5277
qP	0.650 ± 0.015	0.650 ± 0.016	0.9896
NPQ	0.564 ± 0.026	0.523 ± 0.012	0.2018
J_{max}	132.59 ± 10.47	151.65 ± 11.04	0.2571
V_{cmax}	81.75 ±6.56	90.24 ±8.39	0.4557
VTPU	10.87 ± 0.58	12.11 ± 0.57	0.1784
g_m	0.0877 ± 0.01	0.0894 ± 0.01	0.9084

C_i (μmol CO_2 mol^{-1} air) - internal CO_2 concentration; C_c (μmol CO_2 mol^{-1} air) - chloroplastic CO_2 concentration; $F_{v'}/F_{m'}$ - maximum chlorophyll fluorescence ratio (light-state adapted), F_v/F_m - maximum chlorophyll fluorescence ratio (dark-state adapted); ϕPSII - actual PSII photochemical efficiency; ETR – apparent electron transport rate; qP -quenching photochemical; NPQ – quenching non-photochemical; J_{max} (μmol m^{-2} s^{-1}) - maximum capacity for electron transport rate based on C_c; V_{cmax} (μmol m^{-2} s^{-1}) - maximum carboxylation capacity based on C_c; VTPU (μmol m^{-2} s^{-1}) -triose-phosphate utilization; g_m (mol CO_2 m^{-2} s^{-1} bar^{-1}) - mesophyll conductance to CO_2.

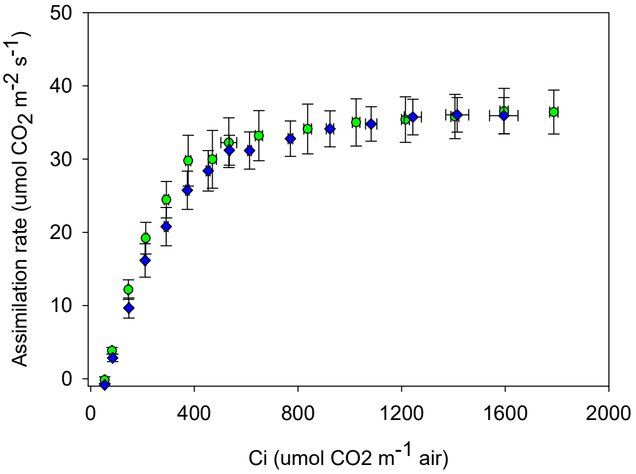

Figure 3: Net photosynthesis (A_N) curves in response to substomatal CO_2 concentration (Ci) in +/+ and +/*epi* plants. Values represented are means ± S.E.M obtained using the fifth fully-expanded leaf from four different plants per genotype (n=4). +/+ represented by blue diamond and +/*epi* by green circle.

The +/*epi* hybrid shows low metabolic and biochemical changes

Vegetative plant growth depends on production, storage, and use of carbon (C) and nitrogen (N) sources (GONZALEZ *et al.*, 2010). To better understand the variation observed in plant biomass, height and growth between +/+ and +/*epi*, a biochemical characterization was performed. Because many metabolites are diurnally regulated, all leaf samples were harvested in the middle of the light period. Firstly, we characterized levels of N compounds. Chlorophyll a (Table 2) and total chlorophyll content (Fig. 4a) per dry weight were higher in the +/*epi* hybrid than in the +/+ parental. For chlorophyll b and the ratio of chlorophyll a/b, we did not find differences between genotypes (Table 2). Regarding N metabolism, the levels of total amino acids (Fig. 4b) and total protein (Fig. 4c) were determined and we only found significant differences between +/+ and +/*epi* in total amino acids content, where +/e*pi* had greater values than +/+. To obtain additional information about C availability, the levels of sucrose, fructose, glucose and starch were determined (Fig. 5). The amount of glucose (Fig. 5a) and sucrose (Fig. 5c) remained unaltered between +/+ and +/*epi*, but we verified large increases in the levels of fructose (approximately 124%) (Fig. 4b) and in the levels of starch (88%) (Fig. 5d).

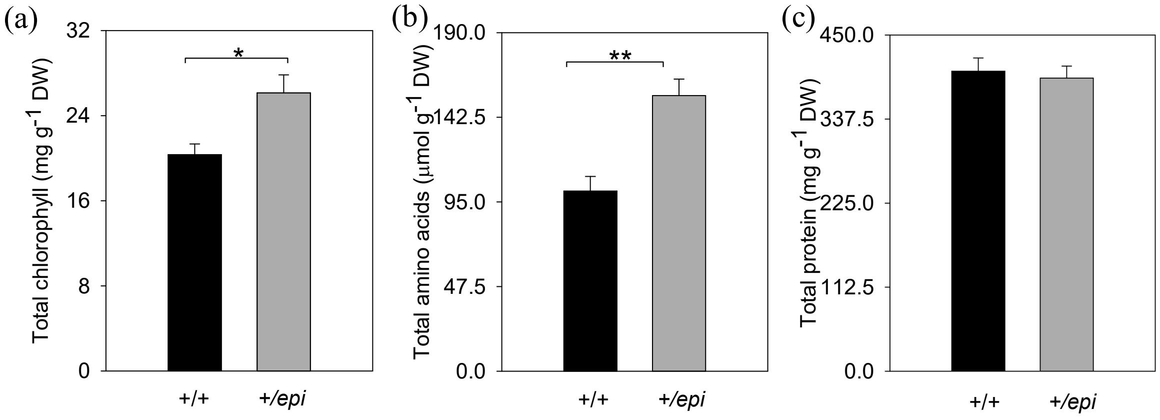

Figure 4: Nitrogen compound contents are altered in the +/*epi* hybrid leaves. (a) Total chlorophyll (*Chl* a + *Chl* b); (b) Total amino acids and (c) Total protein content of +/+ and +/*epi* hybrid at 37 DAG. Values are mean ± S.E.M. * is a significant value at 5% level of significance, **is a significant value at 1% level of significance by t test. (n=7)

Table 2: Discrimination in chlorophylls content in leaves of +/+ and +/*epi* plants. Values presented are means ± S.E.M obtained using the third fully-expanded leaf from 7 different plants per genotype at 38 DAG. Significant values P<0.05 by t test.

Parameters	+/+	+/*epi*	**P-value**
Chlorophyll a (mg g^{-1} FW)	**12.37±0.81**	**17.15±1.38**	**0,015**
Chlorophyll b (mg g^{-1} FW)	7.87±0.25	8.33±0.51	0,439
Ratio chlorophyll a/b	1.56±0.09	2.11±0.25	0,091

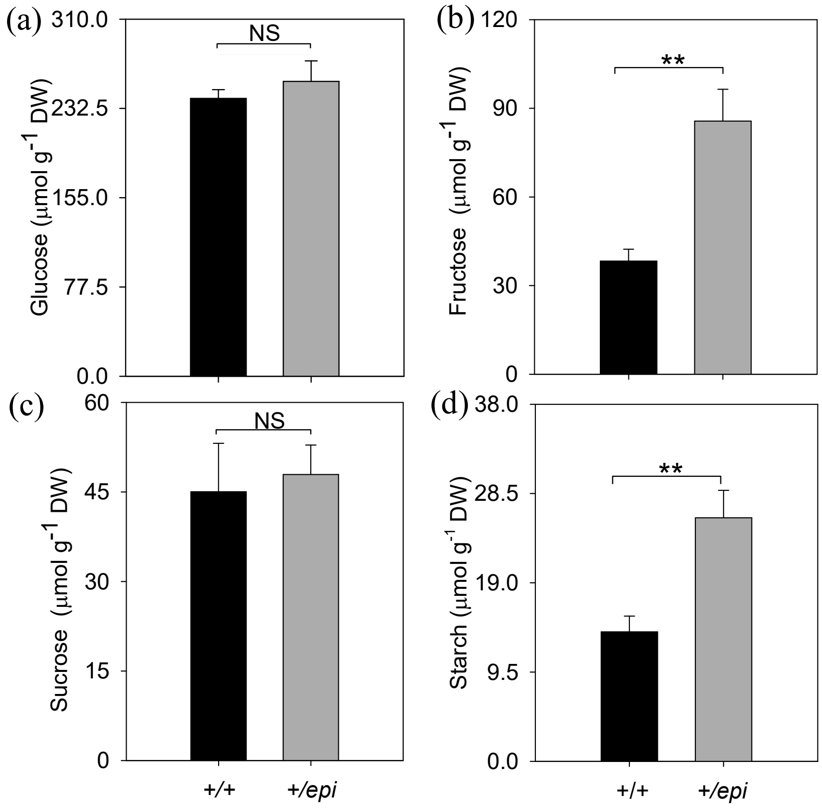

Figure 5: Carbohydrates availability in leaves of +/+ and +/*epi* plants. (a) Glucose, (b) Fructose, (c) Sucrose and (d) Starch content in +/+ and +/*epi* hybrid at 37 DAG. Values are mean ± S.E.M. * is a significant value at 5% level of significance, **is a significant value at 1% level of significance and NS is not significant by t test(n=7).

To analyze if metabolic profile was common in both genotypes, +/+ and +/*epi*, we next extended this study to the metabolomic level by GC-MS analyses. Among 80 successfully annotated compounds, 11 were statistically different between +/*epi* and +/+, (Fig. 6) with positive and negative changes in F_1 hybrids observed for individual metabolites, but no differences detected between +/+ and +/*epi* with respect to number of metabolites. The levels of TCA cycle intermediates succinate and fumarate were higher in +/*epi* hybrid; amino acids analyzed revealed increases in the levels of glycine and homoserine, showing a decrease in +/*epi* hybrid in relation to +/+ (Fig. 6). Furthermore, +/*epi* hybrid showed increase in relative content of cinnamic acid and glycerol and decrease in raffinose, arabinose and lactulose levels.

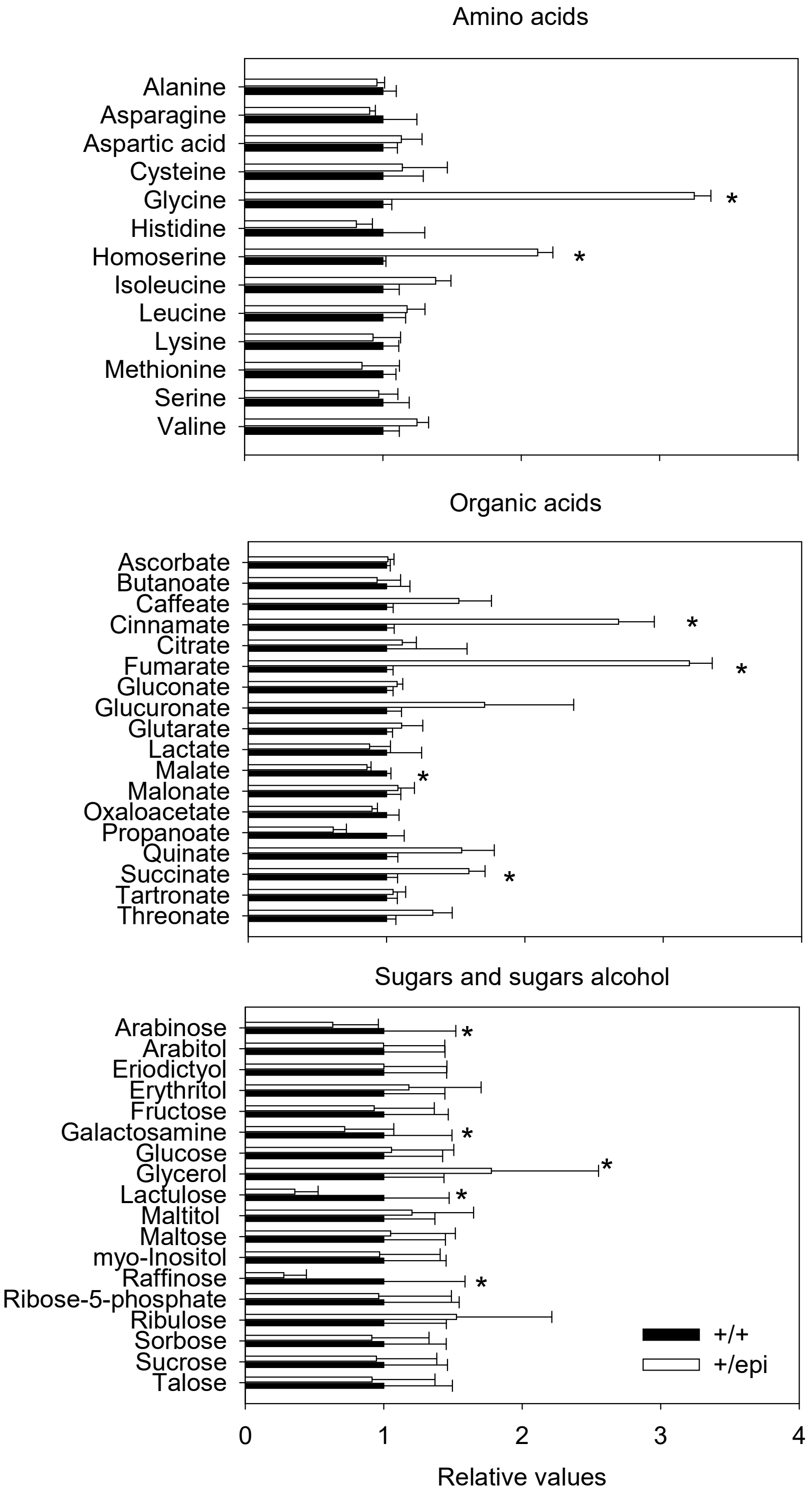

Figure 6: Metabolic alterations identified in GC-MS analyzes in +/*epi* hybrids in relation to +/+ plants. Relative content of organic acids, amino acids and sugars in +/*epi* and +/+ at 37 DAS. Values are mean ± S.E.M. * is a significant value at 5% level of significance (n=6 for +/+ and 7 for +/*epi*)

DISCUSSION

The rate of plant growth is the result of photosynthetic carbon assimilation, developmental cell programs that control carbon conversion into biomass and biomass allocation control (SULPICE *et al.*, 2010, 2013). Assimilation is closely linked to metabolism and plant growth since it provides carbon skeletons for the synthesis of organic molecules. It is proposed that many factors can contribute to higher photosynthesis in plants: increased photosynthesis rate per unit leaf area, increased in photosynthetic area and prolonged photosynthetic period (OFFERMANN & PETERHANSEL, 2014). In fact, heterotic hybrids from crosses of different species showed different mechanisms to increase photosynthesis (BLUM *et al.*, 2013; FUJIMOTO *et al.*, 2012; TOLLENAAR; AHMADZADEH; LEE, 2004). Our results indicate that *epi* hybrid in cv. MT shows two potential mechanisms resulting in higher lifetime carbon gain: increased photosynthetic area by larger leaves and increased photosynthesis per leaf area.

Photosynthetic rate at a whole-leaf level can be analyzed in terms of photochemical and biochemical parameters. The photochemical phase describes electron flow throw photosystem II and I, which generates reducing power and ATP, while biochemical phase consists of reactions to carbon reduction in a Calvin cycle, starting with Rubisco activity. We explored higher A_N in +/*epi* in three ways: stomatal conductance, Calvin cycle capacity and chlorophyll fluorescence parameters. We did not find differences in Calvin cycle capacity, nor in in chlorophyll fluorescence. On the other hand, stomatal conductance was increased in +/*epi* in relation to +/+. The observed effects in g_s without changes in stomatal index and stomatal density indicate that capacity of CO_2 fixation in +/*epi* is mainly controlled by physical limitation, especially in stomatal aperture. One of the factors that strongly regulates stomatal closure is the presence of ABA and it was demonstrated in Arabidopsis that stomatal closure induced by ABA is impaired by ethylene (TANKA *et al.*, 2006), thus, overproduction of ethylene in +/*epi* in relation to +/+ could explain higher stomatal conductance.

ATP produced through mitochondrial respiration and photosynthesis provides energy to increase plant biomass (ARAÚJO; NUNES-NESI; FERNIE, 2011; NUNES-NESI; ARAÚJO; FERNIE, 2011). Despite plant growth being closely related to increases in photosynthesis per leaf area and low respiration rates, we observed higher dark respiration (R_d) in +/*epi* than in +/+. Respiration can be divided into growth respiration, related to the

CO_2 released to provide energy for the biosynthesis of new biomass, and maintenance respiration, which includes costs related to proteins turnover and preservation of cell function (SMITH & DUKES, 2013). This feature can reveal higher metabolic activity of the hybrid at night, since mitochondrial respiration is important to providing intermediates to other pathways and energy for biosynthesis (NUNES-NESI; ARAÚJO; FERNIE, 2011). We propose that higher leaf dark respiration demonstrated here are supported by the increased abundance of starch, a transient source of carbon accumulated during the day and remobilized to support metabolism and growth at night. Analyses of daily cycle of starch and soluble sugars are necessary to confirm this hypothesis.

Lower biochemical changes occur in +/*epi* in relation to +/+

The metabolic and biochemical profile of +/+ and +/*epi* plants, including relative levels of metabolites and quantification of chlorophylls, protein, amino acids, starch and soluble sugars, were analyzed to comprise key metabolic pathways and/or process during the formation of vegetative plant biomass. Despite the expected increase in the levels of metabolites in the hybrid in relation to parental to supply plant growth, low variation in the profile of metabolites was found and this result that agree with the ones for heterotic corn hybrids (LISEC *et al.*, 2011). Lower variation in hybrid metabolite levels could be linked to higher biomass production by optimal metabolite levels theory, that could result in an optimal flux through metabolic system resulting in a faster growth, or other possible hypothesis is that physical and structural changes verified in leaves are the major contributor to plant growth (BLUM *et al.*, 2013; GOFF, 2011; LISEC *et al.*, 2011).

Many studies work the complex relationship between changes in metabolism and identification of metabolites states associated with higher biomass. In this sense, identification of differences in enzymatic activity between plants with different growth behavior (GHAFFARI *et al.*, 2016; SULPICE *et al.*, 2010), metabolic (MEYER *et al.*, 2012; SULPICE *et al.*, 2013) and proteomic profile (VALE *et al.*, 2016) are performed to identify points of plant regulation associated with biomass production, but seem not to have a consensus in levels of metabolites associated with biomass in many species and environmental condition, especially because metabolites levels depend of growth condition. In general, hybrid plants show negative correlation between central metabolites with biomass

(BLUM *et al.*, 2013; GOFF, 2011; SULPICE *et al.*, 2013), supporting the theory of higher source sink to promote growth, but here we find negative contribution of sucrose to plant biomass in +/*epi* and positive correlation to fructose, which was the only soluble sugar with significant difference between +/+ and +/*epi*.

Biochemical changes in carbohydrates verified in +/*epi* in relation to +/+ are closely linked to increases in photosynthesis rate. Increased levels of starch are associated with higher photosynthetic rate in +/*epi* hybrids, since sucrose and starch are the major carbohydrates produced by photosynthesis. In many studies, starch is negatively related to biomass increase (GONZALEZ *et al.*, 2010; SULPICE *et al.*, 2010, 2013), different of our study, where starch is positively associated with growth. However, in these studies starch is analyzed at the end of light period while in ours in middle light period. Thus, at night starch is consumed to support growth, in a negative correlation with biomass production, and in day starch accumulation is in a positive co-relation with growth because more starch accumulated during the day, more carbohydrate available to growth at night.

In relation to fructose, it is derived from sucrose and curiously, similar levels of sucrose and glucose occurred in +/+ and +/*epi* plants, while fructose levels are increased on leaves. Fructose-6-phosphate and glucose-6-phosphate are interconverted by a reaction reversible catalyzed by phosphogluco isomerase (PGI). We observed storage of fructose in +/*epi* hybrid, we can suppose that altered activity of PGI occurs in +/*epi* hybrids. More studies, such as PGI activity and level of expression of gene codifying PGI are necessary to clarify this question.

Total protein content did not differ between both genotypes, +/+ and +/*epi*. In fact, some studies have proposed a negative correlation between protein content and growth (GOFF, 2011; VALE *et al.*, 2016), but here we can hypothesize that protein metabolism in hybrid involves selective protein synthesis more than optimization of energy production by reducing protein metabolism, a common situation in hybrids of other species (BLUM *et al.*, 2013; VALE *et al.*, 2016). Differences in amino acids content without changes in total protein content in +/*epi* hybrid suggest that in +/*epi* there is a pool of free amino acids. Plants can accumulate amino acids and others metabolites as compatible solutes, which serve to store nitrogen that can be used in low nitrogen condition, to protect plants from photoinhibition, reactive oxygen species and other kinds of environment stresses. Our experiment was performed in optimal conditions of light, temperature and nutrition, ergo amino acids storage in hybrid can indicate that +/*epi* hybrid shows better performance under stress condition growth regarding +/+, although we did not examine hybrid in stress condition to prove it.

CONCLUSION

This chapter describes photosynthetic, metabolic and growth parameters for +/*epi* hybrids. The results presented here reinforce the idea that photosynthetic capacity is the key to crop yield in hybrids plants. It was demonstrated that the establishment of leaf area, stomatal conductance and total chlorophyll amount are positively correlated with photosynthesis and plant growth in +/*epi*. However, photosynthesis alone does not explain the differences in +/*epi* increased growth. Although our experiments were restricted to heterosis in the vegetative phase, there is a suggestion of a positive correlation between increased in photosynthetic activity, biomass production and heterosis in reproductive structures of the plants, what in turn contributes to higher yield in crop and non-crops.

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SUPPLEMENTAL INFORMATION OF CHAPTER 3

Table S1: Gas Exchange and photochemical efficiency of +/+ and +/*epi*. Values obtained from 5-7 samples three days before anthesis. ** indicates 1% level of significance and * 5% level of significance by t-test.

Property	+/+	+/*epi*
A_N (μmol CO_2 m^{-2} s^{-1})	**18.37**	**25.08****
R_D (μmol CO_2 m^{-2} s^{-1})	2.03	2.17
g_s (mol H_2O m^{-2} s^{-1})	**0.4**	**0.61***
C_i (μmol CO_2 mol^{-1} air)	**256.62**	**310.97***
F_v'/F_m'	0.63	0.61
ϕPS2	0.39	0.41
ETR	187.14	181.39
qP	0.67	0.67
NPQ	2.72	2,58

CHAPTER 4 – AGRONOMIC ASPECTS OF *epinastic* HYBRIDS

The use of heterosis in plant breeding is one of the most successful crop breeding strategies (SCHNABLE & SPRINGER, 2013; FU *et al.*, 2014; OFFERMANN & PETERHANSEL, 2014). Combination of genes in F_1 hybrids generates a superior phenotype in many traits in relation to the parental types, such as increase in biomass production and growth rate (BARTH *et al.*, 2003; MEYER *et al.*, 2004, 2010; STEINFATH *et al.*, 2010), larger vegetative organs (GOFF, 2011; FUJIMOTO *et al.*, 2012; BLUM *et al.*, 2013) and higher performance in different environmental conditions (LIPPMAN & ZAMIR, 2007). Increased biomass production in the vegetative phase results in heterosis in the reproductive structure of the plants (CHEN, 2010; FUJIMOTO *et al.*, 2012).

In the previous chapters, we described vegetative and reproductive traits and physiological aspects in *epinastic* (*epi*) F_1 hybrids in in the genetic background of tomato cv. Micro-Tom, a model for physiology and molecular biology (CARVALHO *et al.*, 2011). An important feature in a heterosis from an agricultural point of view is that the response can change depending on the environment and genetic background (LIPPMAN & ZAMIR, 2007; KRIEGER *et al.*, 2010). It is therefore recommended that hybrids performance should be tested over many years in various environments and growth conditions, including changed spacing, nutritional supply and others. In the case of a mutated allele, as in *epi*, it has potential use in plant breeding if it is consistently able to promote heterosis in diverse genetic backgrounds (KRIEGER *et al.*, 2010).

Heterosis can be estimated in three different ways: mid-parent heterosis, when performance of hybrids is better than the average of its two parents but worse than one parental (SPRINGER & STUPAR, 2007; SCHNABLE & SPRINGER, 2013); better-parent heterosis, when performance of hybrids to a specific trait having the best value (MEYER *et al.*, 2004; SPRINGER & STUPAR, 2007; SCHNABLE & SPRINGER, 2013), and standard heterosis, when performance of hybrids is over than a standard commercial hybrid (SHARMA *et al.*, 2016).

In tomato plants, traits explored by genetic breeders are diverse, as improved resistances to pests and diseases, tolerance to low water supply and better quality, but increased in yield is the main aim in plant breeding (BAI & LINDHOUT, 2007; KRIEGER *et al.*, 2010). There are multiple yield components in tomatoes: number of fruits per plant, fruit weight and fruit sugar content (brix value). If a tomato plant shows combined improvements in yield and brix value, it is a good indicative of potential for broad agricultural application. To explore if the *epi* heterozygous mutant affects tomato traits to create a new optimum for fruit yield, we analyzed *epi* hybrids in two cultivars with determinate growth, VFN8 and CNPH69. Together, our results suggest that *epi* hybrids show different forms of heterosis depending on the genetic background, and therefore, have potential to be explored in crop breeding to increase fruit yield.

MATERIAL AND METHODS

Plant material and growth conditions

Hybrids were obtained through the crosses between *epinastic* in its original cv. VFN8 background and its corresponding WT, and between *epinastic* in cv. VFN8 background and CNPH069, where pollen of *epi* was used as pollen donor.

For the assays performed in greenhouse conditions, seeds of tomato cv. VFN8 (*Solanum lycopersicum* cv. VFN8), hybrid +/*epi* and homozygous *epi* were germinated and plants grown in 6 L pots with commercial substrate fertilized with 4.5 g L^{-1} of NPK (4-14-8) and lime (5 g L^{-1}) in a greenhouse located at Federal University of Viçosa, Brazil. Plants were watered regularly and fertilized weekly with a foliar solution (Biofert®).

For assays performed in field conditions, tomato hybrids and their parents, in cv. VFN8 and cv. CNPH069, were grown at the experimental fields of the Centro Nacional de Pesquisa de Hortaliças (CNPH, EMBRAPA), Brasília, DF, under the supervision of Dr. Leonardo Boiteux. Cultural practices, including soil improvement, fertilization, irrigation and control of pests and diseases were consistent with established farming practices of the center and with the tomato cultivar used.

Plant biometric parameters

Leaves, stems and roots of the genotypes were collected in paper bags and oven-dried at 60°C for 48 h to dry mass measurement by analytical scales. Additional measurements were determined in specific phases of growth: plant height, fruit size, total soluble solids content in fruits (Brix) and frequency of green and ripe fruits. Total soluble solids content was assessed using a digital refractometer (PR-101, Atago, Tokyo, Japan) in three fruits per plant.

Measurements of leaf gas exchange and chlorophyll parameters

Gas exchange analyses were performed on the last newest fully expanded leaf of non-flowering plants using a portable infrared gas analyzer (IRGA) (Li 6400XT, Li-Cor, Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chamber and using 1000 μmol photons m^{-2} s^{-1} and 10% blue light. Measurements were obtained using the terminal leaflet of fifth fully-expanded leaf in three or five 50 days-old plants (39 DAS). The measurements of net CO_2 assimilation rate (A_N), transpiration rate (E), stomatal conductance (g_s), internal carbon concentration (C_i) and light fluorescence parameters photochemical quenching (qP), non-photochemical quenching (NPQ), electron transport rate (ETR), actual PSII photochemical efficiency (ϕPSII), maximum chlorophyll fluorescence ratio ($F_{v'}/F_{m'}$) were performed on attached leaves in greenhouse conditions; block temperature was set for 25 °C; CO_2 concentration of about 400 μmol mol^{-1} and humidity was kept around 70 % in a light-adapted state (from 8 a.m. until 11 a.m.). Dark respiration (R_D) and F_v/F_m were determined at the same leaves of photosynthesis analyses and environmental conditions but in a dark-adapted state (2 h after sunset). More details about gas exchange and chlorophyll parameters measurements can be obtained in (MARTINS *et al.*, 2014).

Leaf anatomy

Terminal leaflets of fully-expanded leaves were hand cut from the widest part of the leaf using a razor blade and storage in formaldehyde-acetic acid- ethanol (FAA) 70% solution for 24h. After, leaf tissue sample were transferred to ethanol 70% for 24h. Tissues samples were dehydrated in a progressively increasing ethanol concentrations (of 70% to 100%) and later embedded in historesin (Leica), sectioned by ultramicrotome Leica (5 μm) and stained with toluidine blue 5%. Transversal sections were viewed using a photomicroscope (Olympus AX70) and images captured using a AxioCam Hrc digital camera (Zeiss). Four subsamples for each cross-section were selected for measurement of the area occupied by the upper and lower epidermis, the palisade and spongy parenchyma. We also measured the leaf thickness in 5 random places for each cross-section of the leaf. The mean values of all the anatomical measurements were calculated per leaf sample, and subsequently for each genotype. Measurements of traits in transverse section were performed on ImageJ (University of Wisconsin-Madison).

Heterosis measurement

Expressing of heterosis was calculated as previously described (MEYER *et al.*, 2004). Concisely, mid-parent heterosis (MPH) is (mean F_1 − mean *P*)/mean *P* and better-parent heterosis (BPH) is (mean F_1 − maximum *P*)/mean best *P*, both values in %.

Statistical analyses

Experiments in greenhouse-conditions were performed on completely randomized design, and in field-conditions in randomized block design, with the number of replicates varying in each experiment. Values of each trait measured were submitted to variance analyses (ANOVA) and compared by Tukey's test when we were compared more than two genotypes, or all values were compared by t test when we compared two genotypes.

RESULTS

epinastic hybrids increase growth in its original background in controlled conditions

Hybrid plants and their parents in the cultivar VFN8 were grown in the greenhouse in optimal conditions of water, light and nutrients, to verify whether heterosis occurs in F_1 generation in this background. In all analyzed organs, leaf, stem and roots, there was a higher mass production in the hybrid compared to both the parental mutant *epi/epi*, and the wild-type parental, cv VFN8 (+/+). The hybrid +/*epi* showed 12% higher total dry mass relation to +/+ and 41% higher in relation to *epi/epi* (Fig. 1a). As for the visual aspect of plants, we observed a subtle increase in height in +/*epi* hybrids in relation to both parents (Fig. 1b), although this difference turned out not to be significant statistically. Also, height to first inflorescence in +/*epi* (38.1 cm ± 0.9) was not different than in +/+ (38.8 cm ± 2.2).

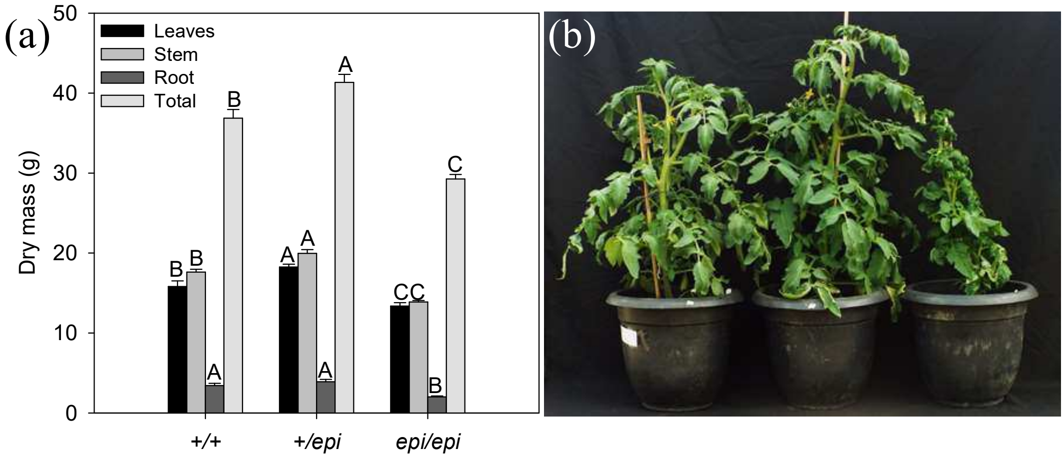

Figure 1: Heterosis in vegetative growth in +/*epi* hybrid. (A) Dry mass of leaves, stem, root an total in 50 days-old +/+, +/*epi* and *epi/epi* plants (n=4-6; means ± S.E.M, same letters do not differ by Tukey's test at 5%), and (B) growth aspect of representative +/+ (left), +/*epi* (center) and *epi/epi* (right) 50-days old plants grown in greenhouse conditions.

Higher biomass production in F_1 hybrids compared to their parents often is associated to an improvement in organ size, specially leaves, by increased cell numbers. To provide the energy necessary for plant growth, it is frequently observed an increase in photosynthesis rate or improved carbon allocation (GOFF, 2011; BLUM *et al.*, 2013; OFFERMANN & PETERHANSEL, 2014). In plants of tomato cv. MT, increased biomass production is coupled to higher net photosynthesis rate, increased starch storage and thicker palisade parenchyma, but in cv. VFN8 the hybrid +/*epi* seems not to have changes in carbon assimilation in relation to either parent, +/+ and *epi/epi*, in two independent assays performed in different seasons and developmental stages (Table1, Table S1), but respiration rate decreased slightly in +/*epi* hybrid (Table 1). In mature and fully-expanded leaves we verified increased thickness in palisade mesophyll in +/*epi* than in +/+ (Fig. 2), similarly to the hybrids in cv. MT (Chapter 2).

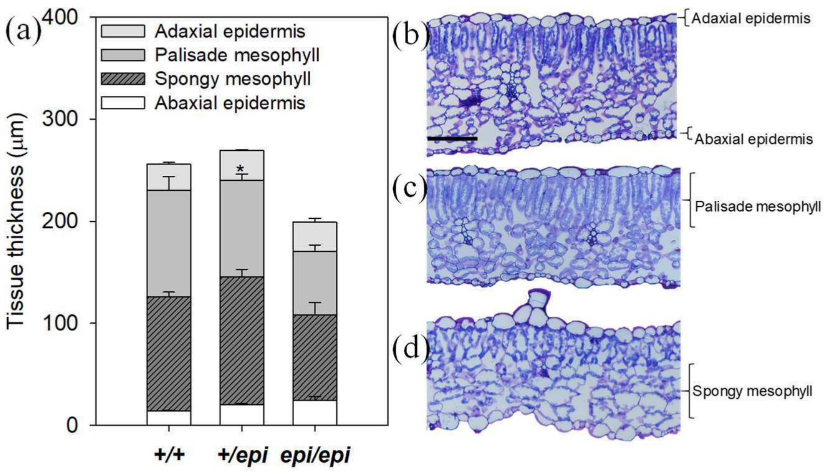

Figure 2: Palisade mesophyll thickness is increased in +/*epi* hybrid. (a) Tissues thickness on fifth fully-expanded leaf in +/+, +/*epi* and *epi/epi* plants (n=6; means ±S.E.M, * indicates significant different from +/+ by t test at 5%), and representative leaf cross-section of (b) +/+, (c) +/*epi* and (d) *epi/epi*.

Table 1: Gas exchange and photochemical parameters in *epinastic* hybrids and their parents. Measurements were taken at 39 DAS in the last fully-expanded leaf (n=3 to +/+ and 5 to +/*epi*). Values are mean ± S.E.M. Same letters do not differ by Tukey's test at 5%

Parameters	**+/+**	**+/*epi***	***epi/epi***
A_{N}	22.9±0.65 A	21.8±1.14 A	15.9±2.05 A
R_{D}	1.1±0.14 AB	0.56±0.10 B	1.47±0.17 A
g_{S}	0.49±0.06 A	0.45±0.02 A	0.35±0.05 A
F_v/F_m	0.66±0.02 A	0.63±0.01 A	0.63±0.01 A
ϕPS2	0.39±0.01 A	0.33±0.02 A	0.35±0.03 A
qP	0.58±0.03 A	0.52±0.03 A	0.55±0.04 A
NPQ	0.58±0.12 A	0.52±0.08 A	0.55±0.12 A
ETR	171.50±4.15 A	144.47±8.47 A	152.68±11.06 A

Heterosis in mature F1 hybrids +/epi resulted in higher fruit yield, (Table 2), although brix-value and fruit diameter do not show differences between +/+ and +/epi. In summary, epinastic hybrids in VFN8 results in heterosis in vegetative and reproductive stages, but lower than in cv. MT (Chapter 2).

Table 2: Fruits parameters in *epinastic* hybrid (cv. VFN8) and their parents. Measurements determined from 5 red fruits per plant (n=3 to +/+ and 5 to +/*epi*). Values are mean ± S.E.M. Same letters do not differ by Tukey's test at 5%.

Parameters	+/+	+/*epi*	**P-value**
Ripe fruit mass (g)	**99.66 ± 12.05**	**130.1 ± 9.12**	**0.044**
Brix value (%)	5.64 ± 0.14	5.88 ±0.14	0.332
Fruit diameter (cm)	20.69 ± 0.87	20.78 ± 0.59	0.939

epinastic hybrids in field-conditions showed increase fruit yield

We next evaluated +/*epi* hybrids and their parents in a field experiment and the traits analyzed were restricted to reproductive stage, since fruit yield and brix, and their product, are the most important features in processing tomato farming (BERNACCHI *et al.*, 1998). In the field, we explored heterosis in two genetic backgrounds, VFN8 and CNPH069.

We consistently detected significant heterosis in +/*epi* hybrids for both backgrounds, VFN8 and CNPH69 in relation to yield of ripe fruits (Fig. 3). Hybrids shower higher number of fruits at the same age (Figs. 3a, 3b, Table 3, Table 4) than their parents, and more yield occurred in hybrids on CHNP69 (Fig. 3d) than in VFN8 (Fig. 3c). It is a good indicative that gene *epinastic* in heterozygosity provides increase in fruit yield.

Hybrids from crosses between *epinastic* and CNPH69 exhibited significant improvement in number of fruits, ripe fruit weight and total fruit weight in relation to the mean values observed in the parents and significant increase only in number of fruits in relation to +/+ parents (Table 3), which we considered the better parent because *epi/epi* shows an aberrant phenotype and development. Therefore, although we observed an increase in yield traits in +/*epi* hybrids in cv. CNPH69, there is predominance of mid-parent heterosis.

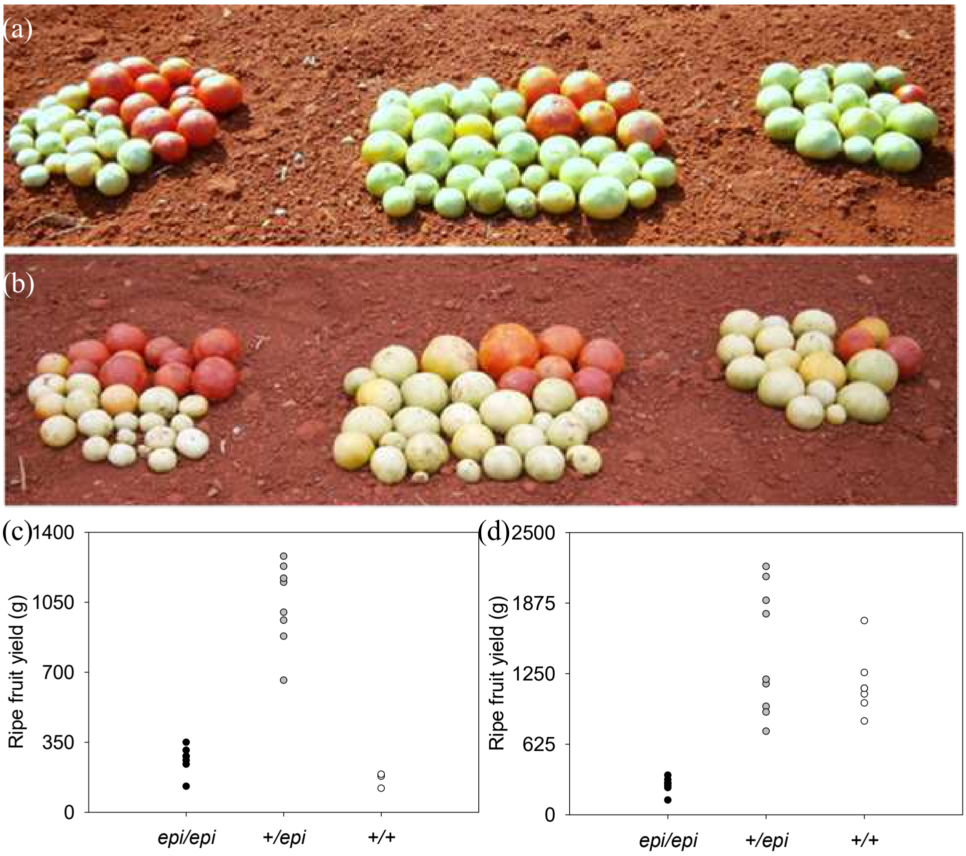

Figure 3: Fruit yield increase in +/*epi* hybrids for VFN8 and CNPH69. (a) Fruit yield in cv. VFN8 and (b) CNPH69, from left to right +/+, +/*epi* and *epi/epi*, (c) Ripe fruit yield in *epinastic* hybrids and their parent in cv. VFN8 and (d) in CNPH69.

Table 3: *epinastic* hybrids in cv. CNPH69 shows largely mid-parent heterosis. % heterosis in relation to mid-parent and better-parent (n=7 to +/+, 9 to *epi/epi* and 11 to +/*epi*) * indicate significant value at 5% level significance by t test, ^{NS} indicates not significance. MP mid-parent mean, BP better-parent mean, MPH mid-parent heterosis, BPH better-parent heterosis.

Parameters				**MPH**	**BPH**
	MP mean	**BP mean**	**Hybrid mean**	**% heterosis**	**% heterosis**
Number of fruits	13.75	12.83	18.78	36.57*	46.32*
Green fruit weight (g)	1042.50	1170.00	1182.50	13.43^{NS}	1.07^{NS}
Ripe fruit weight (g)	757.41	1158.57	1683.33	122.25*	45.30^{NS}
Total fruit weight (g)	1836.88	2402.50	2934.00	59.73*	22.12^{NS}
Fruit size	131.92	132.26	167.33	26.85^{NS}	26.52^{NS}

For hybrid +/*epi* in cv. VFN8, we performed the same experiment showed to cv. CNPH069 and present the results on Table 4. The +/*epi* hybrid in this study showed highly significant increase ($P<0.001$) in green fruit weight, ripe fruit weight and total fruit weight in relation to both, mid-parent means and better-parent means and the increase in all yield traits analyzed exceeded in more than 80% the values of better parent (Table 4).

Table 4: *epinastic* hybrids in cv. VFN8 shows largely better-parent heterosis. % heterosis in relation to mid-parent and better-parent (n=7 to +/+, 9 to *epi/epi* and 11 to +/*epi*) ** indicate significant value at 1% level significance by t test, ^{NS} indicates not significance. MP mid-parent mean, BP better-parent mean, MPH mid-parent heterosis, BPH better-parent heterosis.

Parameters				**MPH**	**BPH**
	MP mean	**BP mean**	**Hybrid mean**	**% heterosis**	**% heterosis**
Number of fruits	15.50	14.67	26.75	72.58*	82.39**
Green fruit weight (g)	828.33	980	1910	130.58**	94.90**
Ripe fruit weight (g)	244.52	325.71	1041.25	325.83**	219.68**
Total fruit weight (g)	1124.17	1285	2974.29	164.58**	131.46**
Fruit size (g)	122.45	31.57	143.12	16.88^{NS}	8.77^{**}

epinastic hybrid in VFN8 background presented higher number of mature fruits at the time of harvest (6.88 ± 0.79 fruits) in relation to both parents, +/+ (1.67 ± 0.22 fruits) and *epi*/*epi* (2.67 ± 0.5 fruits), for this reason we can assume that +/*epi* hybrid presents an early production, and for this reason, number of fruits and greater weight of mature fruits at the harvest time. On the other hand, in cv CNPH69 background, the highest number of fruits at harvest were found in +/+ (10 ± 1.12 fruits), followed by +/*epi* (6.5 ± 0.59) and then *epi/epi* (2.67 ± 0.5 fruits), which leads to the hypothesis that the hybrid in this case has an intermediate production beginning in relation to both parents.

Finally, because high soluble sugar content is an important trait for both, processing-tomato industry and *in natura* consumption, we tested brix value and brix×yield for parents and hybrids, and we observed that the same brix value in hybrids and in WT parent, commercial tomatoes, even though the hybrids exhibited greater amount of fruits and consequently more sinks (Fig. 4a). In relation to brix×yield, which is the product of the ripe fruit mass by brix value, in cv. VFN8 occurred higher brix×yield (Fig. 4b) since there was greater mass of ripe fruits at harvest time and, for CNPH69, same brix×yield value was verified for +/+ and +/*epi*.

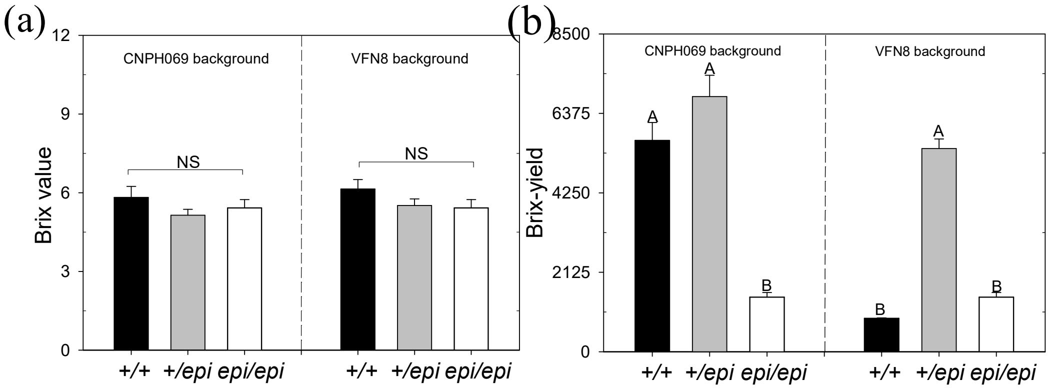

Figure 4: Brix-yield is increased in +/*epi* hybrid. (a) Brix value measured from 3 ripe fruits per plant (n=7-11) and (b) Brix ×yield calculated using brix values and mass of ripe fruits. Values are mean ± S.E.M. Same letters do not differ by Tukey's test at 5%.

DISCUSSION

Increased productivity in tomato is attained only when the correct cultivar is adopted and under adequate cultivation. Crop yield, including tomato, depends on the potential of the genotype used and resources availability. In our experiments, sufficient light, nutrients and water were available to plant growth, plants were cultivated in optimal conditions, thus all traits only represent genotypic differences.

Heterosis represented by the increase of vegetative and reproductive characteristics in +/*epi* hybrid in VFN8 background cultivated in greenhouse presented similarities with +/*epi* hybrid in MT background (as discussed in Chapter 2), as in brix×yield increase and dry mass weight of leaves, stems and roots. Crop yields derive from a complex network of traits exhibited on developmental and physiological mechanisms for organ production and biomass accumulation (SULPICE *et al.*, 2010; POORTER *et al.*, 2012; BLUM *et al.*, 2013; JIANG *et al.*, 2013). In the tomato cv MT background, several mechanisms were found to contribute to this network, such as increased photosynthesis, in stomatal conductance, in palisade mesophyll thickness, and in biochemical parameters, as starch accumulation and total chlorophyll (Chapter 2). In contrast, although heterosis in biomass is clear in VFN8, our experiments were not sufficient to understand the mechanism by which the *epinastic* gene in heterozygosity promotes growth in this cultivar. In fact, genetic background characteristics influence the manifestation of heterosis in each trait, that is, single parent combinations determine the level of heterosis and in what traits it is manifested (LIPPMAN & ZAMIR, 2007; KRIEGER *et al.*, 2010; SCHNABLE & SPRINGER, 2013).

The results found for heterosis manifestation in vegetative and reproductive traits in +/*epi* in MT background suggest that an alteration in leaf structure can be responsible for increases in vegetative growth and thus in source strength to support reproductive growth. Although we cannot explain the mechanisms related to heterosis in VFN8, because we verified changes in leaf structure in +/*epi* hybrids different of both parents and very similar to leaf structure of hybrids in MT, it is seemingly reasonable to speculate that changes in leaf structure are related to biomass increase in both backgrounds.

According to Lippman & Zamir (2006), plant heterosis is better manifested in field conditions, probably because most of the *loci* that contribute to heterosis are not associated with traits of agronomic importance such as production, resistance and morphology, but have a small effect on these mainly in slightly more stressful conditions (SCHNABLE & SPRINGER, 2013), we thus performed experiments in field conditions. Comparing the productive responses of the VFN8 hybrids in greenhouse and in field conditions, it can be noted that greater heterosis was verified in the field, with ripe fruit mass of 23% in the hybrid under controlled conditions and more than 200% under field-condition.

The +/*epi* hybrid in both backgrounds, VFN8 and CNPH69, showed heterosis in yield traits, although for CNPH69 mid-parent heterosis has been dominant and for VFN8 better-parent heterosis. Hybrids superiority over better-parent have more agronomic importance because can be used in commercial exploration of heterosis and help plant breeders to choose the best parental combination to create a hybrid plant while the importance of mid-parent heterosis is restricted to biological studies (SPRINGER & STUPAR, 2007).

VFN8 and CNPH069 are traditional tomato cultivars, because of this they do not show optimal performance, as resistance to some virus and fungal infections and higher fruit yield than others, such as M82. In our experiments, plants in field-condition showed pathogen infection, thus plants were harvested before total fruit ripening. Moreover, the maximum yield potential of hybrids was not expressed, because of this we did not compare yield production of +/*epi* hybrids with standard tomato cultivar neither with the same cultivar in other field conditions. Interestingly, +/*epi* hybrids had increased in fruit yield in relation to parents in both background, VFN8 and CNPH69, in adverse conditions, which makes us think that besides heterotic growth in vegetative and reproductive traits, *epi* hybrids could display better performance in biotic stresses.

CONCLUSION

Heterosis for yield traits in +/*epi* using commercial backgrounds allows us to conclude that the use of the *epinastic* mutation in heterozygosity presents potential of agronomic application, either through the manipulation of the *epinastic* gene in future breeding programs in tomato and other species, which requires studies to fully describe the function of the gene responsible for the mutation, or through the use of the *epi* mutant as a parent in specific tomato breeding programs. Even if the gene function of the *EPINASTIC* gene in tomato is not yet known, our results indicate that the gene is strongly influenced by environmental characteristics, since in field conditions there was an increase in the expression of yield traits in the hybrid.

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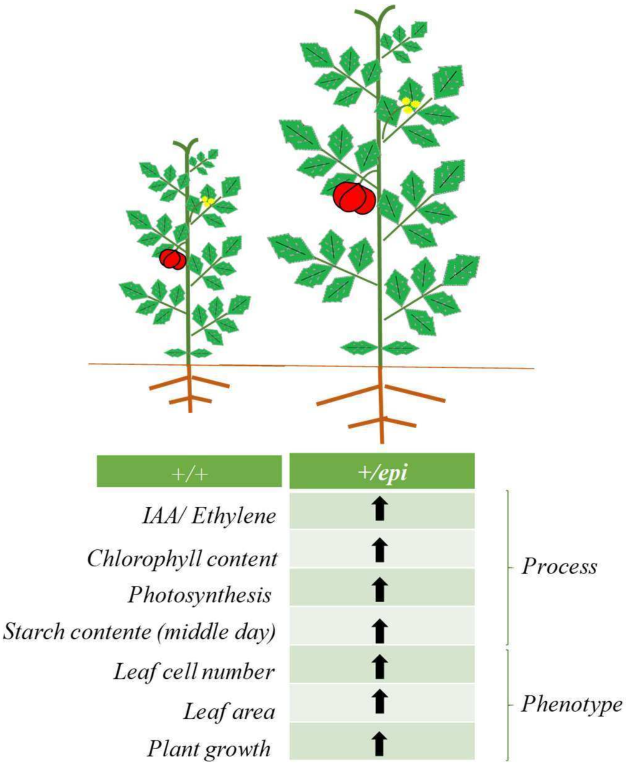
CONCLUSION

The results described in this work suggest a link between +/*epi* heterozygosity with heterosis in vegetative and reproductive traits, and provide a new example of a single gene overdominance for yield in tomato. Furthermore, this is the first case described, to our knowledge, where single gene overdominance affects hormonal balance not related to flowering and growth habit, and results in higher yield and plant growth.

We suggest that +/*epi* shows an epistatic relationship with genes involved in hormonal biosynthesis, increasing endogenous levels of auxin and intermediary levels of ethylene, both classical hormones related to the control of plant growth. According to our model, an adequate auxin:ethylene balance stimulates cell proliferation in leaves, resulting in a higher leaf blade area, the most common heterotic phenotype in plants. Larger leaves improve carbon assimilation per area, adding output to plant growth by increase in carbohydrate levels (Fig. 1).



+/+	*+/epi*	
IAA/ Ethylene	⬆	*Process*
Chlorophyll content	⬆	
Photosynthesis	⬆	
Starch contente (middle day)	⬆	
Leaf cell number	⬆	*Phenotype*
Leaf area	⬆	
Plant growth	⬆	

Figure 1: Summary of processes and phenotype altered in +/*epi* hybrids in relation to +/+. Model proposed to explain higher growth in +/*epi* hybrids in relation to a normal growth of +/+. Results derivate from our observation in hybrids in background MT.

Heterozygosity for *epi* gene mutant has potential to improve crop yields. Although the molecular nature of the *epi* mutation is still not clear, our findings provide good evidence that manipulating auxin:ethylene ratio by *epi* heterozygosity can provide a new path to plant breeding across different backgrounds in different growth conditions.

Given the complexity and variability of heterosis, heterosis research in +/*epi* should focus in these major objectives: (1) clarifying the activity and function of the *epinastic* gene; (2) identifying functional markers associated with *epi* mutant allele for breeding purposes; (3) understanding the environmental and developmental control of heterotic traits; and (4) testing *epi* alleles in different backgrounds and environments. In this way, we took the first step proposing *epi* hybrid and integrating physiological changes networks that contribute to hybrid phenotype. It is clear, however, that research is still necessary to understand the mechanism across *epi* gene product modify diverse metabolic pathway in both, heterozygosity and homozygosity.